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NF 38

THE NATIONAL FORMULARY

Volume 3

By authority of the United States Pharmacopeial Convention.
Prepared by the Council of Experts and its Expert Committees

Official from May 1, 2020

The designation on the cover of this publication, "USP NF 2020," is for ease of identification only. The publication contains two separate compendia: The United States Pharmacopeia, Forty-Third Revision, and The National Formulary, Thirty-Eighth Edition.

THE UNITED STATES PHARMACOPEIAL CONVENTION
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SIX-MONTH IMPLEMENTATION GUIDELINE

The *United States Pharmacopeia–National Formulary* and its supplements become official **six months** after being released to the public. The *USP–NF,* which is released on November 1 of each year, becomes official on May 1 of the following year. This six-month implementation timing gives users more time to bring their methods and procedures into compliance with new and revised *USP–NF* requirements.

The table below describes the official dates of the *USP–NF* and its supplements. The 2019 *USP 42–NF 37,* and its supplements, *Interim Revision Announcements (IRAs)* and *Revision Bulletins* to that edition, will be official until May 1, 2020, at which time the *USP 43–NF 38* becomes official.

Publication	Release Date	Official Date	Official Until
USP 43–NF 38	November 1, 2019	May 1, 2020	May 1, 2021 (except as superseded by supplements, *IRAs,* and *Revision Bulletins*)
First Supplement to the USP 43–NF 38	February 1, 2020	August 1, 2020	May 1, 2021 (except as superseded by *Second Supplement, IRAs,* and *Revision Bulletins*)
Second Supplement to the USP 43–NF 38	June 1, 2020	December 1, 2020	May 1, 2021 (except as superseded by *IRAs* and *Revision Bulletins*)
USP-NF 2021 Issue 1	November 1, 2020	May 1, 2021	May 1, 2022 (except as superseded by supplements, *IRAs,* and *Revision Bulletins*)

The table below gives the details of the *IRAs* that will apply to *USP 43–NF 38.*

IRA	PF Posting Date	Comment Due Date	IRA Posting Date	IRA Official Date
46(1)	January 2, 2020	March 31, 2020	May 29, 2020	July 1, 2020
46(2)	March 2, 2020	May 31, 2020	July 31, 2020	September 1, 2020
46(3)	May 1, 2020	July 31, 2020	September 25, 2020	November 1, 2020
46(4)	July 1, 2020	September 30, 2020	November 20, 2020	January 1, 2021
46(5)	September 1, 2020	November 30, 2020	January 31, 2021	March 1, 2021
46(6)	November 2, 2020	January 31, 2020	March 27, 2021	May 1, 2021

Revision Bulletins are published on the USP website and the *USP–NF Online* product and become official on the date specified in the *Revision Bulletin.*

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GENERAL NOTICES AND REQUIREMENTS

The *General Notices and Requirements* section (the *General Notices*) presents the basic assumptions, definitions, and default conditions for the interpretation and application of the *United States Pharmacopeia* (*USP*) and the *National Formulary* (*NF*).

Requirements stated in these *General Notices* apply to all articles recognized in the *USP* and *NF* (the "compendia") and to all general chapters unless specifically stated otherwise.

1. TITLE AND REVISION

The full title of this publication (consisting of five volumes and including its *Supplements*), is *The Pharmacopeia of the United States of America*, Forty-Third Revision and the *National Formulary*, Thirty-Eighth Edition. These titles may be abbreviated to *USP 43*, to *NF 38*, and to *USP 43–NF 38*. The *United States Pharmacopeia*, Forty-Third Revision, and the *National Formulary*, Thirty-Eighth Edition, supersede all earlier revisions. Where the terms "*USP*," "*NF*," or "*USP–NF*" are used without further qualification during the period in which these compendia are official, they refer only to *USP 43*, *NF 38*, and any *Supplement(s)* thereto. The same titles, with no further distinction, apply equally to print or electronic presentation of these contents. Although *USP* and *NF* are published under one cover and share these *General Notices*, they are separate compendia.

This revision is official beginning May 1, 2020 unless otherwise indicated in specific text.

Supplements to *USP* and *NF* are published periodically.

Accelerated Revisions, published periodically on the *Official Text* section of USP's website (http://www.usp.org/usp-nf/official-text), are designed to make revisions official more quickly than through the routine process for publishing standards in the *USP–NF*. *Interim Revision Announcements* are Accelerated Revisions to *USP* and *NF* that contain official revisions and their effective dates.

Revision Bulletins are Accelerated Revisions to official text or postponements that require expedited publication. They generally are official immediately unless otherwise specified in the *Revision Bulletin*.

Errata are Accelerated Revisions representing corrections to items erroneously published. Announcements of the availability of new USP Reference Standards and announcements of tests or procedures that are held in abeyance pending availability of required USP Reference Standards are also available on the "Official Text" tab of USP's website.

2. OFFICIAL STATUS AND LEGAL RECOGNITION

2.10. Official Text

Official text of the *USP* and *NF* is published in the *USP–NF Online* (www.uspnf.com) in the edition identified as "CURRENTLY OFFICIAL" and in Accelerated Revisions that supersede the *USP–NF Online* as described below.

Routine revisions are published in the *USP–NF Online* and become official on the date indicated, usually six months after publication. Accelerated Revisions supersede the *USP–NF Online* and become official on the date indicated. Links to Accelerated Revisions on the USP website can be found in any superseded monograph or general chapter in the *USP–NF Online*.

Print and USB flash drive versions of the *USP* and *NF* also are available. Routine revisions are provided with the same timing as the *USP–NF Online*. Official text published in *Supplements* supersedes that in the previously published print or USB flash drive versions of *USP–NF*. These versions also are superseded by Accelerated Revisions as described above.

In the event of any disparity between the print or USB flash drive versions and the *USP–NF Online*, the *USP–NF Online* will be deemed to apply.

2.20. Official Articles

An *official article* is an article that is recognized in *USP* or *NF*. An article is deemed to be recognized and included in a compendium when a monograph for the article is published in the compendium and an official date is generally or specifically assigned to the monograph.

The title specified in a monograph is the *official title* for such article. Other names considered to be synonyms of the *official titles* may not be used as substitutes for *official titles*. For drug products that incorporate a sensor to detect that the product has been administered, the *official title* shall be the title specified in the relevant drug product monograph plus the words "with sensor".

Official articles include both *official substances* and *official products*. An *official substance* is a drug substance, excipient, dietary ingredient, other ingredient, or component of a finished device for which the monograph title includes no indication of the nature of the finished form.

An *official product* is a drug product, dietary supplement, compounded preparation, or finished device for which a monograph is provided.

2.30. Legal Recognition

The *USP* and *NF* are recognized in the laws and regulations of many countries throughout the world. Regulatory authorities may enforce the standards presented in the *USP* and *NF*, but because recognition of the *USP* and *NF* may vary by country, users should understand applicable laws and regulations. In the United States under the Federal Food, Drug, and Cosmetic Act (FDCA), both *USP* and *NF* are recognized as official compendia. A drug with a name recognized in *USP–NF* must comply with compendial identity standards or be deemed adulterated, misbranded, or both. See, e.g., FDCA § 501(b) and 502(e)(3)(b); also FDA regulations, 21 CFR § 299.5(a&b). To avoid being deemed adulterated, such drugs must also comply with compendial standards for strength, quality, and purity, unless labeled to show all respects in which the drug differs. See, e.g., FDCA § 501(b) and 21 CFR § 299.5(c). In addition, to avoid being deemed misbranded, drugs recognized in *USP–NF* must also be packaged and

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labeled in compliance with compendial standards. See FDCA § 502(g).

A dietary supplement represented as conforming to specifications in *USP* will be deemed a misbranded food if it fails to so conform. See FDCA § 403(s)(2)(D).

Enforcement of *USP* standards is the responsibility of FDA and other government authorities in the U.S. and elsewhere. USP has no role in enforcement.

3. CONFORMANCE TO STANDARDS

3.10. Applicability of Standards

Standards for an article recognized in the compendia (*USP–NF*) are expressed in the article's monograph, applicable general chapters, and *General Notices*. The identity, strength, quality, and purity of an article are determined by the official tests, procedures, and acceptance criteria, and other requirements incorporated in the monograph, in applicable general chapters, or in the *General Notices*. "Applicable general chapters" means general chapters numbered below 1000 or above 2000 that are made applicable to an article through reference in *General Notices*, a monograph, or another applicable general chapter numbered below 1000. Where the requirements of a monograph differ from the requirements specified in these *General Notices* or an applicable general chapter, the monograph requirements apply and supersede the requirements of the *General Notices* or applicable general chapters, whether or not the monograph explicitly states the difference.

General chapters numbered 1000 to 1999 are for informational purposes only. They contain no mandatory tests, assays, or other requirements applicable to any official article, regardless of citation in a general chapter numbered below 1000, a monograph, or these *General Notices*. General chapters numbered above 2000 apply only to articles that are intended for use as dietary ingredients and dietary supplements. General chapter citations in *NF* monographs refer to *USP* general chapters.

Early adoption of revised standards in advance of the official date is allowed by USP unless specified otherwise at the time of publication. Where revised standards for an existing article have been published as final approved "official text" (as approved in section *2.10 Official Text*) but have not yet reached the official date (6 months after publication, unless otherwise specified; see "official date", section *2.20 Official Articles*), compliance with the revised standard shall not preclude a finding or indication of conformance with compendial standards, unless USP specifies otherwise by prohibiting early adoption in a particular standard.

The standards in the relevant monograph, general chapter(s), and *General Notices* apply at all times in the life of the article from production to expiration. It is also noted that the manufacturer's specifications, and manufacturing practices (e.g., Quality by Design, Process Analytical Technology, and Real Time Release Testing initiatives), generally are followed to ensure that the article will comply with compendial standards until its expiration date, when stored as directed. Every compendial article in commerce shall be so constituted that when examined in accordance with these assays and test procedures, it meets all applicable pharmacopeial requirements (*General Notices*, monographs, and general chapters). Thus, any official article is expected to meet the compendial standards if tested, and any official article actually tested as directed in the relevant monograph must meet such standards to demonstrate compliance.

Some tests, such as those for *Dissolution* and *Uniformity of Dosage Units*, require multiple dosage units in conjunction with a decision scheme. These tests, albeit using a number of dosage units, are in fact one determination. These procedures should not be confused with statistical sampling plans. The similarity to statistical procedures may seem to suggest an intent to make inference to some larger group of units, but in all cases, statements about whether the compendial standard is met apply only to the units tested. Repeats, replicates, statistical rejection of outliers, or extrapolations of results to larger populations, as well as the necessity and appropriate frequency of batch testing, are neither specified nor proscribed by the compendia; such decisions are based on the objectives of the testing. Frequency of testing and sampling are left to the preferences or direction of those performing compliance testing, and other users of *USP–NF*, including manufacturers, buyers, or regulatory authorities.

Official products are prepared according to recognized principles of good manufacturing practice and from ingredients that meet *USP* or *NF* standards, where standards for such ingredients exist (for dietary supplements, see section *3.10.20 Applicability of Standards to Medical Devices, Dietary Supplements, and Their Components and Ingredients*).

Official substances are prepared according to recognized principles of good manufacturing practice and from ingredients complying with specifications designed to ensure that the resultant substances meet the requirements of the compendial monographs.

3.10.10. Applicability of Standards to Drug Products, Drug Substances, and Excipients

The applicable *USP* or *NF* standard applies to any article marketed in the United States that (1) is recognized in the compendium and (2) is intended or labeled for use as a drug or as an ingredient in a drug. Such articles (drug products, drug substances, and excipients) include both human drugs (whether dispensed by prescription, "over the counter," or otherwise), as well as animal drugs. The applicable standard applies to such articles whether or not the added designation "USP" or "NF" is used. The standards apply equally to articles bearing the official titles or names derived by transposition of the definitive words of official titles or transposition in the order of the names of two or more drug substances in official titles, or where there is use of synonyms with the intent or effect of suggesting a significant degree of identity with the official title or name.

3.10.20. Applicability of Standards to Medical Devices, Dietary Supplements, and Their Components and Ingredients

An article recognized in *USP* or *NF* shall comply with the compendial standards if the article is a medical device, component intended for a medical device, dietary supplement, dietary ingredient, or other ingredient that is intended for incorporation into a dietary supplement, and is labeled as conforming to the *USP* or *NF*.

Generally, dietary supplements are prepared from ingredients that meet *USP*, *NF*, or *Food Chemicals Codex* standards. Where such standards do not exist, substances may be used in dietary supplements if they have been shown to be of acceptable food grade quality using other suitable procedures.

3.10.30. Applicability of Standards to the Practice of Compounding

USP compounding practice standards, *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩ and *Pharmaceutical Compounding—Sterile Preparations* ⟨797⟩, as appropriate, apply to compounding practice or activity regardless of whether a monograph exists for the compounded preparation or these chapters are referenced in such a monograph. In the United States, ⟨795⟩ and ⟨797⟩ are not applicable to drugs compounded by entities registered with FDA as outsourcing facilities as defined by FDCA § 503B, because such facilities are required to comply with FDA's current good manufacturing practice requirements. Compounded preparations, including drug products compounded by outsourcing facilities, may also be

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subject to applicable monographs; see section *2.20 Official Articles* and section *4.10 Monographs*.

3.20. Indicating Conformance

A drug product, drug substance, or excipient may use the designation "USP" or "NF" in conjunction with its official title or elsewhere on the label only when (1) a monograph is provided in the specified compendium and (2) the article complies with the identity prescribed in the specified compendium.

When a drug product, drug substance, compounded preparation, or excipient differs from the relevant *USP* or *NF* standard of strength, quality, or purity, as determined by the application of the tests, procedures, and acceptance criteria set forth in the relevant compendium, its difference shall be plainly stated on its label.

When a drug product, drug substance, compounded preparation, or excipient fails to comply with the identity prescribed in *USP* or *NF* or contains an added substance that interferes with the prescribed tests and procedures, the article shall be designated by a name that is clearly distinguishing and differentiating from any name recognized in *USP* or *NF*.

A medical device, dietary supplement, or ingredient or component of a medical device or dietary supplement may use the designation "USP" or "NF" in conjunction with its official title or elsewhere on the label only when (1) a monograph is provided in the specified compendium and (2) the article complies with the monograph standards and other applicable standards in that compendium.

The designation "USP" or "NF" on the label may not and does not constitute an endorsement by USP and does not represent assurance by USP that the article is known to comply with the relevant standards. USP may seek legal redress if an article purports to be or is represented as an official article in one of USP's compendia and such claim is determined by USP not to be made in good faith.

The designation "USP–NF" may be used on the label of an article provided that the label also bears a statement such as "Meets *NF* standards as published by USP," indicating the particular compendium to which the article purports to apply.

When the letters "USP," "NF," or "USP–NF" are used on the label of an article to indicate compliance with compendial standards, the letters shall appear in conjunction with the official title of the article. The letters are not to be enclosed in any symbol such as a circle, square, etc., and shall appear in capital letters.

If a dietary supplement does not comply with all applicable compendial requirements but contains one or more dietary ingredients or other ingredients that are recognized in *USP* or *NF*, the individual ingredient(s) may be designated as complying with *USP* or *NF* standards or being of *USP* or *NF* quality provided that the designation is limited to the individual ingredient(s) and does not suggest that the dietary supplement complies with *USP* standards.

4. MONOGRAPHS AND GENERAL CHAPTERS

4.10. Monographs

Monographs set forth the article's name, definition, specification, and other requirements related to packaging, storage, and labeling. The specification consists of tests, procedures, and acceptance criteria that help ensure the identity, strength, quality, and purity of the article. For general requirements relating to specific monograph sections, see section *5. Monograph Components*.

Because monographs may not provide standards for all relevant characteristics, some official substances may conform to the *USP* or *NF* standard but differ with regard to nonstandardized properties that are relevant to their use in specific preparations. To assure substitutability in such instances, users may wish to ascertain functional equivalence or determine such characteristics before use.

4.10.10. Applicability of Test Procedures

A single monograph may include more than one test, procedure, and/or acceptance criterion for the same attribute. Unless otherwise specified in the monograph, all tests are requirements. In some cases, monograph instructions allow the selection of tests that reflect attributes of different manufacturers' articles, such as different polymorphic forms, impurities, hydrates, and dissolution. Monograph instructions indicate the tests, procedures, and/or acceptance criteria to be used and the required labeling.

The order in which the tests are listed in the monograph is based on the order in which they are approved by the relevant Expert Committee for inclusion in the monograph. Test 1 is not necessarily the test for the innovator or for the reference product. Depending on monograph instructions, a labeling statement is not typically required if Test 1 is used.

4.10.20. Acceptance Criteria

The acceptance criteria allow for analytical error, for unavoidable variations in manufacturing and compounding, and for deterioration to an extent considered acceptable under practical conditions. The existence of compendial acceptance criteria does not constitute a basis for a claim that an official substance that more nearly approaches 100% purity "exceeds" compendial quality. Similarly, the fact that an article has been prepared to tighter criteria than those specified in the monograph does not constitute a basis for a claim that the article "exceeds" the compendial requirements.

An official product shall be formulated with the intent to provide 100% of the quantity of each ingredient declared on the label. Where the minimum amount of a substance present in a dietary supplement is required by law to be higher than the lower acceptance criterion allowed for in the monograph, the upper acceptance criterion contained in the monograph may be increased by a corresponding amount.

The acceptance criteria specified in individual monographs and in the general chapters for compounded preparations are based on such attributes of quality as might be expected to characterize an article compounded from suitable bulk drug substances and ingredients, using the procedures provided or recognized principles of good compounding practice, as described in these compendia.

4.20. General Chapters

Each general chapter is assigned a number that appears in angle brackets adjacent to the chapter name (e.g., *Chromatography* ⟨621⟩). General chapters may contain the following:

- Descriptions of tests and procedures for application through individual monographs,
- Descriptions and specifications of conditions and practices for pharmaceutical compounding,
- General information for the interpretation of the compendial requirements,
- Descriptions of general pharmaceutical storage, dispensing, and packaging practices, or
- General guidance to manufacturers of official substances or official products.

When a general chapter is referenced in a monograph, acceptance criteria may be presented after a colon.

Some chapters may serve as introductory overviews of a test or of analytical techniques. They may reference other general chapters that contain techniques, details of the procedures, and, at times, acceptance criteria.

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5. MONOGRAPH COMPONENTS

5.10. Molecular Formula

The use of the molecular formula for the official substance(s) named in defining the required strength of a compendial article is intended to designate the chemical entity or entities, as given in the complete chemical name of the article, having absolute (100%) purity.

5.20. Added Substances

Added substances are presumed to be unsuitable for inclusion in an official article and therefore prohibited, if their presence impairs the bioavailability, therapeutic efficacy, or safety of the official article; or they interfere with the assays and tests prescribed for determining compliance with the compendial standards (see section *3.20 Indicating Conformance*).

The air in a container of an official article may, where appropriate, be evacuated or be replaced by carbon dioxide, helium, argon, or nitrogen, or by a mixture of these gases. The use of such gas need not be declared in the labeling.

5.20.10. Added Substances in Official Substances

Official substances may contain only the specific added substances that are permitted by the individual monograph. Such added substances shall not exceed the quantity required for providing their intended effect. Where such addition is permitted, the label shall indicate the name(s) and amount(s) of any added substance(s).

5.20.20. Added Substances (Excipients and Ingredients) in Official Products

Suitable substances and excipients such as antimicrobial agents, pharmaceutical bases, carriers, coatings, flavors, preservatives, stabilizers, and vehicles may be added to an official product to enhance its stability, usefulness, or elegance, or to facilitate its preparation, unless otherwise specified in the individual monograph.

Added substances and excipients employed solely to impart color may be incorporated into official products other than those intended for parenteral or ophthalmic use, in accordance with the regulations pertaining to the use of colors issued by the FDA, provided such added substances or excipients are otherwise appropriate in all respects. (See also *Injections and Implanted Drugs Products* ⟨1⟩, *Product Quality Tests Common to Parenteral Dosage Forms, Specific Tests, Vehicles and added substances, Added substances*.)

The proportions of the substances constituting the base in ointment and suppository products and preparations may be varied to maintain a suitable consistency under different climatic conditions, provided that the concentrations of drug substances are not varied and provided that the bioavailability, therapeutic efficacy, and safety of the preparation are not impaired.

5.20.20.1. In Compounded Preparations

Compounded preparations for which a complete composition is given shall contain only the ingredients named in the formulas unless specifically exempted herein or in the individual monograph. Deviation from the specified processes or methods of compounding, although not from the ingredients or proportions thereof, may occur provided that the finished preparation conforms to the relevant standards and to preparations produced by following the specified process.

Where a monograph for a compounded preparation calls for an ingredient in an amount expressed on the dried basis, the ingredient need not be dried before use if due allowance is made for the water or other volatile substances present in the quantity taken.

Specially denatured alcohol formulas are available for use in accordance with federal statutes and regulations of the Internal Revenue Service. A suitable formula of specially denatured alcohol may be substituted for Alcohol in the manufacture of official preparations intended for internal or topical use, provided that the denaturant is volatile and does not remain in the finished product. A preparation that is intended for topical application to the skin may contain specially denatured alcohol, provided that the denaturant is either a usual ingredient in the preparation or a permissible added substance; in either case the denaturant shall be identified on the label of the topical preparation. Where a process is given in the individual monograph, any preparation compounded using denatured alcohol shall be identical to that prepared by the monograph process.

5.20.20.2. In Dietary Supplements

Additional ingredients may be added to dietary supplement products provided that the additional ingredients (1) comply with applicable regulatory requirements, and (2) do not interfere with the assays and tests prescribed for determining compliance with compendial standards.

5.30. Description and Solubility

Only where a quantitative solubility test is given in a monograph and is designated as such is it a test for purity.

A monograph may include information regarding the article's description. Information about an article's "description and solubility" also is provided in the reference table *Description and Relative Solubility of USP and NF Articles*. The reference table merely denotes the properties of articles that comply with monograph standards. The reference table is intended primarily for those who use, prepare, and dispense drugs and/or related articles. Although the information provided in monographs and the information in the reference table may indirectly assist in the preliminary evaluation of an article, it is not intended to serve as a standard or test for purity.

The approximate solubility of a compendial substance is indicated by one of the following descriptive terms:

Descriptive Term	Parts of Solvent Required for 1 Part of Solute
Very soluble	Less than 1
Freely soluble	From 1 to 10
Soluble	From 10 to 30
Sparingly soluble	From 30 to 100
Slightly soluble	From 100 to 1,000
Very slightly soluble	From 1,000 to 10,000
Practically insoluble, or Insoluble	Greater than or equal to 10,000

5.40. Identification

A compendial test titled *Identification* is provided as an aid in verifying the identity of articles as they are purported to be, e.g., those taken from labeled containers, and to establish whether it is the article named in *USP–NF*. The *Identification* test for a particular article may consist of one or more procedures. When a compendial *Identification* test is undertaken, all requirements of all specified procedures in the test must be met to satisfy the requirements of the test. Failure of an article to meet all the requirements of a prescribed *Identification* test (i.e., failure to meet the requirements of all of the specified procedures that are components of that test) indicates that the article is mislabeled and/or adulterated.

5.50. Assay

Assay tests for compounded preparations are not intended for evaluating a compounded preparation before dispensing, but instead are intended to serve as the official test in the event of a question or dispute regarding the preparation's conformance to official standards.

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5.50.10. Units of Potency (Biological)

For substances that cannot be completely characterized by chemical or physical means or that need confirmation of functionality or tertiary structure, it may be necessary to express quantities of biological activity in units of biological potency, each defined by an authoritative, designated reference standard. In cases where international reference materials have been discontinued, international units of potency may be defined in terms of molecular mass, such as in the cases of vitamins A, D, and E.

Where available, World Health Organization (WHO) international biological standards define the International Units (IU). *USP* monographs refer to the units assigned by USP Reference Standards either directly as International Units (IU) or as "USP Units." For some biological products, units of potency are value assigned against a corresponding U.S. Standard established by FDA, whether or not International Units or USP Units have been defined (see *Biologics* ⟨1041⟩). Note that product-related labeling, e.g., on containers, need not use the full phrase "USP [product name] Units" that appears in many *USP* monograph labeling sections. The term "USP Units" can be used on product labeling consistent with USP compendial requirements, provided it is clear from the context that the potency is stated in terms of USP [product name] Units. In such circumstances it should be clear that "USP Units" and "USP [product name] Units" share the same meaning.

5.60. Impurities and Foreign Substances

Tests for the presence of impurities and foreign substances are provided to limit such substances to amounts that are unobjectionable under conditions in which the article is customarily employed (see also *Impurities in Drug Substances and Drug Products* ⟨1086⟩).

Nonmonograph tests and acceptance criteria suitable for detecting and controlling impurities that may result from a change in the processing methods or that may be introduced from external sources should be employed in addition to the tests provided in the individual monograph, where the presence of the impurity is inconsistent with applicable good manufacturing practices or good pharmaceutical practices.

5.60.10. Other Impurities in USP and NF Articles

If a *USP* or *NF* monograph includes an assay or organic impurity test based on chromatography, other than a test for residual solvents, and that monograph procedure does not detect an impurity present in the substance, the amount and identity of the impurity, where both are known, shall be stated in the labeling (certificate of analysis) of the official substance, under the heading *Other Impurity(ies)*.

The presence of any unlabeled other impurity in an official substance is a variance from the standard if the content is 0.1% or greater. The sum of all *Other Impurities* combined with the monograph-detected impurities may not exceed 2.0% (see *Ordinary Impurities* ⟨466⟩), unless otherwise stated in the monograph.

The following categories of drug substances are excluded from *Other Impurities* requirements:

- Fermentation products and semi-synthetics derived therefrom,
- Radiopharmaceuticals,
- Biologics,
- Biotechnology-derived products,
- Peptides,
- Herbals, and
- Crude products of animal or plant origin.

Any substance known to be toxic shall not be listed under *Other Impurities*.

5.60.20. Residual Solvents in USP and NF Articles

All *USP* and *NF* articles are subject to relevant control of residual solvents, even when no test is specified in the individual monograph. If solvents are used during production, they must be of suitable quality. In addition, the toxicity and residual level of each solvent shall be taken into consideration, and the solvents limited according to the principles defined and the requirements specified in *Residual Solvents* ⟨467⟩, using the general methods presented therein or other suitable methods.

5.60.30. Elemental Impurities in USP Drug Products and Dietary Supplements

Elemental impurities in official drug products are controlled according to the principles defined and requirements specified in *Elemental Impurities—Limits* ⟨232⟩. Elemental contaminants in official dietary supplements are controlled according to the principles defined and requirements specified in *Elemental Contaminants in Dietary Supplements* ⟨2232⟩.

5.70. Performance Tests

Where content uniformity determinations have been made using the same analytical methodology specified in the *Assay*, with appropriate allowances made for differences in sample preparation, the average of all of the individual content uniformity determinations may be used as the *Assay* value.

5.80. USP Reference Standards

USP Reference Standards are authentic specimens that have been approved as suitable for use as comparison standards in *USP* or *NF* tests and assays. (See *USP Reference Standards* ⟨11⟩.) Where *USP* or *NF* tests or assays call for the use of a USP Reference Standard, only those results obtained using the specified USP Reference Standard are conclusive. Where a procedure calls for the use of a compendial article rather than for a USP Reference Standard as a material standard of reference, a substance meeting all of the compendial monograph requirements for that article shall be used. If any new *USP* or *NF* standard requires the use of a new USP Reference Standard that is not yet available, that portion of the standard containing the requirement shall not be official until the specified USP reference material is available.

Unless a Reference Standard label bears a specific potency or content, assume the Reference Standard is 100.0% pure in the official application. Unless otherwise directed in the procedure in the individual monograph or in a general chapter, USP Reference Standards are to be used in accordance with the instructions on the label of the Reference Standard.

6. TESTING PRACTICES AND PROCEDURES

6.10. Safe Laboratory Practices

In performing compendial procedures, safe laboratory practices shall be followed, including precautionary measures, protective equipment, and work practices consistent with the chemicals and procedures used. Before undertaking any procedure described in the compendia, the analyst should be aware of the hazards associated with the chemicals and the techniques and means of protecting against them. These compendia are not designed to describe such hazards or protective measures.

6.20. Automated Procedures

Automated and manual procedures employing the same basic chemistry are considered equivalent provided the automated system is properly qualified as being suitable to execute the compendial manual method and the analytical procedure is verified under the new equipment conditions.

6.30. Alternative and Harmonized Methods and Procedures

An alternative method or procedure is defined as any method or procedure other than the compendial method or

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procedure for the article in question. The alternative method or procedure must be fully validated (see *Validation of Compendial Procedures* ⟨1225⟩) and must produce comparable results to the compendial method or procedure within allowable limits established on a case-by-case basis. Alternative methods or procedures can be developed for any one of a number of reasons not limited to simplification of sample preparation, enhanced precision and accuracy, improved (shortened) run time, or being better suited to automation than the compendial method or procedure. Only those results obtained by the methods and procedures given in the compendia are conclusive.

For evaluation as a potential replacement or addition to the standard, alternative methods and procedures should be submitted to USP (see section *4.10 Monographs*).

Certain general chapters contain a statement that the text in question is harmonized with the corresponding text of the *European Pharmacopoeia* and/or the *Japanese Pharmacopoeia* and that these texts are interchangeable. Therefore, if a substance or preparation is found to comply with a requirement using an interchangeable method or procedure from one of these pharmacopeias, it should comply with the requirements of the *USP–NF*. When a difference appears, or in the event of dispute, only the result obtained by the method and/or procedure given in the *USP–NF* is conclusive.

6.40. Dried, Anhydrous, Ignited, or Solvent-Free Basis

All calculations in the compendia assume an "as-is" basis unless otherwise specified.

Test procedures may be performed on the undried or unignited substance and the results calculated on the dried, anhydrous, or ignited basis, provided a test for *Loss on Drying*, or *Water Determination*, or *Loss on Ignition*, respectively, is given in the monograph. Where the presence of moisture or other volatile material may interfere with the procedure, previous drying of the substance is specified in the individual monograph and is obligatory.

The term "solvent-free" signifies that the calculation shall be corrected for the presence of known solvents as determined using the methods described in ⟨467⟩ unless a test for limit of organic solvents is provided in the monograph.

The term "previously dried" without qualification signifies that the substance shall be dried as directed under *Loss on Drying* ⟨731⟩ or *Water Determination* ⟨921⟩ (gravimetric determination).

Where drying in vacuum over a desiccant is directed, a vacuum desiccator, a vacuum drying pistol, or other suitable vacuum drying apparatus shall be used.

6.40.10. Ignite to Constant Weight

"Ignite to constant weight" means that ignition shall be continued at 800 ± 25°, unless otherwise indicated, until two consecutive weighings, the second of which is taken after an additional period appropriate to the nature and quantity of the residue, do not differ by more than 0.50 mg per g of substance taken.

6.40.20. Dried to Constant Weight

"Dried to constant weight" means that drying shall be continued until two consecutive weighings, the second of which is taken after an additional drying period appropriate to the nature and quantity of the residue, do not differ by more than 0.50 mg per g of substance taken.

6.50. Preparation of Solutions

6.50.10. Filtration

Where a procedure gives direction to "filter" without further qualification, the liquid shall be passed through suitable filter paper or equivalent device until the filtrate is clear. Due to the possibility of filter effects, the initial volumes of a filtrate may be discarded.

6.50.20. Solutions

Unless otherwise specified, all solutions shall be prepared with Purified Water. Solutions for quantitative measures shall be prepared using accurately weighed or accurately measured analytes (see section *8.20 About*).

An expression such as "(1 in 10)" means that 1 part *by volume* of a liquid shall be diluted with, or 1 part *by weight* of a solid shall be dissolved in, a sufficient quantity of the diluent or solvent to make the volume of the finished solution 10 parts *by volume*. For example, a 1 in 10 solution is prepared by diluting 1 mL of a liquid or dissolving 1 g of a solid in sufficient solvent to make 10 mL of the solution. An expression such as "(20:5:2)" means that the respective numbers of parts, by volume, of the designated liquids shall be mixed, unless otherwise indicated.

6.50.20.1. Adjustments to Solutions

When a specified concentration is called for in a procedure, a solution of other normality or molarity may be used, provided that allowance is made for the difference in concentration and that the change does not increase the error of measurement.

Proportionately larger or smaller quantities than the specified weights and volumes of assay or test substances and Reference Standards may be taken, provided the measurement is made with at least equivalent accuracy.

Unless otherwise indicated, analyte concentrations shall be prepared to within ten percent (10%) of the indicated value. In the case in which a procedure is adapted to the working range of an instrument, solution concentrations may differ from the indicated value by more than ten percent (10%), with appropriate changes in associated calculations. Any changes shall fall within the validated range of the instrument.

When adjustment of pH is indicated with either an acid or base and the concentration is not indicated, appropriate concentrations of that acid or base may be used.

6.50.20.2. Test Solutions

Information on Test Solutions (TS) is provided in the *Test Solutions* portion of the *Reagents, Indicators, and Solutions* section of the *USP–NF*. Use of an alternative Test Solution or a change in the Test Solution used may require validation.

6.50.20.3. Indicator Solutions

Where a procedure specifies the use of an indicator TS, approximately 0.2 mL, or 3 drops, of the solution shall be added unless otherwise directed.

6.60. Units Necessary to Complete a Test

Unless otherwise specified, a sufficient number of units to ensure a suitable analytical result shall be taken.

6.60.10. Tablets

Where the procedure of a Tablet monograph directs to weigh and finely powder not fewer than a given number of Tablets, a counted number of Tablets shall be weighed and reduced to a powder. The portion of the powdered Tablets taken shall be representative of the whole Tablets and shall, in turn, be weighed accurately.

6.60.20. Capsules

Where the procedure of a Capsule monograph gives direction to remove, as completely as possible, the contents of not fewer than a given number of the Capsules, a counted number of Capsules shall be carefully opened and the contents quantitatively removed, combined, mixed, and weighed accurately. The portion of mixed Capsules contents taken shall be representative of the contents of the Capsules and shall, in turn, be weighed accurately.

6.70. Reagents

The proper conduct of the compendial procedures and the reliability of the results depend, in part, upon the quality of the reagents used in the performance of the procedures. Unless otherwise specified, reagents conforming to the specifications

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set forth in the current edition of *Reagent Chemicals* published by the American Chemical Society (ACS) shall be used. Where such ACS reagent specifications are not available or where the required purity differs, compendial specifications for reagents of acceptable quality are provided (see the *Reagents, Indicators, and Solutions* section of the *USP–NF*). Reagents not covered by any of these specifications should be of a grade suitable to the proper performance of the method of assay or test involved.

Listing of these reagents, including the indicators and solutions employed as reagents, in no way implies that they have therapeutic utility; furthermore, any reference to *USP* or *NF* in their labeling shall include also the term "reagent" or "reagent grade." USP may supply reagents if they otherwise may not be generally commercially available.

6.80. Equipment

Unless otherwise specified, a specification for a definite size or type of container or apparatus in a procedure is given solely as a recommendation. Other dimensions or types may be used if they are suitable for the intended use.

6.80.10. Apparatus for Measurement

Where volumetric flasks or other exact measuring, weighing, or sorting devices are specified, this or other equipment of at least equivalent accuracy shall be employed.

6.80.10.1. Pipet/Pipette

Where a pipet/pipette is specified, a suitable buret may be substituted. Where a "to contain" pipet/pipette is specified, a suitable volumetric flask may be substituted.

6.80.10.2. Light Protection

Where low-actinic or light-resistant containers are specified, either containers specially treated to protect contents from light or clear containers that have been rendered opaque by application of a suitable coating or wrapping may be used.

6.80.20. Instrumental Apparatus

An instrument may be substituted for the specified instrument if the substitute uses the same fundamental principles of operation and is of equivalent or greater sensitivity and accuracy. These characteristics shall be qualified as appropriate. Where a particular brand or source of a material, instrument, or piece of equipment, or the name and address of a manufacturer or distributor, is mentioned (ordinarily in a footnote), this identification is furnished solely for informational purposes as a matter of convenience, without implication of approval, endorsement, or certification.

6.80.20.1. Chromatographic Tubes and Columns

The term "diameter" refers to internal diameter (ID).

6.80.20.2. Tubing

The term "diameter" refers to outside diameter (OD).

6.80.20.3. Steam Bath

Where use of a steam bath is directed, use actively flowing steam or another regulated heat source controlled at an equivalent temperature.

6.80.20.4. Water Bath

A water bath requires vigorously boiling water unless otherwise specified.

6.80.30. Temperature Reading Devices

Temperature reading devices suitable for pharmacopeial tests conform to specifications that are traceable to a National Institute of Standards and Technology (NIST) standard or equivalent. Temperature reading devices may be of the liquid-in-glass type or an analog or digital temperature indicator type, such as a resistance temperature device, thermistor, or thermocouple. Standardization of thermometers is performed on an established testing frequency with a temperature standard traceable to NIST. For example, refer to the current issue of American Society of Testing and Materials (ASTM) standards E1 for liquid-in-glass thermometers.

7. TEST RESULTS

7.10. Interpretation of Requirements

Analytical results observed in the laboratory (or calculated from experimental measurements) are compared with stated acceptance criteria to determine whether the article conforms to compendial requirements.

The reportable value, which often is a summary value for several individual determinations, is compared with the acceptance criteria. The reportable value is the end result of a completed measurement procedure, as documented.

Where acceptance criteria are expressed numerically herein through specification of an upper and/or lower limit, permitted values include the specified values themselves, but no values outside the limit(s). Acceptance criteria are considered significant to the last digit shown.

7.10.5. Nominal Concentrations in Equations

Where a "nominal concentration" is specified, calculate the concentration based on the label claim. In assay procedures, water correction is typically stated in the Definition and on the label of the USP Reference Standard. For other procedures, correction for assayed content, potency, or both is made prior to using the concentration in the equation provided in the monograph.

7.10.10. Equivalence Statements in Titrimetric Procedures

The directions for titrimetric procedures conclude with a statement of the weight of the analyte that is equivalent to each mL of the standardized titrant. In such an equivalence statement, the number of significant figures in the concentration of the titrant should be understood to correspond to the number of significant figures in the weight of the analyte. Corrections to calculations based on the blank determination are to be made for all titrimetric assays where appropriate (see *Titrimetry* ⟨541⟩).

7.20. Rounding Rules

The observed or calculated values shall be rounded off to the number of decimal places that is in agreement with the limit expression. Numbers should not be rounded until the final calculations for the reportable value have been completed. Intermediate calculations (e.g., slope for linearity) may be rounded for reporting purposes, but the original (not rounded) value should be used for any additional required calculations. Acceptance criteria are fixed numbers and are not rounded.

When rounding is required, consider only one digit in the decimal place to the right of the last place in the limit expression. If this digit is smaller than 5, it is eliminated and the preceding digit is unchanged. If this digit is equal to or greater than 5, it is eliminated and the preceding digit is increased by 1.

8. TERMS AND DEFINITIONS

8.10. Abbreviations

- RS refers to a USP Reference Standard.
- CS refers to a Colorimetric Solution.
- TS refers to a Test Solution.
- VS refers to a Volumetric Solution that is standardized in accordance with directions given in the individual monograph or in the *Reagents, Indicators, and Solutions* section of *USP–NF*.

8.20. About

"About" indicates a quantity within 10%.

If the measurement is stated to be "accurately measured" or "accurately weighed," follow the statements in *Volumetric Apparatus* ⟨31⟩ and *Balances* ⟨41⟩, respectively.

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Illustration of Rounding Numerical Values for Comparison with Requirements			
Compendial Requirement	**Unrounded Value**	**Rounded Result**	**Conforms**
Assay limit ≥98.0%	97.96%	98.0%	Yes
	97.92%	97.9%	No
	97.95%	98.0%	Yes
Assay limit ≤101.5%	101.55%	101.6%	No
	101.46%	101.5%	Yes
	101.45%	101.5%	Yes
Limit test ≤0.02%	0.025%	0.03%	No
	0.015%	0.02%	Yes
	0.027%	0.03%	No
Limit test ≤3 ppm	3.5 ppm	4 ppm	No
	3.4 ppm	3 ppm	Yes
	2.5 ppm	3 ppm	Yes

8.30. Alcohol Content

Percentages of alcohol, such as those under the heading *Alcohol Content*, refer to percentage by volume of C_2H_5OH at 15.56°. Where a formula, test, or assay calls for alcohol, ethyl alcohol, or ethanol, the *USP* monograph article Alcohol shall be used. Where reference is made to "C_2H_5OH," absolute (100%) ethanol is intended. Where a procedure calls for dehydrated alcohol, alcohol absolute, or anhydrous alcohol, the *USP* monograph article Dehydrated Alcohol shall be used.

8.40. Atomic Weights

Atomic weights used in computing molecular weights and the factors in the assays and elsewhere are those established by the IUPAC Commission on Isotopic Abundances and Atomic Weights.

8.50. Blank Determinations

Where it is directed that "any necessary correction" be made by a blank determination, the determination shall be conducted using the same quantities of the same reagents treated in the same manner as the solution or mixture containing the portion of the substance under assay or test, but with the substance itself omitted.

8.60. Concomitantly

"Concomitantly" denotes that the determinations or measurements are to be performed in immediate succession.

8.70. Desiccator

The instruction "in a desiccator" indicates use of a tightly closed container of suitable size and design that maintains an atmosphere of low moisture content by means of a suitable desiccant such as anhydrous calcium chloride, magnesium perchlorate, phosphorus pentoxide, or silica gel. See also section *8.220 Vacuum Desiccator.*

8.80. Logarithms

Logarithms are to the base 10.

8.90. Microbial Strain

A microbial strain cited and identified by its American Type Culture Collection (ATCC) catalog number shall be used directly or, if subcultured, shall be used not more than five passages removed from the original strain.

8.100. Negligible

"Negligible" indicates a quantity not exceeding 0.50 mg.

8.110. NLT/NMT

"NLT" means "not less than." "NMT" means "not more than."

8.120. Odor

"Odorless," "practically odorless," "a faint characteristic odor," and variations thereof indicate evaluation of a suitable quantity of freshly opened material after exposure to the air for 15 minutes. An odor designation is descriptive only and should not be regarded as a standard of purity for a particular lot of an article.

8.130. Percent

"Percent" used without qualification means:

- For mixtures of solids and semisolids, percent weight in weight;
- For solutions or suspensions of solids in liquids, percent weight in volume;
- For solutions of liquids in liquids, percent volume in volume;
- For solutions of gases in liquids, percent weight in volume.

For example, a 1 percent solution is prepared by dissolving 1 g of a solid or semisolid, or 1 mL of a liquid, in sufficient solvent to make 100 mL of the solution.

8.140. Percentage Concentrations

Percentage concentrations are expressed as follows:

- *Percent Weight in Weight* (w/w) is defined as the number of g of a solute in 100 g of solution.
- *Percent Weight in Volume* (w/v) is defined as the number of g of a solute in 100 mL of solution.
- *Percent Volume in Volume* (v/v) is defined as the number of mL of a solute in 100 mL of solution.

8.150. Pressure

Pressure is determined by use of a suitable manometer or barometer calibrated in terms of the pressure exerted by a column of mercury of the stated height.

8.160. Reaction Time

Reaction time is 5 minutes unless otherwise specified.

8.170. Specific Gravity

Specific gravity is the weight of a substance in air at 25° divided by the weight of an equal volume of water at the same temperature.

8.180. Temperatures

Temperatures are expressed in centigrade (Celsius) degrees, and all measurements are made at 25° unless otherwise indicated. Where moderate heat is specified, any temperature not higher than 45° (113° F) is indicated.

8.190. Time

Unless otherwise specified, rounding rules, as described in section *7.20 Rounding Rules*, apply to any time specified.

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8.200. Transfer

"Transfer" indicates a quantitative manipulation.

8.210. Vacuum

"Vacuum" denotes exposure to a pressure of less than 20 mm of mercury (2.67 kPas), unless otherwise indicated.

8.220. Vacuum Desiccator

"Vacuum desiccator" indicates a desiccator that maintains a low-moisture atmosphere at a reduced pressure of not more than 20 mm of mercury (2.67 kPas) or at the pressure designated in the individual monograph.

8.230. Water

8.230.10. Water as an Ingredient in an Official Product

As an ingredient in an official product, water meets the requirements of the appropriate water monograph in *USP* or *NF*.

8.230.20. Water in the Manufacture of Official Substances

When used in the manufacture of official substances, water shall meet the requirements for drinking water as set forth in the U.S. Environmental Protection Agency National Primary Drinking Water Regulations or in the drinking water regulations of the European Union or of Japan, or in the World Health Organization's Guidelines for Drinking Water Quality. Additional specifications may be required in monographs.

8.230.30. Water in a Compendial Procedure

When water is called for in a compendial procedure, the *USP* monograph article Purified Water shall be used unless otherwise specified. Definitions for other types of water are provided in *Reagents, Indicators, and Solutions* and in *Water for Pharmaceutical Purposes* ⟨1231⟩.

8.240. Weights and Measures

In general, weights and measures are expressed in the International System of Units (SI) as established and revised by the *Conférence générale des poids et mesures*. For compendial purposes, the term "weight" is considered to be synonymous with "mass."

Molality is designated by the symbol *m* preceded by a number that represents the number of moles of the designated solute contained in 1 kilogram of the designated solvent.

Molarity is designated by the symbol M preceded by a number that represents the number of moles of the designated solute contained in an amount of the designated solvent that is sufficient to prepare 1 liter of solution.

Normality is designated by the symbol N preceded by a number that represents the number of equivalents of the designated solute contained in an amount of the designated solvent that is sufficient to prepare 1 liter of solution.

The symbol for degrees (°) without a qualifying unit of measure represents degrees Celsius.

Chart of Symbols and Prefixes commonly employed for SI metric units and other units:

	Units	Symbol	Notes
Length			
	meter	m	
	centimeter	cm	
	millimeter	mm	
	micrometer	µm	Previously referred to as a micron
	nanometer	nm	Previously the symbol mµ (for millimicron) was used
	Ångström	Å	Equal to 0.1 nm
Mass			
	kilogram	kg	
	gram	g	
	milligram	mg	
	microgram	µg	The symbol µg is used in the *USP* and *NF* to represent micrograms, but micrograms may be represented as "mcg" for labeling and prescribing purposes. The term "gamma," symbolized by γ, frequently is used to represent micrograms in biochemical literature.
	nanogram	ng	
	picogram	pg	
	dalton	Da	Also referred to as the unified atomic mass unit and is equal to 1/12 times the mass of the free carbon 12 atom.
	kilodalton	kDa	
Time			
	second	s	
	minute	min	
	hour	h	
Volume			
	liter	L	1 L is equal to 1000 cm^3 (cubic centimeters)
	deciliter	dL	
	milliliter	mL	1 mL is equal to 1 cm^3, sometimes referred to as cc
	microliter	µL	
Temperature			
	Celsius	°C	
Amount of Substance			
	mole	mol	Historically referred to as gram-molecular weight or gram-atomic weight
	millimole	mmol	
	micromole	µmol	
	femtomole	fmol	
	equivalent	Eq	Also referred to as gram-equivalent weight. It is used in the calculation of substance concentration in units of normality. This unit is no longer preferred for use in analytical chemistry or metrology.
	milli equivalent	mEq	
	osmole	Osmol	Osmotic pressure of a solution, related to substance concentration.
	milliosmole	mOsmol	
Pressure			
	pascal	Pa	
	kilopascal	kPa	

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	Units	Symbol	Notes
	pounds per square inch	psi	
	millimeter of mercury	mmHg	Equal to 133.322 Pa
Electrical units			
	ampere	A	
	volt	V	
	millivolt	mV	
	hertz	Hz	Unit of frequency
	kilohertz	kHz	
	megahertz	MHz	
	electron volt	eV	
	kilo-electron volt	keV	
	mega-electron volt	MeV	
Radiation			
	becquerel	Bq	SI unit of activity for radionuclides
	kilobecquerel	kBq	
	megabecquerel	MBq	
	gigabecquerel	GBq	
	curie	Ci	Non-SI unit of activity for radionuclides
	millicurie	mCi	
	microcurie	μCi	
	nanocurie	nCi	
Other			
	acceleration due to gravity	g	Used to express rate of centrifugation
	revolutions per minute	rpm	Used to express rate of centrifugation

Selected SI Prefixes

Name	Symbol	Factor
giga	G	10^9
mega	M	10^6
kilo	k	10^3
deci	d	10^{-1}
centi	c	10^{-2}
milli	m	10^{-3}
micro	μ	10^{-6}
nano	n	10^{-9}
pico	p	10^{-12}
femto	f	10^{-15}

9. PRESCRIBING AND DISPENSING

9.10. Use of Metric Units

Prescriptions for compendial articles shall be written to state the quantity and/or strength desired in metric units unless otherwise indicated in the individual monograph [see also section *5.50.10 Units of Potency (Biological)* above]. If an amount is prescribed by any other system of measurement, only an amount that is the metric equivalent of the prescribed amount shall be dispensed. Abbreviations for the terms "Units" or "International Units" shall not be used for labeling or prescribing purposes. Apothecary unit designations on labels and labeling shall not be used.

9.20. Changes in Volume

In the dispensing of prescription medications, slight changes in volume owing to variations in room temperatures may be disregarded.

10. PRESERVATION, PACKAGING, STORAGE, AND LABELING

10.10. Packaging and Storage

All articles in *USP* or *NF* are subject to the packaging and storage requirements specified in *Packaging and Storage Requirements* ⟨659⟩, unless different requirements are provided in an individual monograph.

10.20. Labeling

All articles in *USP* or *NF* are subject to the labeling requirements specified in *Labeling* ⟨7⟩, unless different requirements are provided in an individual monograph.

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Guide to General Chapters

(For complete alphabetical list of all general chapters in this Pharmacopeia, see under "General chapters" in the index.)

GENERAL TESTS AND ASSAYS

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Global Health Monographs

Preface

This section contains monographs for articles which are not currently legally marketed in the United States, but which have been approved by a stringent regulatory authority as defined by the World Health Organization and are used for essential purposes in other parts of the world. Selection and prioritization of new entries to this section will be accomplished in close collaboration with stakeholders throughout the global health community. These monographs are not applicable to articles marketed for use in the United States.

Chlorhexidine Gluconate Topical Gel

DEFINITION

Chlorhexidine Gluconate Topical Gel is prepared from Chlorhexidine Gluconate Solution. It contains NLT 90.0% and NMT 110.0% of the labeled amount of chlorhexidine gluconate ($C_{22}H_{30}Cl_2N_{10} \cdot 2C_6H_{12}O_7$). [NOTE—The U.S. Food and Drug Administration has not reviewed the safety and efficacy of Chlorhexidine Gluconate Topical Gel and it is not approved for marketing in the United States.]

IDENTIFICATION

Change to read:

- **A.** ▲The UV spectrum of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.▲ (GH 1-Aug-2019)
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

Delete the following:

▲• **C. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩

Diluent: Acetonitrile and water (1:1)

Standard solution: 10 mg/mL of USP Potassium Gluconate RS in *Diluent*

Sample solution: Nominally 20 mg/mL of chlorhexidine gluconate from Topical Gel, prepared as follows. Transfer a suitable amount of Topical Gel, equivalent to 500 mg of chlorhexidine gluconate, to a 25-mL volumetric flask. Add a sufficient quantity of *Diluent*, sonicate with intermittent shaking for 30 min, and dilute with *Diluent* to volume. Centrifuge the solution for 5 min at 3000 rpm.

Chromatographic system

Adsorbent: 0.25-mm layer of chromatographic silica gel

Application volume: 10 µL

Developing solvent system: Alcohol, ethyl acetate, ammonium hydroxide, and water (5:1:1:3)

Spray reagent: Dissolve 2.5 g of ammonium molybdate in 50 mL of 2 N sulfuric acid in a 100-mL volumetric flask. Add 1.0 g of ceric sulfate, swirl to dissolve, and dilute with 2 N sulfuric acid to volume.

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the chromatogram in the *Developing solvent system* until the solvent front has moved 10 cm from the point of spotting. Remove the plate from the chamber and dry at 110° for 20 min. Allow to cool and spray with *Spray reagent*. Heat the plate at 110° for 10 min.

Acceptance criteria: The principal spot of the *Sample solution* corresponds in color, size, and R_F value to that of the *Standard solution*.▲ (GH 1-Aug-2019)

ASSAY

Change to read:

• **PROCEDURE**

Buffer: Dissolve 27.6 g of sodium dihydrogen phosphate and 10 mL of triethylamine in 1.5 L of water. Adjust with phosphoric acid to a pH of 3.0 and dilute with water to 2 L.

Solution A: Acetonitrile and *Buffer* (30:70)

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
9	100	0
10	45	55
15	45	55
16	100	0
21	100	0

Standard solution: 50 µg/mL of USP Chlorhexidine Acetate RS in *Solution A*

Sample stock solution: Nominally 0.4 mg/mL of chlorhexidine gluconate from Topical Gel, prepared as follows. Transfer a suitable amount of Topical Gel, equivalent to 40 mg of chlorhexidine gluconate, to a 100-mL volumetric flask. Add about 70 mL of *Solution A*, sonicate with intermittent shaking for 30 min, and dilute with *Solution A* to volume.

Sample solution: Nominally 80 µg/mL of chlorhexidine gluconate from the *Sample stock solution* in *Solution A*

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

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Detector: UV 239 nm. ▲For *Identification A*, use a diode array detector in the range of 200–400 nm.▲ (GH 1-Aug-2019)
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 40°
Flow rate: 1.5 mL/min
Injection volume: 50 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Tailing factor: NMT 2.0
Relative standard deviation: NMT 2.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of chlorhexidine gluconate ($C_{22}H_{30}Cl_2N_{10} \cdot 2C_6H_{12}O_7$) in the portion of Topical Gel taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak response of chlorhexidine from the *Sample solution*
r_S = peak response of chlorhexidine from the *Standard solution*
C_S = concentration of USP Chlorhexidine Acetate RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of chlorhexidine gluconate in the *Sample solution* (µg/mL)
M_{r1} = molecular weight of chlorhexidine gluconate, 897.76
M_{r2} = molecular weight of chlorhexidine acetate, 625.55

Acceptance criteria: 90.0%–110.0%

IMPURITIES

Change to read:

• **LIMIT OF *p*-CHLOROANILINE**
Solution A, Solution B, Mobile phase, and **Chromatographic system:** Proceed as directed in the *Assay*.
System suitability solution: 50 µg/mL of USP Chlorhexidine Acetate RS and 1 µg/mL of USP *p*-Chloroaniline RS in *Solution A*
Standard solution: 1.0 µg/mL of USP *p*-Chloroaniline RS in *Solution A*
Sample solution: Nominally 0.4 mg/mL of chlorhexidine gluconate from Topical Gel, prepared as follows. Transfer a suitable amount of Topical Gel, equivalent to 40 mg of chlorhexidine gluconate, to a 100-mL volumetric flask. Add about 70 mL of *Solution A*, sonicate with intermittent shaking for 30 min, and dilute with *Solution A* to volume. Centrifuge the solution.
System suitability
Samples: *System suitability solution* and *Standard solution*
▲[NOTE—The relative retention times for chlorhexidine and *p*-chloroaniline are 1.0 and 1.3, respectively.]▲ (GH 1-Aug-2019)
Suitability requirements
Resolution: NLT 3.0 between chlorhexidine and *p*-chloroaniline, *System suitability solution*
Relative standard deviation: NMT 5.0%, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of *p*-chloroaniline in the portion of Topical Gel taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of *p*-chloroaniline from the *Sample solution*
r_S = peak response of *p*-chloroaniline from the *Standard solution*
C_S = concentration of USP *p*-Chloroaniline RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of chlorhexidine gluconate in the *Sample solution* (mg/mL)

Acceptance criteria: NMT 0.35%

Add the following:

▲• **ORGANIC IMPURITIES**
Solution A: 0.1% (v/v) trifluoroacetic acid in acetonitrile
Solution B: 0.1% (v/v) trifluoroacetic acid in water
Solution C: *Solution A* and *Solution B* (20:80)
Solution D: *Solution A* and *Solution B* (90:10)
Mobile phase: See *Table 2*.

Table 2

Time (min)	Solution C (%)	Solution D (%)
0	100	0
2	100	0
32	80	20
37	80	20
47	70	30
54	70	30
55	100	0
60	100	0

Diluent 1: Acetonitrile, water, and trifluoroacetic acid (10: 90: 0.1)
Diluent 2: Acetonitrile and water (50:50)
System suitability solution: 0.2 mg/mL of USP Chlorhexidine System Suitability Mixture RS in *Diluent 1*
Sensitivity solution: 0.07 µg/mL of USP Chlorhexidine Acetate RS in *Diluent 1*
Sample stock solution: Nominally 0.8 mg/mL of chlorhexidine gluconate in *Diluent 2* from Topical Gel prepared as follows. Transfer a suitable amount of Topical Gel, equivalent to 80 mg of chlorhexidine gluconate, to a 100-mL volumetric flask. Add about 50 mL of *Diluent 2*, sonicate with intermittent shaking for 30 min, and dilute with *Diluent 2* to volume.
Sample solution: Nominally 0.2 mg/mL of chlorhexidine gluconate from *Sample stock solution* in *Diluent 1*. Pass through a suitable filter of 0.45-µm pore size.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; base-deactivated 5-µm packing L1
Column temperature: 30°
Flow rate: 1 mL/min
Injection volume: 50 µL
System suitability
Samples: *System suitability solution* and *Sensitivity solution*
[NOTE—See *Table 3* for relative retention times.]
Suitability requirements
Resolution: NLT 1.5 between any pair of eluting peaks, *System suitability solution*

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Signal-to-noise ratio: NLT 10 for chlorhexidine, *Sensitivity solution*

Analysis

Sample: *Sample solution*

Calculate the percentage of each degradation product in the portion of Topical Gel taken:

$$\text{Result} = (r_U/r_T) \times F \times 100$$

r_U = peak area of each degradation product from the *Sample solution*

r_T = total peak area, determined by summing the chlorhexidine peak and all other peaks above the *Sensitivity solution* level, except *p*-chloroaniline

F = relative response factor (see *Table 3*)

Calculate the percentage of total degradation products as the sum of the percentage of each degradation product and *p*-chloroaniline from the *Limit of p-Chloroaniline* test.

Acceptance criteria: See *Table 3*.

Table 3

Name	Relative Retention Time	Relative Response Factor	Acceptance Criteria, NMT (%)
p-Chloroaniline[a]	0.15	—	—
Chlorhexidine oxazinone analog[b]	0.23	1.0	0.2
Chlorhexidine amine[c]	0.25	1.0	0.6
Chlorhexidine guanidine[d]	0.35	1.0	2.0
Chlorhexidine urea[e]	0.36	1.0	0.2
p-Chlorophenyl urea[f]	0.50	1.34	0.3
Chlorhexidine nitrile[g]	0.60	1.0	0.4
Chlorhexidine dimer[h]	0.85	1.0	0.5
o-Chlorhexidine[i]	0.90	1.0	0.4
Chlorhexidine glucityl triazine[j]	0.96	1.0	0.7
Chlorhexidine	1.0	—	—
Oxochlorhexidine[k]	1.4	1.0	0.7
Any unspecified degradation product	—	1.0	0.2
Total degradation products	—	—	4.0

[a] Do not include this peak in the sum of the total peak areas for the calculation of degradation products.

[b] (5*R*,6*S*)-2-[(4-Chlorophenyl)amino]-5-hydroxy-6-[(1*R*,2*R*)-1,2,3-trihydroxypropyl]-5,6-dihydro-4*H*-1,3-oxazin-4-one.

[c] 1-(6-Aminohexyl)-5-(4-chlorophenyl)biguanide.

[d] 1-[6-(Carbamimidoylamino)hexyl]-5-(4-chlorophenyl)biguanide.

[e] *N*-{[6-({[(4-Chlorophenyl)carbamimidoyl]carbamimidoyl}amino)hexyl]carbamimidoyl}urea.

[f] 1-(4-Chlorophenyl)urea.

[g] 1-(4-Chlorophenyl)-5-[6-[(cyanocarbamimidoyl)amino]hexyl]biguanide.

[h] 1,5-Bis[5-(4-chlorophenyl)biguanidylhexyl]biguanide.

[i] 1-(2-Chlorophenyl)-5-[6-({[(4-chlorophenyl)carbamimidoyl]carbamimidoyl}amino)hexyl]biguanide.

[j] 1-(4-Chlorophenyl)-5-[6-((4-[(4-chlorophenyl)amino]-6-[(1*S*,2*R*,3*R*,4*R*)-1,2,3,4,5-pentahydroxypentyl]-1,3,5-triazin-2-yl}amino)hexyl]biguanide.

[k] *N*-(4-Chlorophenyl)-*N*′-[6-({[(4-chlorophenyl)carbamimidoyl]carbamimidoyl}amino)hexyl]carbamimidoyl}urea.

▲ (GH 1-Aug-2019)

SPECIFIC TESTS

- **pH** ⟨791⟩

Sample solution: Nominally 1% of chlorhexidine gluconate from Topical Gel in water

Acceptance criteria: 5.0–7.0

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in well-closed containers, protected from light. Store at controlled room temperature.

Change to read:

- **USP Reference Standards** ⟨11⟩

USP Chlorhexidine Acetate RS

▲USP Chlorhexidine System Suitability Mixture RS

This is a mixture of chlorhexidine and the following impurities (other impurities may also be present).

Chlorhexidine gluconate;
1,1′-Hexamethylenebis[5-(*p*-chlorophenyl)biguanide] di-D-gluconate.
$C_{22}H_{30}Cl_2N_{10} \cdot 2C_6H_{12}O_7$ 897.76

Chlorhexidine nitrile;
1-(4-Chlorophenyl)-5-[6-[(cyanocarbamimidoyl)amino]hexyl]biguanide.
$C_{16}H_{24}ClN_9$ 377.88

Chlorhexidine urea;
N-{[6-({[(4-Chlorophenyl)carbamimidoyl]carbamimidoyl}amino)hexyl]carbamimidoyl}urea.
$C_{16}H_{26}ClN_9O$ 395.89

p-Chlorophenyl urea;
1-(4-Chlorophenyl)urea.
$C_7H_7ClN_2O$ 170.60

Chlorhexidine amine;
1-(6-Aminohexyl)-5-(4-chlorophenyl)biguanide.
$C_{14}H_{23}ClN_6$ 310.83

Chlorhexidine dimer;
1,5-Bis[5-(4-chlorophenyl)biguanidylhexyl]biguanide.
$C_{30}H_{47}Cl_2N_{15}$ 688.70

Chlorhexidine glucityl triazine;
1-(4-Chlorophenyl)-5-[6-({4-[(4-chlorophenyl)amino]-6-[(1*S*,2*R*,3*R*,4*R*)-1,2,3,4,5-pentahydroxypentyl]-1,3,5-triazin-2-yl}amino)hexyl]biguanide.
$C_{28}H_{38}Cl_2N_{10}O_5$ 665.57

Oxochlorhexidine;
N-(4-Chlorophenyl)-*N*′-{[6-({[(4-chlorophenyl)carbamimidoyl]carbamimidoyl}amino)hexyl]carbamimidoyl}urea.
$C_{22}H_{29}Cl_2N_9O$ 506.43

Chlorhexidine oxazinone analog;
(5*R*,6*S*)-2-[(4-Chlorophenyl)amino]-5-hydroxy-6-[(1*R*,2*R*)-1,2,3-trihydroxypropyl]-5,6-dihydro-4*H*-1,3-oxazin-4-one.
$C_{13}H_{15}ClN_2O_6$ 330.72

Chlorhexidine guanidine;
1-[6-(Carbamimidoylamino)hexyl]-5-(4-chlorophenyl)biguanide.
$C_{15}H_{25}ClN_8$ 352.87

o-Chlorhexidine;
1-(2-Chlorophenyl)-5-[6-({[(4-chlorophenyl)carbamimidoyl]carbamimidoyl}amino)hexyl]biguanide.
$C_{22}H_{30}Cl_2N_{10}$ 505.45▲ (GH 1-Aug-2019)

USP *p*-Chloroaniline RS

▲▲ (GH 1-Aug-2019)

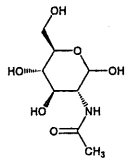
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Dietary Supplement Monographs

N-Acetylglucosamine



$C_8H_{15}NO_6$ 221.21
2-(Acetylamino)-2-deoxy-D-glucose;
N-Acetyl-D-Glucosamine [7512-17-6].

DEFINITION
N-Acetylglucosamine contains NLT 98.0% and NMT 102.0% of *N*-acetylglucosamine ($C_8H_{15}NO_6$), calculated on the dried basis.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
- **B.** It meets the requirements in the test for *Optical Rotation* ⟨781S⟩, *Specific Rotation*.
- **C.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

ASSAY
- **PROCEDURE**

Buffer: Transfer 3.5 g of dibasic potassium phosphate to a 1-L volumetric flask, and add sufficient water to dissolve. Add 0.25 mL of ammonium hydroxide, dilute with water to volume, and mix. Adjust with phosphoric acid to a pH of 7.5.
Mobile phase: Acetonitrile and *Buffer* (75:25)
Diluent: Acetonitrile and water (50:50)
System suitability solution: 1.0 mg/mL of USP *N*-Acetylglucosamine RS and 0.6 mg/mL of USP Glucosamine Hydrochloride RS in *Diluent*
Standard solution: 1.0 mg/mL of USP *N*-Acetylglucosamine RS in *Diluent*
Sample solution: 1.0 mg/mL of *N*-Acetylglucosamine in *Diluent*
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 195 nm
Column: 4.6-mm × 15-cm; 3-µm packing L8
Column temperature: 35°
Flow rate: 1.5 mL/min
Injection volume: 10 µL
System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The relative retention times for *N*-acetylglucosamine and glucosamine are 1.0 and about 2.8, respectively.]
Suitability requirements
Signal-to-noise ratio: NLT 10 for the glucosamine peak, *System suitability solution*
Resolution: NLT 5.0 between the *N*-acetylglucosamine and glucosamine peaks, *System suitability solution*
Tailing factor: NMT 2.0, *Standard solution*
Relative standard deviation: NMT 2.0%, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of *N*-acetylglucosamine ($C_8H_{15}NO_6$) in the portion of *N*-Acetylglucosamine taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP *N*-Acetylglucosamine RS in the *Standard solution* (mg/mL)
C_U = concentration of *N*-Acetylglucosamine in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–102.0% on the dried basis

IMPURITIES
- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%
- **CHLORIDE AND SULFATE,** *Chloride* ⟨221⟩: NMT 0.1%
- **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩

Acceptance criteria
Arsenic: NMT 1 µg/g
Lead: NMT 10 µg/g

- **RELATED COMPOUNDS**

Buffer, Mobile phase, Diluent, System suitability solution, Chromatographic system, and System suitability: Proceed as directed in the *Assay*.
Sample solution: 2.5 mg/mL of *N*-Acetylglucosamine in *Diluent*
Analysis
Sample: *Sample solution*
Calculate the percentage of each impurity in the portion of *N*-Acetylglucosamine taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of each impurity from the *Sample solution*
r_T = sum of the peak responses from the *Sample solution*

Acceptance criteria
Individual impurity: NMT 0.5%
Total impurities: NMT 2.0%

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• **Limit of Glucosamine**
Buffer, Mobile phase, Diluent, System suitability solution, Chromatographic system, and **System suitability:** Proceed as directed in the *Assay*.
Standard solution: 0.6 mg/mL of USP Glucosamine Hydrochloride RS in *Diluent*
Sample solution: 50 mg/mL of *N*-Acetylglucosamine in *Diluent*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of glucosamine in the portion of *N*-Acetylglucosamine taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_1/M_2) \times 100$$

r_U = peak response of glucosamine from the *Sample solution*
r_S = peak response of glucosamine from the *Standard solution*
C_S = concentration of USP Glucosamine Hydrochloride RS in the *Standard solution* (mg/mL)
C_U = concentration of *N*-Acetylglucosamine in the *Sample solution* (mg/mL)
M_1 = molecular weight of glucosamine, 179.17
M_2 = molecular weight of glucosamine hydrochloride, 215.63

Acceptance criteria: NMT 1.0%

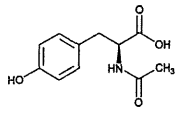
SPECIFIC TESTS

• **Optical Rotation,** *Specific Rotation* ⟨781S⟩
Sample solution: 20 mg/mL in water, perform the measurement 3 h after sample preparation.
Acceptance criteria: +39.0° to +43.0°
• **pH** ⟨791⟩
Sample solution: 10 mg/mL in water
Acceptance criteria: 6.0–8.0
• **Loss on Drying** ⟨731⟩
Analysis: Dry a sample at 105° for 2 h.
Acceptance criteria: NMT 0.5%
• **Melting Range or Temperature** ⟨741⟩**:** 196°–205°
• **Microbial Enumeration Tests** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^3 cfu/g; the total combined molds and yeasts count does not exceed 10^3 cfu/g.
• **Absence of Specified Microorganisms** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Preserve in tight, light-resistant containers.
• **USP Reference Standards** ⟨11⟩
USP *N*-Acetylglucosamine RS
USP Glucosamine Hydrochloride RS

N-Acetyltyrosine



$C_{11}H_{13}NO_4$ 223.2
N-Acetyl-L-tyrosine;
(2*S*)-2-(Acetylamino)-3-(4-hydroxyphenyl)propanoic acid) [537-55-3].

DEFINITION

N-Acetyltyrosine contains NLT 98.5% and NMT 101.0% of *N*-acetyltyrosine ($C_{11}H_{13}NO_4$), as *N*-acetyl-L-tyrosine, calculated on the dried basis.

IDENTIFICATION

Change to read:

• **A.** ▲**Spectroscopic Identification Tests** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
• **B. Optical Rotation,** *Specific Rotation* ⟨781S⟩
Sample solution: 10 mg/mL
Acceptance criteria: NLT +46.0° and NMT +49.0°, determined at 20°
• **C.** The R_F value of the principal spot of the *Sample solution* in the test for *Organic Impurities* corresponds to that of *Standard solution 1*.

ASSAY

• **Procedure**
Sample solution: Dissolve about 180 mg of *N*-Acetyltyrosine, weighed, in 50 mL of carbon dioxide-free water.
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.1 N sodium hydroxide VS
Endpoint detection: Potentiometric
Equivalency: Each mL of 0.1 N sodium hydroxide VS is equivalent to 22.32 mg of *N*-acetyltyrosine ($C_{11}H_{13}NO_4$).

IMPURITIES

• **Residue on Ignition** ⟨281⟩**:** NMT 0.1%
• **Chloride and Sulfate,** *Chloride* ⟨221⟩
Sample: 0.7 g
Standard: 0.40 mL of 0.01 N hydrochloric acid
Acceptance criteria: NMT 200 ppm
• **Chloride and Sulfate,** *Sulfate* ⟨221⟩
Sample: 1.2 g
Standard: 0.25 mL of 0.020 N sulfuric acid
Acceptance criteria: NMT 200 ppm
• **Iron** ⟨241⟩**:** NMT 20 ppm
• **Organic Impurities**
Adsorbent: 0.25-mm layer of chromatographic silica gel mixture
Standard stock solution 1: 8 mg/mL of USP N-Acetyl-L-tyrosine RS in a mixture of water, glacial acetic acid, and alcohol (3:3:94)
Standard solution 1: Dilute *Standard stock solution 1* with alcohol to obtain a solution having a known concentration of about 0.4 mg/mL.
Standard solution 2: 0.8 mg/mL of USP L-Tyrosine RS dissolved in a mixture of glacial acetic acid and water (1:1), and diluted with alcohol
Sample solution: Transfer 0.8 g of *N*-Acetyltyrosine to a 10-mL volumetric flask, dissolve in 6 mL of a mixture of glacial acetic acid and water (1:1), and dilute with alcohol to volume.
Application volume: 5 µL
Developing solvent system: A mixture of ammonia and 2-propanol (3:7)
Spray reagent: Dissolve 0.2 g of ninhydrin in 100 mL of a mixture of butanol and 2 N acetic acid (95:5).
Analysis: Proceed as directed for *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*. After air-drying the plate, repeat the development process. After air-drying a second time, examine the plate under short-wave UV light, and

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record principal and secondary spots. Spray the plate with *Spray reagent*, and heat between 100° and 105° for about 15 min. Examine the plate under white light, and record the principal and secondary spots.

Acceptance criteria: Under the short-wave UV light, any secondary spot observed from the *Sample solution* is not larger or more intense than the principal spot from *Standard solution 1*. After applying the *Spray reagent*, under white light, any secondary spot at the locus of tyrosine from the *Sample solution* is not larger or more intense than the principal spot from *Standard solution 2*.

Individual impurities: NMT 0.5%

Limit of tyrosine: NMT 1.0%

SPECIFIC TESTS

• **LOSS ON DRYING** ⟨731⟩

Analysis: Dry a sample at 105° for 3 h.

Acceptance criteria: NMT 0.1%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at controlled room temperature.

• **USP REFERENCE STANDARDS** ⟨11⟩

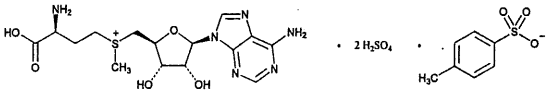
USP N-Acetyl-L-tyrosine RS

USP L-Tyrosine RS

Ademetionine Disulfate Tosylate—*see S-Adenosyl- L-methionine Disulfate Tosylate*

S-Adenosyl- L-methionine Disulfate Tosylate

Former Title: Ademetionine Disulfate Tosylate



$C_{22}H_{34}N_6O_{16}S_4$ 766.80

S-(Adenosyl)-L-methionine disulfate tosylate; (3*S*)-5′-[(3-Amino-3-carboxypropyl)methylsulfonio]-5′-deoxy adenosine, disulfate-methylbenzenesulfonate [97540-22-2].

DEFINITION

S-Adenosyl-L-methionine Disulfate Tosylate is the disulfate–tosylate mixed salt of a mixture of diastereoisomers of the *S*-adenosyl-L-methionine ion. It contains NLT 95.0% and NMT 105.0% of *S*-adenosyl-L-methionine disulfate tosylate ($C_{22}H_{34}N_6O_{16}S_4$) calculated through the content of *S*-adenosyl-L-methionine ($C_{15}H_{23}N_6O_5S^+$), calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

• **A.** ▲**SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

• **B.** The retention time of the major peak of the *Sample solution* corresponds to that of *S*-adenosyl-L-methionine in the *System suitability solution*, as obtained in the *content of s-adenosyl-l-methionine.*

COMPOSITION

• **CONTENT OF *S*-ADENOSYL- L-METHIONINE**

Solution A: 10 mL of glacial acetic acid in 500 mL of water. Add 2.06 g of sodium 1-hexanesulfonate, and dilute with water to 1000 mL.

Mobile phase: Acetronitrile and *Solution A* (15:85)

System suitability solution: 400 µg/mL each of USP *S*-Adenosyl-l-methionine Disulfate Tosylate RS and USP *S*-Adenosyl-l-homocysteine RS

Standard solution A: 400 µg/mL of USP *S*-Adenosyl-l-homocysteine RS

Standard solution B: 200 µg/mL from *Standard solution A*

Standard solution C: 80 µg/mL from *Standard solution A*

Sample solution: 20 mg of *S*-Adenosyl-L-methionine Disulfate Tosylate in 40 mL of water. Stir for 30 min, then dilute with water to 50.0 mL. Transfer 1.0 mL of the solution to a 1.5-mL microcentrifuge tube, and centrifuge for 1 min. Use a portion of the supernatant.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 260 nm

Column: 4.6-mm × 15-cm; 3-µm packing L1

Flow rate: 1 mL/min

Injection size: 10 µL

System suitability

Samples: *System suitability solution* and *Standard solution B*

[NOTE—The relative retention times for *S*-adenosyl-L-homocysteine and *S*-adenosyl-L-methionine disulfate tosylate are about 0.68 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 1.5 between *S*-adenosyl-L-homocysteine and *S*-adenosyl-L-methionine

Tailing factor: NMT 1.5, *Standard solution B*

Relative standard deviation: NMT 2.0% for *S*-adenosyl-L-homocysteine, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

[NOTE—Record the chromatograms, and measure the area of the *S*-adenosyl-L-homocysteine peak in all three *Standard solutions* and the *S*-adenosyl-L-methionine disulfate tosylate peak in the *Sample solution*.]

Plot a calibration curve of the peak area of the *Standard solutions* versus the corresponding *S*-adenosyl-L-homocysteine concentration, in mg/mL, and draw the straight line best fitting the three points. From the calibration curve, and using the peak area of *S*-adenosyl-L-methionine from the chromatogram from the *Sample solution*, determine the concentration, C, in mg/mL, of *S*-adenosyl-L-methionine as *S*-adenosyl-L-homocysteine in the *Sample solution*.

Calculate the percentage of $C_{15}H_{23}N_6O_5S^+$ in the portion of *S*-Adenosyl-L-methionine Disulfate Tosylate taken:

$$\text{Result} = (C/C_U) \times (M_{r1}/M_{r2}) \times 100$$

C = concentration of *S*-adenosyl-L-methionine as *S*-adenosyl-L-homocysteine obtained from the linear regression line (mg/mL)

C_U = concentration of *S*-Adenosyl-L-Methionine Disulfate Tosylate in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of *S*-adenosyl-L-methionine, 399.44

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M_{r2} = molecular weight of *S*-adenosyl-L-homocysteine, 384.41

Acceptance criteria: 49.5%–54.7% on the anhydrous basis, equivalent to 95.0%–105% of *S*-adenosyl-L-methionine disulfate tosylate on the anhydrous basis

• **CONTENT OF SULFATE**
Mobile phase: 8.0 mM sodium carbonate and 1.0 mM sodium bicarbonate in water
Standard solution: 0.18 mg/mL of potassium sulfate
Sample solution: 0.5 mg/mL of *S*-Adenosyl-L-methionine Disulfate Tosylate
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Ion detector with suppressed conductivity
Column: 4.0-mm × 25-cm; 7-µm packing L74
Column temperature: 30°
Flow rate: 1 mL/min
Injection size: 25 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Column efficiency: NLT 8200 theoretical plates
Tailing factor: NMT 1.5
Relative standard deviation: NMT 1.0%
Analysis
Samples: *Standard solution* and *Sample solution*
[NOTE—Measure the area of the sulfate peak.]
Calculate the percentage of sulfate (SO_4) in the portion of *S*-Adenosyl-L-methionine Disulfate Tosylate taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of sulfate from the *Sample solution*
r_S = peak response of sulfate from the *Standard solution*
C_S = concentration of sulfate (SO_4) in the *Standard solution* (mg/mL)
C_U = concentration of *S*-Adenosyl-L-methionine Disulfate Tosylate in the *Sample solution* (mg/mL)

Acceptance criteria: 23.5%–26.5%

SPECIFIC TESTS
• **pH** ⟨791⟩**:** 1.0–2.0, in an aqueous solution (1 in 20)
• **WATER DETERMINATION,** *Method Ia* ⟨921⟩**:** NMT 3.0%
• **ISOMERIC RATIO**
Buffer: Transfer 4.2 g of citric acid monohydrate and 2.03 g of sodium dihydrogen phosphate dihydrate to a 1-L volumetric flask, and dissolve in and dilute with water to volume.
Mobile phase: 4.0 g of sodium dodecyl sulfate and 440 mL of acetonitrile. Dilute with *Buffer* to 1 L.
Standard solution: 1.0 mg/mL of USP S-Adenosyl-l-methionine Disulfate Tosylate RS
Sample solution: 1.0 mg/mL of *S*-Adenosyl-L-methionine Disulfate Tosylate
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Flow rate: 1.2 mL/min
Injection size: 20 µL
System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for *R,S*-isomers and *S,S*-isomers are about 0.94 and 1.0, respectively.]
Suitability requirements
Resolution: NLT 1.0 between the *S,S*-isomer and the *R,S*-isomer
Analysis
Samples: *Standard solution* and *Sample solution*
Identify the peaks of the *S,S*- and *R,S*-isomers of the *Sample solution* by comparison with the *Standard solution*, and calculate the percentage of the *S,S*-isomer:

$$\text{Result} = [r_{SS}/(r_{SS} + r_{RS})] \times 100$$

r_{SS} = areas of the peaks corresponding to the *S,S*-isomer in the *Sample solution*
r_{RS} = areas of the peaks corresponding to the *R,S*-isomer in the *Sample solution*

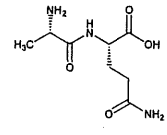
Acceptance criteria: NLT 60% and NLT the labeled amount of the *S,S*-isomer

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store in a refrigerator.
• **LABELING:** Label it to indicate the minimum content of *S,S*-isomer, as a percentage.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP S-Adenosyl-l-methionine Disulfate Tosylate RS
USP *S*-Adenosyl-l-homocysteine RS

Alanine

—see Alanine General Monographs

L-Alanyl-L-glutamine



$C_8H_{15}N_3O_4$ 217.23
L-2-(1-Oxo-2-amino-propylamino)-4-amino-4-oxobutanoic acid [39537-23-0].

DEFINITION
L-Alanyl-L-glutamine contains NLT 98.0% and NMT 101.5% of L-alanyl-L-glutamine ($C_8H_{15}N_3O_4$), calculated on the anhydrous and solvent-free basis, and excluding alanine and glutamine.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197A▲** (CN 1-May-2020)
• **B.** It meets the requirements for *Optical Rotation, Specific Rotation* ⟨781S⟩ in *Specific Tests*.

ASSAY
• **PROCEDURE**
Sample: 300 mg
Blank: Mix 5 mL of formic acid with 50 mL of glacial acetic acid.
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration

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Titrant: 0.1 N perchloric acid VS
Endpoint detection: Potentiometric
Analysis: Dissolve the *Sample* in 5 mL of formic acid, add 50 mL of glacial acetic acid, and titrate with the *Titrant*. Perform a *Blank* determination, and make any necessary correction.
Calculate the percentage of L-alanyl-L-glutamine ($C_8H_{15}N_3O_4$) in the *Sample* taken:

$$\text{Result}_1 = [(V_S - V_B) \times N \times (F/W)] \times 100$$

V_S = *Titrant* volume consumed by the *Sample* (mL)
V_B = *Titrant* volume consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 217.2 mg/mEq
W = *Sample* weight (mg)

Calculate the percentage of L-alanyl-L-glutamine ($C_8H_{15}N_3O_4$) in the *Sample* taken, excluding alanine and glutamine:

$$\text{Result}_2 = [(\text{Result}_1 - a - b)]/[(100 - Ala - Gln)] \times 100$$

$$a = Ala \times (M_{r1}/M_{r2})$$

$$b = Gln \times (M_{r1}/M_{r3})$$

Ala = percentage of alanine from the *Related Compounds* test
Gln = percentage of glutamine from the *Related Compounds* test
M_{r1} = molecular weight of L-alanyl-L-glutamine, 217.2
M_{r2} = molecular weight of alanine, 89.1
M_{r3} = molecular weight of glutamine, 146.1

Acceptance criteria: 98.0%–101.5% on the anhydrous and solvent-free basis, and excluding alanine and glutamine

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 0.1%
- **CHLORIDE AND SULFATE,** *Chloride* ⟨221⟩
Sample: 0.89 g
Standard solution: 0.50 mL of 0.010 N hydrochloric acid
Acceptance criteria: NMT 200 µg/g
- **CHLORIDE AND SULFATE,** *Sulfate* ⟨221⟩
Sample: 0.98 g
Standard solution: 0.20 mL of 0.010 N sulfuric acid
Acceptance criteria: NMT 200 µg/g
- **IRON** ⟨241⟩**:** NMT 10 µg/g
- **RESIDUAL SOLVENTS** ⟨467⟩
Acceptance criteria
Isopropanol: NMT 0.5%
[NOTE—For the *Acceptance criteria* for any other residual solvents, see *Residual Solvents* ⟨467⟩.]
- **LIMIT OF AMMONIUM**
Standard stock solution: Dissolve 1.486 g of ammonium chloride in 500.0 mL of water.
Standard calibration solutions: Transfer 0.01, 0.1, 1.0, and 10.0 mL of *Standard stock solution* into separate 100-mL volumetric flasks, and dilute with water to volume. The final concentrations are 0.1, 1, 10, and 100 µg/mL of ammonium ions (NH_4^+), respectively.
Sample solution: Transfer 1.0 g of L-Alanyl-L-glutamine to a 150-mL beaker containing a plastic-coated stirring bar, add 100.0 mL of water, and stir until dissolved.
Electrode system: Use a gas-sensing, ammonia-specific indicating electrode with internal reference connected to a pH meter capable of measuring potentials with a minimum reproducibility of ±0.1 mV (see *pH* ⟨791⟩). Condition the electrode according to the manufacturer's instructions.
Analysis
Standard response line: Transfer 100 mL of water into a 150-mL beaker containing a plastic-coated stirring bar, insert the electrode into the water, stir, and measure the potential. Add 1 mL of 10 N sodium hydroxide solution, stir, and measure the potential after stabilization (about 3 min). The potential difference must be below 20 mV. Transfer 100.0 mL of each of *Standard calibration solutions* (0.1, 1, 10, 100 µg/mL of ammonium ions) into separate 150-mL beakers, and add 1 mL of 10 N sodium hydroxide. Insert the electrode into each solution, stir, and measure the potential after stabilization (about 3 min). Plot a curve (four calibration points) of the potential (mV) as function of ammonium ion concentrations (µg/mL).
Sample: *Sample solution*
Rinse the electrode, insert it into the *Sample solution*, add 1 mL of 10 N sodium hydroxide, and stir. Check the pH, which must be above 11; if not, adjust with 10 N sodium hydroxide. After 3 min, measure the potential, and determine the corresponding ammonium ion concentration from the calibration curve.
Calculate the content of ammonium in the portion of the *Sample* taken:

$$\text{Result} = (V \times C)/W$$

V = volume of the *Sample solution* (mL)
C = concentration of ammonium ions in the *Sample solution* determined from the *Standard response line* (µg/mL)
W = weight of L-Alanyl-L-glutamine taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 200 µg/g
- **RELATED COMPOUNDS**
Buffer solution: Dissolve 6.84 g of monobasic potassium phosphate in 1000 mL of water.
Mobile phase: Acetonitrile and *Buffer solution* (650:350)
System suitability solution: Transfer 25 mg of USP L-Alanyl-L-glutamine RS and 5 mg of USP L-Alanyl-L-alanine RS into a 25-mL volumetric flask, and dilute with water to volume. Transfer 1.0 mL of this solution into a 10-mL volumetric flask, and dilute with *Mobile phase* to volume.
Standard solution 1: 0.025 mg/mL of USP L-Alanine RS in *Mobile phase*
Standard solution 2: 0.1 mg/mL of USP Glutamine RS in *Mobile phase*
Sample solution: 2.5 mg/mL of L-Alanyl-L-glutamine in *Mobile phase*
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 215 nm
Column: 4.6-mm × 25-cm; 5-µm packing L8
Flow rate: 0.7 mL/min
Injection volume: 20 µL
System suitability
Sample: *System suitability solution*
[NOTE—The relative retention times for L-alanyl-L-alanine and L-alanyl-L-glutamine are 0.86 and 1.0, respectively.]
Suitability requirements
Column efficiency: NLT 8000 theoretical plates for the L-alanyl-L-glutamine peak
Resolution: NLT 2.0 between L-alanyl-L-glutamine and L-alanyl-L-alanine

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Analysis
Samples: *Standard solution 1*, *Standard solution 2*, and *Sample solution*
Calculate the percentage of alanine and glutamine in the portion of the *Sample* taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of alanine or glutamine from the *Sample solution*
r_S = peak response of alanine from *Standard solution 1* or glutamine from *Standard solution 2*
C_S = concentration of USP L-Alanine RS in *Standard solution 1* (mg/mL) or concentration of USP Glutamine RS in *Standard solution 2* (mg/mL)
C_U = concentration of L-Alanyl-L-glutamine in the *Sample solution* (mg/mL)

Calculate the percentage of any other specified and unspecified impurities in the portion of the *Sample* taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of each individual impurity
r_T = sum of the responses of all the peaks, excluding peak responses of alanine and glutamine

Acceptance criteria: See *Table 1*.

Table 1

Name	Relative Retention Time	Acceptance Criteria, NMT (%)
Cyclo(ala-gln)	0.27	0.2
Alanine	0.55	1.0
Glutamine	0.59	0.5
Ala-ala-gln	1.10	0.3
Ala-glu	2.20	0.2
Any unspecified impurity	—	0.1
Total unspecified impurities	—	0.5

SPECIFIC TESTS
• **OPTICAL ROTATION,** *Specific Rotation* ⟨781S⟩
Sample solution: 50 mg/mL in water. Perform the measurement at 20°.
Acceptance criteria: +9.0° to +11.0°
• **WATER DETERMINATION,** *Method Ia* ⟨921⟩**:** NMT 1.0%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed, tight, light-resistant containers.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP L-Alanine RS
USP L-Alanyl-L-alanine RS
USP L-Alanyl-L-glutamine RS
USP Glutamine RS

Alpha Lipoic Acid—*see Alpha Lipoic Acid under L.*

American Ginseng

DEFINITION
American Ginseng consists of the dried roots of *Panax quinquefolius* L. (Fam. Araliaceae). It contains NLT 4.0% of total ginsenosides, calculated on the dried basis.

IDENTIFICATION
• **A. THIN-LAYER CHROMATOGRAPHY**
Standard solution A: 20 mg/mL of USP Powdered American Ginseng Extract RS in methanol
Standard solution B: 20 mg/mL of USP Powdered Asian Ginseng Extract RS in methanol
Sample solution: Transfer about 1.0 g of finely powdered American Ginseng to a 25-mL flask fitted with a reflux condenser. Add 10.0 mL of a mixture of methanol and water (7:13), and heat under reflux for 15 min. Cool, filter, and dilute the filtrate with methanol to 10.0 mL.
Adsorbent: 0.25-mm layer of silica gel, typically 20 cm long (TLC plates)
Application volume: 20 µL
Developing solvent system A: Chloroform, methanol, and water (13:7:2). Use the lower phase.
Developing solvent system B: Butyl alcohol, ethyl acetate, and water (4:1:5). Use the upper phase.
Derivatization reagent: Dissolve 0.5 mL of anisaldehyde in 10 mL of glacial acetic acid, add 85 mL of methanol, mix, and carefully add 5 mL of sulfuric acid.
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Develop in a chamber containing *Developing solvent system A* until the solvent front has moved 10.5 cm from the origin. Remove the plates, and allow to dry. Turn the plates 90°, and develop in a chamber containing *Developing solvent system B* until the solvent front has moved 10.5 cm from the origin. Remove the plates, and allow to dry. Spray with *Derivatization reagent*. Heat the plates at 105°–110° for 10 min, and examine under white light.
Suitability requirements: The order, from top to bottom, of ginsenosides on the chromatographic plates is Rg_2 (on left) and Rg_1 (on right), Rf, Re, Rd, Rc, Rb_2 (on left) and Rb_1 (on right), and Ro. Ginsenosides Rg_2, Rg_1, Rf, Re, and Rd are found on the upper half of the plates; the remaining ginsenosides are found on the lower half after chromatographing with *Developing solvent system B*. *Standard solution A* does not exhibit a spot for ginsenoside Rf. *Standard solution B* exhibits a spot for ginsenoside Rf.
Acceptance criteria: The spots from the *Sample solution* correspond to those from *Standard solution A*.
• **B.** The retention times of the peaks for ginsenosides Rg_1, Re, Rb_1, Rb_2, Rc_2, and Rd of the *Sample solution* correspond to those of *Standard solution A*, as obtained in the test for *Content of Ginsenosides*. The ratio of the peak responses for ginsenosides Rb_2 to Rb_1 is less than 0.4, and the ratio of the peak responses for ginsenosides Rg_1 to Rb_1 is less than 0.3. The chromatogram shows no significant peak at the retention time corresponding to that for ginsenoside Rf of *Standard solution B*, as obtained in the test for *Content of Ginsenosides*.

COMPOSITION
• **CONTENT OF GINSENOSIDES**
Solution A: Water
Solution B: Acetonitrile and water (4:1)
Mobile phase: See *Table 1*.

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Table 1

Time (min)	Solution A (%)	Solution B (%)
0	76	24
12	76	24
28	65	35
51.5	56.5	43.5
52.5	0	100
64.5	76	24
77	76	24

Diluent: Alcohol and water (4:6)

Standard solution A: Transfer a quantity of USP Powdered American Ginseng Extract RS, equivalent to about 2 mg of ginsenoside Rb_1, to a suitable container, and dissolve in 10.0 mL of *Diluent*.

Standard solution B: Transfer a quantity of USP Powdered Asian Ginseng Extract RS, equivalent to about 2 mg of ginsenoside Rg_1, to a suitable container, and dissolve in 10.0 mL of *Diluent*.

Sample solution: Reduce 100 g of American Ginseng to a powder, and transfer about 1.0 g of the powder, accurately weighed, to a 100-mL round-bottom flask fitted with a reflux condenser. Add 50 mL of *Diluent* and a few grains of pumice, boil on a water bath under reflux for 1 h, cool, and filter. Wash the flask and the residue with 20 mL of *Diluent*, and pass through the same filter. Combine the filtrates, and evaporate in a rotary evaporator at 50° to dryness. Dissolve the residue in 10.0 mL of *Diluent*.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 203 nm

Analytical column: 4.6-mm × 15-cm; 3-µm packing L1

Guard column: 4.6-mm × 2.0-cm; packing L1

Column temperature: 25°

Flow rate: 1.5 mL/min

Injection size: 10 µL

System suitability

Sample: *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram is similar to the reference chromatogram provided with the lot of USP Powdered American Ginseng Extract RS being used.

Relative standard deviation: NMT 2.0%, determined for the sum of the peak areas for the six major ginsenosides, in replicate injections

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Identify ginsenosides Rg_1, Re, Rb_1, Rc, Rb_2, and Rd in the *Standard solutions* and the *Sample solution* by comparing the chromatograms with the reference chromatogram provided with USP Powdered American Ginseng Extract RS, and measure the peak responses.

Calculate the percentages of individual ginsenosides in the portion of American Ginseng taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak response of ginsenoside Rg_1, Re, Rb_1, Rc, Rb_2, or Rd from the *Sample solution*

r_S = peak response of ginsenoside Rg_1, Re, Rb_1, Rc, Rb_2, or Rd from the appropriate *Standard solution*

C_S = concentration of ginsenoside Rg_1, Re, Rb_1, Rc, Rb_2, or Rd in the appropriate *Standard solution* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of American Ginseng taken to prepare the *Sample solution* (mg)

Calculate the percentage of total ginsenosides in the portion of American Ginseng taken by adding the individual percentages.

Acceptance criteria: NLT 4.0% of total ginsenosides on the dried basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^4 cfu/g. The total combined molds and yeasts count does not exceed 10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: Fusiform or cylindrical roots, sometimes branched, typically 1–10 cm, sometimes up to 20 cm, in length and up to 2.5 cm in diameter at the crown, with one or more stem scars. Externally pale yellow to golden, rough-textured, with prominent horizontal rings and fine longitudinal ridges as a result of drying. Root scars or fine rootlets are present. If stem base is present, scales are thin and perishing (differs from *P. ginseng*, in which scales at base of stem are fleshy and persistent). Fracture is short; fractured surface is white to ivory, with distinct aromatic odor and rings of secretory canals present in secondary phloem.

Microscopic

Transverse section of root: Multiple layers of thin-walled cork cells are present. Secondary phloem is characterized by conspicuous air lacunae; abundant, starch-containing storage parenchyma; few sieve elements, found in small groupings; and rings of schizogenous secretory canals. Each secretory canal is lined with 6–8 epithelial cells that lack starch. Xylem is characterized by abundant starch-containing storage parenchyma and a few tracheary elements, composed of nonlignified tracheids and slightly lignified spiral or reticulated vessels lacking secretory canals and found in isolation or in small groupings. Druse crystals are sometimes present within vascular parenchyma cells. Diarch or triarch primary xylem is in center of root.

- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩**:** NMT 2.0%
- **LOSS ON DRYING** ⟨731⟩

Sample: 1.0 g of American Ginseng, finely powdered

Analysis: Dry the *Sample* at 105° for 2 h.

Acceptance criteria: NMT 10.0%

- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 8.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store protected from heat.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.

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• **USP Reference Standards** ⟨11⟩
USP Powdered American Ginseng Extract RS
USP Powdered Asian Ginseng Extract RS

Powdered American Ginseng

DEFINITION

Powdered American Ginseng is American Ginseng reduced to a fine or a very fine powder. It contains NLT 4.0% of total ginsenosides, calculated on the dried basis.

IDENTIFICATION

• **A. Thin-Layer Chromatography**

Standard solution A: 20 mg/mL of USP Powdered American Ginseng Extract RS in methanol

Standard solution B: 20 mg/mL of USP Powdered Asian Ginseng Extract RS in methanol

Sample solution: Transfer about 1.0 g of Powdered American Ginseng to a 25-mL flask fitted with a reflux condenser. Add 10.0 mL of a mixture of methanol and water (7:13), and heat under reflux for 15 min. Cool, filter, and dilute the filtrate with methanol to 10.0 mL.

Adsorbent: 0.25-mm layer of silica gel, typically 20 cm long (TLC plates)

Application volume: 20 µL

Developing solvent system A: Chloroform, methanol, and water (13:7:2). Use the lower phase.

Developing solvent system B: Butyl alcohol, ethyl acetate, and water (4:1:5). Use the upper phase.

Derivatization reagent: Dissolve 0.5 mL of anisaldehyde in 10 mL of glacial acetic acid, add 85 mL of methanol, mix, and carefully add 5 mL of sulfuric acid.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop in a chamber containing *Developing solvent system A* until the solvent front has moved 10.5 cm from the origin. Remove the plates, and allow to dry. Turn the plates 90°, and develop in a chamber containing *Developing solvent system B* until the solvent front has moved 10.5 cm from the origin. Remove the plates, and allow to dry. Spray with *Derivatization reagent*. Heat the plates at 105°–110° for 10 min, and examine under white light.

Suitability requirements: The order, from top to bottom, of ginsenosides on the chromatographic plates is: Rg_2 (on left) and Rg_1 (on right), Rf, Re, Rd, Rc, Rb_2 (on left) and Rb_1 (on right), and Ro. Ginsenosides Rg_2, Rg_1, Rf, Re, and Rd are found on the upper half of the plates; the remaining ginsenosides are found on the lower half after chromatographing with *Developing solvent system B*. *Standard solution A* does not exhibit a spot for ginsenoside Rf. *Standard solution B* exhibits a spot for ginsenoside Rf.

Acceptance criteria: The spots from the *Sample solution* correspond to those from *Standard solution A*.

• **B.** The retention times of the peaks for ginsenosides Rg_1, Re, Rb_1, Rb_2, Rc_2, and Rd of the *Sample solution* correspond to those of *Standard solution A*, as obtained in the test for *Content of Ginsenosides*. The ratio of the peak responses for ginsenosides Rb_2 to Rb_1 is less than 0.4, and the ratio of the peak responses for ginsenosides Rg_1 to Rb_1 is less than 0.3. The chromatogram shows no significant peak at the retention time corresponding to that for ginsenoside Rf of *Standard solution B*, as obtained in the test for *Content of Ginsenosides*.

COMPOSITION

• **Content of Ginsenosides**

Solution A: Water

Solution B: Acetonitrile and water (4:1)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	76	24
12	76	24
28	65	35
51.5	56.5	43.5
52.5	0	100
64.5	76	24
77	76	24

Diluent: Alcohol and water (4:6)

Standard solution A: Transfer a quantity of USP Powdered American Ginseng Extract RS, equivalent to about 2 mg of ginsenoside Rb_1, to a suitable container, and dissolve in 10.0 mL of *Diluent*.

Standard solution B: Transfer a quantity of USP Powdered Asian Ginseng Extract RS, equivalent to about 2 mg of ginsenoside Rg_1, to a suitable container, and dissolve in 10.0 mL of *Diluent*.

Sample solution: Transfer about 1.0 g of Powdered American Ginseng, accurately weighed, to a 100-mL round-bottom flask fitted with a reflux condenser. Add 50 mL of *Diluent* and a few grains of pumice, boil on a water bath under reflux for 1 h, cool, and filter. Wash the flask and the residue with 20 mL of *Diluent*, and pass through the same filter. Combine the filtrates, and evaporate in a rotary evaporator at 50° to dryness. Dissolve the residue in 10.0 mL of *Diluent*.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 203 nm

Analytical column: 4.6-mm × 15-cm; 3-µm packing L1

Guard column: 4.6-mm × 2.0-cm; packing L1

Column temperature: 25°

Flow rate: 1.5 mL/min

Injection size: 10 µL

System suitability

Sample: *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram is similar to the reference chromatogram provided with the lot of USP Powdered American Ginseng Extract RS being used.

Relative standard deviation: NMT 2.0%, determined for the sum of the peak areas for the six major ginsenosides, in replicate injections

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Identify ginsenosides Rg_1, Re, Rb_1, Rc, Rb_2, and Rd in the *Standard solutions* and the *Sample solution* by comparing the chromatograms with the reference chromatogram provided with USP Powdered American Ginseng Extract RS, and measure the peak responses.

Calculate the percentages of individual ginsenosides in the portion of Powdered American Ginseng taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

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r_U = peak response of ginsenoside Rg_1, Re, Rb_1, Rc, Rb_2, or Rd from the *Sample solution*
r_S = peak response of ginsenoside Rg_1, Re, Rb_1, Rc, Rb_2, or Rd from the appropriate *Standard solution*
C_S = concentration of ginsenoside Rg_1, Re, Rb_1, Rc, Rb_2, or Rd in the appropriate *Standard solution* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Powdered American Ginseng taken to prepare the *Sample solution* (mg)

Calculate the percentage of total ginsenosides in the portion of Powdered American Ginseng taken by adding the individual percentages.

Acceptance criteria: NLT 4.0% of total ginsenosides on the dried basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^4 cfu/g. The total combined molds and yeasts count does not exceed 10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli.*

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS:** Pale yellowish-brown powder with a slightly aromatic odor; oval parenchymatous cells packed with starch granules and occasional druse crystals of calcium oxalate; yellowish-brown secretory vessels with yellowish-brown contents
- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩**:** NMT 2.0%
- **LOSS ON DRYING** ⟨731⟩
  Sample: 1.0 g of Powdered American Ginseng
  Analysis: Dry the *Sample* at 105° for 2 h.
  Acceptance criteria: NMT 10.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩
  Sample: 1.0 g of Powdered American Ginseng
  Acceptance criteria: NMT 8%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Store in tight containers, protected from light, moisture, and heat.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Powdered American Ginseng Extract RS
  USP Powdered Asian Ginseng Extract RS

Powdered American Ginseng Extract

DEFINITION

Powdered American Ginseng Extract is prepared from the pulverized dried roots of *Panax quinquefolius* L. (Fam. Araliaceae), using suitable solvents, and dried to a powder. It contains NLT 10.0% of total ginsenosides, calculated on the anhydrous basis. The ratio of starting crude plant material to Powdered American Ginseng Extract is between 3:1 and 7:1.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**
  Standard solution A: 20 mg/mL of USP Powdered American Ginseng Extract RS in methanol
  Standard solution B: 20 mg/mL of USP Powdered Asian Ginseng Extract RS in methanol
  Sample solution: 20 mg/mL in methanol
  Adsorbent: 0.25-mm layer of chromatographic silica gel, typically 20 cm long (TLC plates)
  Application volume: 20 µL
  Developing solvent system A: Chloroform, methanol, and water (13:7:2). Use the lower phase.
  Developing solvent system B: Butyl alcohol, ethyl acetate, and water (4:1:5). Use the upper phase.
  Spray reagent: Dissolve 0.5 mL of anisaldehyde in 10 mL of glacial acetic acid, add 85 mL of methanol, mix, and carefully add 5 mL of sulfuric acid.
  Analysis
  Samples: *Standard solution A, Standard solution B,* and *Sample solution*
  Develop in a chamber containing *Developing solvent system A* until the solvent front has moved 10.5 cm from the origin. Remove the plates, and allow to dry. Turn the plates 90°, and develop in a chamber containing *Developing solvent system B* until the solvent front has moved 10.5 cm from the origin. Remove the plates, and allow to dry. Spray with *Spray reagent.* Heat the plates at 105°–110° for 10 min, and examine.
  Suitability requirements: The order, from top to bottom, of ginsenosides on the chromatographic plates is: Rg_2 (on left) and Rg_1 (on right), Rf, Re, Rd, Rc, Rb_2 (on left) and Rb_1 (on right), and Ro. Ginsenosides Rg_2, Rg_1, Rf, Re, and Rd are found on the upper half of the plates; the remaining ginsenosides are found on the lower half after chromatographing with *Developing solvent system B. Standard solution A* does not exhibit a spot for ginsenoside Rf. *Standard solution B* exhibits a spot for ginsenoside Rf.
  Acceptance criteria: The spots from the *Sample solution* correspond to those from *Standard solution A.*
- **B.** The retention times of the peaks for ginsenosides Rg_1, Re, Rb_1, Rb_2, Rc, and Rd of the *Sample solution* correspond to those of *Standard solution A,* as obtained in the test for *Content of Ginsenosides.* The ratio of the peak responses for ginsenosides Rb_2 to Rb_1 is less than 0.4, and the ratio of the peak responses for ginsenosides Rg_1 to Rb_1 is less than 0.3. The *Sample solution* shows no significant peak at the retention time corresponding to that for ginsenoside Rf of *Standard solution B,* as obtained in the test for *Content of Ginsenosides.*

COMPOSITION

- **CONTENT OF GINSENOSIDES**
  Solution A: Water
  Solution B: Acetonitrile and water (4:1)
  Mobile phase: See *Table 1.*

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	76	24
12	76	24
28	65	35
51.5	56.5	43.5
52.5	0	100
64.5	76	24

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Table 1 *(continued)*

Time (min)	Solution A (%)	Solution B (%)
77	76	24

Diluent: Alcohol and water (4:6)
Standard solution A: Transfer a quantity of USP Powdered American Ginseng Extract RS, equivalent to about 2 mg of ginsenoside Rb_1, to a suitable container, and dissolve in 10.0 mL of *Diluent*.
Standard solution B: Transfer a quantity of USP Powdered Asian Ginseng Extract RS, equivalent to about 2 mg of ginsenoside Rg_1, to a suitable container, and dissolve in 10.0 mL of *Diluent*.
Sample solution: Transfer a quantity of Powdered American Ginseng Extract, equivalent to about 5 mg of ginsenosides, to a suitable container. Dissolve in 10.0 mL of *Diluent*, sonicating for 10 min, and filter.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 203 nm
Analytical column: 4.6-mm × 15-cm; 3-µm packing L1
Guard column: 4.6-mm × 2.0-cm; packing L1
Column temperature: 25°
Flow rate: 1.5 mL/min
Injection size: 10 µL
System suitability
Sample: *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram is similar to the Reference Chromatogram provided with the lot of USP Powdered Asian Ginseng Extract RS being used.
Relative standard deviation: NMT 2.0%, determined for the sum of the peak areas for the 6 major ginsenosides, in replicate injections
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Identify ginsenosides Rg_1, Re, Rb_1, Rc, Rb_2, and Rd in the *Standard solutions* and the *Sample solution* by comparing the chromatograms with the Reference Chromatogram provided with USP Powdered American Ginseng Extract RS, and measure the peak responses.
Calculate the percentages of individual ginsenosides in the portion of Powdered American Ginseng Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times P$$

r_U = peak response of ginsenosides Rg_1, Re, Rb_1, Rc, Rb_2, or Rd from the *Sample solution*
r_S = peak response of ginsenosides Rg_1, Re, Rb_1, Rc, Rb_2, or Rd from the appropriate *Standard solution*
C_S = concentration of ginsenosides Rg_1, Re, Rb_1, Rc, Rb_2, or Rd in the appropriate *Standard solution* (mg/mL)
C_U = concentration of Powdered American Ginseng Extract in the *Sample solution* (mg/mL)
P = labeled amount, in percentage, of each relevant ginsenoside in USP Powdered American Ginseng Extract RS

Calculate the percentage of total ginsenosides in the portion of Powdered American Ginseng Extract taken by adding the individual percentages.
Acceptance criteria: NLT 10.0% of total ginsenosides on the anhydrous basis

CONTAMINANTS

Change to read:

- **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements*, *Pesticide Residues*▲ (CN 1-May-2020) : Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic microbial count does not exceed 10^4 cfu/g. The total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩: It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS
- **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 7.0%
- **BOTANICAL EXTRACTS,** *Residue on Evaporation* ⟨565⟩: Meets the requirements
- **ALCOHOL DETERMINATION,** *Method II* ⟨611⟩: NMT 0.25%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived. Label it to indicate the content of total ginsenosides, the extracting solvent used for preparation, and the ratio of the starting crude plant material to the Powdered Extract. It meets the labeling requirements under *Botanical Extracts* ⟨565⟩.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Powdered American Ginseng Extract RS
  USP Powdered Asian Ginseng Extract RS

American Ginseng Capsules

DEFINITION
American Ginseng Capsules contain Powdered American Ginseng Extract. Capsules contain NLT 90.0% and NMT 110.0% of the labeled amount of Extract, calculated as the sum of ginsenosides Rg_1, Re, Rb_1, Rc, Rb_2, and Rd.

IDENTIFICATION
- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩
Sample solution
Soft-shell gelatin Capsules: Transfer a portion of the contents of the Capsules, equivalent to 100 mg of Powdered Extract, to a separatory funnel containing 30 mL of a mixture of hexanes, methanol, and water (20:15:10), dissolve in this mixture, and collect the lower layer. Wash the upper layer with three 15-mL portions of a mixture of methanol and water (15:10), and combine the washings with the lower layer. Evaporate to dryness under vacuum at 45°–50°. Dissolve the residue in 5 mL of methanol.
Hard-shell gelatin Capsules: Transfer a portion of the contents of the Capsules, equivalent to 100 mg of Powdered Extract, to a conical flask. Extract at 55° with three 20-mL portions of a mixture of methanol and water (2:8). Evaporate the combined extracts to dryness under vacuum at 45°–50°. Dissolve the residue in 5 mL of methanol.
Standard solution A: 20 mg/mL of USP Powdered American Ginseng Extract RS in methanol

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Standard solution B: 20 mg/mL of USP Powdered Asian Ginseng Extract RS in methanol
Application volume: 20 µL
Developing solvent system A: The lower phase of a mixture of chloroform, methanol, and water (13:7:2)
Developing solvent system B: The upper phase of a mixture of butyl alcohol, ethyl acetate, and water (4:1:5)
Spray reagent: Dissolve 0.5 mL of anisaldehyde in 10 mL of glacial acetic acid, add 85 mL of methanol, mix, carefully add 5 mL of sulfuric acid, and mix.
Analysis
Samples: *Sample solution*, *Standard solution A*, and *Standard solution B*
Develop the chromatograms in a chamber containing *Developing solvent system A* until the solvent front has moved 10.5 cm from the origin. Remove the plate from the chamber, and allow to dry. Turn the plate 90°, and develop in a chamber containing *Developing solvent system B* until the solvent front has moved 10.5 cm from the origin. Remove the plate from the chamber, and allow to dry. Spray with *Spray reagent*. Heat the plate at 105°–110° for 10 min, and examine. The order, from top to bottom, of ginsenosides on the plates is Rg_2 (on left) and Rg_1 (on right), Rf, Re, Rd, Rc, Rb_2 (on left) and Rb_1 (on right), and Ro.
Ginsenosides Rg_2, Rg_1, Rf, Re, and Rd are found on the upper half of the plates; the remaining ginsenosides are found on the lower half after chromatographing with *Developing solvent system B*.
Acceptance criteria: *Standard solution A* does not exhibit a spot for ginsenoside Rf. *Standard solution B* exhibits a spot for ginsenoside Rf. The spots from the *Sample solution* correspond to those from *Standard solution A*.

• **B.** The retention times of the peaks for ginsenosides Rg_1, Re, Rb_1, Rb_2, Rc_2, and Rd in the chromatogram of the *Sample solution* correspond to those from the *Standard solution*, as obtained in the test for *Content of Ginsenosides*. The ratio of the peak response for Rb_2 to the peak response for Rb_1 is less than 0.4; and the ratio of the peak response for Rg_1 to the peak response for Rb_1 is less than 0.3. There is no significant peak at the retention time corresponding to that of ginsenoside Rf in the *System suitability solution*, as obtained in the test for *Content of Ginsenosides*.

STRENGTH

• **CONTENT OF GINSENOSIDES**
Method 1
Diluent: Water and alcohol (3:2)
Solution A: Water
Solution B: Acetonitrile and water (4:1)
Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	76	24
12	76	24
28	65	35
51.5	56.5	43.5
52.5	0	100
64.5	76	24
77	76	24

Standard solution: A solution of USP Powdered American Ginseng Extract RS in *Diluent* containing the equivalent of 0.2 mg/mL of ginsenoside Rb_1

Sample solution (soft-gelatin Capsules): Open NLT 20 Capsules, transfer the contents to a suitable container, and mix to homogenize. Transfer a portion, expected to contain an amount of Extract equivalent to 12 mg of ginsenosides, to a suitable flask with a stopper. Add 5.0 mL of tetrahydrofuran, and sonicate for 5 min. Add 25.0 mL of a mixture of methanol and water (4:6), and shake for 50 min in an automatic shaker. Transfer 15.0 mL of the obtained emulsion to a centrifuge tube with a stopper, add 800 mg of sodium chloride, shake for 30 s, and centrifuge to obtain a clear upper phase. Dilute 1.0 mL of the upper phase with 4 mL of water in a suitable tube, and transfer the solution to a column containing 360 mg of packing L2 that has been previously treated with 3.0 mL of methanol followed by 8.0 mL of water. [NOTE—Elute slowly, not faster than 1 drop/s, in all elution steps. Do not use vacuum.] Rinse the tube with 5 mL of water, transfer to the column taking the precaution of slow elution, and discard the eluate. Repeat the elution with 5 mL of a mixture of methanol and water (4:6), and discard the eluate. Elute the ginsenosides with 5.0 mL of methanol. Evaporate the solution under a stream of nitrogen at 40° (50 min), and dissolve the residue with 1.0 mL of a solution of acetonitrile and water (1:4).
System suitability solution: 24 mg/mL of USP Powdered Asian Ginseng Extract RS in *Diluent*. Filter.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 203 nm
Column
Guard column: 4.6-mm × 2.0-cm; packing L1
Analytical column: 4.6-mm × 15-cm; 3-µm packing L1
Column temperature: 25°
Flow rate: 1.5 mL/min
Injection size: 10 µL
System suitability
Sample: *System suitability solution* (inject 20 µL)
Suitability requirements
Chromatogram similarity: The *System suitability solution* chromatogram is similar to the Reference Chromatogram provided with the lot of USP Powdered Asian Ginseng Extract RS being used.
Relative standard deviation: NMT 2.0%, determined for the sum of the peak areas for the six major ginsenosides, in repeated injections
Analysis
Samples: *Standard solution* and *Sample solution*
Identify ginsenosides Rg_1, Re, Rb_1, Rc, Rb_2, and Rd in the *Standard solution* and the *Sample solution* by comparing the chromatograms with the Reference Chromatogram provided with USP Powdered American Ginseng Extract RS being used, and measure the peak responses.
Calculate the quantity, in mg, of each relevant ginsenoside (Rg_1, Re, Rb_1, Rc, Rb_2, and Rd) in the portion of Capsule contents taken:

$$\text{Result} = 0.3 \times (r_U/r_S) \times C_S \times P$$

r_U = peak area for each relevant ginsenoside from the *Sample solution*
r_S = peak area for each relevant ginsenoside from the *Standard solution*
C_S = concentration of USP Powdered American Ginseng Extract RS in the *Standard solution* (mg/mL)

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P = labeled amount, in percentage, of each relevant ginsenoside in the USP Powdered American Ginseng Extract RS lot being used

Calculate the content of total ginsenosides, T, in mg, by adding the amounts of individual ginsenoside.
Calculate the percentage of Powdered Extract with respect to the label claim:

$$\text{Result} = T \times (A_{WT}/W) \times (100/L_E) \times (100/L)$$

T = content of total ginsenosides in the portion of Capsule contents taken (mg)
A_{WT} = average weight of Capsule contents (mg/Capsule)
W = weight of the portion of Capsule contents taken (mg)
L_E = content of total ginsenosides, mg, in 100 mg of the Extract used to prepare the Capsules
L = amount of Extract per Capsule according to label claim (mg/Capsule)

Method 2

Diluent, Solution A, Solution B, Mobile phase, System suitability solution, Chromatographic system, and **Suitability requirements:** Proceed as directed under *Method 1*.

Solvent A: Upper phase of a mixture consisting of hexane, methanol, and water (4:3:2)

Solvent B: Lower phase of a mixture consisting of hexane, methanol, and water (4:3:2)

Standard solution: A solution of USP Powdered American Ginseng Extract RS in *Diluent* containing the equivalent of 1 mg/mL of ginsenoside Rb_1

Sample solution A (for soft-gelatin Capsules): Open NLT 20 Capsules and transfer the contents to a suitable container. Mix to homogenize and transfer a portion, expected to contain an amount of Extract equivalent to 15 mg of total ginsenosides, to a 50-mL flask. Add 10.0 mL of *Solvent A*, and sonicate for 3–5 min at 25°–30°. Transfer the solution to a 125-mL separatory funnel. To the residue add 10 mL of *Solvent B*, and sonicate for 3–5 min at 25°–30°. Transfer the solution to the same separatory funnel. Repeat the above procedure twice (the total volume will be about 60 mL). Shake, and then allow the phases to separate. Collect the combined lower phase in a round-bottom flask, and wash the combined upper phase twice with 10 mL of *Solvent B*. Evaporate the combined lower phase to dryness under vacuum at 45°–50°. Transfer the residue to a 10-mL volumetric flask using small volumes of methanol, and dilute with methanol to volume.

Sample solution B (for hard-gelatin Capsules): Weigh the contents of NLT 20 Capsules, and composite the contents. Transfer a portion of the composite, expected to contain an amount of Extract equivalent to 15 mg of total ginsenosides, to a conical flask. Add 15 mL of methanol, and shake to mix. Sonicate the mixture at 25°–30° for 30 min. Cool, pass through filter paper, and return the residue to the conical flask. Add another 15 mL of methanol, sonicate the mixture at 25°–30° for 30 min, and filter. Wash the residue with three 15-mL portions of methanol. Evaporate the combined extracts and washing to dryness under vacuum at 45°–50°. Transfer the residue to a 10-mL volumetric flask using small volumes of methanol, and dilute with methanol to volume.

Analysis

Samples: *Standard solution* and *Sample solution*

Identify ginsenosides Rg_1, Re, Rb_1, Rc, Rb_2, and Rd in the *Standard solution* and the *Sample solution* by comparing the chromatograms with the Reference Chromatogram provided with USP Powdered American Ginseng Extract RS, and measure the peak responses.
Calculate the quantity, in mg, of each relevant ginsenoside (Rg_1, Re, Rb_1, Rc, Rb_2, and Rd) in the portion of Capsule contents taken:

$$\text{Result} = 0.1 \times (r_U/r_S) \times C_S \times P$$

r_U = peak area for each relevant ginsenoside from the *Sample solution*
r_S = peak area for each relevant ginsenoside from the *Standard solution*
C_S = concentration of USP Powdered American Ginseng Extract RS in the *Standard solution* (mg/mL)
P = labeled amount, in percentage, of each relevant ginsenoside in the USP Powdered American Ginseng Extract RS lot being used

Calculate the content of total ginsenosides, T, in mg, by adding the amounts of individual ginsenoside.
Calculate the percentage of Powdered Extract with respect to the label claim:

$$\text{Result} = T \times (A_{WT}/W) \times (100/L_E) \times (100/L)$$

T = content of total ginsenosides in the portion of Capsule contents taken (mg)
A_{WT} = average weight of Capsule contents (mg/Capsule)
W = weight of the portion of Capsule contents taken (mg)
L_E = content of total ginsenosides, mg, in 100 mg of the Extract used to prepare the Capsules
L = amount of Extract per Capsule according to label claim (mg/Capsule)

Acceptance criteria: 90.0%–110.0% of Extract, calculated as the sum of ginsenosides Rg_1, Re, Rb_1, Rc, Rb_2, and Rd

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS ⟨2040⟩:** Meet the requirements for *Disintegration*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS ⟨2091⟩:** Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS ⟨2021⟩:** The total aerobic microbial count does not exceed 10^4 cfu/g. The total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS ⟨2022⟩:** Meet the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light. Store at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the article from which the Capsules were prepared. The label also indicates the amount of Extract, in mg/Capsule. Label the Capsules to indicate the percentage of ginsenosides in the Extract contained in the Capsules. For soft-gelatin Capsules, state the method for *Content of*

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Ginsenosides with which the product complies only if *Method 1* is not used.

• **USP Reference Standards** ⟨11⟩
USP Powdered American Ginseng Extract RS
USP Powdered Asian Ginseng Extract RS

American Ginseng Tablets

DEFINITION

American Ginseng Tablets contain Powdered American Ginseng Extract. Tablets contain NLT 90.0% and NMT 110.0% of Extract, calculated as the sum of ginsenosides Rg_1, Re, Rb_1, Rc, Rb_2, and Rd.

IDENTIFICATION

• **A. Thin-Layer Chromatographic Identification Test** ⟨201⟩

Standard solution A: 20 mg/mL of USP Powdered American Ginseng Extract RS in methanol

Standard solution B: 20 mg/mL of USP Powdered Asian Ginseng Extract RS in methanol

Sample solution: Transfer a quantity of finely powdered Tablets, equivalent to 100 mg of Extract, to a conical flask. Extract at 55° with three 20-mL portions of a mixture of methanol and water (2:8). Evaporate the combined extracts to dryness under vacuum at 45°–50°. Dissolve the residue in 5 mL of methanol.

Application volume: 20 µL

Developing solvent system A: The lower phase of a mixture of chloroform, methanol, and water (13:7:2)

Developing solvent system B: The upper phase of a mixture of butyl alcohol, ethyl acetate, and water (4:1:5)

Spray reagent: Dissolve 0.5 mL of anisaldehyde in 10 mL of glacial acetic acid, add 85 mL of methanol, mix, carefully add 5 mL of sulfuric acid, and mix.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Proceed as directed in the chapter. Develop in a chamber containing *Developing solvent system A* until the solvent front has moved 10.5 cm from the origin. Remove the plates from the chamber, and allow to dry. Turn the plates 90°, and develop in a chamber containing *Developing solvent system B* until the solvent front has moved 10.5 cm from the origin. Remove the plates from the chamber, and allow to dry. Spray with *Spray reagent*. Heat the plates at 105°–110° for 10 min, and examine. The order, from top to bottom, of ginsenosides on the plates is Rg_2 (on left) and Rg_1 (on right), Rf, Re, Rd, Rc, Rb_2 (on left) and Rb_1 (on right), and Ro. Ginsenosides Rg_2, Rg_1, Rf, Re, and Rd are found on the upper half of the plates; the remaining ginsenosides are found on the lower half after chromatographing with *Developing solvent system B*

Acceptance criteria: The chromatogram of *Standard solution A* does not exhibit a spot for ginsenoside Rf. *Standard solution B* exhibits a spot for ginsenoside Rf. The spots from the *Sample solution* correspond to those from *Standard solution A*.

• **B.** The retention times of the peaks for ginsenosides Rg_1, Re, Rb_1, Rb_2, Rc_2, and Rd in the chromatogram of the *Sample solution* correspond to those from the *Standard solution*, as obtained in the test for *Content of Ginsenosides*. The ratio of the peak response for Rb_2 to the peak response for Rb_1 is less than 0.4; and the ratio of the peak response for Rg_1 to the peak response for Rb_1 is less than 0.3. The *Sample solution* chromatogram shows no significant peak at the retention time corresponding to that of ginsenoside Rf in the *System suitability solution*, as obtained in the test for *Content of Ginsenosides*.

STRENGTH

• **Content of Ginsenosides**

Diluent: Water and alcohol (3:2)

Solution A: Water

Solution B: Acetonitrile and water (4:1)

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	76	24
12	76	24
28	65	35
51.5	56.5	43.5
52.5	0	100
64.5	76	24
77	76	24

Standard solution: A solution of USP Powdered American Ginseng Extract RS in *Diluent* containing the equivalent of 1 mg/mL of ginsenoside Rb_1

Sample solution: Accurately weigh and finely powder NLT 20 Tablets. Transfer to a conical flask an accurately weighed portion of the powder expected to contain an amount of Extract equivalent to 15 mg of total ginsenosides, add 15 mL of methanol, and shake to mix. Sonicate the mixture at 25°–30° for 30 min. Cool, pass through filter paper, and return the residue to the conical flask. Add another 15 mL of methanol, sonicate the mixture at 25°–30° for 30 min, and filter. Wash the residue with three 15-mL portions of methanol. Evaporate the combined extracts and washings to dryness under vacuum at 45°–50°. Transfer the residue to a 10.0-mL volumetric flask, using small volumes of methanol, and dilute with methanol to volume.

System suitability solution: 24 mg/mL of USP Powdered Asian Ginseng Extract RS in *Diluent*. Filter.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 203 nm

Column

Guard column: 4.6-mm × 2.0-cm; packing L1

Analytical column: 4.6-mm × 15-cm; 3-µm packing L1

Column temperature: 25°

Flow rate: 1.5 mL/min

Injection size: 10 µL

System suitability

Sample: *System suitability solution* (inject 20 µL)

Suitability requirements

Chromatogram similarity: The *System suitability solution* chromatogram is similar to the Reference Chromatogram provided with the lot of USP Powdered Asian Ginseng Extract RS being used.

Relative standard deviation: NMT 2.0%, determined for the sum of the peak areas for the six major ginsenosides, in repeated injections

Analysis

Samples: *Standard solution* and *Sample solution*

Identify ginsenosides Rg_1, Re, Rb_1, Rc, Rb_2, and Rd in the *Standard solution* and *Sample solution* by comparing the chromatograms with the Reference Chromatogram provided with USP Powdered American Ginseng

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Extract RS lot being used, and measure the peak responses.

Calculate the quantity, in mg, of each relevant ginsenoside (Rg_1, Re, Rb_1, Rc, Rb_2, and Rd) in the portion of Tablets taken:

$$\text{Result} = 0.1 \times (r_U/r_S) \times C_S \times P$$

r_U = peak area for each relevant ginsenoside from the *Sample solution*

r_S = peak area for each relevant ginsenoside from the *Standard solution*

C_S = concentration of USP Powdered American Ginseng Extract RS in the *Standard solution* (mg/mL)

P = labeled amount, in percentage, of each relevant ginsenoside in the USP Powdered American Ginseng Extract RS lot being used

Calculate the content of total ginsenosides, T, in mg, by adding the amounts of individual ginsenoside.

Calculate the percentage of Powdered Extract with respect to the label claim:

$$\text{Result} = T \times (A_{WT}/W) \times (100/L_E) \times (100/L)$$

T = content of total ginsenosides in the portion of Tablets taken (mg)

A_{WT} = average weight of Tablets (mg/Tablet)

W = weight of the portion of Tablets taken (mg)

L_E = content of total ginsenosides, mg, in 100 mg of the Extract used to prepare the Tablets

L = amount of Extract per Tablet according to label claim (mg/Tablet)

Acceptance criteria: 90.0%–110.0% of Powdered Extract, calculated as the sum of ginsenosides Rg_1, Re, Rb_1, Rc, Rb_2, and Rd

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** ⟨2040⟩**:** Meet the requirements for *Disintegration*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩**:** Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS—NUTRITIONAL AND DIETARY SUPPLEMENTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^4 cfu/g. The total combined molds and yeasts count does not exceed 10^3 cfu/g. Meet the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light. Store at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the article from which the Tablets were prepared. The label also indicates the amount of Extract, in mg/Tablet. Label the Tablets to indicate the percentage of total ginsenosides in the Extract contained in the Tablets.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Powdered American Ginseng Extract RS
  USP Powdered Asian Ginseng Extract RS

DS Monographs

Andrographis

DEFINITION

Andrographis consists of the dried stems and leaves of *Andrographis paniculata* (Burm. f.) Nees (Fam. Acanthaceae). It contains NLT 1.0% of diterpene lactones, calculated on the dried basis as the sum of andrographolide, neoandrographolide, 14-deoxy-11,12-didehydroandrographolide, and andrograpanin.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩

Standard solution 1: Use *Standard solution A*, prepared as directed in the test for *Content of Diterpene Lactones*.

Standard solution 2: Sonicate an amount of USP Powdered Andrographis Extract RS, equivalent to about 15 mg of diterpene lactones, for 10–15 min in 25 mL of methanol, centrifuge, and use the supernatant.

Sample solution: Use *Sample stock solution*, prepared as directed in the test for *Content of Diterpene Lactones*.

Adsorbent: Chromatographic silica gel mixture with an average particle size of 10–15 µm (TLC plates)

Application volume: 10 µL, as 5–10 mm bands

Developing solvent system: Chloroform, acetone, and toluene (2:2:1)

Derivatization reagent: A mixture of 1% vanillin in alcohol and 10% sulfuric acid in alcohol (1:1)

Analysis

Samples: *Standard solution 1*, *Standard solution 2*, and *Sample solution*

Use a saturated chamber. Develop until the solvent front has moved up about 90% of the length of the plate. Remove the plate from the chamber, dry, treat with *Derivatization reagent*, heat for 5–10 min at 100°, and examine under white light.

Acceptance criteria: The *Sample solution* exhibits three main grayish-blue zones with R_F values of approximately 0.4, 0.6, and 0.8 that correspond in position and color to zones in *Standard solution 2*. *Standard solution 1* exhibits a grayish-blue zone due to andrographolide at an R_F of about 0.4. The *Sample solution* exhibits a zone similar in color and R_F value to that due to andrographolide in *Standard solution 1*.

- **B.** The retention time of the main peak of the *Sample solution* obtained in the test for *Content of Diterpene Lactones* corresponds to that of andrographolide in *Standard solution A*. Identify other diterpene lactone peaks in the *Sample solution* by comparison with *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Andrographis Extract RS being used. The *Sample solution* shows additional peaks corresponding to neoandrographolide, 14-deoxy-11,12-didehydroandrographolide, and andrograpanin.

COMPOSITION

- **CONTENT OF DITERPENE LACTONES**

Solution A: Dissolve 0.14 g of potassium dihydrogen phosphate in 900 mL of water, add 0.5 mL of phosphoric acid, dilute with water to 1000 mL, mix, filter, and degas.

Solution B: Acetonitrile, filtered and degassed

Standard solution A: Dissolve a weighed quantity of USP Andrographolide RS in methanol to obtain a 1.0-mg/mL solution. Transfer 5.0 mL of this solution to a 10-mL volumetric flask, dilute with acetonitrile to volume, and mix.

Standard solution B: Transfer an amount of USP Powdered Andrographis Extract RS, equivalent to about 25 mg of diterpene lactones, to a 50-mL volumetric flask, add 25 mL

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of methanol, heat gently for 15–20 min, dilute with acetonitrile to volume, and mix. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first 5 mL of the filtrate.

Sample stock solution: Transfer about 2.0 g of finely powdered Andrographis to a 250-mL flask fitted with a reflux condenser. Add 50 mL of methanol, reflux for 15 min, cool to room temperature, and decant the supernatant. Repeat until the extract is colorless. Combine the extracts, filter, concentrate under vacuum, and adjust the volume to 50.0 mL using methanol.

Sample solution: Transfer 25.0 mL of *Sample stock solution* to a 50-mL volumetric flask, dilute with acetonitrile to volume, and mix. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first 5 mL of the filtrate.

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	95	5
18	55	45
25	20	80
28	20	80
35	55	45
40	95	5
45	95	5

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 223 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Flow rate: 1.5 mL/min
Injection volume: 20 µL

System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Andrographis Extract RS being used.
Column efficiency: NLT 5000 theoretical plates, *Standard solution A*
Tailing factor: NMT 1.5 for the andrographolide peak, *Standard solution A*
Relative standard deviation: NMT 2.0%, determined for the andrographolide peak in replicate injections, *Standard solution A*
Resolution: NLT 5 between the neoandrographolide and 14-deoxy-11,12-didehydroandrographolide peaks, *Standard solution B*

Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Using the chromatogram of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Andrographis Extract RS being used, identify the retention times of the peaks corresponding to the different diterpene lactones. The approximate relative retention times of the different diterpene lactones are provided in *Table 2*.

Table 2

Analyte	Relative Retention Time
Andrographolide	1.00
Neoandrographolide	1.16
14-Deoxy-11,12-didehydroandrographolide	1.31
Andrograpanin	1.50

Separately calculate the percentages of andrographolide, neoandrographolide, 14-deoxy-11,12-didehydroandrographolide, and andrograpanin in the portion of Andrographis taken:

$$\text{Result} = (r_U/r_S) \times (C_S/W) \times 10F$$

r_U = peak area of each identified diterpene lactone in the *Sample solution*
r_S = peak area of andrographolide in *Standard solution A*
C_S = concentration of USP Andrographolide RS in *Standard solution A* (mg/mL)
W = weight of Andrographis taken to prepare the *Sample solution* (g)
F = conversion factor: 1.00 for andrographolide, 3.90 for neoandrographolide, 1.45 for 14-deoxy-11,12-didehydroandrographolide, and 2.65 for andrograpanin

Acceptance criteria: NLT 1.0% for the sum of the percentages of andrographolide, neoandrographolide, 14-deoxy-11,12-didehydroandrographolide, and andrograpanin, on the dried basis

IMPURITIES

- **Articles of Botanical Origin,** *Limits of Elemental Impurities* ⟨561⟩: Meets the requirements
- **Articles of Botanical Origin,** *Foreign Organic Matter* ⟨561⟩: NMT 2.0%
- **Articles of Botanical Origin,** *Pesticide Residue Analysis* ⟨561⟩: Meets the requirements

SPECIFIC TESTS

- **Botanical Characteristics**

Macroscopic: Stem is dark green, woody, 2–6 mm in diameter, bearing numerous branches, showing slightly swollen nodes, the upper part is distinctly quadrangular with four bulges in the four corners, and the lower part is somewhat rounded; texture is fragile, easily broken; branches are quadrangular, often narrowly winged in the upper part. Leaves are simple, opposite, short, petiolated or nearly sessile; lamina is crumpled and easily broken, lanceolate or ovate-lanceolate when whole, 2–7 cm long, 1–3 cm wide, with acuminate apex, reticulate venation, and cuneate-decurrent base, margin entire or undulate; the upper surface is green, the lower surface grayish-green; both surfaces are glabrous. Pharmacopeial article consists of dry mixtures of crisp, dark-green broken leaves and quadrangular stems; leaves are brittle; stems are fracture short, fibrous.

Microscopic
Transverse section of stems: Epidermal layer shows cells containing round, long-elliptical or clavate calcium carbonate deposits (cystoliths), 1–4 celled nonglandular hairs and multicellular, disk-shaped glandular hairs; collenchyma is below the epidermis and in the bulges; endodermis is distinct; vascular bundles surround the

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parenchyma of the central pith; small acicular crystals of calcium oxalate are present in the cortex and pith.

Transverse section of leaves: Subsquare or rectangular upper and lower epidermal cells; lower epidermal cells are relatively smaller; both epidermal layers show cells containing cystoliths, nonglandular hairs and glandular hairs similar to those of the stem; mesophyll is composed of 1–2 layers of palisade parenchyma and spongy parenchyma; loosely arranged spongy parenchyma appear across the upper part of the midrib; vascular bundles of midrib are collateral and grooved; cells containing cystoliths appear above the xylem.

- **Loss on Drying** ⟨731⟩
  Sample: 1.0 g of finely powdered Andrographis
  Analysis: Dry the *Sample* at 105° for 3 h.
  Acceptance criteria: NMT 12.0%
- **Articles of Botanical Origin,** *Total Ash* ⟨561⟩
  Sample: 1.0 g of finely powdered Andrographis
  Acceptance criteria: NMT 15%
- **Articles of Botanical Origin,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 3.0%
- **Articles of Botanical Origin,** *Alcohol-Soluble Extractives, Method 2* ⟨561⟩**:** NLT 8.0%
- **Microbial Enumeration Tests** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^5 cfu/g; the total combined molds and yeasts count does not exceed 10^3 cfu/g; and the bile-tolerant Gram-negative bacterial count does not exceed 10^3 cfu/g.
- **Absence of Specified Microorganisms** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **Labeling:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP Reference Standards** ⟨11⟩
  USP Andrographolide RS
  USP Powdered Andrographis Extract RS

Powdered Andrographis

DEFINITION

Powdered Andrographis is Andrographis reduced to a fine or very fine powder. It contains NLT 1.0% of diterpene lactones, calculated on the dried basis as the sum of the andrographolide, neoandrographolide, 14-deoxy-11,12-didehydroandrographolide, and andrograpanin.

IDENTIFICATION

- **A. Thin-Layer Chromatographic Identification Test** ⟨201⟩
  Standard solution 1: Use *Standard solution A*, prepared as directed in the test for *Content of Diterpene Lactones*.
  Standard solution 2: Sonicate an amount of USP Powdered Andrographis Extract RS, equivalent to about 15 mg of diterpene lactones, for 10–15 min in 25 mL of methanol, centrifuge, and use the supernatant.
  Sample solution: Use *Sample stock solution*, prepared as directed in the test for *Content of Diterpene Lactones*.
  Adsorbent: Chromatographic silica gel mixture with an average particle size of 10–15 µm (TLC plates)
  Application volume: 10 µL, as 5–10 mm bands
  Developing solvent system: Chloroform, acetone, and toluene (2:2:1)
  Derivatization reagent: A mixture of 1% vanillin in alcohol and 10% sulfuric acid in alcohol (1:1)
  Analysis
  Samples: *Standard solution 1*, *Standard solution 2*, and *Sample solution*
  Use a saturated chamber. Develop until the solvent front has moved up about 90% of the length of the plate. Remove the plate from the chamber, dry, treat with *Derivatization reagent*, heat for 5–10 min at 100°, and examine under white light.
  Acceptance criteria: The *Sample solution* exhibits three main grayish-blue zones with R_F values of approximately 0.4, 0.6, and 0.8 that correspond in position and color to the main zones of *Standard solution 2*. *Standard solution 1* exhibits a grayish-blue zone due to andrographolide at an R_F of about 0.4. The *Sample solution* exhibits a zone similar in color and R_F value to that due to andrographolide in *Standard solution 1*.
- **B.** The retention time of the main peak of the *Sample solution* obtained in the test for *Content of Diterpene Lactones* corresponds to that of andrographolide in *Standard solution A*. Identify other diterpene lactone peaks in the *Sample solution* by comparison with *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Andrographis Extract RS being used. The *Sample solution* shows additional peaks corresponding to neoandrographolide, 14-deoxy-11,12-didehydroandrographolide, and andrograpanin.

COMPOSITION

- **Content of Diterpene Lactones**
  Solution A: Dissolve 0.14 g of potassium dihydrogen phosphate in 900 mL of water, add 0.5 mL of phosphoric acid, dilute with water to 1000 mL, mix, filter, and degas.
  Solution B: Acetonitrile, filtered and degassed
  Standard solution A: Dissolve a weighed quantity of USP Andrographolide RS in methanol to obtain a 1.0-mg/mL solution. Transfer 5.0 mL of this solution to a 10-mL volumetric flask, dilute with acetonitrile to volume, and mix.
  Standard solution B: Transfer an amount of USP Powdered Andrographis Extract RS, equivalent to about 25 mg of diterpene lactones, to a 50-mL volumetric flask, add 25 mL of methanol, heat gently for 15–20 min, dilute with acetonitrile to volume, and mix. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first 5 mL of the filtrate.
  Sample stock solution: Transfer about 2.0 g of Powdered Andrographis to a 250-mL flask fitted with a reflux condenser. Add 50 mL of methanol, reflux for 15 min, cool to room temperature, and decant the supernatant. Repeat until the extract is colorless. Combine the extracts, filter, concentrate under vacuum, and adjust the volume to 50.0 mL using methanol.
  Sample solution: Transfer 25.0 mL of *Sample stock solution* to a 50-mL volumetric flask, dilute with acetonitrile to volume, and mix. Before injection, pass through a membrane filter of 0.45-µm or finer pore size discarding the first 5 mL of the filtrate.
  Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	95	5
18	55	45

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Table 1 *(continued)*

Time (min)	Solution A (%)	Solution B (%)
25	20	80
28	20	80
35	55	45
40	95	5
45	95	5

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 223 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Flow rate: 1.5 mL/min
Injection volume: 20 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Andrographis Extract RS being used.
Column efficiency: NLT 5000 theoretical plates, *Standard solution A*
Tailing factor: NMT 1.5 for the andrographolide peak, *Standard solution A*
Relative standard deviation: NMT 2.0%, determined for the andrographolide peak in replicate injections, *Standard solution A*
Resolution: NLT 5 between the neoandrographolide and 14-deoxy-11,12-didehydroandrographolide peaks, *Standard solution B*
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Using the chromatogram of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Andrographis Extract RS being used, identify the retention times of the peaks corresponding to the different diterpene lactones. The approximate relative retention times of the different diterpene lactones are provided in *Table 2*.

Table 2

Analyte	Relative Retention Time
Andrographolide	1.00
Neoandrographolide	1.16
14-Deoxy-11,12-didehydroandrographolide	1.31
Andrograpanin	1.50

Separately calculate the percentages of andrographolide, neoandrographolide, 14-deoxy-11,12-didehydroandrographolide, and andrograpanin in the portion of Powdered Andrographis taken:

$$\text{Result} = (r_U/r_S) \times (C_S/W) \times 10F$$

r_U = peak area of each identified diterpene lactone from the *Sample solution*
r_S = peak area of andrographolide from *Standard solution A*
C_S = concentration of USP Andrographolide RS in *Standard solution A* (mg/mL)
W = weight of Powdered Andrographis taken to prepare the *Sample solution* (g)
F = conversion factor: 1.00 for andrographolide, 3.90 for neoandrographolide, 1.45 for 14-deoxy-11,12-didehydroandrographolide, and 2.65 for andrograpanin

Acceptance criteria: NLT 1.0% for the sum of the percentages of andrographolide, neoandrographolide, 14-deoxy-11,12-didehydroandrographolide, and andrograpanin, on the dried basis

IMPURITIES
- **Articles of Botanical Origin,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
- **Articles of Botanical Origin,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements

SPECIFIC TESTS
- **Botanical Characteristics**
Macroscopic: It is a grayish-brown powder.
Microscopic: It reveals cells of the upper and lower epidermis of the leaves, some cells containing large cystoliths, up to 36 µm in diameter and 180 µm long, with a hilum-shaped scar in the large end; 1- to 4-celled nonglandular hairs; disk-shaped glandular hairs, 8-celled head and very short stalk; diacytic stomata mostly on the lower epidermis; stem epidermal cells, some cells containing cystoliths, stomata, nonglandular hairs and glandular hairs similar to those of the leaves; thin-walled parenchyma cells; collenchyma cells; acicular phloem fibers; tracheids; vessels, with spiral and scalariform thickening.
- **Loss on Drying** ⟨731⟩
Sample: 1.0 g of Powdered Andrographis
Analysis: Dry the *Sample* at 105° for 3 h.
Acceptance criteria: NMT 12.0%
- **Articles of Botanical Origin,** *Total Ash* ⟨561⟩
Sample: 1.0 g of Powdered Andrographis
Acceptance criteria: NMT 15%
- **Articles of Botanical Origin,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 3.0%
- **Articles of Botanical Origin,** *Alcohol-Soluble Extractives, Method 2* ⟨561⟩**:** NLT 8.0%
- **Microbial Enumeration Tests** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^5 cfu/g; the total combined molds and yeasts count does not exceed 10^3 cfu/g; and the bile-tolerant Gram-negative bacterial count does not exceed 10^3 cfu/g.
- **Absence of Specified Microorganisms** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS
- **Packaging and Storage:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **Labeling:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP Reference Standards** ⟨11⟩
USP Andrographolide RS
USP Powdered Andrographis Extract RS

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Powdered Andrographis Extract

DEFINITION

Powdered Andrographis Extract is prepared from Andrographis by extraction with methanol or alcohol. The ratio of plant material to extract is between 15:1 and 10:1. It contains NLT 90.0% and NMT 110.0% of the labeled amount of diterpene lactones, calculated on the dried basis as the sum of andrographolide, neoandrographolide, 14-deoxy-11,12-didehydroandrographolide, and andrograpanin. The content of 14-deoxy-11,12-didehydroandrographolide is NMT 15% of the total diterpene lactones. It may contain suitable added substances as carriers.

IDENTIFICATION

• **A. Thin-Layer Chromatographic Identification Test** ⟨201⟩

Standard solution 1: Use *Standard solution A*, prepared as directed in the test for *Content of Diterpene Lactones*.

Standard solution 2: Sonicate for 10–15 min a quantity of USP Powdered Andrographis Extract RS, equivalent to about 15 mg of diterpene lactones, in 25 mL of methanol. Centrifuge, and use the supernatant.

Sample solution: Sonicate for 10–15 min a quantity of Powdered Andrographis Extract, equivalent to about 15 mg of diterpene lactones, in 25 mL of methanol. Centrifuge, and use the supernatant.

Adsorbent: Chromatographic silica gel mixture with an average particle size of 10–15 µm (TLC plates)

Application volume: 10 µL, as 5–10 mm bands

Developing solvent system: Chloroform, acetone, and toluene (2:2:1)

Spray reagent: A mixture containing 1% vanillin in alcohol and 10% sulfuric acid in alcohol (1:1)

Analysis

Samples: *Standard solution 1*, *Standard solution 2*, and *Sample solution*

Use a saturated chamber. Develop until the solvent front has moved up about 90% of the plate. Remove the plate from the chamber. Dry, and spray with *Spray reagent*. Heat for 5–10 min at 100°, and examine under visible light.

Acceptance criteria: The *Sample solution* exhibits three main grayish blue zones with R_F values of approximately 0.4, 0.6, and 0.8 that correspond in position and color to zones in *Standard solution 2*. *Standard solution 1* exhibits a grayish blue zone due to andrographolide at an R_F of about 0.4. The *Sample solution* exhibits a zone similar in color and R_F value to that due to andrographolide in *Standard solution 1*.

• **B.** The *Sample solution* in the test for *Content of Diterpene Lactones* shows a main peak at a retention time corresponding to that of andrographolide in *Standard solution A*. Identify other diterpene lactone peaks in the *Sample solution* by comparison with *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Andrographis Extract RS. The *Sample solution* shows additional peaks corresponding to neoandrographolide, 14-deoxy-11,12-didehydroandrographolide, and andrograpanin.

COMPOSITION

• **Content of Diterpene Lactones**

Solution A: Dissolve 0.14 g of potassium dihydrogen phosphate in 900 mL of water, add 0.5 mL of phosphoric acid, dilute with water to 1000 mL, mix, filter, and degas.

Solution B: Use filtered and degassed acetonitrile.

Standard solution A: Dissolve a weighed quantity of USP Andrographolide RS in methanol to obtain a solution having a known concentration of about 1.0 mg/mL. Transfer 5.0 mL of this solution to a 10-mL volumetric flask, dilute with acetonitrile to volume, and mix.

Standard solution B: Transfer an amount of USP Powdered Andrographis Extract RS, equivalent to about 25 mg of diterpene lactones, to a 50-mL volumetric flask, add 25 mL of methanol, heat gently for 15–20 min, dilute with acetonitrile to volume, and mix. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first 5 mL of the filtrate.

Sample solution: Transfer a weighed amount of Powdered Andrographis Extract, equivalent to about 25 mg of diterpene lactones, to a 50-mL volumetric flask, add 25 mL of methanol, heat gently for 15–20 min, dilute with acetonitrile to volume, and mix. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first 5 mL of the filtrate.

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	95	5
18	55	45
25	20	80
28	20	80
35	55	45
40	95	5
45	95	5

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 223 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Flow rate: 1.5 mL/min

Injection size: 20 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Andrographis Extract RS.

Column efficiency: NLT 5000 theoretical plates, *Standard solution A*

Tailing factor: NMT 1.5 for the andrographolide peak, *Standard solution A*

Relative standard deviation: NMT 2.0%, determined from the andrographolide peak for replicate injections, *Standard solution A*

Resolution: NLT 5 between the neoandrographolide and 14-deoxy-11,12-didehydroandrographolide peaks, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Andrographis Extract RS being used, identify the retention times of the peaks corresponding to different diterpene lactones. The approximate relative retention times of the different diterpene lactones are provided in the following table:

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Analyte	Relative Retention Time
Andrographolide	1.00
Neoandrographolide	1.16
14-Deoxy-11,12-didehydroandrographolide	1.31
Andrograpanin	1.50

Separately calculate the percentages of andrographolide, neoandrographolide, 14-deoxy-11,12-didehydroandrographolide, and andrograpanin in the portion of Powdered Andrographis Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/W) \times 5F$$

r_U = peak response for each diterpene lactone from the *Sample solution*
r_S = peak response for andrographolide from *Standard solution A*
C_S = concentration of USP Andrographolide RS in *Standard solution A* (mg/mL)
W = weight of Powdered Andrographis Extract taken to prepare the *Sample solution* (g)
F = conversion factor for each analyte (1.00 for andrographolide, 3.90 for neoandrographolide, 1.45 for 14-deoxy-11,12-didehydroandrographolide, and 2.65 for andrograpanin)

Acceptance criteria: 90.0%–110.0%, on the dried basis, of the labeled amount of diterpene lactones calculated as the sum of the percentages of andrographolide, neoandrographolide, 14-deoxy-11,12-didehydroandrographolide, and andrograpanin

IMPURITIES

Change to read:

ORGANIC IMPURITIES

• **▲Botanical Extracts** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020)
: Meets the requirements

SPECIFIC TESTS

• **LOSS ON DRYING** ⟨731⟩**:** Dry 2.0 g at 105° for 3 h: it loses NMT 5.0% of its weight.
• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^4 cfu/g. The total combined yeasts and molds count does not exceed 10^3 cfu/g.
• **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.
• **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. It meets other labeling requirements under *Botanical Extracts* ⟨565⟩.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Andrographolide RS
USP Powdered Andrographis Extract RS

Arginine

—see Arginine General Monographs

Arginine Hydrochloride—*see Arginine Hydrochloride General Monographs*

Arginine Capsules

DEFINITION

Arginine Capsules contain NLT 90.0% and NMT 110.0% of the labeled amount of arginine or arginine hydrochloride in an amount equivalent to arginine ($C_6H_{14}N_4O_2$).

IDENTIFICATION

• **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩
Standard solution: 1.5 mg/mL of USP L-Arginine RS or USP Arginine Hydrochloride RS in water
Sample solution: Weigh the content of NLT 20 Capsules, mix, and transfer a portion of the content, equivalent to about 150 mg of arginine, to a 100-mL volumetric flask, add 80 mL of water, sonicate for 15 min, dilute with water to volume, mix, and filter.
Application volume: 5 µL
Developing solvent system: Isopropyl alcohol and ammonium hydroxide (7:3)
Spray reagent: 2 mg/mL of ninhydrin in a mixture of butyl alcohol and 2 N acetic acid (95:5)
Analysis: Proceed as directed for *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*. Dry the plate at 100°–105° until the ammonia disappears completely. Spray with the *Spray reagent*, and heat at 100°–105° for about 15 min. Examine the plate under white light.
Acceptance criteria: The principal spot from the *Sample solution* corresponds in appearance and R_F value to that of the *Standard solution*.
• **B.** The retention time of the major peak of the *Sample solution* corresponds to that from the *Standard solution*, as obtained in the *Strength*.

STRENGTH

• **PROCEDURE**
Buffer: 6.9 mg/mL of monobasic sodium phosphate in water. Adjust with phosphoric acid to a pH of 3.5.
Solution A: 0.5 mg/mL of 1-octanesulfonic acid sodium salt in *Buffer*
Mobile phase: *Solution A* and acetonitrile (95:5)
Standard solution: 1.5 mg/mL of USP L-Arginine RS or USP Arginine Hydrochloride RS in *Buffer*
Sample solution: Weigh the content of NLT 20 Capsules, mix, and transfer a portion of the content, equivalent to about 150 mg of arginine, to a 100-mL volumetric flask, add 80 mL of *Buffer*, sonicate for 15 min, dilute with *Buffer* to volume, mix, and filter.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 215 nm
Column: 4.6-mm × 25-cm; packing L7
Flow rate: 0.8 mL/min
Injection size: 10 µL
System suitability
Sample: *Standard solution*

DS Monographs

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Suitability requirements
Column efficiency: NLT 1500 theoretical plates
Relative standard deviation: NMT 2.0% from the arginine peak, in repeated injections
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of arginine in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of the *Standard solution* (mg/mL)
C_U = nominal concentration of Arginine in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–110.0% of the labeled amount of arginine ($C_6H_{14}N_4O_2$)

PERFORMANCE TESTS
• **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** ⟨2040⟩**:** Meet the requirements for *Dissolution*
Medium: 0.1 N hydrochloric acid; 900 mL
Apparatus 2: 100 rpm
Time: 60 min
Standard solution: Proceed as directed in the *Procedure* for *Strength*.
Sample solution: Sample per *Disintegration and Dissolution of Dietary Supplements* ⟨2040⟩. Dilute with *Medium* to a concentration similar to that of the *Standard solution*.
Analysis: Determine the amount of arginine dissolved in the *Procedure* for *Strength*, making any necessary modifications.
Tolerances: NLT 75% of the labeled amount of arginine ($C_6H_{14}N_4O_2$) is dissolved.
• **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩**:** Meet the requirements

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
• **LABELING:** The label states the form of arginine that is used and the equivalent amount of arginine.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP L-Arginine RS
USP Arginine Hydrochloride RS

Arginine Tablets

DEFINITION
Arginine Tablets contain NLT 90.0% and NMT 110.0% of the labeled amount of arginine or arginine hydrochloride in an amount equivalent to arginine ($C_6H_{14}N_4O_2$).

IDENTIFICATION
• **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩
Standard solution: 1.5 mg/mL of USP L-Arginine RS or USP Arginine Hydrochloride RS in water
Sample solution: Weigh and finely powder NLT 20 Tablets, mix, and transfer a portion of the powder, equivalent to about 150 mg of arginine, to a 100-mL volumetric flask, add 80 mL of water, sonicate for 15 min, dilute with water to volume, mix, and filter.
Application volume: 5 µL
Developing solvent system: Isopropyl alcohol and ammonium hydroxide (7:3)
Spray reagent: 2 mg/mL of ninhydrin in a mixture of butyl alcohol and 2 N acetic acid (95:5)
Analysis: Proceed as directed for *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*. Dry the plate at 100°–105° until the ammonia disappears completely. Spray with the *Spray reagent*, and heat at 100°–105° for about 15 min. Examine the plate under white light.
Acceptance criteria: The principal spot from the *Sample solution* corresponds in appearance and R_F value to that from the *Standard solution*.
• **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in *Strength*.

STRENGTH
• **PROCEDURE**
Buffer: 6.9 mg/mL of monobasic sodium phosphate in water. Adjust with phosphoric acid to a pH of 3.5.
Solution A: 0.5 mg/mL of 1-octanesulfonic acid sodium salt in *Buffer*
Mobile phase: *Solution A* and acetonitrile (95:5)
Standard solution: 1.5 mg/mL of USP L-Arginine RS or USP Arginine Hydrochloride RS in *Buffer*
Sample solution: Weigh and finely powder NLT 20 Tablets, mix, and transfer a portion of the powder, equivalent to about 150 mg of arginine, to a 100-mL volumetric flask, add 80 mL of *Buffer*, sonicate for 15 min, dilute with *Buffer* to volume, mix, and filter.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 215 nm
Column: 4.6-mm × 25-cm; packing L7
Flow rate: 0.8 mL/min
Injection size: 10 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Column efficiency: NLT 1500 theoretical plates
Relative standard deviation: NMT 2.0% from the arginine peak, in repeated injections
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of arginine in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of the *Standard solution* (mg/mL)
C_U = nominal concentration of Arginine in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–110.0% of the labeled amount of arginine ($C_6H_{14}N_4O_2$)

PERFORMANCE TESTS
• **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** ⟨2040⟩**:** Meet the requirements for *Dissolution*
Medium: 0.1 N hydrochloric acid; 900 mL
Apparatus 2: 100 rpm
Time: 60 min
Standard solution: Proceed as directed in the *Procedure* for *Strength*.

DS Monographs

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Sample solution: Sample per *Disintegration and Dissolution of Dietary Supplements* ⟨2040⟩. Dilute with *Medium* to a concentration similar to that of the *Standard solution*.
Analysis: Determine the amounts of arginine dissolved in the *Procedure* for *Strength*, making any necessary modifications.
Tolerances: NLT 75% of the labeled amount of arginine ($C_6H_{14}N_4O_2$) is dissolved.
• **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩: Meet the requirements

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
• **LABELING:** The label states the form of arginine that is used and the equivalent amount of arginine.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP L-Arginine RS
USP Arginine Hydrochloride RS

Ascorbic Acid—*see Ascorbic Acid General Monographs*

Ascorbic Acid Chewable Gels

DEFINITION
Ascorbic Acid Chewable Gels contain ascorbic acid in the form of ascorbic acid ($C_6H_8O_6$), sodium ascorbate ($C_6H_7NaO_6$), calcium ascorbate dihydrate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$), or their mixture in an amount equivalent to NLT 90.0% and NMT 150.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$).

IDENTIFICATION
• **A.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Strength*.

STRENGTH
• **PROCEDURE**
[NOTE—Use amber, low-actinic glassware. Use cryogenic gloves when handling liquid nitrogen.]
Extracting solution: Dissolve 73 g of metaphosphoric acid in 800 mL of water, add 84 mL of glacial acetic acid, and dilute with water to 1000 mL.
Mobile phase: 7.8 g/L of monobasic sodium phosphate dihydrate in water. Adjust with phosphoric acid to a pH of 2.5.
[NOTE—Wash the column periodically with methanol and water using appropriate gradients to eliminate retained substances and avoid carryover interferences.]
Standard stock solution: 1 mg/mL of USP Ascorbic Acid RS in *Extracting solution*. [NOTE—Sonicate with intermittent shaking to help dissolve, if necessary. Prepare fresh every time.]
Standard solution: Dilute *Standard stock solution* with *Extracting solution* to obtain a solution containing 0.05 mg/mL of USP Ascorbic Acid RS.
Sample solution: Immerse 25–30 Chewable Gels in liquid nitrogen in a cryogenic vessel for 10 min. Cool a blender jar by swirling liquid nitrogen for about 1 min and discard the contents. Add frozen Chewable Gels to the cooled blender jar and grind to a fine powder. Transfer a portion of the powder, nominally equivalent to 25 mg of ascorbic acid, into a 25-mL volumetric flask. [NOTE—Proceed to this step immediately or keep the powdered Chewable Gels frozen until use.] Add 15 mL of *Extracting solution*, mix on a vortex mixer until well mixed, and sonicate for 10 min or until the sample has completely dissolved. Cool the solution to room temperature, dilute with *Extracting solution* to volume, and mix well. Quantitatively dilute a portion of the solution with *Extracting solution* to obtain a solution containing 0.05 mg/mL of ascorbic acid. Mix and pass through a 0.45-µm glass microfiber filter, discarding the first few milliliters of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 245 nm
Column: 4.6-mm × 15-cm; 3.5-µm packing L7
Flow rate: 0.8 mL/min
Injection volume: 10 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 2.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of vitamin C, as ascorbic acid ($C_6H_8O_6$), in the portion of sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of ascorbic acid from the *Sample solution*
r_S = peak area of ascorbic acid from the *Standard solution*
C_S = concentration of USP Ascorbic Acid RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of ascorbic acid in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0%

PERFORMANCE TESTS

Change to read:

• **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩, *Dissolution*
Medium: 0.1 N hydrochloric acid; 900 mL
Apparatus 2: 75 rpm
Time: 15 min
Sample solution: Withdraw a portion of the solution under test, pass through a 0.45-µm glass microfiber filter, and use the pooled sample as the test specimen.
Analysis: Proceed as directed in *Strength*, making any necessary modifications.
▲ ▲ (USP 1-Dec-2019)
Calculate the percentage of the labeled amount of ascorbic acid ($C_6H_8O_6$) dissolved:

$$\text{Result} = (r_U/r_S) \times (C_S \times V/L) \times 100$$

r_U = peak area of ascorbic acid from the *Sample solution*
r_S = peak area of ascorbic acid from the *Standard solution*
C_S = concentration of USP Ascorbic Acid RS in the *Standard solution* (mg/mL)
V = volume of *Medium*, 900 mL
L = label claim of ascorbic acid (mg/Chewable Gel)

Tolerances: NLT 75% of the labeled amount of ascorbic acid is dissolved.

DS Monographs

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Change to read:

• **WEIGHT VARIATION** ⟨2091⟩▲ ▲(USP 1-Dec-2019)**:** Meet the requirements

SPECIFIC TESTS

• **pH** ⟨791⟩

Sample solution: Cut the Chewable Gels into small pieces. Transfer about 15 g of the cut Chewable Gels into a 50-mL centrifuge tube, add 15 g of hot (80°–90°) water, close the cap, place the centrifuge tube into a shaker water bath at 55°, and shake for 50–60 min or until all of the Chewable Gels are dissolved. Cool down to 30° and measure the pH.

Acceptance criteria: NMT 4.5

• **WATER ACTIVITY**

Sample: Slice the Chewable Gels into pieces about 2 mm thick.

Procedure: Measure the water activity using the AOAC's *Official Methods of Analysis*, Official Method #978.18.

Acceptance criteria: NMT 0.75

CONTAMINANTS

• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count is NMT 10^3 cfu/g, and the total combined yeasts and molds count is NMT 10^2 cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species*, and *Test for Absence of Escherichia coli***:** Meet the requirements

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers; protect from heat.

• **LABELING:** The label states the quantity of ascorbic acid in mg/Chewable Gel, and the chemical form of ascorbic acid present in the Chewable Gels.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Ascorbic Acid RS

Ascorbic Acid Oral Solution—*see Ascorbic Acid Oral Solution General Monographs*

Ascorbic Acid Tablets—*see Ascorbic Acid Tablets General Monographs*

DS Monographs

Ashwagandha Root

DEFINITION

Ashwagandha Root is the dried mature root of *Withania somnifera* (L.) Dunal (Fam. Solanaceae). It contains NLT 0.3% of withanolides, calculated on the dried basis as the sum of withanolide aglycones, calculated as withanolide A, and withanolide glycosides, calculated as withanoside IV.

IDENTIFICATION

• **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 0.2 mg/mL each of USP β-Sitosterol RS and USP Withanolide A RS in methanol

Standard solution B: 20 mg/mL of USP Powdered Ashwagandha Root Extract RS in methanol. Sonicate for 10 min, centrifuge, and use the supernatant. [NOTE—Retain the supernatant for use in the test for *Content of Withanolides*.]

Sample solution: Suspend about 1 g of Ashwagandha Root, finely powdered, in 10 mL of methanol and sonicate for 15 min. Centrifuge, and use the supernatant.

Chromatographic system

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plate)[1]

Application volume: 2 µL each of *Standard solution A* and *Standard solution B*, and 10 µL of the *Sample solution*, as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of 33%.

Temperature: Ambient, not to exceed 30°

Developing solvent system: Toluene, ethyl acetate, and glacial acetic acid (55:45:3)

Developing distance: 6 cm

Derivatization reagent: 20 mL of sulfuric acid combined with 180 mL of ice-cold methanol

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands and dry in air. Develop in a saturated chamber and dry in air. Treat the plate with the *Derivatization reagent*, heat at 100° for 5 min, and examine under UV light at 365 nm and under white light.

System suitability: Under UV light at 365 nm, the derivatized chromatogram of *Standard solution A* displays in its lower third a blue band due to withanolide A and in its middle third a grayish-blue band due to β-sitosterol. The chromatogram of *Standard solution B* displays a light gray to whitish band due to withanone below the withanolide A band, and a faint light gray band above the β-sitosterol band; there is also a light gray band close to the solvent front. A reddish band slightly above the application line is due to withaferin A. Under white light, the bands due to β-sitosterol and withanolide A appear violet–gray. Additional faint bands may appear.

Acceptance criteria: Under UV light at 365 nm and under white light, the chromatogram of the *Sample solution* displays the bands similar in position and color to those seen in *Standard solution B*. Additional bands may be observed, in particular a band just above that due to withanolide A (light brown under UV light at 365 nm, dark brown under white light), and a thin band below that due to β-sitosterol (light blue under UV light at 365 nm, violet–gray under white light). Bands vary in intensity, and some of those seen in the chromatogram of *Standard solution B* may be very faint or absent from the *Sample solution*.

• **B. HPLC**

Analysis: Proceed as directed in the test for *Content of Withanolides*.

Acceptance criteria: The *Sample solution* shows main peaks at retention times corresponding to those of withanolide A and withanoside IV in *Standard solution A*. The *Sample solution* shows some of the withanolides listed in *Table 2*.

COMPOSITION

Change to read:

• **CONTENT OF WITHANOLIDES**

Solution A: Dissolve 0.14 g of potassium dihydrogen phosphate in 900 mL of water, add 0.5 mL of phosphoric acid, dilute with water to 1000 mL, and mix.

Solution B: Acetonitrile, filtered and degassed

Mobile phase: See *Table 1*.

[1] Suitable commercially available plates are HPTLC Silica Gel 60 F_{254} from EMD Millipore (e.g., Part #1.05642.0001).

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Table 1

Time (min)	Solution A (%)	Solution B (%)
0	95	5
18	55	45
25	20	80
28	20	80
30	95	5
40	95	5

Standard solution A: A composite solution containing 0.1 mg/mL of USP Withanolide A RS and 0.1 mg/mL of USP Withanoside IV RS in methanol, accurately calculated. Use gentle heat to aid dissolution.

Standard solution B: Dilute a portion of *Standard solution B* from *Identification A* with methanol (1:1), and mix well. Before injection, pass through a ▲polyethersulfone▲ (USP 1-May-2019) membrane filter of 0.45-µm or finer pore size.

Sample solution: Transfer about 5.00 g of Ashwagandha Root, finely powdered and accurately weighed, to a 250-mL round-bottom flask fitted with a reflux condenser. Add 50 mL of methanol, reflux on a water bath for 15 min, cool to room temperature, decant, and retain the solvent. Repeat until the solvent is colorless. Combine the retained solvents, filter, concentrate under vacuum to about 40 mL, transfer to a 50-mL volumetric flask, and adjust with methanol to volume. Before injection, pass through a ▲polyethersulfone▲ (USP 1-May-2019) filter of 0.45-µm or finer pore size, discarding the first few milliliters of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 227 nm

Column: 4.6-mm × 25-cm, end-capped; ▲5-µm▲ (USP 1-May-2019) packing L1

Column temperature: 27°

Flow rate: 1.5 mL/min

Injection volume: 20 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Resolution: NLT 1.0 between the withanolide A and withanone peaks, and NLT 3.0 between the withaferin A peak and the peak corresponding to coeluting withanoside V and withanoside VI, *Standard solution B*

Tailing factor: NMT 1.5 for the withanolide A peak, *Standard solution A*

Relative standard deviation: NMT 2.0% for the withanolide A peak in replicate injections, *Standard solution A*

Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Ashwagandha Root Extract RS being used.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Ashwagandha Root Extract RS being used, identify the retention times of the peaks corresponding to withanolide aglycones and glycosides. The approximate relative retention times are provided in *Table 2*.

Table 2

Analyte	Relative Retention Time
Withanoside IV	0.70
Physagulin D	0.75
27-Hydroxywithanone	0.80
Withanoside V	0.89
Withanoside VI	0.89
Withaferin A	0.92
▲12-Deoxywithastramonolide▲ (USP 1-May-2019)	0.96
Withanolide A	1.00
Withanone	1.01
Withanolide B	1.14

Calculate the percentage of withanolide aglycones in the portion of Ashwagandha Root taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = sum of the peak areas of withaferin A, withanolide A, ▲12-deoxywithastramonolide,▲ (USP 1-May-2019) and withanolide B from the *Sample solution*

r_S = peak area of withanolide A from *Standard solution A*

C_S = concentration of USP Withanolide A RS in *Standard solution A* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Ashwagandha Root taken to prepare the *Sample solution* (mg)

Calculate the percentage of withanolide glycosides in the portion of Ashwagandha Root taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = sum of the peak areas of withanoside IV, withanoside V, and withanoside VI from the *Sample solution*

r_S = peak area of withanoside IV from *Standard solution A*

C_S = concentration of USP Withanoside IV RS in *Standard solution A* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Ashwagandha Root taken to prepare the *Sample solution* (mg)

Add the percentages of withanolide aglycones and withanolide glycosides.

Acceptance criteria: NLT 0.3% on the dried basis. [NOTE—Because of inherent variation, some of the withanolides mentioned in this test may be present in minor quantities or may be totally absent. The sample will be deemed compliant if the sum of the withanolides is NLT 0.3%.]

IMPURITIES

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Limits of Elemental Impurities*: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Pesticide Residue Analysis*: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Foreign Organic Matter*: NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Test for Aflatoxins*: Meets the requirements

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- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant gram-negative bacterial count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: Primary roots are not branched and are straight, conical, or finger-like in shape and variable in thickness with age; some carry a crown, consisting of a number of remains of stem base; the outer surface is buff to grayish yellow with longitudinal wrinkles; fracture is short and uneven; secondary roots are thin and fibrous.

Microscopic: Transverse section of root shows a narrow band of yellowish crumpled cork, moderate-size cortex, and a wide wood. The cork cells are rectangular, radially flattened, nonlignified, and filled with starch grains and reddish-brown content; cork cambium is 2–4 diffused rows of cells; secondary cortex is formed of 20–25 rows of thin-walled parenchymatous cells, filled with starch grains, and shows occasional microsphenoidal crystals of calcium oxalate; phloem consists of sieve tubes, companion cells, and phloem parenchyma; vascular cambium consists of tangentially elongated parenchymatous cells; vessels and tracheids are in radial rows toward the periphery of the wood; medullary rays are uniseriate to 2- to 3-seriate, and are filled with starch grains; scattered vessels in groups are embedded in the parenchyma; vessels have pitted and scalariform thickenings, and generally the end walls are perforated; a few fibers with thick lignified walls are also found scattered in the wood.

- **LOSS ON DRYING** ⟨731⟩

Sample: 1.0 g of finely powdered Ashwagandha Root
Analysis: Dry the *Sample* at 105° for 3 h.
Acceptance criteria: NMT 12.0%

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Total Ash*

Sample: 1.0 g of finely powdered Ashwagandha Root
Acceptance criteria: NMT 7.0%

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Acid-Insoluble Ash*: NMT 1.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Alcohol-Soluble Extractives, Method 2*: NLT 10.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.

Change to read:

- **LABELING:** The label states the Latin binomial and ▲the official article name.▲ (USP 1-May-2019)
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Powdered Ashwagandha Root Extract RS
USP β-Sitosterol RS
USP Withanolide A RS
USP Withanoside IV RS

Ashwagandha Root Dry Extract

DEFINITION

Ashwagandha Root Dry Extract is prepared from the dried mature root of *Withania somnifera* (L.) Dunal (Fam. Solanaceae), using methanol, alcohol, water, or mixtures of these solvents. It contains NLT 2.5% of withanolides, calculated on the dried basis as the sum of withanolide aglycones, calculated as withanolide A, and withanolide glycosides, calculated as withanoside IV. It may contain suitable added substances.

IDENTIFICATION

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 0.2 mg/mL each of USP β-Sitosterol RS and USP Withanolide A RS in methanol
Standard solution B: 20 mg/mL of USP Powdered Ashwagandha Root Extract RS in methanol. Sonicate for 10 min, centrifuge, and use the supernatant. [NOTE—Retain the supernatant for use in the test for *Content of Withanolides*.]
Sample solution: Suspend about 200 mg of Ashwagandha Root Dry Extract in 10 mL of methanol, and sonicate for 10 min. Centrifuge, and use the supernatant.
Chromatographic system
Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plate)[1]
Application volume: 2 µL each of *Standard solution A*, *Standard solution B*, and *Sample solution*, as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of 33%.
Temperature: Ambient, not to exceed 30°
Developing solvent system: Toluene, ethyl acetate, and glacial acetic acid (55:45:3)
Developing distance: 6 cm
Derivatization reagent: 20 mL of sulfuric acid combined with 180 mL of ice-cold methanol
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Apply the *Samples* as bands and dry in air. Develop in a saturated chamber and dry in air. Treat the plate with the *Derivatization reagent*, heat at 100° for 5 min, and examine under UV light at 365 nm and under white light.
System suitability: Under UV light at 365 nm, the derivatized chromatogram of *Standard solution A* displays in its lower third a blue band due to withanolide A and in its middle third a grayish-blue band due to β-sitosterol. The chromatogram of *Standard solution B* displays a light gray to whitish band due to withanone below the withanolide A band, and a faint light gray band above the β-sitosterol band; there is also a light gray band close to the solvent front. A reddish band slightly above the application line is due to withaferin A. Under white light, the bands due to β-sitosterol and withanolide A appear violet–gray. Additional faint bands may appear.
Acceptance criteria: Under UV light at 365 nm and under white light, the chromatogram of the *Sample solution* displays the bands similar in position and color to those seen in *Standard solution B*. Additional bands may be observed, in particular a band just above that due to withanolide A (light brown under UV light at 365 nm, dark brown under white light), and a thin band below that due to β-sitosterol (light blue under UV light at 365 nm, violet–gray under white light). Bands vary in intensity, and some of those seen in the chromatogram of *Standard*

[1] Suitable commercially available plates are HPTLC Silica Gel 60 F_{254} from EMD Millipore (e.g., Part #1.05642.0001).

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solution B may be very faint or absent from the *Sample solution*.

• **B. HPLC**

Analysis: Proceed as directed in the test for *Content of Withanolides*.

Acceptance criteria: The *Sample solution* shows main peaks at retention times corresponding to those of withanolide A and withanoside IV in *Standard solution A*. The *Sample solution* shows some of the withanolides listed in *Table 2*.

COMPOSITION

• **CONTENT OF WITHANOLIDES**

Solution A: Dissolve 0.14 g of potassium dihydrogen phosphate in 900 mL of water, add 0.5 mL of phosphoric acid, dilute with water to 1000 mL, and mix.

Solution B: Acetonitrile, filtered and degassed

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	95	5
18	55	45
25	20	80
28	20	80
30	95	5
40	95	5

Standard solution A: A composite solution containing 0.1 mg/mL of USP Withanolide A RS and 0.1 mg/mL of USP Withanoside IV RS in methanol, accurately calculated. Use gentle heat to aid dissolution.

Standard solution B: Dilute a portion of *Standard solution B* from *Identification A* with methanol (1:1), and mix well. Before injection, pass through a polyethersulfone membrane filter of 0.45-µm or finer pore size.

Sample solution: Transfer about 100 mg of Ashwagandha Root Dry Extract, accurately weighed, to a 10-mL volumetric flask, add about 7 mL of methanol, heat gently on a water bath for 20 min, cool, dilute with methanol to volume, and mix. Before injection, pass through a polyethersulfone membrane filter of 0.45-µm or finer pore size, discarding the first few milliliters of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 227 nm

Column: 4.6-mm × 25-cm, end-capped; 5-µm packing L1

Column temperature: 27°

Flow rate: 1.5 mL/min

Injection volume: 20 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Resolution: NLT 1.0 between the withanolide A and withanone peaks, and NLT 3.0 between the withaferin A peak and the peak corresponding to coeluting withanoside V and withanoside VI, *Standard solution B*

Tailing factor: NMT 1.5 for the withanolide A peak, *Standard solution A*

Relative standard deviation: NMT 2.0% for the withanolide A peak in replicate injections, *Standard solution A*

Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Ashwagandha Root Extract RS being used.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Withanolide A RS being used, identify the retention times of the peaks corresponding to withanolide aglycones and glycosides. The approximate relative retention times are provided in *Table 2*.

Table 2

Analyte	Relative Retention Time
Withanoside IV	0.70
Physagulin D	0.75
27-Hydroxywithanone	0.80
Withanoside V	0.89
Withanoside VI	0.89
Withaferin A	0.92
12-Deoxywithastramonolide	0.96
Withanolide A	1.00
Withanone	1.01
Withanolide B	1.14

Calculate the percentage of withanolide aglycones in the portion of Ashwagandha Root Dry Extract taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = sum of the peak areas of withaferin A, 12-deoxywithastramonolide, withanolide A, withanone and withanolide B from the *Sample solution*

r_S = peak area of withanolide A from *Standard solution A*

C_S = concentration of USP Withanoside IV RS in *Standard solution A* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of taken Ashwagandha Root Dry Extract to prepare the *Sample solution* (mg)

Calculate the percentage of withanolide glycosides in the portion of Ashwagandha Root Dry Extract taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = sum of the peak areas of withanoside IV, withanoside V, and withanoside VI from the *Sample solution*

r_S = peak area of withanoside IV from *Standard solution A*

C_S = concentration of USP Withanoside IV RS in *Standard solution A* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Ashwagandha Root Dry Extract taken to prepare the *Sample solution* (mg)

Add the percentages of withanolide aglycones and withanolide glycosides.

Acceptance criteria: NLT 2.5% on the dried basis.

[NOTE—Because of inherent variation, some of the withanolides mentioned in this test may be present in minor quantities or may be totally absent. The sample will

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be deemed compliant if the sum of the withanolides is NLT 2.5%.]

IMPURITIES

Change to read:

- **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020) : Meets the requirements
- **BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Residual Solvents*: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Test for Aflatoxins*: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS

- **LIMIT OF FLAVONOL GLYCOSIDES DERIVED FROM AERIAL PARTS**

Diluent: Methanol and water (1:1)
Solution A: Dissolve 0.14 g of potassium dihydrogen phosphate in 900 mL of water, add 0.5 mL of phosphoric acid, dilute with water to 1000 mL, and mix.
Solution B: Acetonitrile, filtered and degassed
Mobile phase: See *Table 3*.

Table 3

Time (min)	Solution A (%)	Solution B (%)
0	90	10
12	80	20
14	10	90
18	10	90
20	90	10
25	90	10

Standard solution A: 20 µg/mL of USP Kaempferol 3-*O*-Robinoside-7-*O*-Glucoside RS in *Diluent*. Mix well.
Standard solution B: 10 mg/mL of USP Ashwagandha Aerial Parts Dry Extract RS in *Diluent*. Mix well. Before injection, pass through a polyethylsulfone filter of 0.45-µm or finer pore size.
Sample solution: Transfer about 2.0 g of Ashwagandha Root Dry Extract into a suitable container, add 50 mL of *Diluent*, and sonicate for 30 min with intermittent shaking. Centrifuge to sediment the solids, decant, and save the supernatant. Repeat the procedure two more times with fresh 50-mL aliquots of *Diluent*. Combine the supernatants and reduce volume to less than 100 mL using a rotary evaporator. Transfer the resulting solution into a 100-mL volumetric flask, adjust with *Diluent* to volume, and mix well. Pass through a polyethylsulfone filter of 0.45-µm or finer pore size. [NOTE—For the extracts soluble in the *Diluent*, triplicate extraction is not recommended. Instead, accurately transfer the dissolved extract into a 100-mL volumetric flask, and adjust with *Diluent* to volume.]
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 350 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 25°
Flow rate: 1.5 mL/min
Injection volume: 20 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Column efficiency: NLT 5,000 for the kaempferol 3-*O*-robinoside-7-*O*-glucoside peak, *Standard solution A*
Tailing factor: NMT 1.5 for the kaempferol 3-*O*-robinoside-7-*O*-glucoside peak, *Standard solution A*
Relative standard deviation: NMT 2.0% for the kaempferol 3-*O*-robinoside-7-*O*-glucoside peak in replicate injections, *Standard solution A*
Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Ashwagandha Aerial Parts Dry Extract RS being used.
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Using the chromatogram of *Standard solution B* and the reference chromatogram supplied with the lot of USP Ashwagandha Aerial Parts Dry Extract RS being used, identify the retention times of the three flavonol glycosides indicative of aerial parts (see *Table 4*).

Table 4

Flavonol Glycoside	Relative Retention Time	Conversion Factor
Quercetin 3-*O*-robinoside-7-*O*-glucoside	0.86	1.25
Quercetin 3-*O*-rutinoside-7-*O*-glucoside	0.90	1.39
Kaempferol 3-*O*-robinoside-7-*O*-glucoside	1.00	1.00

Calculate the percentage of the three specified flavonol glycosides in the portion of Ashwagandha Root Dry Extract taken:

$$\text{Result} = C_S \times [\Sigma(r_i \times F_i)]/r_S \times (V/W) \times 100$$

C_S = concentration of kaempferol 3-*O*-robinoside-7-*O*-glucoside in *Standard solution A* (mg/mL)
r_i = peak area of each specified flavonol glycoside from the *Sample solution*
F_i = respective conversion factor for each specified flavonol glycoside in the *Sample solution*
r_S = peak area of kaempferol 3-*O*-robinoside-7-*O*-glucoside from *Standard solution A*
V = volume of the *Sample solution* (mL)
W = weight of Ashwagandha Root Dry Extract taken to prepare the *Sample solution* (mg)

Acceptance criteria: NMT 0.04% on the dried basis.

- **LOSS ON DRYING** ⟨731⟩

Sample: 2.0 g of Ashwagandha Root Dry Extract
Analysis: Dry the *Sample* at 105° for 3 h.
Acceptance criteria: NMT 6.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial and the official article name. It meets other labeling requirements under *Botanical Extracts* ⟨565⟩.

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• **USP REFERENCE STANDARDS** ⟨11⟩
USP Ashwagandha Aerial Parts Dry Extract RS
USP Powdered Ashwagandha Root Extract RS
USP Kaempferol 3-*O*-Robinoside-7-*O*-Glucoside RS
USP β-Sitosterol RS
USP Withanolide A RS
USP Withanoside IV RS

Change to read:

▲Ashwagandha Root Powder▲ (USP 1-May-2019)

DEFINITION

Change to read:

▲Ashwagandha Root Powder is the dried mature root of *Withania somnifera* (L.) Dunal (Fam. Solanaceae),▲ (USP 1-May-2019) reduced to a fine or very fine powder. It contains NLT 0.3% of withanolides, calculated on the dried basis as the sum of withanolide aglycones, calculated as withanolide A, and withanolide glycosides, calculated as withanoside IV.

IDENTIFICATION

Change to read:

• **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩
Standard solution A: 0.2 mg/mL each of USP β-Sitosterol RS and USP Withanolide A RS in methanol
Standard solution B: 20 mg/mL of USP Powdered Ashwagandha Root Extract RS in methanol. Sonicate for 10 min, centrifuge, and use the supernatant. [NOTE—Retain the supernatant for use in the test for *Content of Withanolides*.]
Sample solution: Suspend about 1 g of ▲Ashwagandha Root Powder▲ (USP 1-May-2019) in 10 mL of methanol, and sonicate for 15 min. Centrifuge, and use the supernatant.
Chromatographic system
Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plate)[1]
Application volume: 2 µL each of *Standard solution A* and *Standard solution B*, and 10 µL of the *Sample solution*, as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of 33%.
Temperature: Ambient, not to exceed 30°
Developing solvent system: Toluene, ethyl acetate, and glacial acetic acid (55:45:3)
Developing distance: 6 cm
Derivatization reagent: 20 mL of sulfuric acid combined with 180 mL of ice-cold methanol
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Apply the *Samples* as bands and dry in air. Develop in a saturated chamber and dry in air. Treat the plate with the *Derivatization reagent*, heat at 100° for 5 min, and examine under UV light at 365 nm and under white light.
System suitability: Under UV light at 365 nm, the derivatized chromatogram of *Standard solution A* displays in its lower third a blue band due to withanolide A and in its middle third a grayish-blue band due to β-sitosterol. The chromatogram of *Standard solution B* displays a light gray to whitish band due to withanone below the withanolide A band, and a faint light gray band above the β-sitosterol band; there is also a light gray band close to the solvent front. A reddish band slightly above the application line is due to withaferin A. Under white light, the bands due to β-sitosterol and withanolide A appear violet–gray. Additional faint bands may appear.
Acceptance criteria: Under UV light at 365 nm and under white light, the chromatogram of the *Sample solution* displays the bands similar in position and color to those seen in *Standard solution B*. Additional bands may be observed, in particular a band just above that due to withanolide A (light brown under UV light at 365 nm, dark brown under white light), and a thin band below that due to β-sitosterol (light blue under UV light at 365 nm, violet–gray under white light). Bands vary in intensity, and some of those seen in the chromatogram of *Standard solution B* may be very faint or absent from the *Sample solution*.

• **B. HPLC**
Analysis: Proceed as directed in the test for *Content of Withanolides*.
Acceptance criteria: The *Sample solution* shows main peaks at retention times corresponding to those of withanolide A and withanoside IV in *Standard solution A*. The *Sample solution* shows some of the withanolides listed in *Table 2*.

COMPOSITION

Change to read:

• **CONTENT OF WITHANOLIDES**
Solution A: Dissolve 0.14 g of potassium dihydrogen phosphate in 900 mL of water, add 0.5 mL of phosphoric acid, dilute with water to 1000 mL, and mix.
Solution B: Acetonitrile, filtered and degassed
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	95	5
18	55	45
25	20	80
28	20	80
30	95	5
40	95	5

Standard solution A: A composite solution containing 0.1 mg/mL of USP Withanolide A RS and 0.1 mg/mL of USP Withanoside IV RS in methanol, accurately calculated. Use gentle heat to aid dissolution.
Standard solution B: Dilute a portion of *Standard solution B* from *Identification A* with methanol (1:1), and mix well. Before injection, pass through a ▲polyethersulfone▲ (USP 1-May-2019) membrane filter of 0.45-µm or finer pore size.
Sample solution: Transfer about 5.00 g of accurately weighed ▲Ashwagandha Root Powder▲ (USP 1-May-2019) to a 250-mL round-bottom flask fitted with a reflux condenser. Add 50 mL of methanol, reflux on a water bath for 15 min, cool to room temperature, decant, and retain the solvent. Repeat until the solvent is colorless. Combine the retained

[1] Suitable commercially available plates are HPTLC Silica Gel 60 F_{254} from EMD Millipore (e.g., Part #1.05642.0001).

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solvents, filter, concentrate under vacuum to about 40 mL, transfer to a 50-mL volumetric flask, and adjust with methanol to volume. Before injection, pass through a ▲polyethersulfone▲ (USP 1-May-2019) filter of 0.45-µm or finer pore size, discarding the first few milliliters of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 227 nm

Column: 4.6-mm × 25-cm, end-capped; ▲5-µm▲ (USP 1-May-2019) packing L1

Column temperature: 27°

Flow rate: 1.5 mL/min

Injection volume: 20 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Resolution: NLT 1.0 between the withanolide A and withanone peaks, and NLT 3.0 between the withaferin A peak and the peak corresponding to coeluting withanoside V and withanoside VI, *Standard solution B*

Tailing factor: NMT 1.5 for the withanolide A peak, *Standard solution A*

Relative standard deviation: NMT 2.0% for the withanolide A peak in replicate injections, *Standard solution A*

Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Ashwagandha Root Extract RS being used.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Ashwagandha Root Extract RS being used, identify the retention times of the peaks corresponding to withanolide aglycones and glycosides. The approximate relative retention times are provided in *Table 2*.

Table 2

Analyte	Relative Retention Time
Withanoside IV	0.70
Physagulin D	0.75
27-Hydroxywithanone	0.80
Withanoside V	0.89
Withanoside VI	0.89
Withaferin A	0.92
▲12-Deoxywithastramonolide▲ (USP 1-May-2019)	0.96
Withanolide A	1.00
Withanone	1.01
Withanolide B	1.14

Calculate the percentage of withanolide aglycones in the portion of ▲Ashwagandha Root Powder▲ (USP 1-May-2019) taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = sum of the peak areas of withaferin A, ▲12-deoxywithastramonolide,▲ (USP 1-May-2019) withanolide A, withanone, and withanolide B from the *Sample solution*

r_S = peak area of withanolide A from *Standard solution A*

C_S = concentration of USP Withanolide A RS in *Standard solution A* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of ▲Ashwagandha Root Powder▲ (USP 1-May-2019) taken to prepare the *Sample solution* (mg)

Calculate the percentage of withanolide glycosides in the portion of ▲Ashwagandha Root Powder▲ (USP 1-May-2019) taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = sum of the peak areas of withanoside IV, withanoside V, and withanoside VI from the *Sample solution*

r_S = peak area of withanoside IV from *Standard solution A*

C_S = concentration of USP Withanoside IV RS in *Standard solution A* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of ▲Ashwagandha Root Powder▲ (USP 1-May-2019) taken to prepare the *Sample solution* (mg)

Add the percentages of withanolide aglycones and withanolide glycosides.

Acceptance criteria: NLT 0.3% on the dried basis. [NOTE—Because of inherent variation, some of the withanolides mentioned in this test may be present in minor quantities or may be totally absent. The sample will be deemed compliant if the sum of the withanolides is NLT 0.3%.]

IMPURITIES

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Limits of Elemental Impurities*: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Pesticide Residue Analysis*: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Test for Aflatoxins*: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant gram-negative bacteria count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures*, *Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: Dusty white or gray to light brown powder with a characteristic odor and a mucilaginous, bitter, acrid taste

Microscopic: Collapsed cork cells filled with starch grains and reddish-brown content; thin-walled cortex parenchyma cells filled with starch grains and occasional microsphenoidal crystals of calcium oxalate; vessels have pitted and scalariform thickenings, and generally with end walls perforated; a few fibers with thick lignified walls and simple pits; abundant starch grains, mostly simple, sometimes compound, spherical, reniform-oval with central hilum.

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Add the following:

▲• **LIMIT OF FLAVONOL GLYCOSIDES DERIVED FROM AERIAL PARTS**

Diluent: Methanol and water (1:1)

Solution A: Dissolve 0.14 g of potassium dihydrogen phosphate in 900 mL of water, add 0.5 mL of phosphoric acid, dilute with water to 1000 mL, and mix.

Solution B: Acetonitrile, filtered and degassed

Mobile phase: See *Table 3*.

Table 3

Time (min)	Solution A (%)	Solution B (%)
0	90	10
12	80	20
14	10	90
18	10	90
20	90	10
25	90	10

Standard solution A: 20 µg/mL of USP Kaempferol 3-*O*-Robinoside-7-*O*-Glucoside RS in *Diluent*. Mix well.

Standard solution B: 10 mg/mL of USP Ashwagandha Aerial Parts Dry Extract RS in *Diluent*. Mix well. Before injection, pass through a polyethylsulfone filter of 0.45-µm or finer pore size.

Sample solution: Transfer about 2.0 g of Ashwagandha Root Powder into a suitable container. Add 50 mL of *Diluent*, sonicate for 30 min with intermittent shaking, and reflux for 10 min. Centrifuge to sediment the solids, decant, and save the supernatant. Repeat the procedure two more times with fresh 50-mL aliquots of the *Diluent*. Combine the supernatants and reduce volume to less than 100 mL using a rotary evaporator. Transfer the resulting solution into a 100-mL volumetric flask, adjust with *Diluent* to volume, and mix well. Pass through a polyethylsulfone filter of 0.45-µm or finer pore size.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 350 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 25°

Flow rate: 1.5 mL/min

Injection volume: 20 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Column efficiency: NLT 5,000 for the kaempferol 3-*O*-robinoside-7-*O*-glucoside peak, *Standard solution A*

Tailing factor: NMT 1.5 for the kaempferol 3-*O*-robinoside-7-*O*-glucoside peak, *Standard solution A*

Relative standard deviation: NMT 2.0% for the kaempferol 3-*O*-robinoside-7-*O*-glucoside peak in replicate injections, *Standard solution A*

Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Ashwagandha Aerial Parts Dry Extract RS being used.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatogram of *Standard solution B* and the reference chromatogram supplied with the lot of USP Ashwagandha Aerial Parts Dry Extract RS being used, identify the retention times of the three flavonol glycosides indicative of aerial parts (see *Table 4*).

Table 4

Flavonol Glycoside	Relative Retention Time	Conversion Factor
Quercetin 3-*O*-robinoside-7-*O*-glucoside	0.86	1.25
Quercetin 3-*O*-rutinoside-7-*O*-glucoside	0.90	1.39
Kaempferol 3-*O*-robinoside-7-*O*-glucoside	1.00	1.00

Calculate the percentage of the three specified flavonol glycosides in the portion of Ashwagandha Root Powder taken:

$$\text{Result} = C_S \times [\Sigma(r_i \times F_i)]/r_S \times (V/W) \times 100$$

C_S = concentration of kaempferol 3-*O*-robinoside-7-*O*-glucoside in *Standard solution A* (mg/mL)

r_i = peak area of each specified flavonol glycoside from the *Sample solution*

F_i = respective conversion factor for each specified flavonol glycoside in the *Sample solution*

r_S = peak area of kaempferol 3-*O*-robinoside-7-*O*-glucoside from *Standard solution A*

V = volume of the *Sample solution* (mL)

W = weight of Ashwagandha Root Powder taken to prepare the *Sample solution* (mg)

Acceptance criteria: NMT 0.01% on the dried basis. ▲ (USP 1-May-2019)

Change to read:

• **LOSS ON DRYING** ⟨731⟩

Sample: 1.0 g of ▲Ashwagandha Root Powder▲ (USP 1-May-2019)

Analysis: Dry the *Sample* at 105° for 3 h.

Acceptance criteria: NMT 12.0%

Change to read:

• **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Total Ash*

Sample: 1.0 g of ▲Ashwagandha Root Powder▲ (USP 1-May-2019)

Acceptance criteria: NMT 7.0%

• **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Acid-Insoluble Ash*: NMT 1.0%

• **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Alcohol-Soluble Extractives, Method 2*: NLT 10.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.

Change to read:

• **LABELING:** The label states the Latin binomial and ▲the official article name.▲ (USP 1-May-2019)

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Change to read:

- **USP REFERENCE STANDARDS** ⟨11⟩
  - ▲USP Ashwagandha Aerial Parts Dry Extract RS▲ (USP 1-May-2019)
  - USP Powdered Ashwagandha Root Extract RS
  - ▲USP Kaempferol 3-*O*-Robinoside-7-*O*-Glucoside RS▲ (USP 1-May-2019)
  - USP β-Sitosterol RS
  - USP Withanolide A RS
  - USP Withanoside IV RS

Asian Ginseng

DEFINITION

Asian Ginseng consists of the dried roots of *Panax ginseng* C.A. Mey. (Fam. Araliaceae). It contains NLT 0.2% of ginsenoside Rg_1 and NLT 0.1% of ginsenoside Rb_1, both calculated on the dried basis.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHY**

Standard solution: 5 mg/mL each of arbutin and escin, in methanol

Sample solution: 1.0 g of finely powdered Asian Ginseng in a 25-mL flask fitted with a reflux condenser. Add 10.0 mL of a mixture of methanol and water (7:3), and heat under reflux for 15 min. Cool, filter, and dilute the filtrate with methanol to 10.0 mL.

Adsorbent: 0.25-mm layer of chromatographic silica gel, typically 20 cm long (TLC plates)

Application volume: 20 µL, as bands

Developing solvent system: The upper layer of a mixture of butyl alcohol, ethyl acetate, and water (10: 2.5: 5) in an unsaturated chamber

Spray reagent: Dissolve 0.5 mL of anisaldehyde in 10 mL of glacial acetic acid, add 85 mL of methanol, mix, and carefully add 5 mL of sulfuric acid, and mix.

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the chromatograms until the solvent front has moved up about three-fourths of the length of the plate. Remove the plate from the chamber, mark the solvent front, and allow the plate to dry. Spray with *Spray reagent*. Heat the plate at 105°–110° for 10 min, and examine the plate under white light.

System suitability: The *Standard solution* chromatogram shows, in the upper third, a brown zone corresponding to arbutin, and in the lower third, a gray zone corresponding to escin.

Acceptance criteria: The *Sample solution* exhibits violet-gray zones corresponding to ginsenoside Rg_1 in the upper portion and to ginsenoside Re in the middle and in between the zones corresponding to arbutin and escin in the *Standard solution*. A violet-gray zone corresponding to ginsenoside Rb_1 is located at the same R_F value as the gray zone corresponding to escin in the *Standard solution*. Other, less intense bands may be observed between the zones due to ginsenosides Rb_1 and Re, and the zone closest to the origin corresponds to ginsenoside Rc. Other spots may be visible in the lower third of the chromatogram.

- **B.** The retention times of the peaks for ginsenosides Rg_1, Re, Rf, Rb_1, Rc, and Rd in the *Sample solution* chromatogram correspond to those in the *Standard solution*, as obtained in the test for *Content of Ginsenosides Rb_1 and Rg_1*. The ratio of the peak area for ginsenoside Rb_2 to the peak area for ginsenoside Rb_1 is NLT 0.4 (differentiation from American ginseng).

COMPOSITION

- **CONTENT OF GINSENOSIDES Rb_1 AND Rg_1**

Solution A: Water

Solution B: Acetonitrile and water (4:1)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	76	24
12	76	24
28	65	35
51.5	56.5	43.5
52.5	0	100
64.5	76	24
77	76	24

Diluent: Alcohol and water (4:6)

Standard solution: Transfer a quantity of USP Powdered Asian Ginseng Extract RS, equivalent to 2 mg of ginsenoside Rg_1, to a suitable container, and dissolve in 10.0 mL of *Diluent*. [NOTE—The concentrations of ginsenoside Rg_1 and ginsenoside Rb_1 in this solution are not expected to be equal and are determined on the basis of the labeled quantities present in USP Powdered Asian Ginseng Extract RS.]

Sample solution: Reduce 100 g of Asian Ginseng to a powder, and transfer about 1.0 g of the powder, accurately weighed, to a 100-mL, round-bottom flask fitted with a reflux condenser. Add 50 mL of *Diluent* and a few grains of pumice, and boil on a water bath under reflux for 1 h. Cool, and filter. Wash the flask and the residue with 20 mL of *Diluent*, and pass through the same filter. Combine the filtrates, and evaporate in a rotary evaporator at 50° to dryness. Dissolve the residue in 10.0 mL of *Diluent*.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 203 nm

Analytical column: 4.6-mm × 15-cm; 3-µm packing L1

Guard column: 4.6-mm × 2.0-cm; packing L1

Column temperature: 25°

Flow rate: 1.5 mL/min

Injection volume: 10 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Chromatogram similarity: The chromatogram is similar to the reference chromatogram provided with the lot of USP Powdered Asian Ginseng Extract RS being used.

Relative standard deviation: NMT 2.0%, determined for the sum of the peak areas for the 6 major ginsenosides, in replicate injections

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentages of ginsenosides Rb_1 and Rg_1 in the portion of Asian Ginseng taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak response of ginsenoside Rg_1 or ginsenoside Rb_1 from the *Sample solution*

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r_S = peak response of ginsenoside Rg_1 or ginsenoside Rb_1 from the *Standard solution*
C_S = concentration of ginsenoside Rg_1 or ginsenoside Rb_1 in the *Standard solution* (mg/mL)
V = final volume of the *Sample solution* (mL)
W = weight of Asian Ginseng taken to prepare the *Sample solution* (mg)

Acceptance criteria
Ginsenoside Rg_1: NLT 0.2% on the dried basis
Ginsenoside Rb_1: NLT 0.1% on the dried basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^4 cfu/g. The total combined molds and yeasts count does not exceed 10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli.*

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: Fusiform or cylindrical roots, with distinct aromatic odor, sometimes branched, typically 1–10 cm, sometimes up to 20 cm in length and up to 2.5 cm in diameter at the crown, with one or more stem scars. Externally pale yellow to golden, rough textured in the lower part, with prominent horizontal rings and fine longitudinal ridges as a result of drying. Root scars or fine rootlets are present. Fractures are short, with the fractured surface, white to ivory, exposing a ring of secretory canals present in secondary phloem.

Microscopic: Transverse section of root presents multiple layers of thin-walled cork cells. Secondary phloem characterized by conspicuous air lacunae, abundant starch-containing storage parenchyma, few sieve elements, and rings of schizogenous secretory canals. Xylem characterized by abundant starch-containing storage parenchyma, few tracheary elements, and a lack of secretory canals. Druse crystals are sometimes present with vascular parenchyma cells.

- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩**:** NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Alcohol-Soluble Extractives, Method 2* ⟨561⟩**:** NLT 14.0%
- **LOSS ON DRYING** ⟨731⟩

Sample: 1.0 g of Asian Ginseng, finely powdered
Analysis: Dry the *Sample* at 105° for 2 h.
Acceptance criteria: NMT 12.0%

- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩

Sample: 1.0 g of Asian Ginseng, finely powdered
Acceptance criteria: NMT 8.0%

- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store in a cool, dry place.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Powdered Asian Ginseng Extract RS

Powdered Asian Ginseng

DEFINITION

Powdered Asian Ginseng is Asian Ginseng reduced to a fine or very fine powder. It contains NLT 0.2% of ginsenoside Rg_1 and NLT 0.1% of ginsenoside Rb_1, both calculated on the dried basis.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHY**

Standard solution: 5 mg/mL each of arbutin and escin, in methanol
Sample solution: 1.0 g of Powdered Asian Ginseng in a 25-mL flask fitted with a reflux condenser. Add 10.0 mL of a mixture of methanol and water (7:3), and heat under reflux for 15 min. Cool, filter, and dilute the filtrate with methanol to 10.0 mL.
Adsorbent: 0.25-mm layer of chromatographic silica gel, typically 20 cm long (TLC plates)
Application volume: 20 µL, as bands
Developing solvent system: The upper layer of a mixture of butyl alcohol, ethyl acetate, and water (10: 2.5: 5) in an unsaturated chamber
Spray reagent: Dissolve 0.5 mL of anisaldehyde in 10 mL of glacial acetic acid, and add 85 mL of methanol, mix, and carefully add 5 mL of sulfuric acid to this mixture.
Analysis
Samples: *Standard solution* and *Sample solution*
Develop the chromatograms until the solvent front has moved up about three-fourths of the length of the plate. Remove the plate from the chamber, mark the solvent front, and allow the plate to dry. Spray with *Spray reagent.* Heat the plate at 105°–110° for 10 min, and examine the plate under white light.
System suitability: The *Standard solution* chromatogram shows, in the upper third, a brown zone corresponding to arbutin, and in the lower third, a gray zone corresponding to escin.
Acceptance criteria: The *Sample solution* exhibits violet-gray zones corresponding to ginsenoside Rg_1 in the upper portion and to ginsenoside Re in the middle and in between the zones corresponding to arbutin and escin in the *Standard solution.* A violet-gray zone corresponding to ginsenoside Rb_1 is located at the same R_F value as the gray zone corresponding to escin in the *Standard solution.* Other, less intense bands may be observed between the zones due to ginsenosides Rb_1 and Re, and the zone closest to the origin corresponds to ginsenoside Rc. Other spots may be visible in the lower third of the chromatogram.

- **B.** The retention times of the peaks for ginsenosides Rg_1, Re, Rf, Rb_1, Rc, and Rd in the *Sample solution* chromatogram correspond to those in the *Standard solution,* as obtained in the test for *Content of Ginsenosides Rb_1 and Rg_1.* The ratio of the peak area for ginsenoside Rb_2 to the peak area for ginsenoside Rb_1 is NLT 0.4 (differentiation from American ginseng).

COMPOSITION

- **CONTENT OF GINSENOSIDES Rb_1 AND Rg_1**

Solution A: Water
Solution B: Acetonitrile and water (4:1)
Mobile phase: See *Table 1.*

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	76	24

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Table 1 *(continued)*

Time (min)	Solution A (%)	Solution B (%)
12	76	24
28	65	35
51.5	56.5	43.5
52.5	0	100
64.5	76	24
77	76	24

Diluent: Alcohol and water (4:6)

Standard solution: Transfer a quantity of USP Powdered Asian Ginseng Extract RS, equivalent to 2 mg of ginsenoside Rg_1, to a suitable container, and dissolve in 10.0 mL of *Diluent*. [NOTE—The concentrations of ginsenoside Rg_1 and ginsenoside Rb_1 in this solution are not expected to be equal and are determined on the basis of the labeled quantities present in USP Powdered Asian Ginseng Extract RS.]

Sample solution: Transfer about 1.0 g of Powdered Asian Ginseng, accurately weighed, to a 100-mL, round-bottom flask fitted with a reflux condenser. Add 50 mL of a mixture of *Diluent* and a few grains of pumice, and boil on a water bath under reflux for 1 h. Cool, and filter. Wash the flask and the residue with 20 mL of *Diluent*, and pass through the same filter. Combine the filtrates, and evaporate in a rotary evaporator at 50° to dryness. Dissolve the residue in 10.0 mL of *Diluent*.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 203 nm
Analytical column: 4.6-mm × 15-cm; 3-µm packing L1
Guard column: 4.6-mm × 2.0-cm; packing L1
Column temperature: 25°
Flow rate: 1.5 mL/min
Injection volume: 10 µL

System suitability
Sample: *Standard solution*
Suitability requirements
Chromatogram similarity: The chromatogram is similar to the reference chromatogram provided with the lot of USP Powdered Asian Ginseng Extract RS being used.
Relative standard deviation: NMT 2.0%, determined for the sum of the peak areas for the 6 major ginsenosides, in replicate injections

Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentages of ginsenosides Rb_1 and Rg_1 in the portion of Powdered Asian Ginseng taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak response of ginsenoside Rg_1 or ginsenoside Rb_1 from the *Sample solution*
r_S = peak response of ginsenoside Rg_1 or ginsenoside Rb_1 from the *Standard solution*
C_S = concentration of ginsenoside Rg_1 or ginsenoside Rb_1 in the *Standard solution* (mg/mL)
V = final volume of the *Sample solution* (mL)
W = weight of Powdered Asian Ginseng used to prepare the *Sample solution* (mg)

Acceptance criteria
Ginsenoside Rg_1: NLT 0.2% on the dried basis
Ginsenoside Rb_1: NLT 0.1% on the dried basis

CONTAMINANTS
- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^4 cfu/g. The total combined molds and yeasts count does not exceed 10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS
- **BOTANICAL CHARACTERISTICS:** Pale yellowish-brown powder with a slightly aromatic odor. Under a microscope, the powder shows traces of cork composed of thin-walled polygonal cells but mainly with phelloderm on the outside; wide cortex of parenchymatous cells with numerous secretory canals arranged in concentric zones; parenchymatous xylem with nonlignified tracheids and slightly lignified vessels with spiral and reticulate thickening, isolated or in small groups; small granules of starch 0.5–1.0 µm in diameter in all of the parenchymatous cells; and occasional cluster crystals of calcium oxalate in the cells of the central region.
- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩**:** NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Alcohol-Soluble Extractives, Method 2* ⟨561⟩**:** NLT 14.0%
- **LOSS ON DRYING** ⟨731⟩
  Sample: 1.0 g of Powdered Asian Ginseng
  Analysis: Dry the *Sample* at 105° for 2 h.
  Acceptance criteria: NMT 12.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩
  Sample: 1.0 g of Powdered Asian Ginseng
  Acceptance criteria: NMT 8.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 1.0%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store in a cool, dry place.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant source from which the article was derived.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Powdered Asian Ginseng Extract RS

Powdered Asian Ginseng Extract

DEFINITION
Powdered Asian Ginseng Extract is prepared from Asian Ginseng by maceration, percolation, or both processes performed at room temperature with suitable solvents such as alcohol, methanol, water, or mixtures of these solvents, and by concentrating the fluidextract at temperatures below 50°. The ratio of the starting crude plant material to Powdered Asian Ginseng Extract is between 3:1 and 7:1. It contains NLT 3.0% of ginsenosides Rg_1, Re, Rb_1, Rc, Rb_2, and Rd combined, calculated on the anhydrous basis. It may contain other added substances.

USP Monographs

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IDENTIFICATION

• **A. Thin-Layer Chromatographic Identification Test**

Extraction column: Use a solid-phase extraction column that contains C18 packing with 55- to 105-µm particle size and a ratio of sorbent mass to column volume of 360 mg/0.85 mL, or equivalent. Condition the column before use by washing with 3 mL of methanol and 8 mL of water.

Standard solution: Transfer about 0.1 g of USP Powdered Asian Ginseng Extract RS to a 5-mL volumetric flask, and proceed as directed for the *Sample solution*, beginning with "Dissolve in water".

Sample solution: About 1.0 g of Powdered Asian Ginseng Extract in a 25-mL volumetric flask. Dissolve in water, sonicating if necessary. Dilute with water to volume. Transfer 4.0 mL of this solution to the *Extraction column*, wash with 10 mL of water, and discard the eluate. Elute the column with 2 mL of methanol. [NOTE—Do not use vacuum, elute manually and slowly.] Collect the eluate in a suitable vial.

Adsorbent: 0.2-mm layer of chromatographic silica gel mixture on a high-performance thin-layer plate

Application volume: 10 µL, as bands

Developing solvent system: Chloroform, methanol, and water (65:35:10). Use the lower phase.

Spray reagent: Alcohol, acetic anhydride, and sulfuric acid (18:1:1)

Analysis

Samples: *Standard solution* and *Sample solution*

Saturate the chamber with *Developing solvent system* for 2 h. Develop the chromatograms until the solvent front has moved up about three-fourths of the length of the plate. Remove the plate from the chamber, mark the solvent front, and allow the plate to dry. Spray with *Spray reagent*, and heat in an oven at 105° for 10 min. Immediately examine the plate in white light.

Acceptance criteria: The *Sample solution* exhibits, among other spots, eight brown-violet spots at the R_F values of about 0.70, 0.60, 0.50, 0.36, 0.30, 0.28, 0.20, and 0.18, corresponding in color and R_F values to those obtained for the *Standard solution*.

• **B.** Add 2 mL of glacial acetic acid to 0.1 g of Powdered Asian Ginseng Extract, warm for 5 min in a hot water bath, and filter. Gently add 0.5 mL of sulfuric acid to 1.0 mL of the filtrate.

Acceptance criteria: A red-brown color develops at the zone of contact.

• **C.** The retention times of the peaks for ginsenosides Rg_1, Re, Rf, Rb_1, Rb_2, Rc, and Rd in the *Sample solution* chromatogram correspond to those in the *Standard solution*, as obtained in the test for *Content of Ginsenosides*. The ratio of the peak area of Rb_2 to the peak area of Rb_1 is NLT 0.4 (differentiation from American Ginseng).

COMPOSITION

• **Content of Ginsenosides**

Solution A: Water

Solution B: Acetonitrile and water (4:1)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	76	24
12	76	24
28	65	35
51.5	56.5	43.5
52.5	0	100
64.5	76	24
77	76	24

Diluent: Alcohol and water (4:6)

Standard solution: 24 mg/mL of USP Powdered Asian Ginseng Extract RS in *Diluent*. Dissolve by sonicating for 10 min, mix, and filter.

Sample solution: Proceed as directed for *Standard solution*, except use Powdered Asian Ginseng Extract.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 203 nm

Analytical column: 4.6-mm × 15-cm; 3-µm packing L1

Guard column: 4.6-mm × 2.0-cm; packing L1

Column temperature: 25°

Flow rate: 1.5 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Chromatogram similarity: The chromatogram is similar to the Reference Chromatogram provided with the lot of USP Powdered Asian Ginseng Extract RS being used.

Relative standard deviation: NMT 2.0%, determined for the sum of the peak areas for the 6 major ginsenosides, in replicate injections

Analysis

Samples: *Standard solution* and *Sample solution*

Identify the peaks for the ginsenosides by comparison with the Reference Chromatogram provided with the lot of USP Powdered Asian Ginseng Extract RS being used, and measure the peak areas for the 6 major ginsenosides.

Calculate the percentage of each relevant ginsenoside (Rg_1, Re, Rb_1, Rc, Rb_2, and Rd) in the portion of Powdered Asian Ginseng Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times P$$

r_U = peak area for each relevant ginsenoside from the *Sample solution*

r_S = peak area for each relevant ginsenoside from the *Standard solution*

C_S = concentration of USP Powdered Asian Ginseng Extract RS in the *Standard solution* (mg/mL)

C_U = concentration of Powdered Asian Ginseng Extract in the *Sample solution* (mg/mL)

P = labeled amount, in percentage, of each relevant ginsenoside in the USP Powdered Asian Ginseng Extract RS

Calculate the percentage of ginsenosides by adding the percentages of each relevant ginsenoside.

Acceptance criteria: NLT 3.0% on the anhydrous basis

CONTAMINANTS

Change to read:

• **▲Botanical Extracts** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020)

: Meets the requirements

• **Microbial Enumeration Tests** ⟨2021⟩**:** The total aerobic microbial count does not exceed 300 cfu/g. The total

DS Monographs

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combined molds and yeasts count does not exceed 100 cfu/g.

- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩: It meets the requirements of the tests for absence of *Salmonella* species, *Escherichia coli*, and *Staphylococcus aureus*.

SPECIFIC TESTS

- **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 7.0%, determined on a 0.15-g specimen
- **ALCOHOL DETERMINATION,** *Method II* ⟨611⟩: NMT 0.25%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Meets the requirements in *Botanical Extracts* ⟨565⟩
- **LABELING:** Meets the requirements in *Botanical Extracts* ⟨565⟩
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Powdered Asian Ginseng Extract RS

Asian Ginseng Tablets

DEFINITION

Asian Ginseng Tablets are prepared from Powdered Asian Ginseng Extract. They contain NLT 90.0% and NMT 110.0% of Powdered Extract, calculated as the sum of ginsenosides Rg_1, Re, Rb_1, Rc, Rb_2, and Rd.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩

Standard solution: 5 mg/mL each of arbutin and escin, in methanol

Sample solution: Transfer the equivalent of 100 mg of Powdered Extract from powdered Tablets to a conical flask, and extract three times, each with a 20-mL portion of a mixture of methanol and water (4:1), in a 55° bath for 30 min, stirring with a magnetic stirrer. Evaporate the combined extracts to dryness in vacuum between 45° and 50°, and dissolve the residue in 10 mL of a mixture of methanol and water (3:2).

Application volume: 20 µL, as bands

Developing solvent system: The upper layer of a mixture of butyl alcohol, ethyl acetate, and water (4:1:2) in an unsaturated chamber

Spray reagent: 0.5 mL of anisaldehyde in 10 mL of glacial acetic acid. Add 85 mL of methanol, carefully add 5 mL of sulfuric acid, and mix.

Analysis

Samples: *Standard solution* and *Sample solution*

Proceed as directed in the chapter. Remove the plate from the developing chamber, and allow it to dry. Spray with *Spray reagent*. Heat the plate at 105°–110° for 10 min, and examine the plate.

Acceptance criteria: The chromatogram of the *Standard solution* shows, in the upper third, a brown zone corresponding to arbutin and, in the lower third, a gray zone corresponding to escin. Between these two zones, the chromatogram of the *Sample solution* exhibits violet-gray zones corresponding to ginsenoside Rg_1 in the upper portion and to ginsenoside Re in the middle. A violet-gray zone corresponding to ginsenoside Rb_1 is located at the same R_F value as the gray zone corresponding to escin in the chromatogram of the *Standard solution*. Other, less intense bands may be observed between the zones due to ginsenosides Rb_1 and Re, and the zone closest to the origin corresponds to ginsenoside Rc. Other spots may be visible in the lower third of the chromatogram.

- **B.** The retention times of the relevant analytes of the *Sample solution* correspond to those of the *Standard solution*, as obtained in the test for *Content of Ginsenosides*. The retention time of the peak for ginsenoside Rf of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Ginsenosides*.

STRENGTH

- **CONTENT OF GINSENOSIDES**

Diluent: Water and alcohol (3:2)

Solution A: Water

Solution B: Acetonitrile and water (4:1)

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	76	24
12	76	24
28	65	35
51.5	56.5	43.5
52.5	0	100
64.5	76	24
77	76	24

Standard solution: 40 mg/mL of USP Powdered Asian Ginseng Extract RS in *Diluent*. Filter.

Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer a quantity of the powder, equivalent to 200 mg of Powdered Extract to a conical flask, and extract three times, each with a 20-mL portion of a mixture of methanol and water (4:1), in a 55° bath for 30 min, stirring with a magnetic stirrer. Evaporate the combined extracts to dryness in a vacuum between 45° and 50°. Dissolve the residue in 5.0 mL of *Diluent*, and filter.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 203 nm

Column

Guard: 4.6-mm × 2.0-cm; packing L1

Analytical: 4.6-mm × 15-cm; 3-µm packing L1

Column temperature: 25°

Flow rate: 1.5 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Chromatogram similarity: The *Standard solution* chromatogram is similar to the Reference Chromatogram provided with the lot of USP Powdered Asian Ginseng Extract RS being used.

Relative standard deviation: NMT 2.0%, determined for the sum of the peak areas for the six major ginsenosides, in repeated injections

Analysis

Samples: *Standard solution* and *Sample solution*

Record the chromatograms, identify the peaks for the ginsenosides by comparison with the Reference Chromatogram provided with the lot of USP Powdered Asian Ginseng Extract RS being used, and measure the peak areas for the six major ginsenosides.

Calculate the quantity, in mg, of each relevant ginsenoside (Rg_1, Re, Rb_1, Rc, Rb_2, and Rd) in the portion of Tablets taken:

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$$\text{Result} = 0.05 \times (r_U/r_S) \times C_S \times P$$

r_U = peak areas for each relevant ginsenoside from the *Sample solution*
r_S = peak areas for each relevant ginsenoside from the *Standard solution*
C_S = concentration of USP Powdered Asian Ginseng Extract RS in the *Standard solution* (mg/mL)
P = labeled amount, in percentage, of each relevant ginsenoside in the USP Powdered Asian Ginseng Extract RS lot being used

Calculate the content of total ginsenosides, T, in mg, by adding the amounts of individual ginsenoside.
Calculate the percentage of Powdered Extract with respect to the label claim:

$$\text{Result} = T \times (A_{WT}/W) \times (100/L_E) \times (100/L)$$

T = content of total ginsenosides in the portion of Tablets taken (mg)
A_{WT} = average weight of Tablets (mg/Tablet)
W = weight of the portion of Tablets taken (mg)
L_E = content of total ginsenosides in 100 mg of the Extract used to prepare the Tablets (mg)
L = amount of Extract per Tablet according to label claim (mg/Tablet)

Acceptance criteria: 90.0%–110.0% of Powdered Extract, calculated as the sum of ginsenosides Rg_1, Re, Rb_1, Rc, Rb_2, and Rd

PERFORMANCE TESTS
- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** ⟨2040⟩**:** Meet the requirements for *Disintegration*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩**:** Meet the requirements

CONTAMINANTS
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 1000 cfu/g. Tablets meet the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light.
- **LABELING:** The label states the Latin binomial and, following the official name, the article from which the Tablets were prepared. The label also indicates the amount of Powdered Extract, in mg/Tablet, and the content, in mg, of ginsenosides per 100 mg of Powdered Extract.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Powdered Asian Ginseng Extract RS

Aspartic Acid—*see Aspartic Acid General Monographs*

Astaxanthin Esters

Astaxanthin esters;
Astaxanthin fatty acid esters;
Fatty acid esters of (3*S*,3′*S*)-3,3′-dihydroxy-β,β-carotene-4,4′-dione.

DEFINITION
Astaxanthin Esters is obtained by extraction with either supercritical carbon dioxide or acetone from cultures of *Haematococcus pluvialis*. It consists mainly of 3*S*,3′*S* stereoisomers of astaxanthin in the monoester, diester, and free forms. The monoester form is the most abundant, followed by the diester form. The free form is a minor component. Suitable antioxidants may be added. It contains NLT 5% of total astaxanthin, calculated as free astaxanthin on the anhydrous basis.

IDENTIFICATION
- **A. THIN-LAYER CHROMATOGRAPHY**
  Standard solution: 10 mg/mL of USP Astaxanthin Esters from *Haematococcus pluvialis* RS in acetone
  Sample solution: 10 mg/mL of Astaxanthin Esters in acetone
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *General Procedures, Thin-Layer Chromatography*.)
  Adsorbent: 0.25-mm layer of chromatographic silica gel mixture. Dry the adsorbent at 110° for 1 h before use.
  Application volume: 5 µL
  Developing solvent system: Hexane and acetone (70:30)
  System suitability
  Sample: *Standard solution*
  Suitability requirements: The chromatogram of the *Standard solution* exhibits three clearly separated zones, with astaxanthin diester having the highest R_F value, followed by astaxanthin monoester (the most intense) and free astaxanthin (the least intense).
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Develop the chromatogram in the *Developing solvent system* until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the chamber, and dry in a current of air. Examine the plates under white light.
  Acceptance criteria: The *Sample solution* exhibits three main zones corresponding in R_F value to those obtained from the *Standard solution*. The zone in the middle (monoester) is the most intense, and the zone with the lower R_F is the least intense.
- **B. HPLC:** The *Sample solution* exhibits three major peaks with the retention times corresponding to those of 13-*cis*-astaxanthin, *all-trans*-astaxanthin, and 9-*cis*-astaxanthin peaks in the *Standard solution*, as obtained in the *Assay* for *Content of Total Astaxanthin*.

ASSAY

Change to read:

- **CONTENT OF TOTAL ASTAXANTHIN**
  [NOTE—Astaxanthin determined by this method is total astaxanthin, including the free astaxanthin, the monoester, and the diester.]
  Buffer solution: Dissolve 6.06 g of tris(hydroxymethyl) aminomethane in 750 mL of water, adjust with 1 N hydrochloric acid to a pH of 7.0, and dilute with water to 1000 mL.
  Cholesterol esterase solution: 4 units/mL of cholesterol esterase[1] in *Buffer solution*. Prepare fresh daily.
  Solution A: Methanol
  Solution B: *t*-Butylmethylether

[1] Use Wako Pure Chemicals catalog #037-11221, available from www.wakousa.com; Sigma catalog #C9281, available from www.sigmaaldrich.com; or equivalent.

DS Monographs

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Solution C: Phosphoric acid, 1% aqueous
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)	Solution C (%)
0	81	15	4
15	66	30	4
23	16	80	4
27	16	80	4
27.1	81	15	4
35	81	15	4

Internal standard solution: 37.5 µg/mL of USP Apocarotenal RS in acetone
Standard stock solution: Transfer 30 mg of USP Astaxanthin Esters from *Haematococcus pluvialis* RS to a 100-mL volumetric flask. Dissolve in 30 mL of acetone, shake by mechanical means, and dilute with acetone to volume.
Standard solution: Combine 2.0 mL of the *Standard stock solution* and 1.0 mL of the *Internal standard solution* in a glass centrifuge tube. Add 3.0 mL of *Cholesterol esterase solution* to the tube, and mix gently by inversion. Place the tube in a block heater set to 37°, and allow the reaction to continue for 45 min, gently and slowly inverting the tube every 10 min. After 45 min, add 1 g of sodium sulfate and 2 mL of petroleum ether to the tube. Mix on a vortex mixer for 30 s, and then centrifuge at 3000 rpm for 3 min. Carefully transfer the petroleum ether layer to a 10-mL glass centrifuge tube containing 1 g of anhydrous sodium sulfate. Be careful to avoid pipetting the intermediate emulsive layer. Evaporate the petroleum ether layer using a vacuum or a stream of inert gas at room temperature. Add 3 mL of acetone, sonicate, and filter the mixture. The filtered solution is the *Standard solution*.
Sample stock solution: Warm a quantity of the sample in a water bath at 50°–60° for 30 min. Shake the sample well at 10-min intervals. After 30 min, transfer 30 mg of the sample to a 100-mL volumetric flask. Dissolve in 30 mL of acetone, shake by mechanical means, and dilute with acetone to volume.
Sample solution: Combine 2.0 mL of the *Sample stock solution* and 1.0 mL of the *Internal standard solution* in a glass centrifuge tube. Add 3.0 mL of *Cholesterol esterase solution* to the tube, and mix gently by inversion. Place the tube in a block heater set to 37°, and allow the reaction to continue for 45 min, gently and slowly inverting the tube every 10 min. After 45 min, add 1 g of sodium sulfate and 2 mL of petroleum ether to the tube. Mix on a vortex mixer for 30 s, and then centrifuge at 3000 rpm for 3 min. Carefully transfer the petroleum ether layer to a 10-mL glass centrifuge tube containing 1 g of anhydrous sodium sulfate. Be careful to avoid pipetting the intermediate emulsive layer. Evaporate the petroleum ether layer using a vacuum or a stream of inert gas at room temperature. Add 3 mL of acetone, sonicate, and filter the mixture. The filtered solution is the *Sample solution*.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 474 nm
Column: ▲▲ (USP 1-Aug-2019) 4.6-mm × 25-cm; 5-µm packing L62
Flow rate: 1.0 mL/min
Injection volume: 20 µL

System suitability
Sample: *Standard solution*
[NOTE—See *Table 2* for approximate relative retention times.]

Table 2

Name	Relative Retention Time	Relative Response Factor
13-*cis*-Astaxanthin	0.9	1.3
all-trans-Astaxanthin	1.0	1.0
9-*cis*-Astaxanthin	1.4	1.1
Apocarotenal (internal standard)	1.7	—

Suitability requirements
Chromatogram similarity: The chromatogram of the *Standard solution* is similar to the reference chromatogram provided with the lot of USP Astaxanthin Esters from *Haematococcus pluvialis* RS being used.
Resolution: NLT 2.0 between 13-*cis*-astaxanthin and *all-trans*-astaxanthin
Relative standard deviation: NMT 2.0% for *all-trans*-astaxanthin
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of total astaxanthin content in the portion of sample taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times P$$

R_U = ratio of [(1.3 × the peak area of 13-*cis*-astaxanthin) + the peak area of *all-trans*-astaxanthin + (1.1 × the peak area of 9-*cis*-astaxanthin)] to the peak area of the internal standard from the *Sample solution*
R_S = ratio of [(1.3 × the peak area of 13-*cis*-astaxanthin) + the peak area of *all-trans*-astaxanthin + (1.1 × the peak area of 9-*cis*-astaxanthin)] to the peak area of the internal standard from the *Standard solution*
C_S = concentration of USP Astaxanthin Esters from *Haematococcus pluvialis* RS in the *Standard solution* (mg/mL)
C_U = concentration of the *Sample solution* (mg/mL)
P = labeled amount of total astaxanthin as free astaxanthin in USP Astaxanthin Esters from *Haematococcus pluvialis* RS (%)

Acceptance criteria: NLT 5% of total astaxanthin, calculated as free astaxanthin on the anhydrous basis

CONTAMINANTS

• **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩
Acceptance criteria
Arsenic: NMT 2.0 µg/g
Cadmium: NMT 1.0 µg/g
Lead: NMT 1.0 µg/g
Mercury: NMT 1.0 µg/g

USP Monographs

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Add the following:

▲• Limit of Benzo[*a*]pyrene, Benzo[*b*]fluoranthene, Benzo[*a*]anthracene, and Chrysene

Internal standard stock solution: 10 µg/mL each of benzo[*a*]pyrene-d_{12}-isooctane,[2] benzo[*b*]fluoranthene-d_{12}-isooctane,[2] benzo[*a*]anthracene-d_{12}-isooctane,[2] and chrysene-d_{12}-toluene-d_8[2] in *n*-hexane

Internal standard solution: 0.1 µg/mL each of benzo[*a*]pyrene-d_{12}-isooctane, benzo[*b*]fluoranthene-d_{12}-isooctane, benzo[*a*]anthracene-d_{12}-isooctane, and chrysene-d_{12}-toluene-d_8 in *n*-hexane, by serially diluting the *Internal standard stock solution* with *n*-hexane

Standard stock solution: 0.1 µg/mL each of benzo[*a*]pyrene-dichloromethane,[3] benzo[*b*]fluoranthene-acetone,[3] benzo[*a*]anthracene-dichloromethane,[3] and chrysene-dichloromethane[3] in *n*-hexane

Standard solution: 0.01 µg/mL each of benzo[*a*]pyrene-dichloromethane, benzo[*b*]fluoranthene-acetone, benzo[*a*]anthracene-dichloromethane, and chrysene-dichloromethane in *n*-hexane, by serially diluting the *Standard stock solution* with *n*-hexane

Working standard solutions: See *Table 3*.

Table 3

Working Standard Concentration (ng/mL)	Volume Taken (mL)			Final Volume after Dilution with n-hexane (mL)
	Standard Stock Solution	Standard Solution	Internal Standard Solution	
10	2	—	2	20
5	1	—	2	20
2	—	4	2	20
1	—	2	2	20
0.5	—	1	2	20

Sample solution: Transfer 1 g of Astaxanthin Esters to a suitable refluxing flask. Add 100 µL of the *Internal standard solution*, 10 mL of water, 5 g of potassium hydroxide, and 80 mL of ethanol. The mixture is refluxed at 90° for 1 h. Cool, filter the solution through glass wool, and collect the filtrate in a suitable separator. Add 100 mL each of water and *n*-hexane to the separator, and shake for 10 min. Transfer the upper layer (hexane extract) to a second separator. Add 50 mL of *n*-hexane to the first separator and repeat the extraction again. Combine the upper layer with the extract in the second separator. Wash the combined extracts twice, each with 50 mL of water. Pass the washed extracts through anhydrous sodium sulfate to remove water. Evaporate the extracts to dryness using a stream of nitrogen. Dissolve the residue in 100 mL of *n*-hexane. Transfer this solution to a separator. Add 50 mL of dimethyl sulfoxide (DMSO) to the separator and shake it for 10 min. Transfer the lower layer to a second separator. Repeat the extraction with the solution in the first separator 2 more times. Combine the second and third extracts with the first extract in the second separator. To the combined extracts add 150 mL of sodium chloride solution (1 in 75), mix, and allow to cool. Add 50 mL of *n*-hexane to the extracts and shake the separator for 10 min. Transfer the upper layer to a third separator. Repeat the hexane extraction 2 more times. Combine the second and third extracts with the first extract in the third separator. Wash the combined extracts twice with 150 mL of water each time. Pass the washed extracts through anhydrous sodium sulfate to remove water. Evaporate the extracts to dryness using a stream of nitrogen. Dissolve the residue in 10 mL of *n*-hexane. Quantitatively transfer this solution to a chromatographic column (prepared by packing 5 g of silica gel in a brown glass column and wash with 30 mL of *n*-hexane), wash the column with 20 mL of *n*-hexane, and then elute the sample with 90 mL of a mixture of *n*-hexane and acetone (99:1). Evaporate the eluant to dryness with a stream of nitrogen, and dissolve the residue in 1 mL of *n*-hexane. Quantitatively transfer this solution to a molecular imprinted polymer cartridge that has been prewashed with 1 mL of cyclohexane. Wash the cartridge 3 times with 1 mL of *n*-hexane each time. Elute the sample with 4 mL of ethyl acetate. Evaporate the eluant to dryness with a stream of nitrogen, and dissolve the residue in 1.0 mL of *n*-hexane. This is the *Sample solution*.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability* and *Mass Spectrometry* ⟨736⟩.)

Mode: GC

Detector: Mass spectrometer, electron energy 70 eV

Ionization method: Electron impact (EI)

Column: 0.15-mm × 15-m capillary; coated with a 0.1-µm film of phase G51

Temperatures

Injection port: 320°

Ion source: 300°

Transfer line: 320°

Column: See *Table 4*.

Table 4

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
100	50	230	7
230	50	280	7
280	30	350	10

Carrier gas: Helium

Flow rate: 1.2 mL/min

Injection volume: 2 µL

Injection type: Pulsed splitless

Pulse pressure: 400 kPa

Pulse time: 2 min

Solvent delay: 9.2 min

System suitability

Sample: *Standard solution*

[Note—The relative retention times for benzo[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, and benzo[*a*]pyrene are about 1.00, 1.02, 1.23, and 1.35 min, respectively.]

Suitability requirements

Resolution: NLT 1.5 between benzo[*a*]anthracene and chrysene

Analysis

Samples: *Working standard solutions* and *Sample solution*

[2] Use Supelco Certified Reference Material, available from www.sigmaaldrich.com, or equivalent.

[3] Use Polycyclic Aromatic Hydrocarbon (PAH) Standards and Standard Mixtures, available from www.isotope.com/products/index.cfm, or equivalent.

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Use the following monitoring ions for peak detection:
- m/z 254: benzo[*b*]fluoranthene-d_{12} and benzo[*a*]pyrene-d_{12}
- m/z 252 and 253: benzo[*b*]fluoranthene and benzo[*a*]pyrene
- m/z 240: benzo[*a*]anthracene-d_{12} and chrysene-d_{12}
- m/z 228 and 229: benzo[*a*]anthracene and chrysene

Construct a calibration curve for each benzo[*a*]pyrene, benzo[*b*]fluoranthene, benzo[*a*]anthracene, and chrysene standard by plotting the concentrations of the *Working standard solutions* versus the ratios of the peak response of the benzo[*a*]pyrene, benzo[*b*]fluoranthene, benzo[*a*]anthracene, or chrysene standard to that of the internal standard. Calculate the slope and *y*-intercept for each standard calibration curve using linear regression analysis.

Separately calculate the content of benzo[*a*]pyrene, benzo[*b*]fluoranthene, benzo[*a*]anthracene, and chrysene in the portion of Astaxanthin Esters taken:

$$\text{Result} = [(R_U - Y)/S] \times (V/W)$$

R_U = ratio of the peak response of benzo[*a*]pyrene, benzo[*b*]fluoranthene, benzo[*a*]anthracene, or chrysene to that of the internal standard from the *Sample solution*
Y = *y*-intercept of the calibration curve for benzo[*a*]pyrene, benzo[*b*]fluoranthene, benzo[*a*]anthracene, or chrysene from the *Working standard solutions*
S = slope of the calibration curve for benzo[*a*]pyrene, benzo[*b*]fluoranthene, benzo[*a*]anthracene, or chrysene from the *Working standard solutions*
V = total volume used to dissolve the sample (mL)
W = weight of the sample (g)

Acceptance criteria
Benzo[*a*]pyrene: NMT 2 ppb
Sum of benzo[*a*]pyrene, benzo[*b*]fluoranthene, benzo[*a*]anthracene, and chrysene: NMT 10 ppb ▲(USP 1-Aug-2019)

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements
- **PHEOPHORBIDE CONTENT**

Solution A: 50 mg/mL of sodium sulfate
Solution B: Saturated solution of sodium sulfate
Sample stock solution: Transfer 100 mg of the sample to a 10-mL test tube, add 10 mL of acetone, and dissolve with sonication. Quantitatively transfer this solution to a separatory funnel, rinsing the test tube 3 times with 10-mL portions of acetone and adding the rinsings to the funnel. Add 30 mL of ethyl ether to the separatory funnel, followed by 50 mL of *Solution A*. Mix the contents of the separatory funnel by shaking gently, then draw off and discard the lower layer. Repeat washing with *Solution A* 3 times. Dehydrate the remaining extract with anhydrous sodium sulfate, then transfer the extract to a 50-mL volumetric flask, and dilute with ethyl ether to volume.
Sample solution: Transfer 20 mL of the *Sample stock solution* to a small beaker. Add 20 mL of 17% hydrochloric acid, and mix the solution vigorously. Transfer the hydrochloric acid layer to a separatory funnel, and repeat the extraction with a second 10-mL portion of 17% hydrochloric acid, adding the hydrochloric acid layer to the separatory funnel. Add 150 mL of *Solution B*, 20 mL of ethyl ether, and mix the contents of the separatory funnel by shaking. Transfer the ethyl ether layer to a 20-mL volumetric flask, and dilute with ethyl ether to volume.
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: UV-Vis
Analytical wavelength: 667 nm
Cell path: 1 cm
Blank: Ethyl ether
Analysis
Sample: *Sample solution*
Calculate the percentage of pheophorbide in the portion of sample taken:

$$\text{Result} = A/(C \times F)$$

A = absorbance of the *Sample solution*
C = concentration of the *Sample solution* (g/mL)
F = coefficient of extinction ($\varepsilon^{1\%}$) of pure pheophorbide in ethyl ether (100 mL · g^{-1} · cm^{-1}), 702

Acceptance criteria: NMT 0.02%

SPECIFIC TESTS
- **WATER DETERMINATION** ⟨921⟩, *Method I*: NMT 0.5%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Apocarotenal RS
trans-beta-Apo-8′-carotenal.
$C_{30}H_{40}O$
USP Astaxanthin Esters from *Haematococcus pluvialis* RS

Astragalus Root

DEFINITION
Astragalus Root consists of the dried root of *Astragalus membranaceus* var. *mongholicus* (Bunge) P.K.Hsiao or *Astragalus membranaceus* (Fisch.) Bunge (Fam. Fabaceae). Astragalus root is typically harvested from a 2- to 3-year-old plant in early fall. It contains NLT 0.04% of cycloartane saponins and NLT 0.03% of isoflavonoids calculated on the dried basis.

IDENTIFICATION
- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 1 mg/mL of USP Astragaloside IV RS in methanol
Standard solution B: 2 mg/mL of USP Daidzin RS and 1 mg/mL of USP Daidzein RS in methanol
Standard solution C: 50 mg/mL of USP Astragalus Root Dry Extract RS in methanol. Sonicate for about 10 min, centrifuge, and use the supernatant.
Sample solution: Heat 3 g of Astragalus Root, finely powdered, in 50 mL of methanol for 50 min under reflux. Centrifuge, withdraw the supernatant, and evaporate to dryness under reduced pressure. Dissolve the residue in 1.0 mL of water. Transfer the resulting solution onto a 6-mL solid-phase extraction column containing 500 mg of sorbent previously conditioned with 3 mL of methanol and

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3 mL of water.[1] Wash with 15 mL of water followed by 15 mL of 30% methanol, and discard the rinsate. Elute with 20 mL of methanol, collect the eluate, evaporate to dryness under reduced pressure, and dissolve the residue in 2 mL of methanol.

Chromatographic system

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plate)[2]

Application volume: 3 µL each of *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution* as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of 33%.

Temperature: Ambient, not to exceed 30°

Developing solvent system: Ethyl acetate, methanol, and water (100: 13.5: 10)

Developing distance: 6 cm

Derivatization reagent: 10% Sulfuric acid in methanol. [NOTE—Prepare fresh. Slowly and gradually add sulfuric acid to ice-cold methanol, and mix well.]

System suitability

Samples: *Standard solution A*, *Standard solution B*, and *Standard solution C*

Suitability requirements

Chromatographic pattern: Under long-wave UV light (365 nm), following derivatization, *Standard solution A* exhibits an orange band in the middle of the lower third of the plate due to astragaloside IV, with a retardation factor (R_F) of approximately 0.15. In *Standard solution B*, daidzin and daidzein form bluish-grey bands with R_F of approximately 0.34 and 0.76, respectively; the proximal band is sharper, while the distal is somewhat diffuse. In *Standard solution C*, four orange bands are seen in the lower third of the plate, corresponding to astragalosides IV, III, II, and I with R_F of approximately 0.15, 0.18, 0.24, and 0.34, respectively. The R_F of the astragaloside I band approximates that of daidzin in *Standard solution B*. The upper two-thirds of the plate typically display a number of bluish, greenish, and pinkish diffuse bands, one of which corresponds to that of daidzein in *Standard solution B*.

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Apply the *Samples* as bands and dry in air. Develop in a saturated chamber. Air-dry, treat with *Derivatization reagent*, heat for 5 min at 100°, and examine under long-wave UV light (365 nm).

Acceptance criteria: Under long-wave UV light (365 nm), the *Sample solution* exhibits bands corresponding in color and R_F to similar bands from *Standard solution C*. In the lower third of the chromatogram, a number of orange bands are present; the most prominent ones corresponding to astragalosides I and II, with R_F of approximately 0.34 and 0.24, respectively. In the upper part of the chromatogram, a number of diffuse bands are present, and additional weak bands may appear with respect to those seen in *Standard solution C*. [NOTE—The root of *Hedysarum polybotros*, a common adulterant, does not show orange bands corresponding to astragalosides I and II.]

• **B. HPLC**

Analysis: Proceed as directed in the test for *Content of Isoflavonoids and Saponins*.

Acceptance criteria: The *Sample solution* exhibits peaks at the retention times corresponding to those of calycosin 7-*O*-β-D-glucopyranoside, ononin, calycosin, formononetin, astragaloside IV, astragaloside I, and astragaloside II from *Standard solution F*.

COMPOSITION

• **CONTENT OF ISOFLAVONOIDS AND SAPONINS**

Solution A: 0.3% Formic acid

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	80	20
15	80	20
25	68	32
35	66	34
45	55	45
55	50	50
75	25	75
80	80	20
100	80	20

Standard solution A: Prepare a composite solution containing 0.4 mg/mL of USP Astragaloside IV RS, 0.1 mg/mL of USP Calycosin RS, 0.2 mg/mL of USP Calycosin 7-*O*-β-D-Glucopyranoside RS, 0.05 mg/mL of USP Formononetin RS, and 0.1 mg/mL of USP Ononin RS in methanol.

Standard solutions B, C, D, E: Prepare four consecutive two-fold serial dilutions of *Standard solution A* in methanol.

Standard solution F: Sonicate 150 mg of USP Astragalus Root Dry Extract RS in 5 mL of methanol. Pass through a nylon filter of 0.45-µm pore size, and discard the initial 1 mL of the filtrate.

Sample solution: Accurately weigh 1.5 g of Astragalus Root reduced to fine powder, and transfer into a 100-mL round-bottomed flask. Attach the condenser and reflux in 60 mL of methanol for 3 h. Filter and evaporate methanol to dryness under reduced pressure. Dissolve the residue in a small amount of methanol, and transfer quantitatively into a 5-mL volumetric flask. Adjust with methanol to volume and mix well. Pass through a nylon filter of 0.45-µm pore size, and discard the initial 1 mL of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: HPLC

Detectors: UV 280 nm and ELSD, connected in series

ELSD drift tube temperature: Optimize according to the manufacturer's recommendations to achieve optimal signal-to-noise ratio, typically 105°.

ELSD carrier gas flow: Optimize according to the manufacturer's recommendations to achieve optimal signal-to-noise ratio, typically 2.70 L/min.

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 25°

Flow rate: 0.8 mL/min

Injection volume: 15 µL

System suitability

Samples: *Standard solutions A–E* and *Standard solution F*

Suitability requirements

Chromatographic similarity: The chromatogram of *Standard solution F* is similar to the reference

[1] Suitable commercially available SPE columns are Bakerbond Octadecyl C_{18}.

[2] Suitable commercially available plates are HPTLC Silica Gel 60 F_{254} from EMD Millipore (e.g., part no. 1.05642.0001).

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chromatogram provided with the lot of USP Astragalus Root Dry Extract RS being used.

Theoretical plates: NLT 3,000 for calycosin 7-*O*-β-D-glucopyranoside (UV) and astragaloside IV (ELSD) peaks, *Standard solution A*

Correlation coefficient: NLT 0.995 for each regression line as determined in *Analysis*

Analysis

Samples: *Standard solutions A–F* and *Sample solution*

Using the UV absorbance chromatograms of *Standard solutions A–E*, *Standard solution F*, and the reference chromatogram provided with the lot of USP Astragalus Root Dry Extract RS being used, identify the specified isoflavonoids in the *Sample solution* chromatogram. Measure the areas of the isoflavonoid peaks. Plot the areas of the relevant peaks against the respective concentrations (mg/mL) of each analyte in *Standard solutions A–E*, and determine the equations of the resulting least-squares regression lines.

Using the equations of the relevant least-squares lines, determine the concentrations of each specified isoflavonoid (calycosin 7-*O*-β-D-glucopyranoside, ononin, calycosin, and formononetin) in the *Sample solution*.

Separately calculate the percentages of each isoflavonoid in the portion of Astragalus Root taken:

$$\text{Result} = C_I \times (V/W) \times 100$$

C_I = concentration of the relevant isoflavonoid in the *Sample solution* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Astragalus Root taken to prepare the *Sample solution* (mg)

Calculate the sum of percentages of isoflavonoids.

Using the ELSD chromatograms of *Standard solutions A–E*, *Standard solution F*, and the reference chromatogram, provided with the lot of USP Astragalus Root Dry Extract RS being used, identify all specified saponins in the *Sample solution* chromatogram. The approximate relative retention times for astragalosides I and II, with respect to astragaloside IV, are provided in *Table 2*.

Table 2

Analyte	Relative Retention Time
Astragaloside IV	1.00
Astragaloside II	1.10
Astragaloside I	1.28

Measure the areas of the saponin peaks. Plot the logarithms of astragaloside IV peak areas against the logarithms of their respective concentrations (mg/mL) in *Standard solutions A–E*, and determine the equation of a least-squares regression line. Using the equation of the least-squares line for astragaloside IV, calculate the concentrations of each specified saponin (astragaloside I, astragaloside II, and astragaloside IV) in the *Sample solution*.

Separately calculate the percentages of each saponin in the portion of Astragalus Root taken:

$$\text{Result} = C_S \times (V/W) \times 100$$

C_S = concentration of the relevant saponin in the *Sample solution* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Astragalus Root taken to prepare the *Sample solution* (mg)

Calculate the sum of percentages of saponins.

Acceptance criteria

Sum of isoflavonoids: NLT 0.03% on the dried basis

Sum of saponins: NLT 0.04% on the dried basis

CONTAMINANTS

- **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩

Acceptance criteria

Arsenic: NMT 1.5 µg/g
Cadmium: NMT 0.3 µg/g
Lead: NMT 5.0 µg/g
Mercury: NMT 0.1 µg/g

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Pesticide Residue Analysis*: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^5 cfu/g, total combined yeasts and molds count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of* Salmonella *Species* and *Test for Absence of* Escherichia coli: Meets the requirements

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: Astragalus Root is cylindrical, some upper branches relatively thick, 30–90 cm long, 0.5–3.5 cm in diameter. Externally pale brownish yellow or pale brown (but not red), with irregular, longitudinal wrinkles or furrows. Texture hard and tenacious, broken with difficulty, fracture highly fibrous and starchy; bark yellowish-white; wood pale yellow, with radiate striations and fissures, the center part of old root occasionally looking like rotten wood, blackish brown or hollowed.

Microscopic: The transverse section shows cork consisting of many rows of tangentially elongated cells. Phelloderm, 3–7 rows of collenchymatous cells. Outer part of phloem rays often curved and fissured, fibers in bundles from 6–22 µm in diameter, with longitudinal fissures and truncate or brush-like ends. The walls are thickened and lignified or slightly lignified, arranged alternately with sieve tube groups; stone cells sometimes visible near phelloderm. Cambium in a ring. Xylem vessels scattered singly or 2–3 aggregated in groups; wood fibers among vessel stone cells singly or 2–4 in groups, sometimes visible in rays. Parenchymatous cells contain starch granules. In the longitudinal section, no solitary calcium oxalate crystals are seen outside the fiber bundle (a distinction from *Hedysarum polybotros* and other *Hedysarum* species, common adulterants).

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Foreign Organic Matter*: NMT 2.0%
- **LOSS ON DRYING** ⟨731⟩

Sample: 1.0 g of finely powdered Astragalus Root
Analysis: Dry the *Sample* at 105° for 3 h.
Acceptance criteria: NMT 10.0%

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Total Ash*

Sample: 1.0 g of powdered Astragalus Root
Acceptance criteria: NMT 5.0%

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Acid-Insoluble Ash*

Sample: 1.0 g of powdered Astragalus Root
Acceptance criteria: NMT 1.0%

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Alcohol-Soluble Extractives, Method 1*

Sample: 2–4 g of powdered Astragalus Root
Acceptance criteria: NLT 2.0%

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Water-Soluble Extractives, Method 1*

Sample: 2–4 g of powdered Astragalus Root
Acceptance criteria: NLT 17.0%

Supplement 2

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ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial of the species from which the article was derived.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Astragaloside IV RS
  USP Astragalus Root Dry Extract RS
  USP Calycosin RS
  USP Calycosin 7-*O*-β-D-Glucopyranoside RS
  USP Daidzein RS
  USP Daidzin RS
  USP Formononetin RS
  USP Ononin RS

Astragalus Root Powder

DEFINITION

Astragalus Root Powder consists of the dried root of *Astragalus membranaceus* var. *mongholicus* (Bunge) P.K.Hsiao or *Astragalus membranaceus* (Fisch.) Bunge (Fam. Fabaceae) reduced to powder or very fine powder. Astragalus root is typically harvested from a 2- to 3-year-old plant in early fall. It contains NLT 0.04% of cycloartane saponins and NLT 0.03% of isoflavonoids calculated on the dried basis.

IDENTIFICATION

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 1 mg/mL of USP Astragaloside IV RS in methanol

Standard solution B: 2 mg/mL of USP Daidzin RS and 1 mg/mL of USP Daidzein RS in methanol

Standard solution C: 50 mg/mL of USP Astragalus Root Dry Extract RS in methanol. Sonicate for about 10 min, centrifuge, and use the supernatant.

Sample solution: Heat 3 g of Astragalus Root Powder in 50 mL of methanol for 50 min under reflux. Centrifuge, withdraw the supernatant, and evaporate to dryness under reduced pressure. Dissolve the residue in 1.0 mL of water. Transfer the resulting solution onto a 6-mL solid-phase extraction column containing 500 mg of sorbent previously conditioned with 3 mL of methanol and 3 mL of water.[1] Wash with 15 mL of water followed by 15 mL of 30% methanol, and discard the rinsate. Elute with 20 mL of methanol, collect the eluate, evaporate to dryness under reduced pressure, and dissolve the residue in 2 mL of methanol.

Chromatographic system

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plate)[2]

Application volume: 3 µL each of *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution* as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of 33%.

Temperature: Ambient, not to exceed 30°

Developing solvent system: Ethyl acetate, methanol, and water (100: 13.5: 10)

Developing distance: 6 cm

Derivatization reagent: 10% Sulfuric acid in methanol. [NOTE—Prepare fresh. Slowly and gradually add sulfuric acid to ice-cold methanol, and mix well.]

System suitability

Samples: *Standard solution A*, *Standard solution B*, and *Standard solution C*

Suitability requirements

Chromatographic pattern: Under long-wave UV light (365 nm), following derivatization, *Standard solution A* exhibits an orange band in the middle of the lower third of the plate due to astragaloside IV, with a retardation factor (R_F) of approximately 0.15. In *Standard solution B*, daidzin and daidzein form bluish-grey bands with R_F of approximately 0.34 and 0.76, respectively; the proximal band is sharper, while the distal is somewhat diffuse. In *Standard solution C*, four orange bands are seen in the lower third of the plate, corresponding to astragalosides IV, III, II, and I with R_F of approximately 0.15, 0.18, 0.24, and 0.34, respectively. The R_F of the astragaloside I band approximates that of daidzin in *Standard solution B*. The upper two-thirds of the plate typically display a number of bluish, greenish, and pinkish diffuse bands, one of which corresponds to that of daidzein in *Standard solution B*.

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Apply the *Samples* as bands and dry in air. Develop in a saturated chamber. Air-dry, treat with *Derivatization reagent*, heat for 5 min at 100°, and examine under long-wave UV light (365 nm).

Acceptance criteria: Under long-wave UV light (365 nm), the *Sample solution* exhibits bands corresponding in color and R_F to similar bands from *Standard solution C*. In the lower third of the chromatogram, a number of orange bands are present; the most prominent ones corresponding to astragalosides I and II, with R_F of approximately 0.34 and 0.24, respectively. In the upper part of the chromatogram, a number of diffuse bands are present, and additional weak bands may appear with respect to those seen in *Standard solution C*. [NOTE—The root of *Hedysarum polybotros*, a common adulterant, does not show orange bands corresponding to astragalosides I and II.]

- **B. HPLC**

Analysis: Proceed as directed in the test for *Content of Isoflavonoids and Saponins*.

Acceptance criteria: The *Sample solution* exhibits peaks at the retention times corresponding to those of calycosin 7-*O*-β-D-glucopyranoside, ononin, calycosin, formononetin, astragaloside IV, astragaloside I, and astragaloside II from *Standard solution F*.

COMPOSITION

- **CONTENT OF ISOFLAVONOIDS AND SAPONINS**

Solution A: 0.3% Formic acid

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	80	20
15	80	20
25	68	32
35	66	34
45	55	45

[1] Suitable commercially available SPE columns are Bakerbond Octadecyl C_{18}.

[2] Suitable commercially available plates are HPTLC Silica Gel 60 F_{254} from EMD Millipore (e.g., part no. 1.05642.0001).

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Table 1 *(continued)*

Time (min)	Solution A (%)	Solution B (%)
55	50	50
75	25	75
80	80	20
100	80	20

Standard solution A: Prepare a composite solution containing 0.4 mg/mL of USP Astragaloside IV RS, 0.1 mg/mL of USP Calycosin RS, 0.2 mg/mL of USP Calycosin 7-*O*-β-D-Glucopyranoside RS, 0.05 mg/mL of USP Formononetin RS, and 0.1 mg/mL of USP Ononin RS in methanol.

Standard solutions B, C, D, E: Prepare four consecutive two-fold serial dilutions of *Standard solution A* in methanol.

Standard solution F: Sonicate 150 mg of USP Astragalus Root Dry Extract RS in 5 mL of methanol. Pass through a nylon filter of 0.45-µm pore size, and discard the initial 1 mL of the filtrate.

Sample solution: Accurately weigh 1.5 g of Astragalus Root Powder, and transfer into a 100-mL round-bottomed flask. Attach the condenser and reflux in 60 mL of methanol for 3 h. Filter and evaporate methanol to dryness under reduced pressure, dissolve the residue in a small amount of methanol, and transfer quantitatively into a 5-mL volumetric flask. Adjust with methanol to volume and mix well. Pass through a nylon filter of 0.45-µm pore size, and discard the initial 1 mL of the filtrate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: HPLC
Detectors: UV 280 nm and ELSD, connected in series
ELSD drift tube temperature: Optimize according to the manufacturer's recommendations to achieve optimal signal-to-noise ratio, typically 105°.
ELSD carrier gas flow: Optimize according to the manufacturer's recommendations to achieve optimal signal-to-noise ratio, typically 2.70 L/min.
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 25°
Flow rate: 0.8 mL/min
Injection volume: 15 µL

System suitability
Samples: *Standard solutions A–E* and *Standard solution F*
Suitability requirements
Chromatographic similarity: The chromatogram of *Standard solution F* is similar to the reference chromatogram provided with the lot of USP Astragalus Root Dry Extract RS being used.
Theoretical plates: NLT 3,000 for calycosin 7-*O*-β-D-glucopyranoside (UV) and astragaloside IV (ELSD) peaks, *Standard solution A*
Correlation coefficient: NLT 0.995 for each regression line as determined in *Analysis*

Analysis
Samples: *Standard solutions A–F* and *Sample solution*
Using the UV absorbance chromatograms of *Standard solutions A–E*, *Standard solution F*, and the reference chromatogram, provided with the lot of USP Astragalus Root Dry Extract RS being used, identify the specified isoflavonoids in the *Sample solution* chromatogram.
Measure the areas of the isoflavonoid peaks. Plot the areas of the relevant peaks against the respective concentrations (mg/mL) of each analyte in *Standard solutions A–E*, and determine the equations of the resulting least-squares regression lines.
Using the equations of the relevant least-squares lines, determine the concentrations of each specified isoflavonoid (calycosin 7-*O*-β-D-glucopyranoside, ononin, calycosin, and formononetin) in the *Sample solution*.
Separately calculate the percentages of each isoflavonoid in the portion of Astragalus Root Powder taken:

$$\text{Result} = C_I \times (V/W) \times 100$$

C_I = concentration of the relevant isoflavonoid in the *Sample solution* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Astragalus Root Powder taken to prepare the *Sample solution* (mg)

Calculate the sum of percentages of isoflavonoids.
Using the ELSD chromatograms of *Standard solutions A–E*, *Standard solution F*, and the reference chromatogram, provided with the lot of USP Astragalus Root Dry Extract RS being used, identify all specified saponins in the *Sample solution* chromatogram. The approximate relative retention times for astragalosides I and II, with respect to astragaloside IV, are provided in *Table 2*.

Table 2

Analyte	Relative Retention Time
Astragaloside IV	1.00
Astragaloside II	1.10
Astragaloside I	1.28

Measure the areas of the saponin peaks. Plot the logarithms of astragaloside IV peak areas against the logarithms of their respective concentrations (mg/mL) in *Standard solutions A–E*, and determine the equation of least-squares regression line. Using the equation of the least-squares line for astragaloside IV, calculate the concentrations of each specified saponin (astragaloside I, astragaloside II, and astragaloside IV) in the *Sample solution*.
Separately calculate the percentages of each saponin in the portion of Astragalus Root Powder taken:

$$\text{Result} = C_S \times (V/W) \times 100$$

C_S = concentration of the relevant saponin in the *Sample solution* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Astragalus Root Powder taken to prepare the *Sample solution* (mg)

Calculate the sum of percentages of saponins.

Acceptance criteria
Sum of isoflavonoids: NLT 0.03% on the dried basis
Sum of saponins: NLT 0.04% on the dried basis

CONTAMINANTS

- **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩
  Acceptance criteria
  Arsenic: NMT 1.5 µg/g
  Cadmium: NMT 0.3 µg/g
  Lead: NMT 5.0 µg/g
  Mercury: NMT 0.1 µg/g
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Pesticide Residue Analysis*: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^5 cfu/g, total combined yeasts and molds count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria count does not exceed 10^3 cfu/g.

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• **Absence of Specified Microorganisms** ⟨2022⟩, *Test Procedures, Test for Absence of* Salmonella *Species* and *Test for Absence of* Escherichia coli**:** Meets the requirements

SPECIFIC TESTS

• **Botanical Characteristics**

Macroscopic: White to pale-yellow powder, splintery and fibrous

Microscopic: Fibers occur in bundles or scattered, 3–30 µm in diameter, walls thickened, the primary walls often separated from secondary, with longitudinal fissures and truncate or brush-like ends, polychromatic in polarized light. Stone cells occasionally visible, subrounded, oblong or irregular, slightly thick-walled, bright yellowish-white in polarized light. Cork cells irregular or polygonal, sometimes with sinuous anticlinal walls. In *Astragalus membranaceus* var. *mongholicus* (Bunge) P.K.Hsiao, reticulated vessels abundant, bordered-pitted vessels few, 16–150 µm in diameter. In *Astragalus membranaceus* (Fisch.) Bunge, bordered-pitted vessels abundant, bordered pits arranged closely, up to 200 µm in diameter. Simple starch granules spheroidal or ellipsoid, 3–23 µm in diameter, with visible linear or punctate hilum. Occasional compound starch granules composed of 2–4 components. Observed in polarized light, starch granules are black and cruciate. Calcium oxalate crystals are absent.

• **Articles of Botanical Origin** ⟨561⟩, *Foreign Organic Matter***:** NMT 2.0%

• **Loss on Drying** ⟨731⟩

Sample: 1.0 g of Astragalus Root Powder

Analysis: Dry the *Sample* at 105° for 3 h.

Acceptance criteria: NMT 10.0%

• **Articles of Botanical Origin** ⟨561⟩, *Total Ash*

Sample: 1.0 g of Astragalus Root Powder

Acceptance criteria: NMT 5.0%

• **Articles of Botanical Origin** ⟨561⟩, *Acid-Insoluble Ash*

Sample: 1.0 g of Astragalus Root Powder

Acceptance criteria: NMT 1.0%

• **Articles of Botanical Origin** ⟨561⟩, *Alcohol-Soluble Extractives, Method 1*

Sample: 2–4 g of Astragalus Root Powder

Acceptance criteria: NLT 2.0%

• **Articles of Botanical Origin** ⟨561⟩, *Water-Soluble Extractives, Method 1*

Sample: 2–4 g of Astragalus Root Powder

Acceptance criteria: NLT 17.0%

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.

• **Labeling:** The label states the Latin binomial of the species from which the article was derived.

• **USP Reference Standards** ⟨11⟩

USP Astragaloside IV RS
USP Astragalus Root Dry Extract RS
USP Calycosin RS
USP Calycosin 7-*O*-β-D-Glucopyranoside RS
USP Daidzein RS
USP Daidzin RS
USP Formononetin RS
USP Ononin RS

Astragalus Root Dry Extract

DEFINITION

Astragalus Root Dry Extract is prepared from the dried root of *Astragalus membranaceus* var. *mongholicus* (Bunge) P.K.Hsiao or *Astragalus membranaceus* (Fisch.) Bunge (Fam. Fabaceae) subjected to aqueous or hydroalcoholic extraction. It contains NLT 90.0% and NMT 110.0% of the labeled amounts of cycloartane saponins and isoflavonoids calculated on the anhydrous basis. It may contain suitable substances added as carriers.

IDENTIFICATION

• **A. HPTLC for Articles of Botanical Origin** ⟨203⟩

Standard solution A: 1 mg/mL of USP Astragaloside IV RS in methanol

Standard solution B: 2 mg/mL of USP Daidzin RS and 1 mg/mL of USP Daidzein RS in methanol

Standard solution C: 50 mg/mL of USP Astragalus Root Dry Extract RS in methanol. Sonicate for about 10 min, centrifuge, and use the supernatant.

Sample solution: 50 mg/mL of Astragalus Root Dry Extract in methanol. Sonicate for about 10 min, centrifuge, and use the supernatant.

Chromatographic system

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plate)[1]

Application volume: 3 µL each of *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution* as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of 33%.

Temperature: Ambient, not to exceed 30°

Developing solvent system: Ethyl acetate, methanol, and water (100: 13.5: 10)

Developing distance: 6 cm

Derivatization reagent: 10% Sulfuric acid in methanol. [Note—Prepare fresh. Slowly and gradually add sulfuric acid to ice-cold methanol, and mix well.]

System suitability

Samples: *Standard solution A*, *Standard solution B*, and *Standard solution C*

Suitability requirements

Chromatographic pattern: Under long-wave UV light (365 nm), following derivatization, *Standard solution A* exhibits an orange band in the middle of the lower third of the plate due to astragaloside IV, with a retardation factor (R_F) of approximately 0.15. In *Standard solution B*, daidzin and daidzein form bluish-grey bands with R_F of approximately 0.34 and 0.76, respectively; the proximal band is sharper, while the distal is somewhat diffuse. In *Standard solution C*, four orange bands are seen in the lower third of the plate, corresponding to astragalosides IV, III, II, and I with R_F of approximately 0.15, 0.18, 0.24, and 0.34, respectively. The R_F of the astragaloside I band approximates that of daidzin in *Standard solution B*. The upper two-thirds of the plate typically display a number of bluish, greenish, and pinkish bands, one of which corresponds to that of daidzein in *Standard solution B*.

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Apply the *Samples* as bands and dry in air. Develop in a saturated chamber. Air-dry, treat with *Derivatization reagent*, heat for 5 min at 105°, and examine under long-wave UV light (365 nm).

[1] Suitable commercially available plates are HPTLC Silica Gel 60 F_{254} from EMD Millipore (e.g., part no. 1.05642.0001).

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Acceptance criteria: Under long-wave UV light (365 nm), the *Sample solution* exhibits bands corresponding in color and R_F to similar bands from *Standard solution C*, at the R_F values listed in *Chromatographic pattern*. [NOTE—The extract of *Hedysarum polybotros*, a common adulterant, does not show orange bands corresponding to astragalosides I and II.]

• **B. HPLC**

Analysis: Proceed as directed in the test for *Content of Isoflavonoids and Saponins*.

Acceptance criteria: The *Sample solution* exhibits peaks at the retention times corresponding to those of calycosin 7-*O*-β-D-glucopyranoside, ononin, calycosin, formononetin, astragaloside IV, astragaloside I, and astragaloside II from *Standard solution F*.

COMPOSITION

• **CONTENT OF ISOFLAVONOIDS AND SAPONINS**

Solution A: 0.3% Formic acid

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	80	20
15	80	20
25	68	32
35	66	34
45	55	45
55	50	50
75	25	75
80	80	20
100	80	20

Standard solution A: Prepare a composite solution containing 0.4 mg/mL of USP Astragaloside IV RS, 0.1 mg/mL of USP Calycosin RS, 0.2 mg/mL of USP Calycosin 7-*O*-β-D-Glucopyranoside RS, 0.05 mg/mL of USP Formononetin RS, and 0.1 mg/mL of USP Ononin RS in methanol.

Standard solutions B, C, D, E: Prepare four consecutive two-fold serial dilutions of *Standard solution A* in methanol.

Standard solution F: Sonicate 150 mg of USP Astragalus Root Dry Extract RS in 5 mL of methanol. Pass through a nylon filter of 0.45-µm pore size, and discard the initial 1 mL of the filtrate.

Sample solution: Accurately weigh about 300 mg of Astragalus Root Dry Extract into a 10-mL volumetric flask. Add 5 mL of methanol and sonicate for 10 min. Cool, adjust with methanol to volume, and mix well.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: HPLC

Detectors: UV 280 nm and ELSD, connected in series

ELSD drift tube temperature: Optimize according to the manufacturer's recommendations to achieve optimal signal-to-noise ratio, typically 105°.

ELSD carrier gas flow: Optimize according to the manufacturer's recommendations to achieve optimal signal-to-noise ratio, typically 2.70 L/min.

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 25°

Flow rate: 0.8 mL/min

Injection volume: 15 µL

System suitability

Samples: *Standard solutions A–E* and *Standard solution F*

Suitability requirements

Chromatographic similarity: The chromatogram of *Standard solution F* is similar to the reference chromatogram provided with the lot of USP Astragalus Root Dry Extract RS being used.

Theoretical plates: NLT 3,000 for calycosin 7-*O*-β-D-glucopyranoside (UV) and astragaloside IV (ELSD) peaks, *Standard solution A*

Correlation coefficient: NLT 0.995 for each regression line as determined in *Analysis*

Analysis

Samples: *Standard solutions A–F* and *Sample solution*

Using the UV absorbance chromatograms of *Standard solutions A–E*, *Standard solution F*, and the reference chromatogram, provided with the lot of USP Astragalus Root Dry Extract RS being used, identify the specified isoflavonoids in the *Sample solution* chromatogram. Measure the areas of the isoflavonoid peaks. Plot the areas of the relevant peaks against the respective concentrations (mg/mL) of each analyte in *Standard solutions A–E*, and determine the equations of the resulting least-squares regression lines.

Using the equations of the relevant least-squares lines, determine the concentrations of each specified isoflavonoid (calycosin 7-*O*-β-D-glucopyranoside, ononin, calycosin, and formononetin) in the *Sample solution*.

Separately calculate the percentages of each isoflavonoid in the portion of Astragalus Root Dry Extract taken:

$$\text{Result} = C_I \times (V/W) \times 100$$

C_I = concentration of the relevant isoflavonoid in the *Sample solution* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Astragalus Root Dry Extract taken to prepare the *Sample solution* (mg)

Calculate the sum of percentages of isoflavonoids.

Calculate the percentage of the labeled amount of isoflavonoids in the portion of Astragalus Root Dry Extract taken:

$$\text{Result} = (P/L) \times 100$$

P = sum of percentages of isoflavonoids in the Astragalus Root Dry Extract, as calculated above (%)

L = labeled amount of isoflavonoids in the Astragalus Root Dry Extract (%)

Using the ELSD chromatograms of *Standard solutions A–E*, *Standard solution F*, and the reference chromatogram, provided with the lot of USP Astragalus Root Dry Extract RS being used, identify all specified saponins in the *Sample solution* chromatogram. The approximate relative retention times for astragalosides I and II, with respect to astragaloside IV, are provided in *Table 2*.

Table 2

Analyte	Relative Retention Time
Astragaloside IV	1.00
Astragaloside II	1.10
Astragaloside I	1.28

Measure the areas of the saponin peaks. Plot the logarithms of Astragaloside IV peak areas against the logarithms of

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their respective concentrations (mg/mL) in *Standard solutions A–E*, and determine the equation of a least-squares regression line. Using the equation of the least-squares line for astragaloside IV, calculate the concentrations of each specified saponin (astragaloside I, astragaloside II, and astragaloside IV) in the *Sample solution*.

Separately calculate the percentages of each saponin in the portion of Astragalus Root Dry Extract taken:

$$\text{Result} = C_S \times (V/W) \times 100$$

C_S = concentration of the relevant saponin in the *Sample solution* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Astragalus Root Dry Extract taken to prepare the *Sample solution* (mg)

Calculate the sum of percentages of saponins.

Calculate the percentage of the labeled amount of saponins in the portion of Astragalus Root Dry Extract taken:

$$\text{Result} = (P/L) \times 100$$

P = sum of percentages of saponins in Astragalus Root Dry Extract, as calculated above (%)
L = labeled amount of saponins in Astragalus Root Dry Extract (%)

Acceptance criteria

Sum of isoflavonoids: 90.0%–110.0% of the labeled amount of isoflavonoids on the anhydrous basis

Sum of saponins: 90.0%–110.0% of the labeled amount of saponins on the anhydrous basis

CONTAMINANTS

- **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩

Acceptance criteria

Arsenic: NMT 1.5 µg/g
Cadmium: NMT 0.3 µg/g
Lead: NMT 5.0 µg/g
Mercury: NMT 0.1 µg/g

Change to read:

- **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020) **:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^4 cfu/g, and total combined yeasts and molds count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of* Salmonella *Species* and *Test for Absence of* Escherichia coli**:** Meets the requirements

SPECIFIC TESTS

- **RESIDUE ON IGNITION** ⟨281⟩

Sample: 1.0 g of Astragalus Root Dry Extract
Acceptance criteria: NMT 5.0%

- **BOTANICAL EXTRACTS** ⟨565⟩, *Residual Solvents***:** Meets the requirements
- **WATER DETERMINATION** ⟨921⟩, *Method Ia*

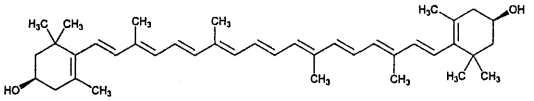
Acceptance criteria: NMT 6.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial of the species from which the article was derived. The label also indicates the content of isoflavonoids and cycloartane saponins, the solvent used in extract preparation, and the ratio of the starting crude plant material to dry extract. It meets the labeling requirements of *Botanical Extracts* ⟨565⟩.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Astragaloside IV RS
USP Astragalus Root Dry Extract RS
USP Calycosin RS
USP Calycosin 7-*O*-β-D-Glucopyranoside RS
USP Daidzein RS
USP Daidzin RS
USP Formononetin RS
USP Ononin RS

Aztec Marigold Zeaxanthin Extract



$C_{40}H_{56}O_2$ 568.87

(all-*E*)-1,1′-(3,7,12,16-Tetramethyl-1,3,5,7,9,11,13,15,17-octadecanonaene-1,18-diyl)bis[2,6,6-trimethylcyclohexene-3-ol];
3*R*,3′*R*-β,β-Carotene-3,3′-diol [148-68-3].

DEFINITION

Aztec Marigold Zeaxanthin Extract is a purified extract, derived from the flowers of *Tagetes erecta* L., grown from seeds of varieties of the Scarletade cultivar rich in zeaxanthin. The extract contains NLT 36.0% of total carotenoids calculated as zeaxanthin ($C_{40}H_{56}O_2$), NLT 30.0% of all-*trans*-zeaxanthin, and NMT 8.0% of lutein, calculated on the dried basis.

IDENTIFICATION

- **A.**

Sample solution: Use the *Sample solution* from the test for *Content of Total Carotenoids*.
Analysis: Record the UV-Vis spectrum from 300–600 nm.
Acceptance criteria: The *Sample solution* shows a shoulder at about 428 nm, an absorption maximum at about 450 nm, and another maximum at about 478 nm.

- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Zeaxanthin*.
- **C.** The retention time of the major peak of the *Sample solution* corresponds to that of 3*R*,3′*R*-β,β-carotene-3,3′-diol from the *Standard solution*, as obtained in the test for *Stereoisomeric Composition*.

COMPOSITION

- **CONTENT OF TOTAL CAROTENOIDS**

[NOTE—Use low-actinic glassware.]
Sample stock solution: Use the *Sample stock solution* from the test for *Content of Zeaxanthin*.
Sample solution: Transfer 2.0 mL of the *Sample stock solution* to a 100-mL volumetric flask, dilute with ethanol to volume, and mix well.
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Analytical wavelength: 450 nm
Cell path: 1 cm
Blank: Ethanol
Analysis
Sample: *Sample solution*

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[NOTE—The absorbance reading should be between 0.2 AU to 1.0 AU. If not, readjust the dilution of the solution.]

Calculate the percentage of the total carotenoids as zeaxanthin ($C_{40}H_{56}O_2$):

$$\text{Result} = A/(C \times F)$$

A = absorbance of the *Sample solution*
C = concentration of the *Sample solution* (g/mL)
F = coefficient of extinction ($E^{1\%}$) of zeaxanthin in ethanol (100 mL · g^{-1} · cm^{-1}), 2480

Acceptance criteria: NLT 36.0% of total carotenoids (*T*) as zeaxanthin ($C_{40}H_{56}O_2$) on the dried basis

• **CONTENT OF ZEAXANTHIN**

[NOTE—Use low-actinic glassware.]

Solution A: Methyl *tert*-butyl ether
Solution B: Water
Solution C: Methanol
Diluent: Mixture of hexane, ethanol, acetone, and toluene (10:6:7:7)
Mobile phase: Gradient elution. See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)	Solution C (%)
0.0	5	7	88
15	15	7	78
30	45	7	48
60	75	6.5	18.5
66	75	6	19
76	5	7	88
86	5	7	88

Standard stock solution: Transfer 20.0 mg of USP Aztec Marigold Zeaxanthin Extract RS into a 100-mL volumetric flask, add 75 mL of *Diluent* to the flask to suspend the sample, and sonicate for 5 min. Dilute with *Diluent* to volume, and mix well. Allow the insolubles to settle for at least 10 min. Pass the supernatant through a membrane filter of 0.45-µm pore size.

Standard solution: Transfer 0.5 mL of the *Standard stock solution* to an 8-mL vial, evaporated to dryness with an aid of a stream of nitrogen. Dissolve the residue in 4.0 mL of a mixture of methyl *tert*-butyl ether and methanol (5:95).

Sample stock solution: Transfer 20.0 mg of Extract to a 100-mL volumetric flask, add 75 mL of *Diluent* to the flask to suspend the sample, and sonicate for 5 min. [**CAUTION**—Electrostatic charges may cause the sample to sputter and stick to the sides of the flask. Carefully wash down the product into the flask using *Diluent*.] Dilute with *Diluent* to volume, and mix well. Allow the insolubles to settle for at least 10 min. Pass the supernatant through a membrane filter of 0.45-µm pore size.

Sample solution: Transfer 0.5 mL of the *Sample stock solution* to an 8-mL vial, and evaporate to dryness with the aid of a stream of nitrogen. Dissolve the residue in a 4.0-mL mixture of methyl *tert*-butyl ether and methanol (5:95).

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: HPLC
Detector: 450 nm
Column: 2.0-mm × 15-cm; 3-µm packing L62
Flow rate: 0.4 mL/min
Injection volume: 10 µL

System suitability
Sample: *Standard solution*

[NOTE—The approximate relative retention times for lutein and zeaxanthin are 0.87 and 1.0, respectively.]

Suitability requirements
Chromatographic similarity: The chromatogram from the *Standard solution* is similar to the reference chromatogram provided with the USP Aztec Marigold Zeaxanthin Extract RS being used.
Resolution: NLT 1.0 between zeaxanthin and lutein
Tailing factor: NMT 2.0 for the zeaxanthin peak
Relative standard deviation: NMT 2.0% for the zeaxanthin peak

Analysis
Sample: *Sample solution*

Calculate the percentage of all-*trans*-zeaxanthin ($C_{40}H_{56}O_2$) in the portion of the sample taken:

$$\text{Result} = (r_U/r_T) \times T$$

r_U = peak response of all-*trans*-zeaxanthin from the *Sample solution*
r_T = sum of the responses for all the peaks from the *Sample solution*
T = percentage of total carotenoids as determined in the test for *Content of Total Carotenoids*

Acceptance criteria: NLT 30.0% of all-*trans*-zeaxanthin on the dried basis

• **LUTEIN AND OTHER RELATED COMPOUNDS**

[NOTE—Use low-actinic glassware.]

Mobile phase, Standard solution, Sample solution, Chromatographic system, and System suitability: Proceed as directed in the test for *Content of Zeaxanthin*.

Analysis
Sample: *Sample solution*

Calculate the percentage of lutein in the portion of the sample taken:

$$\text{Result} = (r_U/r_T) \times T$$

r_U = peak response of lutein from the *Sample solution*
r_T = sum of the responses for all the peaks from the *Sample solution*
T = percentage of total carotenoids as determined in the test for *Content of Total Carotenoids*

Calculate the percentage of other related compounds in the portion of the sample taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of individual related compounds peaks from the *Sample solution*
r_T = sum of the responses for all the peaks from the *Sample solution*

Acceptance criteria
Lutein: NMT 8.0% on the dried basis
Other related compounds: NMT 2.0%

• **STEREOISOMERIC COMPOSITION**

[NOTE—Use low-actinic glassware.]

Mobile phase: Gradient elution. See *Table 2*.

Table 2

Time (min)	*n*-Hexane (%)	2-Propanol (%)
0.0	95	5

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Table 2 *(continued)*

Time (min)	*n*-Hexane (%)	2-Propanol (%)
50	95	5
55	50	50
63	50	50
65	95	5
75	95	5

Standard stock solution: 0.1 mg/mL of USP Aztec Marigold Zeaxanthin Extract RS in methylene chloride. Sonicate if necessary to dissolve the sample.

Standard solution: Transfer 1.0 mL of the *Standard stock solution* into a 15-mL test tube, and evaporate to dryness with the aid of a stream of nitrogen. Dissolve the residue in a 10.0-mL mixture of 2-propanol and hexane (5:95). Pass through a membrane filter of 0.45-µm pore size.

Sample stock solution: Weigh 10 mg of Extract into a 100-mL volumetric flask, add 85 mL of methylene chloride, sonicate, and swirl to dissolve. Dilute with methylene chloride to volume.

Sample solution: Transfer 1.0 mL of the *Sample stock solution* into a 15-mL test tube, and evaporate to dryness with the aid of a nitrogen stream. Dissolve the residue in a 10.0-mL mixture of 2-propanol and hexane (5:95). Pass the solution through a membrane filter of 0.45-µm pore size.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: HPLC

Detector: 450 nm

Column: 4.6-mm × 25-cm; 5-µm packing L51

Flow rate: 0.8 mL/min

Injection volume: 20 µL

System suitability

Sample: *Standard solution*

[NOTE—The approximate relative retention times for (3*R*,3′*S meso*)-zeaxanthin, (3*R*,3′*R*)-zeaxanthin, and (3*R*,3′*R*,6′*R*)-lutein are 0.92, 1.00, and 1.12, respectively.]

Suitability requirements

Resolution: The resolution between each pair of peaks due to (3*R*,3′*S meso*)-zeaxanthin, (3*R*,3′*R*)-zeaxanthin, and (3*R*,3′*R*,6′*R*)-lutein is NLT 1.0.

Chromatogram similarity: The chromatogram from the *Standard solution* is similar to the reference chromatogram provided with the USP Aztec Marigold Zeaxanthin Extract RS being used.

Analysis

Samples: *Standard solution* and *Sample solution*

Identify the peaks of the relevant analytes in the chromatogram of the *Sample solution* by comparing with those in the chromatogram of the *Standard solution* obtained from the *System suitability*.

Calculate the percentages of (3*R*,3′*S meso*)-zeaxanthin and (3*R*,3′*R*)-zeaxanthin in the portion of the sample taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of the corresponding analyte

r_T = sum of the responses for two peaks

Acceptance criteria

(3*R*,3′*R*)-Zeaxanthin: NLT 99.0%

(3*R*,3′*S meso*)-Zeaxanthin: NMT 1.0%

CONTAMINANTS

• **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩

Acceptance criteria

Arsenic: NMT 0.5 µg/g

Cadmium: NMT 1.0 µg/g

Lead: NMT 5.0 µg/g

Mercury: NMT 0.1 µg/g

SPECIFIC TESTS

• **LOSS ON DRYING** ⟨731⟩

Analysis: Dry a sample under vacuum at 105° for 3 h.

Acceptance criteria: NMT 25.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Aztec Marigold Zeaxanthin Extract RS

Bacillus coagulans

DEFINITION

The *Bacillus coagulans* species is a lactic-acid producing, Gram-positive, spore-forming, rod-shaped bacterium that is aerobic to microaerophilic.

The strain *Bacillus coagulans* GBI-30, 6086 is a pure, specific strain of *Bacillus coagulans* Hammer. *Bacillus coagulans* GBI-30, 6086 occurs as a white to beige powder that is manufactured as a spore preparation consisting solely of *Bacillus coagulans* GBI-30, 6086 that has been freeze-dried or spray-dried. The formulated product may be blended with food-grade diluents and/or bulking agents. It contains NLT 100% of the labeled viable cell count of the *Bacillus coagulans* GBI-30, 6086 strain.

IDENTIFICATION

• **NUCLEIC ACID-BASED IDENTIFICATION**

[NOTE—In all cases for *Identification*, "sterile water" refers to sterile, nuclease-free water acceptable for use in molecular biology.[1]]

Sample preparation reagent: Use PrepMan™ Ultra Sample Preparation Reagent.[2]

Sample: The preparation of the *Sample* requires isolation of colonies grown on plates in the test for *Enumeration*. Surface colonies grown in the *Enumeration* test should be carefully removed from incubated *BC agar medium* plates following counting of the colonies as described in the *Assay* as follows. Dispense 100 µL of *Sample preparation reagent* into a 2.0-mL microcentrifuge tube with a screw cap closure. [NOTE—Dispense the *Sample preparation reagent* according to the manufacturer's instructions.] Using a sterile loop, carefully remove the cells from a single surface colony and suspend the cells in the microcentrifuge tube. Cap the tube, and vortex for 10–30 s or until the cells are homogeneously suspended in the *Sample preparation reagent*. Heat the mixture for 10 min in a water bath held at 100°, then centrifuge the mixture for 3 min at 16,000 x g. Carefully transfer the supernatant to a new microcentrifuge tube. This solution contains the *Bacillus coagulans* GBI-30, 6086 genomic DNA. Before analysis, prepare the *Sample* by diluting an aliquot of this solution with sterile water (1:100, v/v).

[1] Suitable PCR-Certified Waters, RNase and DNase Free, are available from www.teknova.com.

[2] Applied Biosystems PrepMan™ Ultra Sample Preparation Reagent is available from Applied Biosystems (www.appliedbiosystems.com).

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Primer set 301: Use a set of 301_Left, primer sequence (5′–3′) AAGAAGTGGATGTGGGCCGTTC and 301_Right, primer sequence (5′–3′) CTTTTGCCATGCCAGCATCC.[3] Primers should be diluted to 2 µg/mL in sterile water and stored between –20° and –80°. Immediately before use, dilute an aliquot of each primer with sterile water (1:5, v/v). The annealing temperature for *Primer set 301* is 65°.

Primer set 210243: Use a set of 210243_Left, primer sequence (5′–3′) GCCTGATGCAGGGCTTTTCCT and 210243_Right, primer sequence (5′–3′) CCGTCCGCTTCCGTTAAGCCG. Primers should be diluted to 2 µg/mL in sterile water and stored between –20° and –80°. Immediately before use, dilute an aliquot of each primer with sterile water (1:5, v/v). The annealing temperature for *Primer set 210243* is 70°.

Primer set 31125: Use a set of 31125_Left, primer sequence (5′–3′) TTATAGGCGGTAGCAGGGATC and 31125_Right, primer sequence (5′–3′) CGATTGTTTTTCCGAAGCA. Primers should be diluted to 2 µg/mL in sterile water and stored between –20° and –80°. Immediately before use, dilute an aliquot of each primer with sterile water (1:5, v/v). The annealing temperature for *Primer set 31125* is 61°.

Polymerase chain reaction (PCR) sample preparations: For each primer set, prepare a PCR sample preparation that contains 7.5 µL of polymerase master mix,[4] 1 µL of diluted primer left (approximately 400 ng), 1 µL of diluted primer right (approximately 400 ng), 1 µL of diluted *Sample* containing template DNA (approximately 10–100 ng), and 4.5 µL of sterile water.

PCR negative control: Prepare as directed for the *PCR sample preparations*, replacing the 1 µL of diluted *Sample* with 1 µL of nuclease-free water.

PCR amplification: Perform PCR on each *PCR sample preparation* and the *PCR negative control* using a thermal cycler.[5] Incubate at 98° for 30 s (1 cycle), followed by 30 cycles at 98° for 10 s, annealing temperature for 10 s, and 1 cycle at 72° for 45 s, followed by a final incubation at 72° for 5 min with a hold at 4°.

Analysis: Analyze the products of the *PCR amplification* for each *PCR sample preparation* and for the *PCR negative control* using an automated on-chip electrophoresis system with a DNA kit.[6] Follow the manufacturer's instructions for analysis. Analysis of the *PCR negative control* must result in the absence of any amplification products or the preparation of the *PCR sample preparations* and the *PCR negative control* must be repeated, followed by *PCR amplification* and *Analysis*.

Acceptance criteria

Primer set 301: The *PCR sample preparation* prepared with *Primer set 301* produces an amplification product of 376–399 base pairs.

Primer set 210243: The *PCR sample preparation* prepared with *Primer set 210243* does not produce an amplification product of 1253–1331 base pairs.

Primer set 31125: The *PCR sample preparation* prepared with *Primer set 31125* produces an amplification product of 329–339 base pairs.

[3] DNA primers are commercially available (custom manufacture) through Invitrogen via Life Technologies™ (www.thermofisher.com) and other commercial sources.

[4] Use New England Biolabs Phusion® High-Fidelity PCR Master Mix with GC Buffer (www.neb.com), or equivalent mixture.

[5] Eppendorf Mastercycler® ep gradient S (www.eppendorf.com), or equivalent.

[6] Suitable automated on-chip electrophoresis systems with a DNA kit are available from Agilent [Agilent 2100 Bioanalyzer with Agilent DNA 1000 Kit (www.genomics.agilent.com)].

ASSAY

Change to read:

- **ENUMERATION**

Peptone diluent: Prepare a solution of 0.1% peptone[7] in water (w/v) and adjust with a solution of lactic acid to a pH of 7.0. Using an autoclave, steam sterilize the solution at 121° for NLT 15 min, then allow to cool in the unopened autoclave. Dispense into sterile containers as needed for preparing samples.

Trace mineral solution: ▲(ERR 1-Jun-2019)▲ Prepare a solution containing the mineral concentrations shown in *Table 1* in deionized water.

Table 1. Preparation of Trace Mineral Solution

Reagent	Quantity (mg/mL)
Sodium chloride	10
Iron(II) sulfate, heptahydrate	18
Manganese(II) sulfate, monohydrate	16
Zinc sulfate, heptahydrate	1.6
Copper(II) sulfate, pentahydrate	1.6
Cobalt(II) sulfate, heptahydrate	1.6

[NOTE—The solution will be slightly pink in color. It may be refrigerated for up to 2 months. In the case of hydrated salts, users may substitute other hydration forms so long as the mineral salt concentration is maintained in the final solution.]

BC agar medium: Prepare as shown in *Table 2*.

Table 2. Preparation of Glucose Yeast Extract BC Agar Medium

Reagent	Quantity
Yeast extract powder	5.0 g
Peptone	5.0 g
D-Glucose	5.0 g
Dibasic potassium phosphate (K_2HPO_4)	0.5 g
Monobasic potassium phosphate (KH_2PO_4)	0.5 g
Magnesium sulfate	0.3 g
Trace mineral solution	1.0 mL
Water	1000.0 mL

Adjust the mixture with a solution of lactic acid to a pH of 6.3. Transfer the mixture to a large conical flask, add 15.0 g of bacteriological agar to the flask, cover the flask with aluminum foil, and bring to boiling on a hot plate with stirring. Allow the mixture to boil until the agar has completely dissolved, then sterilize in an autoclave at 121° for NLT 15 min. Once the autoclave can safely be opened, remove the flask and incubate in a water bath at 50° until needed for plating. [NOTE—Can be stored at 4° (allow the agar to come to room temperature before use).]

Sample preparation: Transfer 1.00 g of sample into a sterile stomacher bag. Add 199 mL of previously sterilized *Peptone diluent* to the bag and mix at about 150–200 rpm for 5 min

[7] BD Bacto™ Peptone (www.bd.com), or equivalent peptone suitable for microbiological analysis.

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in a stomacher. [NOTE—In some cases a larger sample size is required for accurate enumeration. For samples with a serving size ranging from 2 g to 50 g, add from 198 mL to 150 mL, respectively, to get a total volume of 200 mL (i.e., dilutions ranging from 2 g/200 mL to 50 g/200 mL), or add from 298 mL to 250 mL of ▲*Peptone diluent*,▲ (ERR 1-Jun-2019) respectively, to get a total volume of 300 mL (i.e., dilutions ranging from 2 g/300 mL to 50 g/300 mL). For samples with a serving size larger than 50 g, take a half-serving size or use a representative sample of around 50 g or less, and add ▲*Peptone diluent*▲ (ERR 1-Jun-2019) to get a total volume of 200–300 mL. In these cases, the term "serving size" refers to the sample size representing the declared enumeration value for the product.] Check the pH of the suspension. If the pH is below 7.0, adjust with 5 N sodium hydroxide solution to a pH of 8.5 ± 0.2. If the pH is above 8.7, adjust with 5 N lactic acid solution to a pH of 8.5 ± 0.2. Transfer 20–30 mL of the homogenized suspension to a sterile 50-mL conical centrifuge tube with a cap. Incubate the tube in a water bath held at 75° for exactly 30 min, then immediately cool to below 45°. Transfer 1.0 mL of the cooled suspension into a sterile test tube containing 9 mL of *Peptone diluent*, previously prepared. Mix thoroughly by vortexing. This suspension represents a 0.5×10^{-3} dilution of the sample. Repeat dilution in a succession of test tubes until the final dilution is expected to contain about 30 cfu/mL. The final three dilutions will be used in the *Analysis*. [NOTE—The *Sample preparation* should be performed in duplicate. Take care to plate the *Sample preparation* dilutions within 10–20 min of preparation.]

Analysis: For each *Sample preparation* tube to be plated, prepare Petri plates as follows. Aseptically transfer 1.0 mL of the *Sample preparation* separately into three sterile 15-mm × 100-mm Petri plates, then pour 15–20 mL of the molten *BC agar medium* into each plate. Place the lid on each plate after adding the molten *BC agar medium*, then gently swirl the plates to mix the *Sample preparation* and the *BC agar medium*. [NOTE—Be careful to avoid spillage onto the lid of the dish when swirling the plates.] Repeat this procedure for the additional two dilutions of the *Sample preparation* (and all duplicate tubes). Prepare one blank plate that contains only *BC agar medium* and a second blank plate in which 1.0 mL of *Peptone diluent* has been mixed with *BC agar medium*. Allow the plates to sit at room temperature until the *BC agar medium* solidifies, then invert the plates and incubate them at 40 ± 2° for 48 h. After 48 h of incubation, count the colonies on the prepared plates, including both blank plates. Plates containing between 30 and 300 colonies are considered ideal for counting. Count only colonies with the following appearance. Surface colonies should be 1–5 mm in diameter, white to cream in color, convex, with entire margins and smooth surfaces. Colonies inside the *BC agar medium* should be 0.5–1 mm in diameter and should appear as cream colored pinpoints in the *BC agar medium*. Report results in cfu/g. Calculate the average number of colonies per plate, then multiply the average number of colonies counted by the reciprocal of the dilution factor to obtain the cfu/g of the sample. For samples larger than 1 g, consider the sample size as 1 unit. Calculate the average number of colonies per plate, and then multiply the average number of colonies counted by the reciprocal of the dilution factor to obtain cfu/sample. To get cfu/g of the sample, divide cfu/sample by actual gram weight sample size. For example, if the sample size is 50 g and the final volume is 300 mL (50-g sample plus 250 mL of ▲*Peptone diluent*),▲ (ERR 1-Jun-2019) the first dilution factor would be 1/300 (consider the whole sample size as 1 unit). If 5 additional 1-in-10 dilutions are made, the total dilution factor would be $1/300 \times 1/10 \times 1/10 \times 1/10 \times 1/10 \times 1/10 = 1/300 \times 1/10^{-5}$. If the average colonies counted per plate of the last dilution is 200, the cfu/sample size would be $200 \times 300 \times 10^5 = 6 \times 10^9$ cfu/50 g and $6/50 \times 10^9 = 1.2 \times 10^8$ cfu/g. The presence of any colonies not conforming to this description suggests a contaminated sample that must be investigated. Depending on the outcome of the investigation, the test may be rejected or repeated. Both blank plates should be entirely free of any type of colonies. [NOTE—In the case of blank plates that contain colonies, the entire procedure must be repeated, potentially including the preparation of the *Diluent* and the *BC agar medium*, depending on which plate(s) contain colonies.]

Acceptance criteria: NLT 100% of the labeled viable cfu/g

SPECIFIC TESTS

• **Loss on Drying** ⟨731⟩

Sample: 2–3 g, mixed for homogeneity

Analysis: Weigh the *Sample* directly into a weighed, round, flat-bottom metal dish (NMT 5 cm in diameter) with a tightly fit, slip-in cover. Loosely cover the dish and place it directly on the metal shelf of a vacuum oven set at 100°. Dry to constant weight (about 4 h) under a pressure of NLT 100 mmHg. During drying, admit a slow current of air (about 2 bubbles/s) that has been dried by passing it through sulfuric acid into the oven. Stop the vacuum pump and carefully admit dried air into the oven. Press the cover tightly into the dish, remove the dish from the oven, cool in a desiccator, and weigh to determine the moisture content.

Acceptance criteria: NMT 6%

CONTAMINANTS

• **Yeasts**

Peptone diluent: Prepare a solution of 0.1% peptone in water (w/v) and adjust to a pH of 7.0 with a solution of hydrochloric acid. Transfer a quantity of the *Peptone diluent* to media bottles in 225-mL aliquots and cap each bottle loosely. Transfer the remainder of the *Peptone diluent* to 15-mL sterile test tubes in 9-mL aliquots and cap tubes loosely or cover with aluminum foil. Using an autoclave, steam sterilize the solution in the media bottles and test tubes at 121° for NLT 15 min, then allow to cool in the unopened autoclave. Tighten the loose caps on the media bottles and test tubes immediately after opening the autoclave.

Yeast agar medium: Mix the reagents shown in *Table 3* in a 1-L conical flask.

Table 3. Preparation of Dichloran 18% Glycerol (DG-18)[a] Yeast Agar Medium

Reagent	Quantity
Peptone	5.0 g
D-Glucose	10.0 g
Monobasic potassium phosphate (KH_2PO_4)	1.0 g
Magnesium sulfate heptahydrate	0.5 g
Dichloran[b]	1.0 mL
Bacteriological agar	15.0 g
Chloramphenicol	0.1 g

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Table 3. Preparation of Dichloran 18% Glycerol (DG-18)[a] Yeast Agar Medium *(continued)*

Reagent	Quantity
Water	800 mL

[a] An equivalent commercial preparation may be used in place of this mixture. Prepare as directed by the manufacturer.
[b] Dichloran is 2,6-dichloro-4-nitroaniline. (0.2% in ethanol, w/v)

Heat the mixture to boiling (with stirring) on a hot plate and allow the mixture to boil until the agar is completely dissolved. Once the agar has dissolved, add water to bring the volume in the flask to 1000 mL. Add 220 g of glycerol to the flask, cover it with aluminum foil, and sterilize in an autoclave at 121° for NLT 15 min. Once the autoclave can safely be opened, remove the flask and incubate in a water bath at 50° until needed for plating. The final pH of this solution should be 5.6.

Sample preparation: The number of samples necessary for analysis should follow a pre-determined sampling plan based on the quantity of material available and the number and size of containers. For each sample to be tested, transfer 25 g of sample into a sterile stomacher bag. Transfer the contents of one sterile bottle of *Peptone diluent* (225 mL) to the bag, and mix at about 150–200 rpm for 2 min in a stomacher. This suspension represents a 10^{-1} dilution of the sample. Transfer 1.0 mL of the suspension into a sterile test tube containing 9 mL of *Peptone diluent*, previously prepared. Mix thoroughly by vortexing. This suspension represents a 10^{-2} dilution of the sample. Use both the 10^{-1} and 10^{-2} dilutions of the *Sample preparation* in the *Analysis*. [NOTE—Plate the *Sample preparation* dilutions within 10–20 min of preparation.]

Analysis: For each *Sample preparation* dilution to be plated, prepare Petri plates as follows. Aseptically transfer 1.0 mL of the *Sample preparation* separately into three sterile 15-mm × 100-mm Petri plates, then pour 20–25 mL of the molten *Yeast agar medium* into each plate. Place the lid on each plate after adding the molten *Yeast agar medium*, then gently swirl the plates to mix the *Sample preparation* and the *Yeast agar medium*. [NOTE—Be careful to avoid spillage onto the lid of the dish when swirling the plates.] Prepare one blank plate that contains only *Yeast agar medium* and a second blank plate in which 1.0 mL of *Peptone diluent* has been mixed with *Yeast agar medium*. Allow the plates to sit at room temperature until the *Yeast agar medium* solidifies, then incubate them (in the dark) at 25° for 5 days.

Count colonies present after 5 days of incubation. If there is no growth after 5 days, incubate the plates for an additional 48 h and count colonies at that time. Yeast colonies grow within the *Yeast agar medium* and appear as discrete disk-shaped units. If plates contain more than 150 colonies, repeat the *Analysis* on a further diluted portion of the *Sample preparation* in order to obtain an accurate count. Both blank plates should be free of any colonies. Report results for each dilution of the *Sample preparation* in cfu/g based on the average count of the plates multiplied by the reciprocal of the dilution factor. When all plates from both dilutions have no colonies, report the number of colonies as <10 cfu/g.

Acceptance criteria: NMT 100 cfu/g

- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Escherichia coli, Test for Absence of Staphylococcus aureus,* and *Test for Absence of Salmonella Species*: It meets the requirements of the tests for the absence of *Escherichia coli* in 25 g, *Staphylococcus aureus* in 50 g, and *Salmonella* species in 25 g.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers in a cool, dry place.
- **LABELING:** This ingredient should be labeled with the genus, species, and strain name and with the formulated enumeration in cfu/g (or similar units).

Bacillus coagulans Capsules

DEFINITION

Bacillus coagulans Capsules contain NLT 100% of the labeled viable cell count of the *Bacillus coagulans* GBI-30, 6086 strain.

IDENTIFICATION

- **NUCLEIC ACID-BASED IDENTIFICATION**

[NOTE—In all cases for *Identification*, "sterile water" refers to sterile, nuclease-free water acceptable for use in molecular biology.[1]]

Sample preparation reagent: Use PrepMan™ Ultra Sample Preparation Reagent.[2]

Sample: The preparation of the *Sample* requires isolation of colonies grown on plates in the test for *Enumeration*. Surface colonies grown in the *Enumeration* test should be carefully removed from incubated *BC agar medium* plates following counting of the colonies as described in the *Assay* as follows. Dispense 100 µL of *Sample preparation reagent* into a 2.0-mL microcentrifuge tube with a screw cap closure. [NOTE—Dispense the *Sample preparation reagent* according to the manufacturer's instructions.] Using a sterile loop, carefully remove the cells from a single surface colony and suspend the cells in the microcentrifuge tube. Cap the tube, and vortex for 10–30 s or until the cells are homogeneously suspended in the *Sample preparation reagent*. Heat the mixture for 10 min in a water bath held at 100°, then centrifuge the mixture for 3 min at 16,000 × g. Carefully transfer the supernatant to a new microcentrifuge tube. This solution contains the *Bacillus coagulans* GBI-30, 6086 genomic DNA. Before analysis, prepare the *Sample* by diluting an aliquot of this solution with sterile water (1:100, v/v).

Primer set 301: Use a set of 301_Left, primer sequence (5′–3′) AAGAAGTGGATGTGGGCCGTTC and 301_Right, primer sequence (5′–3′) CTTTTGCCATGCCAGCATCC.[3] Primers should be diluted to 2 µg/mL in sterile water and stored between –20° and –80°. Immediately before use, dilute an aliquot of each primer with sterile water (1:5, v/v). The annealing temperature for *Primer set 301* is 65°.

Primer set 210243: Use a set of 210243_Left, primer sequence (5′–3′) GCCTGATGCAGGGCTTTTCCT and 210243_Right, primer sequence (5′–3′) CCGTCCGCTTCCGTTAAGCCG. Primers should be diluted to 2 µg/mL in sterile water and stored between –20° and –80°. Immediately before use, dilute an aliquot of each primer with sterile water (1:5, v/v). The annealing temperature for *Primer set 210243* is 70°.

Primer set 31125: Use a set of 31125_Left, primer sequence (5′–3′) TTATAGGCGGTAGCAGGGATC and 31125_Right, primer sequence (5′–3′) CGATTGTTTTTCCGAAGCA. Primers should be diluted to 2 µg/mL in sterile water and

[1] Suitable PCR-Certified Waters, RNase and DNase Free, are available from www.teknova.com.
[2] Applied Biosystems PrepMan™ Ultra Sample Preparation Reagent is available from Applied Biosystems (www.appliedbiosystems.com).
[3] DNA primers are commercially available (custom manufacture) through Invitrogen via Life Technologies™ (www.thermofisher.com) and other commercial sources.

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stored between −20° and −80°. Immediately before use, dilute an aliquot of each primer with sterile water (1:5, v/v). The annealing temperature for *Primer set 31125* is 61°.

Polymerase chain reaction (PCR) sample preparations: For each primer set, prepare a PCR sample preparation that contains 7.5 µL of polymerase master mix,[4] 1 µL of diluted primer left (approximately 400 ng), 1 µL of diluted primer right (approximately 400 ng), 1 µL of diluted *Sample* containing template DNA (approximately 10–100 ng), and 4.5 µL of sterile water.

PCR negative control: Prepare as directed for the *PCR sample preparations*, replacing the 1 µL of diluted *Sample* with 1 µL of nuclease-free water.

PCR amplification: Perform PCR on each PCR sample preparation and the *PCR negative control* using a thermal cycler.[5] Incubate at 98° for 30 s (1 cycle), followed by 30 cycles at 98° for 10 s, annealing temperature for 10 s, and 1 cycle at 72° for 45 s, followed by a final incubation at 72° for 5 min with a hold at 4°.

Analysis: Analyze the products of the *PCR amplification* for each PCR sample preparation and for the *PCR negative control* using an automated on-chip electrophoresis system with a DNA kit.[6] Follow the manufacturer's instructions for analysis. Analysis of the *PCR negative control* must result in the absence of any amplification products or the preparation of the *PCR sample preparations* and the *PCR negative control* must be repeated, followed by *PCR amplification* and *Analysis*.

Acceptance criteria

Primer set 301: The PCR sample preparation prepared with *Primer set 301* produces an amplification product of 376–399 base pairs.

Primer set 210243: The PCR sample preparation prepared with *Primer set 210243* does not produce the expected amplification product of 1253–1331 base pairs.

Primer set 31125: The PCR sample preparation prepared with *Primer set 31125* produces an amplification product of 329–339 base pairs.

ASSAY

Change to read:

- **ENUMERATION**

Peptone diluent: Prepare a solution of 0.1% peptone[7] in water (w/v) and adjust with a solution of lactic acid to a pH of 7.0. Using an autoclave, steam sterilize the solution at 121° for NLT 15 min, then allow to cool in the unopened autoclave. Dispense into sterile containers as needed for preparing samples.

▲Trace mineral solution:▲ (ERR 1-Jun-2019) Prepare a solution containing the mineral concentrations shown in *Table 1* in deionized water.

Table 1. Preparation of Trace Mineral Solution

Reagent	Quantity (mg/mL)
Sodium chloride	10
Iron(II) sulfate, heptahydrate	18
Manganese(II) sulfate, monohydrate	16
Zinc sulfate, heptahydrate	1.6
Copper(II) sulfate, pentahydrate	1.6
Cobalt(II) sulfate, heptahydrate	1.6

[NOTE—The solution will be slightly pink in color. It may be refrigerated for up to 2 months. In the case of hydrated salts, users may substitute other hydration forms so long as the mineral salt concentration is maintained in the final solution.]

BC agar medium: Prepare as shown in *Table 2*.

Table 2. Preparation of Glucose Yeast Extract BC Agar Medium

Reagent	Quantity
Yeast extract powder	5.0 g
Peptone	5.0 g
D-Glucose	5.0 g
Dibasic potassium phosphate (K_2HPO_4)	0.5 g
Monobasic potassium phosphate (KH_2PO_4)	0.5 g
Magnesium sulfate	0.3 g
Trace mineral solution	1.0 mL
Water	1000.0 mL

Adjust the mixture with a solution of lactic acid to a pH of 6.3. Transfer the mixture to a large conical flask, add 15.0 g of bacteriological agar to the flask, cover the flask with aluminum foil, and bring to boiling on a hot plate with stirring. Allow the mixture to boil until the agar has completely dissolved, then sterilize in an autoclave at 121° for NLT 15 min. Once the autoclave can safely be opened, remove the flask and incubate in a water bath at 50° until needed for plating. [NOTE—Can be stored at 4° (allow the agar to come to room temperature before use).]

Sample preparation: Weigh the contents of NLT 20 Capsules and determine the average weight. Empty the Capsules and transfer 1.00 g of the Capsule contents into a sterile stomacher bag. Add 199 mL of previously sterilized *Peptone diluent* to the bag and mix at about 150–200 rpm for 5 min in a stomacher. Check the pH of the suspension. If the pH is below 7.0, adjust with 5 N sodium hydroxide solution to a pH of 8.5 ± 0.2. If the pH is above 8.7, adjust with 5 N lactic acid solution to a pH of 8.5 ± 0.2. Transfer 20–30 mL of the homogenized suspension to a sterile 50-mL conical centrifuge tube with a cap. Incubate the tube in a water bath held at 75° for exactly 30 min, then immediately cool to below 45°. Transfer 1.0 mL of the cooled suspension into a sterile test tube containing 9 mL of *Peptone diluent*, previously prepared. Mix thoroughly by vortexing. This suspension represents a 0.5 x 10^{-3} dilution of the sample. Repeat dilution in a succession of test tubes until the final dilution is expected to contain about 30 cfu/mL. The final three dilutions will be used in the *Analysis*. [NOTE—The *Sample preparation* should be performed in duplicate. Take care to plate the *Sample preparation* dilutions within 10–20 min of preparation.]

Analysis: For each *Sample preparation* tube to be plated, prepare Petri plates as follows. Aseptically transfer 1.0 mL

[4] Use New England Biolabs Phusion® High-Fidelity PCR Master Mix with GC Buffer (www.neb.com), or equivalent mixture.

[5] Eppendorf Mastercycler® ep gradient S (www.eppendorf.com), or equivalent..

[6] Suitable automated on-chip electrophoresis systems with a DNA kit are available from Agilent [Agilent 2100 Bioanalyzer with Agilent DNA 1000 Kit (www.genomics.agilent.com)].

[7] BD Bacto™ Peptone (www.bd.com), or equivalent peptone suitable for microbiological analysis.

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of the *Sample preparation* separately into three sterile 15-mm × 100-mm Petri plates, then pour 15–20 mL of the molten *BC agar medium* into each plate. Place the lid on each plate after adding the molten *BC agar medium*, then gently swirl the plates to mix the *Sample preparation* and the *BC agar medium*. [NOTE—Be careful to avoid spillage onto the lid of the dish when swirling the plates.] Repeat this procedure for the additional two dilutions of the *Sample preparation* (and all duplicate tubes). Prepare one blank plate that contains only *BC agar medium* and a second blank plate in which 1.0 mL of *Peptone diluent* has been mixed with *BC agar medium*. Allow the plates to sit at room temperature until the *BC agar medium* solidifies, then invert the plates and incubate them at 40 ± 2° for 48 h. After 48 h of incubation, count the colonies on the prepared plates, including both blank plates. Plates containing between 30 and 300 colonies are considered ideal for counting. Count only colonies with the following appearance. Surface colonies should be 1–5 mm in diameter, white to cream in color, convex, with entire margins and smooth surfaces. Colonies inside the *BC agar medium* should be 0.5–1 mm in diameter and should appear as cream colored pinpoints in the *BC agar medium*. Calculate the average number of colonies per plate, then multiply the average number of colonies counted by the reciprocal of the dilution factor to obtain the cfu/g of the sample. Determine the cell counts in cfu/Capsule. The presence of any colonies not conforming to this description suggests a contaminated sample that must be investigated. Depending on the outcome of the investigation, the test may be rejected or repeated. Both blank plates should be entirely free of any type of colonies. [NOTE—In the case of blank plates that contain colonies, the entire procedure must be repeated, potentially including the preparation of the *Diluent* and the *BC agar medium*, depending on which plate(s) contain colonies.]

Acceptance criteria: NLT 100% of the labeled viable cfu/Capsule.

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩, *Disintegration*: Meet the requirements
- **WEIGHT VARIATION** ⟨2091⟩: Meet the requirements

CONTAMINANTS

- **YEASTS**

Peptone diluent: Prepare a solution of 0.1% peptone in water (w/v) and adjust with a solution of hydrochloric acid to a pH of 7.0. Transfer a quantity of the *Peptone diluent* to media bottles in 225-mL aliquots and cap each bottle loosely. Transfer the remainder of the *Peptone diluent* to 15-mL sterile test tubes in 9-mL aliquots and cap tubes loosely or cover with aluminum foil. Using an autoclave, steam sterilize the solution in the media bottles and test tubes at 121° for NLT 15 min, then allow to cool in the unopened autoclave. Tighten the loose caps on the media bottles and test tubes immediately after opening the autoclave.

Yeast agar medium: Mix the reagents shown in *Table 3* in a 1-L conical flask.

Table 3. Preparation of Dichloran 18% Glycerol (DG-18)[a] Yeast Agar Medium

Reagent	Quantity
Peptone	5.0 g
D-Glucose	10.0 g
Monobasic potassium phosphate (KH_2PO_4)	1.0 g
Magnesium sulfate heptahydrate	0.5 g
Dichloran[b] (0.2% in ethanol, w/v)	1.0 mL
Bacteriological agar	15.0 g
Chloramphenicol	0.1 g
Water	800 mL

[a] An equivalent commercial preparation may be used in place of this mixture. Prepare as directed by the manufacturer.
[b] Dichloran is 2,6-dichloro-4-nitroaniline.

Heat the mixture to boiling (with stirring) on a hot plate and allow the mixture to boil until the agar is completely dissolved. Once the agar has dissolved, add water to bring the volume in the flask to 1000 mL. Add 220 g of glycerol to the flask, cover it with aluminum foil, and sterilize in an autoclave at 121° for NLT 15 min. Once the autoclave can safely be opened, remove the flask and incubate in a water bath at 50° until needed for plating. The final pH of this solution should be 5.6.

Sample preparation: The number of samples necessary for analysis should follow a pre-determined sampling plan based on the quantity of material available and the number and size of containers. For each sample to be tested, transfer 25 g of the Capsule content into a sterile stomacher bag. Transfer the contents of one sterile bottle of *Peptone diluent* (225 mL) to the bag, and mix at about 150–200 rpm for 2 min in a stomacher. This suspension represents a 10^{-1} dilution of the sample. Transfer 1.0 mL of the suspension into a sterile test tube containing 9 mL of *Peptone diluent*, previously prepared. Mix thoroughly by vortexing. This suspension represents a 10^{-2} dilution of the sample. Use both the 10^{-1} and 10^{-2} dilutions of the *Sample preparation* in the *Analysis*. [NOTE—Plate the *Sample preparation* dilutions within 10–20 min of preparation.]

Analysis: For each *Sample preparation* dilution to be plated, prepare Petri plates as follows. Aseptically transfer 1.0 mL of the *Sample preparation* separately into three sterile 15-mm × 100-mm Petri plates, then pour 20–25 mL of the molten *Yeast agar medium* into each plate. Place the lid on each plate after adding the molten *Yeast agar medium*, then gently swirl the plates to mix the *Sample preparation* and the *Yeast agar medium*. [NOTE—Be careful to avoid spillage onto the lid of the dish when swirling the plates.] Prepare one blank plate that contains only *Yeast agar medium* and a second blank plate in which 1.0 mL of *Peptone diluent* has been mixed with *Yeast agar medium*. Allow the plates to sit at room temperature until the *Yeast agar medium* solidifies, then incubate them (in the dark) at 25° for 5 days. Count colonies present after 5 days of incubation. If there is no growth after 5 days, incubate the plates for an additional 48 h and count colonies at that time. Yeast colonies grow within the *Yeast agar medium* and appear as discrete disk-shaped units. If plates contain more than 150 colonies, repeat the *Analysis* on a further diluted portion of the *Sample preparation* in order to obtain an accurate count. Both blank plates should be free of any colonies. Report results for each dilution of the *Sample preparation* in cfu/g based on the average count of the plates multiplied by the reciprocal of the dilution factor. When all plates from both dilutions have no colonies, report the number of colonies as <10 cfu/g.

Acceptance criteria: NMT 100 cfu/g

- **COLIFORMS:** See *Food Chemicals Codex, General Tests and Assays, Appendix XV, Coliforms*.

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Acceptance criteria: NMT 10 cfu/g

- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Escherichia coli, Test for Absence of Staphylococcus aureus,* and *Test for Absence of Salmonella Species*: It meets the requirements of the tests for the absence of *Escherichia coli* in 25 g, *Staphylococcus aureus* in 50 g, and *Salmonella* species in 25 g.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store in a cool, dry place.
- **LABELING:** The Capsules should be labeled with the genus and species names, or genus, species, and strain names and with the formulated enumeration in cfu/Capsule.

Bacopa

DEFINITION

Bacopa consists of the dried stems and leaves of *Bacopa monnieri* (L.) Pennell (Fam. Scrophulariaceae). It contains NLT 2.5% of triterpene glycosides, calculated on the dried basis as the sum of bacopaside I, bacoside A_3, bacopaside II, the jujubogenin isomer of bacopasaponin C, and bacopasaponin C.

IDENTIFICATION

- **A.** Bacopa meets the requirements for *Specific Tests, Botanical Characteristics.*
- **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩

Standard solution: Transfer about 10 mg of USP Powdered Bacopa Extract RS to a 10-mL volumetric flask, and add about 8 mL of methanol. Sonicate and heat gently for 15–20 min, dilute with methanol to volume, mix, centrifuge, and use the supernatant.

Sample solution: Use the *Sample solution*, prepared as directed in the test for *Content of Triterpene Glycosides.*

Adsorbent: Chromatographic silica gel mixture with an average particle size of 10–15 µm (TLC plates)

Application volume: 15 µL, as 5–10 mm bands

Developing solvent system: Ethyl acetate, methanol, and water (7:2:1)

Spray reagent: 1% Vanillin in alcohol and 10% sulfuric acid in alcohol (1:1)

Analysis

Samples: *Standard solution* and *Sample solution*

Apply the samples as bands (see *Chromatography* ⟨621⟩). Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about three-fourths of the plate. Remove the plate from the chamber, dry, spray with *Spray reagent*, heat for 5–10 min at 70°, and examine under white light.

Acceptance criteria: The *Sample solution* exhibits a main dark blue zone due to a mixture of bacoside A_3, bacopaside II, the jujubogenin isomer of bacopasaponin C, and bacopasaponin C at an R_F value of approximately 0.6 and a faint pink spot due to bacopaside I at an R_F value of approximately 0.4, both of which correspond in position and color to zones in the chromatogram of the *Standard solution.* Other zones are observed for the *Sample solution* and *Standard solution.*

- **C. HPLC IDENTIFICATION TEST:** The *Sample solution* from the test for *Content of Triterpene Glycosides* shows a main peak at the retention time corresponding to that of bacoside A_3 in the chromatogram of *Standard solution A.* Identify other triterpene glycoside peaks in the *Sample solution* by comparison with the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Bacopa Extract RS. The *Sample solution* shows additional peaks corresponding to bacopaside I, bacopaside II, the jujubogenin isomer of bacopasaponin C, and bacopasaponin C.

COMPOSITION

- **CONTENT OF TRITERPENE GLYCOSIDES**

Solution A: Dissolve 0.14 g of anhydrous potassium dihydrogen phosphate in 900 mL of water, add 0.5 mL of phosphoric acid, dilute with water to 1000 mL, mix, filter, and degas.

Solution B: Use filtered and degassed acetonitrile.

Mobile phase: See the *gradient table* below.

Time (min)	Solution A (%)	Solution B (%)
0	70	30
25	60	40
26	70	30
30	70	30

Standard solution A: Sonicate an accurately weighed quantity of USP Bacoside A_3RS in methanol to obtain a solution with a concentration of about 0.5 mg/mL.

Standard solution B: Transfer about 10 mg of USP Powdered Bacopa Extract RS to a 10-mL volumetric flask, and add about 8 mL of methanol. Sonicate and heat gently for 15–20 min, dilute with methanol to volume, and mix. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first 5 mL of the filtrate.

Sample solution: Transfer about 2.5 g of Bacopa, finely powdered, to a 100-mL round-bottom flask fitted with a reflux condenser. Add 25 mL of methanol, reflux on a water bath for 10 min, cool to room temperature, and decant the supernatant. Repeat until the last extract is colorless. Combine the extracts, filter, concentrate under vacuum, and adjust the volume to 100 mL using methanol. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first 5 mL of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability.*)

Mode: LC

Detector: UV 205 nm

Column: 4.6-mm × 25-cm; 5-µm, endcapped, base-deactivated packing L1

Column temperature: 27°

Flow rate: 1.5 mL/min

Injection volume: 20 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Bacopa Extract RS being used.

Resolution: NLT 1.0 between the bacopaside II and bacoside A_3 peaks, *Standard solution B*

Tailing factor: NMT 1.5 for the bacoside A_3 peak, *Standard solution A*

Relative standard deviation: NMT 2% determined from the bacoside A_3 peak for replicate injections, *Standard solution A*

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Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatograms of *Standard solution A* and *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Bacopa Extract RS being used, identify the retention times of the peaks corresponding to different triterpene glycosides. The approximate relative retention times of the relevant triterpene glycosides are provided in the following *table.*

Analyte	Relative Retention Time
Bacopaside I	0.73
Bacoside A_3	1.00
Bacopaside II	1.04
The jujubogenin isomer of bacopasaponin C	1.15
Bacopasaponin C	1.22

Separately calculate the percentages of bacopaside I, bacoside A_3, bacopaside II, the jujubogenin isomer of bacopasaponin C, and bacopasaponin C in the portion of Bacopa taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak response for each triterpene glycoside from the *Sample solution*

r_S = peak response for bacoside A_3 from *Standard solution A*

C_S = concentration of USP Bacoside A_3RS in *Standard solution A* (mg/mL)

V = final volume of the *Sample solution* (mL)

W = weight of Bacopa used to prepare the *Sample solution* (mg)

F = conversion factor for each analyte: 1.00 for bacoside A_3, 1.03 for bacopaside I, 0.81 for bacopaside II, 0.99 for the jujubogenin isomer of bacopasaponin C, and 0.75 for bacopasaponin C

Acceptance criteria: Add the percentages of bacopaside I, bacoside A_3, bacopaside II, the jujubogenin isomer of bacopasaponin C, and bacopasaponin C: NLT 2.5% is found, calculated on the dried basis.

IMPURITIES

- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩: NMT 6.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩: NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩: Meets the requirements

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: Stem is creeping, succulent, glabrous, soft, obtuse-angular; with long internodes, rooting at nodes; devoid of leaves toward the base; branches ascending. Leaves are simple, sessile or short petiolate, opposite, succulent, 1–2 mm thick; oblong-obovate or spatulate, margin entire or rarely dentate, apex rounded, midrib indistinct, 0.6–2.5 cm long, 3–8 mm wide; the upper surface is green, the lower surface is green and dotted. Pharmacopeial article is yellowish in color; consists of dry mixtures of broken leaves and stems, with majority of leaves detached; mild and hay-like odor, and very bitter taste.

Microscopic

Transverse section of stems: Epidermal layer; a wide cortex composed of thin-wall parenchyma cells and large intercellular spaces; xylem vessels radially arranged, uniseriate medullary rays; pith composed of thin-wall, round or isodiametric cells with distinct intercellular spaces. Resin canals and pericyclic sclereids are absent.

Transverse section of leaves: Shows a more or less isobilateral structure; epidermis with glandular hair and stomata; upper surface has more hairs and less stomata than the lower surface; mesophyll composed of spongy tissue, a few prisms of calcium oxalate, and vascular bundles are present.

- **LOSS ON DRYING** ⟨731⟩

Sample: 1.0 g of finely powdered Bacopa

Analysis: Dry the *Sample* at 105° for 3 h.

Acceptance criteria: NMT 12.0%

- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩

Sample: 1.0 g of finely powdered Bacopa

Acceptance criteria: NMT 18%

- **ARTICLES OF BOTANICAL ORIGIN,** *Alcohol-Soluble Extractives, Method 2* ⟨561⟩: NLT 6.0%
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩: Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Bacoside A_3RS

USP Powdered Bacopa Extract RS

Powdered Bacopa

DEFINITION

Powdered Bacopa is Bacopa reduced to a powder or very fine powder. It contains NLT 2.5% of triterpene glycosides, calculated on the dried basis as the sum of bacopaside I, bacoside A_3, bacopaside II, the jujubogenin isomer of bacopasaponin C, and bacopasaponin C.

IDENTIFICATION

- **A.** Powdered Bacopa meets the requirements under *Specific Tests, Botanical Characteristics.*
- **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩

Standard solution: Transfer about 10 mg of USP Powdered Bacopa Extract RS to a 10-mL volumetric flask, and add about 8 mL of methanol. Sonicate and heat gently for 15–20 min, dilute with methanol to volume, mix, centrifuge, and use the supernatant.

Sample solution: Use the *Sample solution*, prepared as directed in the test for *Content of Triterpene Glycosides.*

Adsorbent: Chromatographic silica gel mixture with an average particle size of 10–15 µm (TLC plates)

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Application volume: 15 µL, as 5–10 mm bands
Developing solvent system: Ethyl acetate, methanol, and water (7:2:1)
Spray reagent: 1% Vanillin in alcohol and 10% sulfuric acid in alcohol (1:1)
Analysis
Samples: *Standard solution* and *Sample solution*
Apply the samples as bands (see *Chromatography* ⟨621⟩). Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about three-fourths of the plate. Remove the plate from the chamber, dry, spray with *Spray reagent*, heat for 5–10 min at 70°, and examine under white light.
Acceptance criteria: The *Sample solution* exhibits a main dark blue zone due to mixture of bacoside A_3, bacopaside II, the jujubogenin isomer of bacopasaponin C, and bacopasaponin C at an R_F value of approximately 0.6 and a faint pink spot due to bacopaside I at an R_F value of approximately 0.4, both of which correspond in position and color to zones in the chromatogram of the *Standard solution*. Other zones are observed for the *Sample solution* and *Standard solution*.

• **C. HPLC IDENTIFICATION TEST:** The *Sample solution* from the test for *Content of Triterpene Glycosides* shows a main peak at a retention time corresponding to that of bacoside A_3 in the chromatogram of *Standard solution A*. Identify other triterpene glycoside peaks in the *Sample solution* by comparison with the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Bacopa Extract RS being used. The *Sample solution* shows additional peaks corresponding to bacopaside I, bacopaside II, the jujubogenin isomer of bacopasaponin C, and bacopasaponin C.

COMPOSITION

• **CONTENT OF TRITERPENE GLYCOSIDES**
Solution A: Dissolve 0.14 g of anhydrous potassium dihydrogen phosphate in 900 mL of water, add 0.5 mL of phosphoric acid, dilute with water to 1000 mL, mix, filter, and degas.
Solution B: Use filtered and degassed acetonitrile.
Mobile phase: See the *gradient table* below.

Time (min)	Solution A (%)	Solution B (%)
0	70	30
25	60	40
26	70	30
30	70	30

Standard solution A: Sonicate an accurately weighed quantity of USP Bacoside A_3RS in methanol to obtain a solution having a known concentration of about 0.5 mg/mL.
Standard solution B: Transfer about 10 mg of USP Powdered Bacopa Extract RS to a 10-mL volumetric flask, and add about 8 mL of methanol. Sonicate and heat gently for 15–20 min, dilute with methanol to volume, and mix. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first 5 mL of the filtrate.
Sample solution: Transfer about 2.5 g of Powdered Bacopa, accurately weighed, to a 100-mL round-bottom flask fitted with a reflux condenser. Add 25 mL of methanol, reflux on a water bath for 10 min, cool to room temperature, and decant the supernatant. Repeat until the last extract is colorless. Combine the extracts, filter, concentrate under vacuum, and adjust the volume to 100 mL using methanol. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first 5 mL of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 205 nm
Column: 4.6-mm × 25-cm; 5-µm, endcapped, base-deactivated packing L1
Column temperature: 27°
Flow rate: 1.5 mL/min
Injection volume: 20 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Bacopa Extract RS being used.
Resolution: NLT 1.0 between the bacopaside II and bacoside A_3 peaks, *Standard solution B*
Tailing factor: NMT 1.5 for the bacoside A_3 peak, *Standard solution A*
Relative standard deviation: NMT 2% determined from the bacoside A_3 peak for replicate injections, *Standard solution A*
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Using the chromatograms of *Standard solution A* and *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Bacopa Extract RS being used, identify the retention times of the peaks corresponding to different triterpene glycosides. The approximate relative retention times of the relevant triterpene glycosides are provided in the following *table*.

Analyte	Relative Retention Time
Bacopaside I	0.73
Bacoside A_3	1.00
Bacopaside II	1.04
The jujubogenin isomer of bacopasaponin C	1.15
Bacopasaponin C	1.22

Separately calculate the percentages of bacopaside I, bacoside A_3, bacopaside II, the jujubogenin isomer of bacopasaponin C, and bacopasaponin C in the portion of Powdered Bacopa taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak response for each triterpene glycoside from the *Sample solution*
r_S = peak response for bacoside A_3 from *Standard solution A*
C_S = concentration of USP Bacoside A_3RS in *Standard solution A* (mg/mL)
V = final volume of the *Sample solution* (mL)
W = weight of Powdered Bacopa used to prepare the *Sample solution* (mg)
F = conversion factor for each analyte: 1.00 for bacoside A_3, 1.03 for bacopaside I, 0.81 for

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bacopaside II, 0.99 for the jujubogenin isomer of bacopasaponin C, and 0.75 for bacopasaponin C

Acceptance criteria: Add the percentages of bacopaside I, bacoside A_3, bacopaside II, the jujubogenin isomer of bacopasaponin C, and bacopasaponin C: NLT 2.5% is found on the dried basis.

IMPURITIES

- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩: NMT 6.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residues Analysis* ⟨561⟩: Meets the requirements

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS:** Yellowish in color; mild and hay-like odor, and very bitter taste. Under a microscope, it shows fragments of upper and lower epidermal cells of the leaves in surface view, having sessile glandular trichomes with 4–8 cells and diacytic or anomocytic stomata; upper epidermis has more trichomes and less stomata than the lower epidermis; lower epidermis cells with sinuous anticlinal walls and at places striated cuticle; fragments of epidermal cells of the stem in surface view; parenchyma cells enclosing air cavities and some contain rosette and prismatic crystals of calcium oxalate; fragments of longitudinally cut annular and spiral vessels; fragments of cortical cells of the stem; and crystals of calcium oxalate.
- **LOSS ON DRYING** ⟨731⟩
  Sample: 1.0 g of Powdered Bacopa
  Analysis: Dry the *Sample* at 105° for 3 h.
  Acceptance criteria: 12.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩
  Sample: 1.0 g of Powdered Bacopa
  Acceptance criteria: NMT 18%
- **ARTICLES OF BOTANICAL ORIGIN,** *Alcohol-Soluble Extractives, Method 2* ⟨561⟩: NLT 6.0%
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩: Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Bacoside A_3RS
  USP Powdered Bacopa Extract RS

Powdered Bacopa Extract

DEFINITION

Powdered Bacopa Extract is prepared from Bacopa by extraction with water, alcohol, methanol, or a mixture of these solvents. The ratio of plant material to extract is between 20:1 and 10:1. It contains NLT 90.0% and NMT 110.0% of the labeled amount of triterpene glycosides, calculated on the dried basis as the sum of bacopaside I, bacoside A_3, bacopaside II, the jujubogenin isomer of bacopasaponin C, and bacopasaponin C. It may contain suitable added substances as carriers.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**
  Standard solution: Transfer about 10 mg of USP Powdered Bacopa Extract RS to a 10-mL volumetric flask, and add about 8 mL of methanol. Sonicate and heat gently for 15–20 min, dilute with methanol to volume, mix, centrifuge, and use the supernatant.
  Sample solution: Sonicate for about 10 min an amount of Powdered Bacopa Extract equivalent to about 40 mg of triterpene glycosides in 10 mL of methanol, centrifuge, and use the supernatant.
  Adsorbent: Chromatographic silica gel mixture with an average particle size of 10–15 µm (TLC plates)
  Application volume: 15 µL, as 5–10 mm bands
  Developing solvent system: Ethyl acetate, methanol, and water (7:2:1)
  Spray reagent: 1% vanillin in alcohol and 10% sulfuric acid in alcohol (1:1)
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Apply the samples as bands to a suitable thin-layer chromatographic plate (see *Chromatography* ⟨621⟩). Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about three-fourths of the plate. Remove the plate from the chamber, dry, spray with *Spray reagent*, heat for 5–10 min at about 70°, and examine under visible light.
  Acceptance criteria: The *Sample solution* exhibits a main dark blue zone due to a mixture of bacoside A_3, bacopaside II, the jujubogenin isomer of bacopasaponin C, and bacopasaponin C at an R_F value of approximately 0.6 and a faint pink spot due to bacopaside I at an R_F value of approximately 0.4, both of which correspond in position and color to zones in the chromatogram of the *Standard solution*. Other zones are observed for the *Sample solution* and *Standard solution*.
- **B. HPLC IDENTIFICATION TEST:** The *Sample solution* from the test for *Content of Triterpene Glycosides* shows a main peak at a retention time corresponding to that of bacoside A_3 in the chromatogram of *Standard solution A*. Identify other triterpene glycoside peaks in the *Sample solution* by comparison with the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Bacopa Extract RS being used. The *Sample solution* shows additional peaks corresponding to bacopaside I, bacopaside II, the jujubogenin isomer of bacopasaponin C, and bacopasaponin C.

COMPOSITION

- **CONTENT OF TRITERPENE GLYCOSIDES**
  Solution A: Dissolve 0.14 g of anhydrous potassium dihydrogen phosphate in 900 mL of water, add 0.5 mL of phosphoric acid, dilute with water to 1000 mL, mix, filter, and degas.
  Solution B: Use filtered and degassed acetonitrile.
  Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	70	30
25	60	40
26	70	30
30	70	30

DS Monographs

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Standard solution A: Sonicate a weighed quantity of USP Bacoside A_3RS in methanol to obtain a solution with a concentration of about 0.5 mg/mL.

Standard solution B: Transfer about 10 mg of USP Powdered Bacopa Extract RS to a 10-mL volumetric flask, and add about 8 mL of methanol. Sonicate and heat gently for 15–20 min, dilute with methanol to volume, and mix. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first 5 mL of the filtrate.

Sample solution: Transfer an amount of Powdered Bacopa Extract, equivalent to about 25 mg triterpene glycosides, to a 25-mL volumetric flask, and add 15 mL of methanol. Sonicate and heat gently for 15–20 min, dilute with methanol to volume, and mix. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first 5 mL of the filtrate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 205 nm
Column: 4.6-mm × 25-cm; 5-µm, endcapped, base-deactivated packing L1
Column temperature: 27 ± 1°
Flow rate: 1.5 mL/min
Injection size: 20 µL

System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Bacopa Extract RS being used.
Resolution: NLT 1.0 between the bacopaside II and bacoside A_3 peaks, *Standard solution B*
Tailing factor: NMT 1.5 for the bacoside A_3 peak, *Standard solution A*
Relative standard deviation: NMT 2% determined from the bacoside A_3 peak for replicate injections, *Standard solution A*

Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Using the chromatograms of *Standard solution A* and *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Bacopa Extract RS being used, identify the retention times of the peaks corresponding to different triterpene glycosides. The approximate relative retention times of the different triterpene glycosides are provided in the following table.

Analyte	Relative Retention Time
Bacopaside I	0.73
Bacoside A_3	1.00
Bacopaside II	1.04
The jujubogenin isomer of bacopasaponin C	1.15
Bacopasaponin C	1.22

Separately calculate the percentages of bacopaside I, bacoside A_3, bacopaside II, jujubogenin isomer of bacopasaponin C, and bacopasaponin C in the portion of Powdered Bacopa Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak response for each triterpene glycoside from the *Sample solution*
r_S = peak response for bacoside A_3 in *Standard solution A*
C_S = concentration of USP Bacoside A_3RS in *Standard solution A* (mg/mL)
C_U = concentration of Powdered Bacopa Extract in the *Sample solution* (mg/mL)
F = conversion factor for each analyte: 1.00 for bacoside A_3, 1.03 for bacopaside I, 0.81 for bacopaside II, 0.99 for the jujubogenin isomer of bacopasaponin C, and 0.75 for bacopasaponin C

Acceptance criteria: Add the percentages of bacopaside I, bacoside A_3, bacopaside II, the jujubogenin isomer of bacopasaponin C, and bacopasaponin C: NLT 90.0%–NMT 110.0% of the labeled amount of triterpene glycosides is found on the dried basis.

IMPURITIES

Change to read:

ORGANIC IMPURITIES
• ▲**Botanical Extracts** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020)
: Meets the requirements

SPECIFIC TESTS
• **LOSS ON DRYING** ⟨731⟩**:** Dry 1.0 g of Powdered Bacopa Extract at 105° for 3 h: it loses NMT 5% of its weight.
• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^4 cfu/g. The total combined molds and yeasts count does not exceed 10^3 cfu/g.
• **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.
• **OTHER REQUIREMENTS:** It meets the requirements of the test for *Residual Solvents* under *Botanical Extracts* ⟨565⟩.

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.
• **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived. It meets other labeling requirements under *Botanical Extracts* ⟨565⟩.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Bacoside A_3RS
USP Powdered Bacopa Extract RS

Banaba Leaf

DEFINITION
Banaba Leaf consists of the dried leaves of *Lagerstroemia speciosa* (L.) Pers. (Fam. Lythraceae). It contains NLT 0.2% of corosolic acid ($C_{30}H_{48}O_4$), calculated on the dried basis.

IDENTIFICATION
• **A.** Meets the requirements for *Specific Tests*, *Botanic Characteristics*

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• **B. Thin-Layer Chromatography**

Standard solution A: 0.2 mg/mL of USP Corosolic Acid RS in methanol

Standard solution B: 10 mg/mL of USP *Lagerstroemia speciosa* Leaf Dry Extract RS in methanol. Sonicate for 10 min, centrifuge, and use the supernatant.

Sample solution: About 0.2 g of Banaba Leaf, finely powdered, in 10 mL of methanol. Sonicate for 15 min, centrifuge, and use supernatant.

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography.*)

Mode: HPTLC

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)

Application volume: 6 µL each of *Standard solution A* and *Standard solution B* and 8 µL of the *Sample solution* as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

Developing solvent system: A mixture of toluene, ethyl acetate, and acetic acid (55: 45: 0.5)

Derivatization reagent: 85 mL of ice-cooled methanol mixed with 10 mL of glacial acetic acid, 5 mL of sulfuric acid, and 0.5 mL of *p*-anisaldehyde

Analysis

Samples: *Standard solution A, Standard solution B,* and *Sample solution*

Apply the samples as bands to a suitable HPTLC plate, and dry in air. Develop the chromatograms in an unsaturated chamber, remove the plate from the chamber, and dry. Treat with *Derivatization reagent*, heat for 3 min at 100°, and examine under visible light.

System suitability: Under visible light, the chromatogram of *Standard solution B* exhibits the most intense band, a violet or blue band, with similar R_F and color as the corosolic acid band in the chromatogram of *Standard solution A*; a blue band close to the start (about R_F 0.1), consistent with asiatic acid; two minor blue bands in between the corosolic and asiatic bands; a minor blue band due to virgatic acid, above the band due to corosolic acid; and just below the asiatic band, a minor brown band. *Standard solution B* also exhibits two minor violet bands, separated, at about three-fourths of the chromatogram; the band with the lower R_F corresponds to oleanolic acid.

Acceptance criteria: Under visible light, the chromatogram of the *Sample solution* exhibits the most intense band as a violet band corresponding in color and R_F to the band due to corosolic acid in the chromatogram of *Standard solution A*, as well as the following bands corresponding to similar bands of *Standard solution B*: a minor blue band close to the start (about R_F 0.1), a minor brownish band above the corosolic acid, and a minor violet band at about three-fourths of the chromatogram.

• **C. HPLC**

Analysis: Proceed as directed in *Content of Corosolic Acid.*

Acceptance criteria: The chromatogram of the *Sample solution* exhibits a group of three peaks. The one in the center is the most intense of the group and occurs at a retention time corresponding to that of corosolic acid in the chromatogram of *Standard solution A*. The peak that elutes before corosolic acid has about one-half to one-third of the intensity of that of corosolic acid, and the peak that elutes after corosolic acid has the lesser intensity of the three and is consistent with virgatic acid. A minor peak due to oleanolic acid elutes later in the chromatogram.

COMPOSITION

• **Content of Corosolic Acid**

Solution A: Dilute 0.1% phosphoric acid in water.

Solution B: Acetonitrile

Mobile phase: A mixture of *Solution A* and *Solution B* (4:6)

Standard solution A: 0.1 mg/mL of USP Corosolic Acid RS in methanol

Standard solution B: 5.0 mg/mL of USP *Lagerstroemia speciosa* Leaf Dry Extract RS in methanol. Sonicate if necessary. Before injection, pass through a membrane filter of 0.45-µm or finer pore size. Discard the first few mL of the filtrate.

Sample solution: Transfer about 5.0 g of Banaba Leaf, finely powdered and accurately weighed, to a round-bottom flask. Add 75 mL of methanol, reflux for 15 min, set aside to settle, and decant the supernatant. Repeat the extraction three more times, then combine the extracts. Filter, and concentrate under reduced pressure. Transfer to a 100-mL volumetric flask, adjust with methanol to volume, and mix. Before injection, pass through a membrane filter of 0.45-µm or finer pore size. Discard the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability.*)

Mode: LC

Detector: UV 205 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 25°

Flow rate: 1.6 mL/min

Injection volume: 20 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

[Note—The approximate relative retention times of the individual peaks for corosolic acid, virgatic acid, and oleanolic acid are 1.0, 1.1, and 3.2, respectively.]

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP *Lagerstroemia speciosa* Leaf Dry Extract RS being used.

Resolution: NLT 1.5 between the corosolic acid peak and the preceding peak, *Standard solution B*

Tailing factor: NMT 2.0 for the corosolic acid peak, *Standard solution A*

Relative standard deviation: NMT 2.0% determined from the corosolic acid peak in repeated injections, *Standard solution A*

Analysis

Samples: *Standard solution A, Standard solution B,* and *Sample solution*

Identify the relative retention times of the peaks for corosolic acid, virgatic acid, and oleanolic acid in the *Sample solution.*

Calculate the percentage of corosolic acid in the portion of Banaba Leaf taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of corosolic acid from *Sample solution*

r_S = peak area of corosolic acid from *Standard solution A*

C_S = concentration of corosolic acid in *Standard solution A* (mg/mL)

V = volume of *Sample solution* (mL)

W = weight of Banaba Leaf taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 0.2% of corosolic acid on the dried basis

DS Monographs

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CONTAMINANTS

- **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩

Acceptance criteria

Arsenic: NMT 2.0 µg/g
Cadmium: NMT 0.5 µg/g
Lead: NMT 5 µg/g
Mercury: NMT 0.2 µg/g

Change to read:

- **▲ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩▲ (CN 1-May-2019)**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

- **BOTANIC CHARACTERISTICS**

Macroscopic: Banaba leaves vary in shape, including lanceolate, oblong-lanceolate, oblong, and elliptic ovate. They are up to 34 cm long and 11 cm wide; olive green to yellowish brown; entirely or slightly wavy at the margins; base acute; apex acute to acuminate; leathery in texture; and petiolate with petioles up to 1 cm long.

Microscopic

Transverse section of the midrib: A layer of upper epidermis composed of rectangular to round cells covered with thin cuticle; a few layers of collenchyma cells; numerous layers of parenchyma cells, some containing cluster crystals of calcium oxalate, with large intercellular spaces; groups of lignified fiber bundles; and secretory canals scattered in the parenchyma zone. Bicollateral vascular bundle encircled by a continuous sheath of fibers accompanied by sclerenchyma and cells containing cluster crystals of calcium oxalate; secretory canals between the vascular bundles; numerous layers of parenchyma cells, some containing cluster crystals of calcium oxalate, with large intercellular spaces; a few layers of collenchyma; a layer of lower epidermal cells.

Transverse section of the lamina: A layer of upper epidermis composed of rectangular cells about twice as large as those of the lower epidermis. Some cells are secretory cells, which tend to protrude into the mesophyll and sometimes appear to be below the upper epidermis. Two layers of rectangular palisade cells; 4–6 layers of parenchyma cells, some containing prisms of calcium oxalate and others containing clusters crystals of calcium oxalate, with large intercellular spaces; groups of vascular bundles scattered in the parenchyma zone; lower epidermis showing stomata.

- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩**:** NMT 2.0%
- **LOSS ON DRYING** ⟨731⟩

Analysis: Dry 2.0 g of Banaba Leaf, finely powdered, at 105° for 2 h.
Acceptance criteria: NMT 10%

- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩

Analysis: 2.0 g of Banaba Leaf, finely powdered
Acceptance criteria: NMT 7.0%

- **ARTICLES OF BOTANICAL ORIGIN,** *Alcohol-Soluble Extractives, Method 1* ⟨561⟩**:** NMT 10.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩

Analysis: 4.0 g of Banaba Leaf, finely powdered
Acceptance criteria: NMT 2%

- **ARTICLES OF BOTANICAL ORIGIN,** *Water-Soluble Extractives, Method 1* ⟨561⟩**:** NLT 18.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part(s) of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Corosolic Acid RS
USP *Lagerstroemia speciosa* Leaf Dry Extract RS

Banaba Leaf Powder

DEFINITION

Banaba Leaf Powder consists of the dried leaves of *Lagerstroemia speciosa* (L.) Pers. (Fam. Lythraceae) reduced to powder or very fine powder. It contains NLT 0.2% of the labeled amount of corosolic acid ($C_{30}H_{48}O_4$), calculated on the dried basis.

IDENTIFICATION

- **A.** Meets the requirements for *Specific Tests, Botanic Characteristics*
- **B. THIN-LAYER CHROMATOGRAPHY**

Standard solution A: 0.2 mg/mL of USP Corosolic Acid RS in methanol
Standard solution B: 10 mg/mL of USP *Lagerstroemia speciosa* Leaf Dry Extract RS in methanol. Sonicate for 10 min, centrifuge, and use the supernatant.
Sample solution: About 0.2 g of Banaba Leaf Powder in 10 mL of methanol. Sonicate for 15 min, centrifuge, and use the supernatant.
Chromatographic system
(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography.*)
Mode: HPTLC
Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)
Application volume: 6 µL each of *Standard solution A* and *Standard solution B* and 8 µL of the *Sample solution* as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
Column temperature: 25°
Developing solvent system: A mixture of toluene, ethyl acetate, and acetic acid (55: 45: 0.5)
Derivatization reagent: 85 mL of ice-cooled methanol mixed with 10 mL of glacial acetic acid, 5 mL of sulfuric acid, and 0.5 mL of *p*-anisaldehyde
Analysis
Samples: *Standard solution A, Standard solution B,* and *Sample solution*
Apply the samples as bands to a suitable HPTLC plate, and dry in air. Develop the chromatograms in an unsaturated chamber, remove the plate from the chamber, and dry. Treat with *Derivatization reagent,* heat for 3 min at 100°, and examine under visible light.
System suitability: Under visible light, the chromatogram of *Standard solution B* exhibits the most intense band, a violet or blue band, with similar R_F and color as the corosolic acid band in the chromatogram of *Standard solution A;* a blue band close to the start (about R_F 0.1), consistent with asiatic acid; two minor blue bands in between the corosolic and asiatic bands; a minor blue band due to virgatic acid, above the band due to corosolic acid; and just below the

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asiatic band, a minor brown band. *Standard solution B* also exhibits two minor violet bands, separated, at about three-fourths of the chromatogram; the band with the lower R_F corresponds to oleanolic acid.

Acceptance criteria: Under visible light, the chromatogram of the *Sample solution* exhibits the most intense band as a violet band corresponding in color and R_F to the band due to corosolic acid in the chromatogram of *Standard solution A*, as well as the following bands corresponding to similar bands of *Standard solution B*: a minor blue band close to the start (about R_F 0.1), a minor brownish band above the corosolic acid, and a minor violet band at about three-fourths of the chromatogram.

• **C. HPLC**

Analysis: Proceed as directed in *Content of Corosolic Acid*.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits a group of three peaks. The one in the center is the most intense of the group and occurs at a retention time corresponding to that of corosolic acid in the chromatogram of *Standard solution A*. The peak that elutes before corosolic acid has about one-half to one-third of the intensity of that of corosolic acid, and the peak that elutes after corosolic acid has the lesser intensity of the three and is consistent with virgatic acid. A minor peak due to oleanolic acid elutes later in the chromatogram.

COMPOSITION

• **CONTENT OF COROSOLIC ACID**

Solution A: Dilute 0.1% phosphoric acid in water.

Solution B: Acetonitrile

Mobile phase: A mixture of *Solution A* and *Solution B* (4:6)

Standard solution A: 0.1 mg/mL of USP Corosolic Acid RS in methanol

Standard solution B: 5.0 mg/mL of USP *Lagerstroemia speciosa* Leaf Dry Extract RS in methanol. Sonicate if necessary. Before injection, pass through a membrane filter of 0.45-µm or finer pore size. Discard the first few mL of the filtrate.

Sample solution: Transfer about 5.0 g of Banaba Leaf Powder, accurately weighed, to a round-bottom flask. Add 75 mL of methanol, reflux for 15 min, set aside to settle, and decant the supernatant. Repeat the extraction three more times, then combine the extracts. Filter, and concentrate under reduced pressure. Transfer to a 100-mL volumetric flask, adjust with methanol to volume, and mix. Before injection, pass through a membrane filter of 0.45-µm or finer pore size. Discard the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 205 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Flow rate: 1.6 mL/min

Injection volume: 20 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

[NOTE—The approximate relative retention times of the individual peaks for corosolic acid, virgatic acid, and oleanolic acid are 1.0, 1.1, and 3.2 min, respectively.]

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP *Lagerstroemia speciosa* Leaf Dry Extract RS being used.

Resolution: NLT 1.5 between the corosolic acid peak and the preceding peak, *Standard solution B*

Tailing factor: NMT 2.0 for the corosolic acid peak, *Standard solution A*

Relative standard deviation: NMT 2.0% determined from the corosolic acid peak in repeated injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Identify the relative retention times of the peaks for corosolic acid, virgatic acid, and oleanolic acid in the *Sample solution*.

Calculate the percentage of the labeled amount of corosolic acid in the portion of Banaba Leaf Powder taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of corosolic acid from the *Sample solution*

r_S = peak area of corosolic acid from *Standard solution A*

C_S = concentration of corosolic acid in *Standard solution A* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Banaba Leaf Powder taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 0.2% of the labeled amount of corosolic acid on the dried basis

CONTAMINANTS

• **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩

Acceptance criteria

Arsenic: NMT 2.0 µg/g

Cadmium: NMT 0.5 µg/g

Lead: NMT 5 µg/g

Mercury: NMT 0.2 µg/g

Change to read:

• **▲ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩▲ (CN 1-May-2019)**:** Meets the requirements

• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^2cfu/g.

• **MICROBIAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

• **BOTANIC CHARACTERISTICS**

Macroscopic: Grayish-green powder

Microscopic: Fragments of upper epidermis with polygonal cells, some containing calcium oxalate crystals, and no stomata; fragments of lower epidermis cells with irregular shapes and slightly wavy walls, anomocytic stomata; fragments of upper epidermal cells with underlying palisade cells; fragments of parenchyma cells, some containing prisms of calcium oxalate and others containing cluster crystals of calcium oxalate; oil cells; fragments of lignified fibers; fragments of spiral vessels; fragments of pitted vessels associated with fibers

Change to read:

• **▲ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩▲ (CN 1-May-2019)**:** Meets the requirements

• **LOSS ON DRYING** ⟨731⟩

Sample: 2.0 g of Banaba Leaf, finely powdered

Analysis: Dry the *Sample* at 105° for 2 h.

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Acceptance criteria: NMT 10%

- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩
  Analysis: 2.0 g of Banaba Leaf, finely powdered
  Acceptance criteria: NMT 7.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩
  Analysis: 4.0 g of Banaba Leaf, finely powdered
  Acceptance criteria: NMT 2%
- **ARTICLES OF BOTANICAL ORIGIN,** *Alcohol-Soluble Extractives, Method 1* ⟨561⟩**:** NLT 10.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Water-Soluble Extractives, Method 1* ⟨561⟩**:** NLT 18.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part(s) of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Corosolic Acid RS
  USP *Lagerstroemia speciosa* Leaf Dry Extract RS

Banaba Leaf Dry Extract

DEFINITION

Banaba Leaf Dry Extract consists of the dried leaves of *Lagetroemia speciosa* (L.) Pers. (Fam. Lythraceae) by extraction with hydroalcoholic mixtures. It contains NLT 90.0% and NMT 110.0% of the labeled amount of corosolic acid ($C_{30}H_{48}O_4$), calculated on the dried basis.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHY**
  Standard solution A: 0.2 mg/mL of USP Corosolic Acid RS in methanol
  Standard solution B: 10 mg/mL of USP *Lagerstroemia speciosa* Leaf Dry Extract RS in methanol. Sonicate for 10 min, centrifuge, and use the supernatant.
  Sample solution: Banaba Leaf Dry Extract in methanol at a concentration equivalent to 0.2 mg/mL of corosolic acid according to the label claim. Sonicate if necessary.
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
  Mode: HPTLC
  Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)
  Application volume: 6 µL as 8-mm bands
  Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
  Column temperature: 25°
  Developing solvent system: A mixture of toluene, ethyl acetate, and acetic acid (55: 45: 0.5)
  Developing distance: 6 cm
  Derivatization reagent: 85 mL of ice-cooled methanol mixed with 10 mL of glacial acetic acid, 5 mL of sulfuric acid, and 0.5 mL of *p*-anisaldehyde
  Analysis
  Samples: *Standard solution A, Standard solution B,* and *Sample solution*
  Apply the samples as bands to a suitable HPTLC plate, and dry in air. Develop the chromatograms in an unsaturated chamber, remove the plate from the chamber, and dry. Treat with *Derivatization reagent,* heat for 3 min at 100°, and examine under visible light.
  System suitability: Under visible light, the chromatogram of *Standard solution B* exhibits the most intense band, a violet or blue band, with similar R_F and color as the corosolic acid band in the chromatogram of *Standard solution A*; a blue band close to the start (about R_F 0.1), consistent with asiatic acid; two minor blue bands in between the corosolic and asiatic bands; a minor blue band due to virgatic acid, above the band due to corosolic acid; and just below the asiatic band, a minor brown band. *Standard solution B* also exhibits two minor violet bands, separated, at about three-fourths of the chromatogram; the band with the lower R_F corresponds to oleanolic acid.
  Acceptance criteria: Under visible light, the chromatogram of the *Sample solution* exhibits the most intense band as a violet band corresponding in color and R_F to the band due to corosolic acid in the chromatogram of *Standard solution A,* as well as the following bands corresponding to similar bands of *Standard solution B*: a minor blue band close to the start (about R_F 0.1), two minor purple bands below the corosolic acid, two minor blue bands above the corosolic acid, and two minor violet bands, which are separated, at about three-fourths of the chromatogram.
- **B. HPLC**
  Analysis: Proceed as directed in *Content of Corosolic Acid.*
  Acceptance criteria: The chromatogram of the *Sample solution* exhibits a group of three peaks. The one in the center is the most intense of the group and occurs at a retention time corresponding to that of corosolic acid in the chromatogram of *Standard solution A.* The peak that elutes before corosolic acid has about one-half to one-third of the intensity of that of corosolic acid, and the peak that elutes after corosolic acid has the lesser intensity of the three and is consistent with virgatic acid. A minor peak due to oleanolic acid elutes later in the chromatogram.

COMPOSITION

- **CONTENT OF COROSOLIC ACID**
  Solution A: Dilute 0.1% phosphoric acid in water.
  Solution B: Acetonitrile
  Mobile phase: A mixture of *Solution A* and *Solution B* (4:6)
  Standard solution A: 0.1 mg/mL of USP Corosolic Acid RS in methanol
  Standard solution B: 5.0 mg/mL of USP *Lagerstroemia speciosa* Leaf Dry Extract RS in methanol. Sonicate if necessary. Before injection, pass through a membrane filter of 0.45-µm or finer pore size. Discard the first few mL of the filtrate.
  Sample solution: Banaba Leaf Dry Extract in methanol at a concentration equivalent to 0.1 mg/mL of corosolic acid according to the label claim. Sonicate if necessary. Before injection, pass through a membrane filter of 0.45-µm or finer pore size. Discard the first few mL of the filtrate.
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: LC
  Detector: UV 205 nm
  Column: 4.6-mm × 25-cm; 5-µm packing L1
  Flow rate: 1.6 mL/min
  Injection volume: 20 µL
  System suitability
  Samples: *Standard solution A* and *Standard solution B*
  [NOTE—The approximate relative retention times of the individual peaks for corosolic acid, virgatic acid, and oleanolic acid are 1.00, 1.1, and 3.2, respectively.]
  Suitability requirements
  Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP *Lagerstroemia speciosa* Leaf Dry Extract RS being used.

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Resolution: NLT 1.5 between the corosolic acid peak and the preceding peak, *Standard solution B*
Tailing factor: NMT 2.0 for the corosolic acid peak, *Standard solution A*
Relative standard deviation: NMT 2.0%, determined from the corosolic acid peak in repeated injections, *Standard solution A*

Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Identify the relative retention times of the peaks for corosolic acid, virgatic acid, and oleanolic acid of the *Sample solution*.
Calculate the percentage of the labeled amount of corosolic acid in the portion of Banaba Leaf Dry Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of corosolic acid from the *Sample solution*
r_S = peak area of corosolic acid from *Standard solution A*
C_S = concentration of corosolic acid in *Standard solution A* (mg/mL)
C_U = concentration of Banaba Leaf Dry Extract in the *Sample solution* (mg/mL)

Calculate the percentage of the labeled amount of corosolic acid in the portion of Extract taken:

$$\text{Result} = (P/L) \times 100$$

P = content of corosolic acid as determined above (%)
L = labeled amount of corosolic acid (%)

Acceptance criteria: 90.0%–110.0% of the labeled amount of corosolic acid on the dried basis

CONTAMINANTS

• **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩
Acceptance criteria
Arsenic: NMT 2.0 µg/g
Cadmium: NMT 0.5 µg/g
Lead: NMT 5 µg/g
Mercury: NMT 0.2 µg/g

Change to read:

• **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020)
: Meets the requirements
• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
• **MICROBIAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

• **LOSS ON DRYING** ⟨731⟩
Sample: 2.0 g of Banaba Leaf Dry Extract
Analysis: Dry the *Sample* at 105° for 2 h.
Acceptance criteria: NMT 8%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
• **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant from which the article was derived. It meets other labeling requirements in *Botanical Extracts* ⟨565⟩.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Corosolic Acid RS
USP *Lagerstroemia speciosa* Leaf Dry Extract RS

Beta Carotene—*see Beta Carotene General Monographs*

Beta Carotene Preparation

DEFINITION

Beta Carotene Preparation is a combination of beta carotene with one or more inert substances. It may be in a solid or a liquid form. It contains NLT 95.0% and NMT 130.0% of the labeled amount of total beta carotene ($C_{40}H_{56}$) on the anhydrous basis.

IDENTIFICATION

• **A.**
Sample solution: Transfer 5.0 mL of *Sample stock solution A* or *Sample stock solution B* from the test for *Content of Beta Carotene* to a 100-mL volumetric flask, and dilute with cyclohexane to volume. Pass the solution through a membrane filter of 0.45-µm pore size.
Analysis: Record the UV-Vis spectrum from 300 to 600 nm.
Acceptance criteria: The *Sample solution* shows a shoulder at about 427 nm, an absorption maximum at about 455 nm, and another maximum at about 483 nm. The absorbance ratio A_{455}/A_{483} is between 1.14 and 1.18.
• **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Beta Carotene*.

COMPOSITION

• **CONTENT OF BETA CAROTENE**
[NOTE—Use low-actinic glassware.]
Mobile phase: Transfer 50 mg of butylated hydroxytoluene to a 1-L volumetric flask, and dissolve with 20 mL of 2-propanol. Add 0.2 mL of *N*-ethyldiisopropylamine, 25 mL of 0.2% ammonium acetate solution, 455 mL of acetonitrile, and about 450 mL of methanol. Allow the solution to reach room temperature, and dilute with methanol to volume.
Diluent: 50 µg/mL of butylated hydroxytoluene in alcohol
System suitability solution: Transfer 20 mg of USP Beta Carotene System Suitability RS to a 50-mL volumetric flask. Add 1 mL of water and 4 mL of tetrahydrofuran, and sonicate for 5 min. Dilute with *Diluent* to volume, and sonicate for 5 min. Cool to room temperature, pass the suspension through a membrane filter of 0.45-µm pore size, and use the clear filtrate.
Standard stock solution: 60 µg/mL of USP Beta Carotene RS in tetrahydrofuran
Standard solution A: Transfer 5.0 mL of the *Standard stock solution* to a 100-mL volumetric flask, add 5.0 mL of tetrahydrofuran, and dilute with *Diluent* to volume. The concentration of the all-*trans*-beta carotene in this solution will be determined by the spectrophotometric procedure using *Standard solution B* as follows.
Standard solution B: Transfer 5.0 mL of the *Standard stock solution* to a 100-mL volumetric flask, and dilute with cyclohexane to volume. Prepare in triplicate.

DS Monographs

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Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Analytical wavelength: 456 nm
Cell path: 1 cm
Blank: Cyclohexane
Analysis
Sample: *Standard solution B*
Calculate the concentration of total beta carotene (mg/mL) as all-*trans*-beta carotene ($C_{40}H_{56}$) in *Standard solution B*:

$$\text{Result} = A/F$$

A = average absorbance of the three preparations of *Standard solution B*
F = absorptivity of pure all-*trans*-beta carotene in cyclohexane, 250.5

Sample stock solution A (for solid Beta Carotene Preparations): Transfer a quantity of Preparation, equivalent to 10 mg of beta carotene, to a 250-mL volumetric flask. Add 250 mg of butylated hydroxytoluene, 0.5 mL of alkaline protease R, and 15 mL of water. Tilt the flask gently to wet the entire contents. Sonicate the solution in an ultrasonic bath at about 50° for 30 min, and swirl at 10-min intervals. Add 100 mL of alcohol to the warm suspension, and shake vigorously. Add 135 mL of methylene chloride, and shake again. Let the mixture stand in the dark until it reaches room temperature (about 2 h). Dilute with methylene chloride to volume, shake vigorously, and allow solids to settle in the dark.
Sample stock solution B (for liquid Beta Carotene suspensions in oil Preparations): Transfer a quantity of Preparation, equivalent to 20 mg of beta carotene, to a 250-mL volumetric flask. Add 250 mg of butylated hydroxytoluene, 120 mL of methylene chloride, and 100 mL of alcohol. Shake the flask until the sample is completely dissolved or suspended. Let the mixture stand in the dark until it reaches room temperature (about 2 h). Add methylene chloride to volume, and shake again vigorously.
Sample solution: Transfer 5.0 mL of *Sample stock solution A* or *Sample stock solution B* to a 50-mL volumetric flask, and dilute with a mixture of methylene chloride and *Diluent* (1:1) to volume. Pass through a membrane filter of 0.45-µm pore size.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 448 nm
Column: 4.6-mm × 25-cm; 5-µm packing L68
Column temperature: 30°
Flow rate: 0.6 mL/min
Injection volume: 20 µL
System suitability
Samples: *System suitability solution* and *Standard solution A*
The approximate relative retention times of the components in the *System suitability solution* are listed in *Table 1*.

Table 1

Name	Relative Retention Time	Relative Response Factor
all-*trans*-Alpha carotene	0.93	1.0
all-*trans*-Beta carotene	1.00	1.0
9-*cis*-Beta carotene	1.07	1.0
13-*cis*-Beta carotene	1.17	1.2
15-*cis*-Beta carotene	1.21	1.4

Suitability requirements
Resolution: NLT 1.2 between beta carotene and alpha carotene and between beta carotene and 9-*cis*-beta carotene, *System suitability solution*
Tailing factor: NMT 2.0 for the beta carotene peak, *Standard solution A*
Relative standard deviation: NMT 2.0% for the beta carotene peak from replicate injections, *Standard solution A*
Chromatogram similarity: The chromatogram from the *System suitability solution* is similar to the reference chromatogram provided with the lot of USP Beta Carotene System Suitability RS being used.
Analysis
Samples: *Standard solution A* and *Sample solution*
Record the chromatograms, and identify the peaks of the relevant analytes of the *Sample solution* by comparing with those of the *System suitability solution*. Measure the peak area responses.
Calculate the percentage of the labeled amount of total beta carotene in the portion of Preparation taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = [(peak area of all-*trans*-beta carotene) + (peak area of 9-*cis*-beta carotene) + (peak area of 13-*cis*-beta carotene × 1.2) + (peak area of 15-*cis*-beta carotene × 1.4) + (sum of peak areas of other *cis*-isomers of beta carotene)] in the *Sample solution*
r_S = peak area of all-*trans*-beta carotene in *Standard solution A*
C_S = concentration of all-*trans*-beta carotene in *Standard solution A* as determined by spectrometric procedure (mg/mL)
C_U = nominal concentration of Preparation in the *Sample solution* (mg/mL)

Calculate the percentage of the labeled amount of all-*trans*-beta carotene in the portion of Preparation taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of all-*trans*-beta carotene in the *Sample solution*
r_S = peak area of all-*trans*-beta carotene in *Standard solution A*
C_S = concentration of all-*trans*-beta carotene in *Standard solution A* as determined by spectrometric procedure (mg/mL)
C_U = nominal concentration of Preparation in the *Sample solution* (mg/mL)

Acceptance criteria: The Preparation contains 95.0%–130.0% of the labeled amount of total beta carotene, calculated as ($C_{40}H_{56}$) on the anhydrous basis.

• **ALPHA CAROTENE AND OTHER RELATED COMPOUNDS**
Mobile phase, System suitability solution, Sample solution, and Chromatographic system: Proceed as directed in the test for *Content of Beta Carotene*.
Injection volume: 20 µL

DS Monographs

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Analysis
Sample: *Sample solution*
Calculate the percentage of alpha carotene and other individual related compounds relative to total beta carotene in the portion of Preparation taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak area of alpha carotene or other individual related compounds from the *Sample solution*
r_T = sum of the peak areas of all the peaks from the *Sample solution*

Acceptance criteria
Alpha carotene: NMT 1.0%
Any other individual related compound: NMT 1.0%
Total related compounds (including alpha carotene): NMT 5%

IMPURITIES
• **RESIDUE ON IGNITION** ⟨281⟩: NMT 2.0%

SPECIFIC TESTS
• **WATER DETERMINATION** ⟨921⟩, *Method I*: NMT 8.0% for solid Preparations; NMT 1.0% for liquid Preparations

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tightly sealed, light- and oxygen-resistant containers. Store in a cool place.
• **LABELING:** The label states the name and content of any carriers and antioxidants added to the formulation, the content of total carotenoids as beta carotene, and the percentages of *cis*- and all-*trans*-isomers in the total beta carotene at the time of product manufacture and release.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Beta Carotene RS
(*all-E*)-1,1′-(3,7,12,16-Tetramethyl-1,3,5,7,9,11,13,15,17-octadecanonaene-1,18- diyl) bis[2,6,6-trimethylcyclohexene].
USP Beta Carotene System Suitability RS

Beta Carotene Capsules—*see Beta Carotene Capsules General Monographs*

Beta Glucan

DEFINITION
Beta Glucan is obtained by extraction from the cell wall of fermented and thermally processed Baker's yeast (*Saccharomyces cervisiae*). It is comprised mainly of β-(1,3)/(1,6) branched glucan polymers. Small amounts of β-(1,6)-glucan and chitin are also expected to be present in the final product. It contains NLT 70% beta glucan, calculated as glucose after enzymatic hydrolysis, on the dried basis.

IDENTIFICATION
• **NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY** ⟨761⟩
Standard solution: Dissolve 10 mg of USP Beta Glucan RS in 0.6 mL of dimethyl sulfoxide-d_6, and heat at 100° for 1 h. Then add 0.1 mL of D_2O, mix the solution, and transfer to an NMR tube.
Sample solution: Dissolve 10 mg of Beta Glucan in 0.6 mL of dimethyl sulfoxide-d_6, and heat at 100° for 1 h. Then add 0.1 mL of D_2O, mix the solution, and transfer to an NMR tube.
Analysis: Collect ^{1}H NMR spectra at 80°, and compare individual resonances from the *Sample solution* to those from the *Standard solution*. The major signals associated with Beta Glucan are shown in *Table 1*.

Table 1

^{1}H NMR Major Signals	USP Beta Glucan RS
H-1 (1,3)-glucan	4.52, d, J = 7.5 Hz, 1H
H-2, 4, and 5 (1,3-)	3.27–3.33, m, 3H
H-3 and 6b (1,3-)	3.45–3.48, m, 2H
H-6a (1,3-)	3.71, d, J = 11 Hz, 1H
H-1 (1,6)-glucan	4.27, d, J = 7.7 Hz, 1H

Integrate the area under the peaks five times for each sample, and average. Determine the relative percentage of (1,6) linked glucan in the portion of Beta Glucan taken:

$$\text{Result} = \{A/(A + B)\} \times 100$$

A = integration values for the signal at 4.27 ppm, corresponding to H-1 from (1,6) glucan
B = integration values for the signal at 4.52 ppm corresponding to H-1 (1,3) glucan

Acceptance criteria: The ^{1}H spectrum of the *Sample solution* exhibits a chemical shift pattern with signal locations and relative intensities that correspond to those of the *Standard solution*. In addition, the relative percentage of (1,6) linked glucan is 10%–18% of the total linkages.

COMPOSITION
• **CONTENT OF BETA GLUCAN**
Buffer A: Dissolve 11.6 mL of glacial acetic acid in approximately 900 mL of water. Adjust the solution with 20% sodium hydroxide solution to a pH of 5, and dilute with water to 1000 mL.
Buffer B: Dissolve 69.6 mL of glacial acetic acid in approximately 800 mL of water. Adjust with 20% sodium hydroxide solution to a pH of 3.8, and dilute with water to 1000 mL.
Buffer C: Dissolve 12.12 g of tris(hydroxymethyl) aminomethane (TRIS), 11.69 g sodium chloride, and 4.16 g of ethylenediaminetetraacetic acid (EDTA) tetrasodium salt dihydrate in approximately 900 mL of water. Adjust with concentrated hydrochloric acid or 20% sodium hydroxide solution to a pH of 7.5, and dilute with water to 1000 mL. [NOTE—*Buffer C* can be stored for 1 year at 2°–8°.]
Buffer D[1]**:** Transfer 45.287 g of dibasic potassium phosphate, 30.382 g of *p*-hydroxybenzoic acid, and 4 g of sodium azide into a 1000-mL volumetric flask, and carefully add 800 mL of water. Mix with a stirring bar and mild heat until completely dissolved. Allow the solution to cool, adjust with 16.7% potassium hydroxide solution to a pH of 7.4, and dilute with water to volume. [NOTE—Store *Buffer D* in an amber bottle with an expiration date of 3 years at 4°.]
Lyticase solution: Prepare the required volume of lyticase from *Arthrobacter luteus*[2] at a concentration of 10 U/µL by dissolving the quantity stated by the manufacturer (U/mg) in a solution containing 10% (v/v) *Buffer C*. [NOTE—Unused solution can be stored at NMT –15° with an expiration date of 1 year. Every time a different lot of lyticase is used, the

[1] This buffer is also available as Bottle #3 of the K-YBGL kit (Megazyme), or Bottle #1 of the GOPOD kit (Megazyme).
[2] Lyticase from *Arthrobacter luteus*, Sigma L4025, or equivalent.

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concentration of lyticase solution required needs to be qualified.]

(1,6)-Glucanase solution: 1U/300 µL solution of lyophilized (1,6)-glucanase[3] in *Buffer A*. [NOTE—Solids may not fully dissolve; therefore, this solution should be handled as a homogeneous suspension. The solution is stable for at least 60 days at NMT –15°.]

Polishing enzyme mix: Mix 2000 U of *exo*-beta-glucanase[4] and 400 U of beta-glucosidase[5] in 100.0 mL of *Buffer A*. A premix of the enzymes may be used as an alternative[6]. [NOTE—Store on ice during the procedure, and for use in a same-day assay. Unused *Polishing enzyme mix* can be refrozen once at NMT –15° with an expiration date of 2 years.]

Glucose oxidase/peroxidase reagent: Dissolve the contents of the Glucose Determination Reagent[7] in 1 L of water containing 50 mL of *Buffer D*. [NOTE—Store the reagent in an amber bottle, and label with an expiration date of 3 months at a temperature between 2° and 8° or 1 year at NMT –17°. Minimize the time spent at room temperature.]

Sample solution: Transfer 15–20 mg of Beta Glucan into a 16- × 100-mm glass vial. Place the vial in an ice bath. Add a 0.4-mL aliquot of cold potassium hydroxide (1 in 6) while mixing on a vortex mixer to disperse the powder. Return the vial to the ice bath. Continue cycling through mixing on a vortex mixer and placing the vials in the ice bath for 20 min. The mixture should turn into a homogenous, translucent dispersion.

Standard solution: Proceed as directed for the *Sample solution* except replace Beta Glucan with USP Beta Glucan RS. [NOTE—Prepare the *Sample solution* and *Standard solution* in triplicate. It is critical for the success of the assay that the sample is well dispersed.]

Lyticase digestion: Upon removal of all vials containing the *Sample solution* or the *Standard solution* from the ice bath, add 1.6 mL of *Buffer B* and 600 µL of *Lyticase solution* to each vial. Incubate the mixture at 50° for 12–18 h, and cool to room temperature.

(1,6)-Glucanase digestion: After cooling of all vials, remove a 130-µL aliquot of each vial, and digest further by adding 25 µL of 16.7% potassium hydroxide solution and 300 µL of *(1,6)-Glucanase solution*. Incubate vials at 80° for 15 min, and cool to room temperature.

Beta glucanase/glucosidase digestion: After cooling of all vials, add 390 µL of the *Polishing enzyme mix* to each vial, and incubate the vials at 40° for 1 h. Cool them to room temperature, centrifuge, and transfer 50-µL aliquots (in duplicate) to new vials.

Enzyme blank solution: Prepare enzyme blanks in triplicate by combining all the reagents used during the digestion steps except the *Sample solution* or *Standard solution*.

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Mode: Vis

Analytical wavelength: 510 nm

Analysis: Dilute the 50-µL aliquots obtained after the *Beta glucanase/glucosidase digestion* with 50 µL of water, and then add 3 mL of *Glucose oxidase/peroxidase reagent*. Incubate the vials for 20 min at 40°. Determine the absorbance of each vial of the *Sample solution* or *Standard solution* against the *Enzyme blank solution*. Prepare a standard curve using the absorbance of similarly treated series of glucose standards (0, 0.1, 0.25, 0.5, and 1.0 mg/mL). From the slope of the standard curve and the absorbance of the digested *Sample solution* and *Standard solutions*, determine the concentration, *C*, in mg/mL, of liberated glucose in the cuvette:

$$\text{Result} = (A_S - A_B)/\text{slope}$$

A_S = average absorbance of the sample or USP Beta Glucan RS

A_B = average absorbance of the *Enzyme blank solution*

Calculate the percentage of beta glucan as glucose in the portion of Beta Glucan taken:

$$\text{Result} = 100 \times C/\{[(WT_S/F1) \times (F2/F3)]/2\}$$

WT_S = original weight of the sample or USP Beta Glucan RS (mg)

$F1$ = total volume in the vial during *Lyticase digestion*, 2.6 mL

$F2$ = volume of the sample or USP Beta Glucan RS transferred to a new vial during *(1,6)-Glucanase digestion*, 0.130 mL

$F3$ = total volume during *Beta glucanase/glucosidase digestion*, 0.845 mL

Acceptance criteria: NLT 70% beta glucan, calculated as glucose after enzymatic hydrolysis, on the dried basis

• **CONTENT OF PROTEIN**

Sample: 1.0 g of Beta Glucan

Analysis: Proceed as directed in *Nitrogen Determination* ⟨461⟩, and multiply the nitrogen content by 6.25.

Acceptance criteria: NMT 10.0%

• **CONTENT OF FAT**

Sample: 2 g of Beta Glucan, previously dried

Analysis: Transfer the *Sample* to an extraction thimble, and mix with an equivalent quantity of dry, clean sand. Place a fat-free cotton or glass wool plug on top of the thimble. Place the thimble in a continuous-extraction apparatus provided with a tared collection flask. Pour 75 mL of solvent hexane through the sample into the collection flask. Extract at a condensation rate of 5–6 drops/s for 4 h, then at a rate of 2–3 drops/s for the next 16 h. Detach the collection flask, carefully evaporate the solvent, and dry the collection flask and its contents in a drying oven at 100° for 30 min to constant weight. Calculate the percentage of the extract (crude fat) in the portion of Beta Glucan taken.

Acceptance criteria: NMT 20.0%

CONTAMINANTS

• **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩

Acceptance criteria

Arsenic: NMT 0.5 µg/g

Cadmium: NMT 0.5 µg/g

Lead: NMT 0.5 µg/g

Mercury: NMT 0.1 µg/g

• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 2×10^4 cfu/g, the total combined molds and yeasts count does not exceed 2.5×10^1 cfu/g, and the bile-tolerant Gram-negative bacteria does not exceed 10 cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

• **GLYCOGEN**

Buffer B: Prepare as directed in *Content of Beta Glucan*.

[3] Commercially available as Pustulanase, Cel136, Prokazyme, or equivalent.

[4] E-EXBGL 200 U/mL, 200 U/bottle, Megazyme, or equivalent.

[5] 200 U/bottle, Megazyme, or equivalent.

[6] E-EXBGOS, Megazyme, or equivalent.

[7] Bottle #4 of K-YBGL kit, or Bottle #2 of GOPOD kit, Megazyme, or equivalent.

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Amyloglucosidase/invertase solution[8]: A mixture of 1630 U/mL of amyloglucosidase and 500 U/mL of invertase in glycerol solution (50% v/v)

Sample: 100 mg

Analysis: Transfer the *Sample* in triplicate into individual 16- × 150-mm glass screw-cap vials. Place the vials in an ice bath, and add to each vial a 2-mL aliquot of cold potassium hydroxide (1 in 6) while mixing on a vortex mixer to disperse the powder. Return the vial to the ice bath. Continue cycling through mixing on a vortex mixer and placing vials in the ice bath for 20 min. The mixture should turn into a homogenous, translucent dispersion. Add 8 mL of *Buffer B*. Mix thoroughly on a vortex mixer, and immediately add 200 µL of *Amyloglucosidase/invertase solution*, and mix again on a vortex mixer. Incubate the mixture at 40° for 30–35 min. Cool to room temperature. Mix again on a vortex mixer, transfer to a suitable centrifuge tube, and centrifuge until a clear supernatant is obtained. Transfer duplicate 50-µL aliquots of supernatant into new vials, and proceed as directed for the *Analysis* in *Content of Beta Glucan*.

Acceptance criteria: NMT 1.0%

• **MANNOSE**

Solution A: 100% purified water

Solution B: 956 mM sodium hydroxide

Mobile phase: See *Table 2*.

Table 2

Time (min)	Solution A (%)	Solution B (%)
0	36.0	64.0
15.0	36.0	64.0
35.0 (sample injection)	59.4	40.6
80.0	59.4	40.6

[NOTE—The run time typically required is 80 min.]

Internal standard solution: 0.8 mg/mL of USP Inositol RS in water

Sample solution: Weigh 2.0–4.0 mg of Beta Glucan in duplicate into vials with stir bars. Add 500 µL of pure trifluoroacetic acid (TFA), and allow the mixture to form a uniform dispersion by stirring for 1 h at room temperature. Incubate in a 80° water bath for 2 h with stirring, and then cool to room temperature. Add 100 µL of the *Internal standard solution* to each vial, and incubate with stirring in a boiling water bath for 15 min. Cool again to room temperature, then add 1.07 mL of water to each vial, and incubate with stirring in a boiling water bath for 1 h. Cool the solutions to room temperature, and dry overnight on a SpeedVac, or equivalent, at low heat with the cryopumping system off. Dissolve the dried preparation in 2.5 mL of deionized water, and pass through a PTFE syringe filter of 0.2-µm pore size. Dilute with an equal volume of water before injection.

Standard solutions: Prepare a 4-mg/mL solution of USP Dextrose RS and a 80-µg/mL solution of USP Mannose RS. Separately transfer aliquots of these solutions to individual vials (see *Table 3*). Prepare each standard in duplicate, and freeze-dry them. Treat the freeze-dried vials as directed in the *Sample solution*, beginning with "Add 500 µL of pure trifluoroacetic acid".

[8] Alternatively, Bottle #2 of K-YBGL kit (Megazyme, or equivalent) could be used directly.

Table 3

Standard Identification Number	µL/Vial of 4 mg/mL Glucose	Content of Glucose (µg)	µL/Vial of 80 µg/mL Mannose	Content of Mannose (µg)
0	0	0	0	0
1	100	400	25	2
2	200	800	50	4
3	300	1200	100	8
4	500	2000	200	16
5	1000	4000	400	32

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Electrochemical detector

Detector mode: Pulsed amperometric detection

Detector range: 3000 µC (may be modified if needed)

Working electrode: Gold

Reference electrode: pH, silver–silver chloride

Electrochemical waveform: See *Table 4*.

Table 4

Time (s)	Potential (V)	Integration
0.00	0.10	
0.20	0.10	Start
0.40	0.10	End
0.41	−2.00	
0.42	−2.00	
0.43	0.60	
0.44	−0.10	
0.50	−0.10	

Column: 4-mm × 25-cm; packing L47

Guard column: 4-mm × 5-cm; packing L47

Column temperature: 30°

Flow rate: 0.4 mL/min

Injection size: 10 µL

System suitability

Sample: *Standard Identification #5*

[NOTE—The relative retention times for inositol, mannose, and glucose are 0.68, 0.95 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 1.5 between mannose and glucose

Analysis

Samples: *Standard solutions* and *Sample solution*

Calculate the ratios of the peak area response of the glucose and mannose to the peak area response of the internal standard from the *Standard solutions*. Make two standard response lines by plotting the peak area response ratio versus the amount (µg) of the glucose and mannose in the *Standard solutions*. Calculate the ratio of the peak area response of the glucose and mannose to the peak area response of the internal standard from the *Sample solution*. From the calculated ratios of peak responses for glucose and mannose and their respective standard response lines, determine the content of glucose, C_G, and mannose, C_M, both in µg, in the *Sample solution*.

Calculate the percentage of mannose in the portion of Beta Glucan taken:

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$$\text{Result} = C_M/(C_M + C_G) \times 100$$

C_M = content of mannose in the *Sample solution* from the mannose regression line (µg)
C_G = content of glucose in the *Sample solution* from the glucose regression line (µg)

Acceptance criteria: NMT 1.0% mannose, as a function of total hexose recovered (glucose and mannose)

- **RESIDUE ON IGNITION** ⟨281⟩: NMT 2.5%
- **LOSS ON DRYING** ⟨731⟩

Sample: 1 g
Analysis: Dry the *Sample* at 105° for 3 h.
Acceptance criteria: NMT 8.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight and light-resistant containers.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Beta Glucan RS
USP Dextrose RS
USP Inositol RS
USP Mannose RS

Bifidobacterium animalis subsp. *lactis*

DEFINITION

Bifidobacterium animalis subsp. *lactis* is a lactic-acid producing, Gram-positive, rod-shaped, non-spore-forming, anaerobic bacterium that is pleomorphic. Various rod shapes may be observed, but they are typically curved/clubbed, and often branched. *Bifidobacterium animalis* subsp. *lactis* occurs as a white- to cream-colored powder that is produced via fermentation of one of three strains of *Bifidobacterium animalis* subsp. *lactis*: Bi-07 (ATCC SD5220), Bl-04 (ATCC SD5219), and HN019 (ATCC SD5674). Suitable cryoprotectants may be added to the concentrated bacteria following fermentation, after which the product is frozen and then freeze-dried. The formulated product may be blended with food-grade diluents and/or bulking agents. It contains NLT 100% of the labeled viable cell count of one of the three *Bifidobacterium animalis* subsp. *lactis* strains.

IDENTIFICATION

- **A. NUCLEIC ACID-BASED IDENTIFICATION**

[NOTE—In all cases for *Identification A*, "sterile water" refers to sterile, nuclease-free water acceptable for use in molecular biology.[1]]

Buffer: Use a molecular biology-grade 10 mM tris-hydrochloride, 1 mM EDTA sodium buffer.[2]

Sample solution: 100 mg/mL of the freeze-dried probiotic powder in *Buffer*

Primer set: Use primer(s) set per strain (see *Table 1*).[3] Primers should be diluted in *Buffer* to a stock concentration of 100 µM, then further diluted to 25 µM in *Buffer*, and stored at −20°. A positive test for each *Primer set* to the strain is expected to give an amplification product specified in *Table 1*.

Table 1. Primer Set

Strain	Primer
Bi-07	Set 1: Forward (5′-3′) CATCGCAACTTCACCCACATTG and Reverse (5′-3′) ATGCCGTACCCCTGAATGAAG. The annealing temperature of set 1: 57.0°. An amplification product of this set is 533 base pairs. Set 2: Forward (5′-3′) ACGGATATGTATAGGTGGCATGC and Reverse (5′-3′) GTATGTTCAATCGTATGCAGCCC. The annealing temperature of set 2: 56.0°. An amplification product of this set is 492 base pairs with a single nucleotide polymorphism (SNP).
Bl-04	Forward (5′-3′) CATCGCAACTTCACCCACATTG and Reverse (5′-3′) ATGCCGTACCCCTGAATGAAG. An amplification product of this set is 479 base pairs.
HN019	Set 1: Forward (5′-3′) ACTCTTCTGTGTCGTTCTGCTTC and Reverse (5′-3′) CAAGGATTGGAACGCGAGAAAAC. The annealing temperature of set 1: 56.0°. An amplification product of this set is 351 base pairs with an SNP. Set 2: Forward (5′-3′) CCTGCTGTGGTGAATACGAAGAA and Reverse (5′-3′) TTGCATCTTGTACAGTTCGGCAT. The annealing temperature of set 2: 57.0°. An amplification product of this set is 531 base pairs with an SNP.

Polymerase chain reaction (PCR) sample preparations: For each *Primer set*, prepare a solution containing 1 µL of the *Sample solution*, 10 µL of mastermix polymerase,[4] 1 µL of diluted forward primer (25 µM), 1 µL of diluted reverse primer (25 µM), and 12 µL of sterile water.

PCR negative control: Prepare as directed for the *PCR sample preparations*, replacing the 1 µL of *Sample solution* with 1 µL of sterile water.

PCR amplification: Perform PCR on each *PCR sample preparation* and the *PCR negative control* using an appropriate thermal cycler (see *Table 2*).[5] Perform the PCR amplification cycle per primer and per strain as specified in *Table 2*.

Table 2. PCR Amplification Cycle

Strain	PCR Amplification Cycle
Bi-07	Incubate at 95° for 7 min (step 1); 95° for 30 s (step 2); the *Primer set* annealing temperature for 30 s (step 3); and at 72° for 1 min (for *Primer set 1*) or 30 s (for *Primer set 2*; step 4). Repeat steps 2–4 for 34 cycles, then incubate at 72° for 5 min and hold at 4°.
Bl-04	Incubate at 95° for 7 min (step 1); 95° for 30 s (step 2); 57.0° for 30 s (step 3); and at 72° for 1 min (step 4). Repeat steps 2–4 for 34 cycles, then incubate at 72° for 5 min and hold at 4°.
HN019	Incubate at 95° for 7 min (step 1); 95° for 30 s (step 2); the *Primer set* annealing temperature for 30 s (step 3); and at 72° for 30 s (for *Primer set 1*) or 1 min (for *Primer set 2*; step 4). Repeat steps 2–4 for 34 cycles, then incubate at 72° for 5 min and hold at 4°.

[1] Suitable PCR-Certified Waters, RNase and DNase Free, are available from www.teknova.com.
[2] Suitable buffers (e.g., TE Buffer 1X, Molecular Biology Grade) are available from www.promega.com.
[3] DNA primers are commercially available (custom manufacture) from Integrated DNA Technologies (www.idtdna.com) and other commercial sources.
[4] 5 Prime MasterMix polymerase from 5 Prime.
[5] Suitable thermal cyclers are available from Eppendorf® (www.eppendorf.com).

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Analysis: Analyze the products of the *PCR amplification* for each *PCR sample preparation* and for the *PCR negative control* using an automated on-chip electrophoresis system with a DNA kit.[6] Follow the manufacturer's instructions for analysis. Alternatively, analysis and visualization may be accomplished using gel electrophoresis. Prepare or use a commercially available 1% (w/v) agarose gel in a 1X tris-acetic acid–EDTA buffer (40 mM tris-hydrochloride, 1% glacial acetic acid, and 1 mM EDTA). Stain the gel with 0.5 mg/mL of ethidium bromide in water and de-stain with deionized water. [**CAUTION**—Ethidium bromide is considered a toxic substance and a potential mutagen. Use appropriate personal protective equipment (including nitrile gloves) when handling this reagent.] Use a DNA ladder standard (1 KB plus)[7] suitable for determining the size of linear double-stranded DNA fragments between 100 and 12,000 base pairs. The ladder standard should be used in the first and last lanes on the gel to allow for proper comparison of amplicons.

Analysis of the *PCR negative control* must result in the absence of any amplification products or the preparation of the *PCR sample preparations*, and the *PCR negative control* must be repeated, followed by *PCR amplification* and *Analysis*.

Acceptance criteria: The *PCR sample preparation* prepared with strain-specific *Primer set* gives an acceptable amplification product per strain (see *Table 3*).

Table 3. Acceptance Criteria of PCR Amplification Products

Strain	Acceptance Criteria per Strain
Bi-07	The *PCR sample preparation* prepared with *Primer set 1* gives an amplification product of 533 base pairs. There should be no amplification product of 479 base pairs. The *PCR sample preparation* prepared with *Primer set 2* gives an amplification product of 492 base pairs with an SNP identified as (underlined in the following 15 base pair sequence) GCGGGCAAGTGCTGG. The SNP location is 218 base pairs from the 5′ end of the forward primer described in *Primer set 2*. The sequence of the amplicon should be determined by validated, standard sequencing technologies.
Bi-04	The *PCR sample preparation* prepared with the *Primer set* gives an amplification product of 479 base pairs. There should be no amplification product of 533 base pairs.
HN019	The *PCR sample preparation* prepared with *Primer set 1* gives an amplification product of 351 base pairs with an SNP identified as (underlined in the following 15 base pair sequence) CTTCAGATTTTAGGC. The SNP location is 44 base pairs from the 5′ end of the forward primer described in *Primer set 1*. The *PCR sample preparation* prepared with *Primer set 2* gives an amplification product of 531 base pairs with an SNP identified as (underlined in the following 15 base pair sequence) GCCCGCTCAAACGAA. The SNP location is 279 base pairs from the 5′ end of the forward primer described in *Primer set 2*. The sequence of the amplicon should be determined by validated, standard sequencing technologies.

ASSAY

• **ENUMERATION**

Agar medium: Prepare as follows or use a suitable commercially available agar (see *Table 4*).[8]

Table 4. Lactobacilli MRS Agar

Reagent	Quantity (g)
Proteose peptone no. 3	10.0
Beef extract	10.0
Yeast extract	5.0
Dextrose	20.0
Polysorbate 80	1.0
Ammonium citrate	2.0
Sodium acetate	5.0
Magnesium sulfate	0.1
Manganese sulfate	0.05
Dipotassium phosphate	2.0
Agar	15.0

Suspend Lactobacilli MRS Agar in 1 L of purified water in an appropriately sized conical flask or beaker (sufficiently large to not boil over). Cover the flask or beaker with aluminum foil and heat with stirring to boiling on a hot plate. Allow to boil for 1 min to completely dissolve the medium, then autoclave the solution at 121° for 15 min. Cool to 45° and use immediately. Boiled *Agar medium* may also be aseptically transferred into individual media bottles in 100- or 200-mL aliquots before sterilizing, and then autoclaved and stored for later use. [NOTE—Can be stored at 4° (heat gently to 45° to melt the agar before use).] Immediately before use, aseptically add 1.0 mL of a sterile 5% (w/v) cysteine hydrochloride solution for each 100 mL of *Agar medium* prepared, such that the final concentration of cysteine hydrochloride in the *Agar medium* is 0.05%.

Sample broth: Prepare as follows or use a suitable commercially available broth (see *Table 5*).[9]

Table 5. Lactobacilli MRS Broth

Reagent	Quantity (g)
Proteose peptone no. 3	10.0
Beef extract	10.0
Yeast extract	5.0
Dextrose	20.0
Polysorbate 80	1.0
Ammonium citrate	2.0
Sodium acetate	5.0
Magnesium sulfate	0.1
Manganese sulfate	0.05
Dipotassium phosphate	2.0

[6] Suitable automated on-chip electrophoresis systems with a DNA kit are available from Agilent (Agilent 2100 Bioanalyzer with Agilent DNA 1000 Kit www.genomics.agilent.com).

[7] Suitable 1 KB plus DNA ladders are available from www.thermofisher.com.

[8] Difco™ Lactobacilli MRS Agar, or equivalent. Suitable Lactobacilli MRS Agars are available from www.vwr.com or other chemical/microbiological suppliers.

[9] Difco™ Lactobacilli MRS Broth, or equivalent are available from www.vwr.com or other chemical/microbiological suppliers.

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Suspend Lactobacilli MRS Broth in 1 L of purified water in an appropriately sized conical flask or beaker (sufficiently large to not boil over). Cover the flask or beaker with aluminum foil and heat with stirring to boiling on a hot plate. Allow to boil for 1 min to completely dissolve the broth ingredients, then autoclave the solution at 121° for 15 min. Broth may also be aseptically transferred into individual media bottles in 100- or 200-mL aliquots before sterilizing, and then autoclaved and stored for later use. [NOTE—Can be stored at 4° (allow broth to come to room temperature before use).]

Peptone diluent: Prepare a solution of 0.1% peptone[10] in water (w/v) and adjust to a pH of 7.0 with a solution of lactic acid. Using an autoclave, steam sterilize the solution at 121° for NLT 15 min, then allow to cool in the unopened autoclave. Dispense into sterile containers as needed for preparing samples.

Sample preparation: Aseptically transfer 11.0 g of freeze-dried probiotic powder into a sterile stomacher bag. Add 99 mL of previously sterilized (room temperature) *Sample broth* to the bag and blend at 230 rpm for 30 s in a stomacher. Hold the mixture at room temperature for 30 min to allow rehydration of the freeze-dried sample, then blend in the stomacher for an additional 30 s at 230 rpm. This is the primary 10^{-1} dilution.

Using sterilized, filtered pipet tips, make serial dilutions by aseptically transferring 1.0 mL of the primary 10^{-1} dilution to sterile media bottles, each containing 99.0 mL of *Peptone diluent* (10^{-3} dilution). Repeat this operation until the desired dilution series is obtained. [NOTE—The dilutions used in the *Analysis* should be expected to contain 25–250 cfu/mL.] Shake the media bottles for complete mixing before proceeding with the *Analysis*.

Analysis: For each *Sample preparation* to be plated, prepare Petri plates as follows. Using three sterile, filtered 1-mL pipet tips, aseptically transfer 1.0 mL of the *Sample preparation* separately into three appropriately labeled sterile 15- × 100-mm Petri plates, then pour about 15 mL of the 45° *Agar medium* into each plate, flaming the lip of the bottle between pours. Place the lid on each plate after adding the *Agar medium*, then gently swirl the plates to mix the *Sample preparation* and the *Agar medium*. [NOTE—Be careful to avoid spillage onto the lid of the dish when swirling the plates.] Repeat this procedure for additional dilutions of the *Sample preparation*. Prepare one blank plate that contains only the *Agar medium* and a second blank plate in which 1.0 mL of *Peptone diluent* has been mixed with the *Agar medium*. Allow the plates to sit at room temperature on a level surface until the *Agar medium* solidifies, then incubate the plates at 38° for 72 h under anaerobic conditions.[11]

After 72 h of incubation, count the colonies and record the results as viable cfu/g, taking into account the appropriate dilution factor of the *Sample preparation*. Only count plates containing 25–250 colonies. Determine the average plate count, in cfu/g.

Acceptance criteria: NLT 100% of the labeled viable cell count, in cfu/g

CONTAMINANTS

[NOTE—The methods of microbial analysis included in this section as examples represent currently accepted methods commonly used in industry. Users may substitute other validated test methods for the methods in this section.]

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total combined molds and yeasts count does not exceed 10^2 cfu/g.
- **NON-LACTIC ACID BACTERIA:** ISO International Standard number 13559 (IDF 153), available from the International Organization for Standardization (www.iso.org). The total non-lactic acid bacteria count is less than 5×10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Escherichia coli*: It meets the requirements of the tests for absence of *Escherichia coli*. It meets the requirements of the tests for absence of *Salmonella* species in 40 g.
- ***Listeria*:** (See *Food Chemicals Codex, Appendix XV*.) It meets the requirements of the tests for absence of *Listeria* in 25 g.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in high-barrier foil laminate bags and store at or below 4°.
- **LABELING:** This ingredient should be labeled with the genus and species names, or genus, species, and strain names and with the formulated enumeration in cfu/g (or similar units). This monograph applies only to three strains of *Bifidobacterium animalis* subsp. *lactis*—Bi-07, Bl-04, and HN019—and no other strains of *Bifidobacterium animalis* subsp. *lactis* cultures.

Powdered Bilberry Extract

DEFINITION

Powdered Bilberry Extract is prepared from the ripe fruits of *Vaccinium myrtillus* L. (Fam. Ericaceae) using suitable solvents such as alcohol, methanol, or water or mixtures of these solvents. The ratio of the starting plant material to Powdered Extract is between 153:1 and 76:1. It contains NLT 36.0% of anthocyanosides, calculated as cyanidin-3-*O*-glucoside chloride, and NMT 1.0% of anthocyanidins, calculated as cyanidin chloride; both are calculated on the anhydrous basis.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution: 4 mg/mL of USP Powdered Bilberry Extract RS in methanol. Centrifuge, and use the clear supernatant.

Sample solution: 4 mg/mL of Powdered Bilberry Extract in methanol. Centrifuge, and use the clear supernatant.

Adsorbent: Use suitable thin-layer chromatographic plates coated with a layer of cellulose.

Application volume: 10 µL

Developing solvent system A: Glacial acetic acid, hydrochloric acid, and water (15:3:82)

Developing solvent system B: Glacial acetic acid and water (6:4)

Analysis

Samples: *Standard solution* and *Sample solution*

Use a saturated chamber. Develop the chromatograms using *Developing solvent system A*, and dry the plate with the aid of a current of warm air. Develop the chromatograms in the same direction using *Developing solvent system B*. Examine the plate under visible light.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits three main red bands with R_F values of approximately 0.55, 0.65, and 0.70 that are similar in position and color to the corresponding main bands in the chromatogram of the *Standard solution*.

- **B.** The retention times of the anthocyanoside peaks in the chromatogram of the *Sample solution* correspond to those

[10] Suitable peptone for microbiological analysis is available from BD Bacto™ (www.bd.com).

[11] Suitable anaerobic systems are available from BD GasPak™ EZ Container System (www.bd.com).

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in the chromatogram of *Standard solution C*, as obtained in the test for *Content of Anthocyanosides and Anthocyanidins*. The peaks due to delphinidin-3-*O*-galactoside chloride and delphinidin-3-*O*-glucoside chloride are the most intense peaks and are of similar intensity, and each is more intense than the peak due to cyanidin-3-*O*-glucoside chloride. The peaks due to cyanidin-3-*O*-galactoside chloride, delphinidin-3-*O*-arabinoside chloride, and cyanidin-3-*O*-glucoside chloride are of similar intensity. Each of the remaining anthocyanoside peaks is of lower intensity than the peak due to cyanidin-3-*O*-glucoside chloride.

COMPOSITION

• **CONTENT OF ANTHOCYANOSIDES AND ANTHOCYANIDINS**

Solvent: Methanol and hydrochloric acid (49:1)

Diluent: 85% phosphoric acid and water (1:9)

Solution A: Formic acid and water (1:9)

Solution B: Acetonitrile, methanol, formic acid, and water (45:45:20:80)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	93	7
35	75	25
45	35	65
46	0	100
50	0	100
51	93	7
60	93	7

Standard stock solution A: 0.4 mg/mL of USP Cyanidin-3-*O*-glucoside Chloride RS in *Solvent*. [NOTE—Dissolve using sonication.]

Standard solution A: 0.08 mg/mL of USP Cyanidin-3-*O*-glucoside Chloride RS from *Standard stock solution A* in *Diluent*. [NOTE—This solution is stable for 48 h at 4°.]

Standard stock solution B: 0.5 mg/mL of USP Cyanidin Chloride RS in *Solvent*. [NOTE—Dissolve using sonication.]

Standard solution B: 0.01 mg/mL of USP Cyanidin Chloride RS from *Standard stock solution B* in *Diluent*. [NOTE—This solution is stable for 36 h at 4°]

Standard solution C: Transfer 125 mg of USP Powdered Bilberry Extract RS to a 100-mL volumetric flask, add 25 mL of *Solvent*, sonicate to dissolve, and dilute with *Diluent* to volume. [NOTE—This solution is stable for 48 h at 4°.]

Sample solution: Transfer 125 mg of Powdered Bilberry Extract to a 100-mL volumetric flask, add 25 mL of *Solvent*, sonicate to dissolve, and dilute with *Diluent* to volume.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

[NOTE—Use deactivated silanized HPLC vials.]

Mode: LC

Detector: UV-Vis 535 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Temperature

Refrigerated autosampler: 4°

Column: 30 ± 1°

Flow rate: 1 mL/min

Injection size: 10 µL

System suitability

Samples: *Standard solution A* and *Standard solution C*

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution C* is similar to the Reference Chromatogram provided with USP Powdered Bilberry Extract RS.

Resolution: NLT 0.8 between the delphinidin-3-*O*-arabinoside, malvidin-3-*O*-galactoside, and petunidin-3-*O*-arabinose peaks and NLT 1.0 for other components, *Standard solution C*

Tailing factor range: 0.8–2.0 for the cyanidin-3-*O*-glucoside chloride peak, *Standard solution A*

Relative standard deviation: NMT 2.0% for cyanidin-3-*O*-glucoside chloride peak in repeated injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Using the chromatogram of *Standard solution C* and the Reference Chromatogram, identify the retention times of the peaks corresponding to the different anthocyanosides and anthocyanidins. The approximate relative retention times, relative to cyanidin-3-*O*-glucoside chloride, are provided for the anthocyanosides in *Table 2* and for the anthocyanidins in *Table 3*.

Separately calculate the percentages of each anthocyanoside (see *Table 2*) in the portion of Powdered Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of each of the anthocyanosides in the *Sample solution*

r_S = peak response of cyanidin-3-*O*-glucoside chloride in *Standard solution A*

C_S = concentration of USP Cyanidin-3-*O*-glucoside Chloride RS in *Standard solution A* (mg/mL)

C_U = concentration of Powdered Extract in the *Sample solution* (mg/mL)

Table 2

Analyte	Relative Retention Time
Delphinidin-3-*O*-galactoside chloride	0.61
Delphinidin-3-*O*-glucoside chloride	0.73
Cyanidin-3-*O*-galactoside chloride	0.84
Delphinidin-3-*O*-arabinoside chloride	0.86
Cyanidin-3-*O*-glucoside chloride	1.00
Petunidin-3-*O*-galactoside chloride	1.08
Cyanidin-3-*O*-arabinoside chloride	1.11
Petunidin-3-*O*-glucoside chloride	1.24
Peonidin-3-*O*-galactoside chloride	1.36
Petunidin-3-*O*-arabinoside chloride	1.39
Peonidin-3-*O*-glucoside chloride	1.55
Malvidin-3-*O*-galactoside chloride	1.58
Peonidin-3-*O*-arabinoside chloride	1.67
Malvidin-3-*O*-glucoside chloride	1.76
Malvidin-3-*O*-arabinoside chloride	1.91

Separately calculate the percentages of anthocyanidins (see *Table 3*) in the portion of Powdered Extract taken:

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$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of each of the anthocyanidins in the *Sample solution*
r_S = peak response of cyanidin chloride in *Standard solution B*
C_S = concentration of USP Cyanidin Chloride RS in *Standard solution B* (mg/mL)
C_U = concentration of Powdered Extract in the *Sample solution* (mg/mL)

Table 3

Analyte	Relative Retention Time
Delphinidin chloride	1.28
Cyanidin chloride	1.82
Petunidin chloride	2.08
Peonidin chloride	2.27
Malvidin chloride	2.30

Acceptance criteria
Sum of all anthocyanosides: NLT 36.0% on the anhydrous basis
Sum of all anthocyanidins: NMT 1.0% on the anhydrous basis

CONTAMINANTS

Change to read:

- **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020)
  : Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩
  Total aerobic microbial count: NMT 10^4 cfu/g
  Total combined yeasts and molds count: NMT 10^3 cfu/g
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

- **ACID INSOLUBLE FRACTION**
  Sample: 5 g of Powdered Extract finely ground
  Analysis: Transfer about 1 g to a 500-mL flask, add 200 mL of 0.1 N hydrochloric acid, and shake vigorously for 2 h. Pass the solution through a previously tared sintered-glass filter, wash the flask with 30 mL of 0.1 N hydrochloric acid, and transfer the washings to the filter. Wash the filter with 30 mL of 0.1 N hydrochloric acid in 5-mL portions. Dry the filter for 3 h at 105°, cool in a desiccator, and weigh. Calculate the percentage of the acid insoluble fraction.
  Acceptance criteria: NMT 5%
- **WATER DETERMINATION,** *Method Ia* ⟨921⟩**:** NMT 4.5%, determined on 0.5 g
- **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 3.0%, determined on 1.0 g
- **BOTANICAL EXTRACTS,** *Residual Solvents* ⟨565⟩**:** Meets the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. It meets other labeling requirements under *Botanical Extracts* ⟨565⟩.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Powdered Bilberry Extract RS
  USP Cyanidin Chloride RS
  USP Cyanidin-3-*O*-glucoside Chloride RS

Biotin—*see Biotin General Monographs*

Biotin Capsules—*see Biotin Capsules General Monographs*

Biotin Tablets—*see Biotin Tablets General Monographs*

Black Cohosh

DEFINITION

Black Cohosh consists of the dried rhizome and roots of *Actaea racemosa* L. [*Cimicifuga racemosa* (L.) Nutt.] (*Ranunculaceae*). It is harvested in the summer. It contains NLT 0.4% of triterpene glycosides, calculated as 23-*epi*-26-deoxyactein[1] ($C_{37}H_{56}O_{10}$) on the dried basis.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHY**
  Standard solution A: 100 mg/mL of USP Powdered Black Cohosh Extract RS in methanol
  Standard solution B: 1 mg/mL each of USP Actein RS, USP 23-*epi*-26-Deoxyactein RS, and isoferulic acid in methanol
  Sample solution: Transfer 5 g of finely powdered Black Cohosh to a screw-cap centrifuge tube, add 10 mL of a mixture of alcohol and water (7:3), and heat on a steam bath for 10 min. Centrifuge, and use the clear supernatant.
  Adsorbent: Chromatographic silica gel mixture with an average particle size of 10–15 µm (TLC plates)
  Application volume: 10 µL
  Developing solvent system: Use the upper phase of a mixture of butyl alcohol, glacial acetic acid, and water (5:1:4).
  Derivatization reagent: Methanol, glacial acetic acid, sulfuric acid, and *p*-anisaldehyde (85: 10: 5: 0.5). [NOTE—Store in a refrigerator. The reagent is colorless; discard if color appears.]
  Analysis
  Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
  Develop the chromatograms until the solvent front has moved about 15 cm, and dry the plate in a stream of air. Examine the plate under UV light at 365 nm. Treat the plate with *Derivatization reagent*, heat at 100° for 5 min, and examine in white light.
  Acceptance criteria: Under UV light at 365 nm, the chromatogram of the *Sample solution* exhibits main zones similar in position and color to the main zones of *Standard solution A*. In the upper third of the plate, the *Sample solution* exhibits a blue fluorescent zone at the level of the zone due to isoferulic acid of *Standard solution B*. Under

[1] 23-*epi*-26-Deoxyactein is sometimes referred to as 27-deoxyactein.

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white light, the *Sample solution* exhibits main zones similar in position and color to the main zones of *Standard solution A*. *Standard solution B* exhibits red-violet zones due to actein and 23-*epi*-26-deoxyactein. The *Sample solution* exhibits several greenish-brown spots in the lower third of the plate and several violet zones above; two of these violet zones occur at R_F values similar to those due to actein and 23-*epi*-26-deoxyactein of *Standard solution B*.

• **B. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)

Standard solution A: 0.5 mL of *Standard solution A* prepared in *Identification* test *A*, diluted with methanol to 2 mL

Standard solution B: 1.0 mL of *Standard solution B* prepared in *Identification* test *A*, diluted with methanol to 5 mL

Sample solution: Transfer 0.5 g of Black Cohosh, finely powdered, to a screw-cap tube, add 5 mL of methanol, sonicate for 10 min, and filter into a 10-mL volumetric flask. Wash the residue on the filter paper four times, using 1 mL of methanol for each washing; add the washings to the volumetric flask; and dilute with methanol to volume.

Application volume: 2 µL as 8-mm bands

Developing solvent system: Toluene, ethyl formate, and formic acid (5:3:2)

Derivatization reagent: Proceed as directed for *Identification* test *A*.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatograms until the solvent front has moved about two-thirds of the length of the plate, and dry the plate in a current of air. Treat the plate with *Derivatization reagent*, heat at 100° for 5 min, and examine in white light.

Acceptance criteria: The *Sample solution* exhibits main zones similar in position and color to the main zones of *Standard solution A*. *Standard solution B* exhibits red-violet zones due to actein and 23-*epi*-26-deoxyactein at R_F values of about 0.5 and 0.4, respectively. The *Sample solution* exhibits zones similar in color and R_F values to those due to actein and 23-*epi*-26-deoxyactein of *Standard solution B*.

• **C.** The *Sample solution* exhibits peaks for cimiracemoside A, 26-deoxycimicifugoside, (26*S*)-actein, 23-*epi*-26-deoxyactein, cimigenol–arabinoside, and cimigenol–xyloside at retention times corresponding to these compounds in the *Standard solution*, as obtained in the test for *Content of Triterpene Glycosides*. The ratio of the peak areas of cimigenol–arabinoside to cimigenol–xyloside is NLT 0.4 (distinction from *Cimicifuga foetida*).

COMPOSITION

• **CONTENT OF TRITERPENE GLYCOSIDES**

Standard solution: Dissolve a quantity of USP Powdered Black Cohosh Extract RS in methanol with shaking for 1 min, and dilute with methanol to obtain a solution having a known concentration of 30 mg/mL. Pass through a membrane filter of 0.45-µm or finer pore size.

23-*epi*-26-Deoxyactein standard solutions: Dissolve USP 23-*epi*-26-Deoxyactein RS in methanol with shaking for 1 min. Dilute quantitatively, and stepwise if necessary, to obtain solutions having concentrations of 500, 100, 50, 25, and 12.5 µg/mL. Pass through a membrane filter of 0.45-µm or finer pore size.

System suitability solution: 0.1 mg/mL each of USP Actein RS and USP 23-*epi*-26-Deoxyactein RS in methanol

Sample solution: Accurately weigh about 750 mg of Black Cohosh, finely powdered, and place in a 20-mL PTFE-capped centrifuge tube. Add 15 mL of methanol, sonicate for 30 min, centrifuge, and retain the supernatant. Repeat the extraction twice. Evaporate the combined extracts under vacuum at 45°–50°. Dissolve the residue in methanol, and quantitatively transfer to a 10-mL volumetric flask. Dilute with methanol to volume, and pass through a membrane filter of 0.45-µm or finer pore size.

Solution A: 0.05% Trifluoroacetic acid in water

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Water (%)	Solution A (%)	Solution B (%)
0	0	80	20
8	0	80	20
15	68	0	32
55	36	0	64
65	5	0	95
70	5	0	95
85	0	80	20

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Evaporative light-scattering

[NOTE—The *Detector* is set up according to the manufacturer's instructions in order to achieve a signal-to-noise ratio of NLT 10 for the 12.5-µg/mL *23-epi-26-Deoxyactein standard solution*.]

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 35°

Flow rate: 1.6 mL/min

Injection volume: 20 µL

System suitability

Samples: *System suitability solution*, *Standard solution*, and 100-µg/mL *23-epi-26-Deoxyactein standard solution*

Suitability requirements

Chromatogram similarity: The chromatogram of the *Standard solution* is similar to the reference chromatogram provided with the lot of USP Powdered Black Cohosh Extract RS being used.

Resolution: NLT 1.0 between the (26*S*)-actein and the 23-*epi*-26-deoxyactein peaks, *System suitability solution*

Tailing factor: NMT 2.0 for the 23-*epi*-26-deoxyactein peak, 100-µg/mL *23-epi-26-Deoxyactein standard solution*

Relative standard deviation: NMT 2.0% of the logarithm of the area response of the 23-epi-26-deoxyactein peak in repeated injections, 100-µg/mL *23-epi-26-Deoxyactein standard solution*

Analysis

Samples: *System suitability solution*, *Standard solution*, *23-epi-26-Deoxyactein standard solutions*, and *Sample solution*

Using the chromatogram of the *Standard solution* and the reference chromatogram provided with the lot of USP Powdered Black Cohosh Extract RS being used, identify the retention times of the peaks corresponding to the triterpene glycosides. The approximate relative retention times of the triterpene glycosides are provided in *Table 2*.

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Table 2

Compound	Relative Retention Time
Cimicifugoside H-1	0.61
Cimiracemoside A	0.78
(26*R*)-Actein	0.94
26-Deoxycimicifugoside	0.96
(26*S*)-Actein	0.98
23-*epi*-26-Deoxyactein	1.00
Acetyl-shengmanol–xyloside	1.03
Cimigenol–arabinoside	1.08
Cimigenol–xyloside (cimicifugoside)	1.13
26-Deoxyactein	1.22
25-Acetyl-cimigenol–arabinoside	1.60
(24*S*)-25-Acetyl-cimigenol–xyloside	1.64
25-*O*-Methyl-cimigenol–arabinoside	1.90
25-*O*-Methyl-cimigenol–xyloside	1.93

Plot the logarithms of the peak areas against the logarithms of the concentrations, in µg/mL, of the *23-epi-26-Deoxyactein standard solutions*, and establish the calibration curve by least-squares regression. The correlation coefficient for the regression line is NLT 0.995. From the plot, determine the concentration, *C*, in µg/mL, of the relevant analytes in the *Sample solution*.

Separately calculate the percentages of the individual triterpene glycosides in *Table 2* as 23-*epi*-26-deoxyactein ($C_{37}H_{56}O_{10}$) in the portion of Black Cohosh taken:

$$\text{Result} = (V \times C)/(F \times W) \times 100$$

V = final volume of the *Sample solution* (mL)
C = concentration of the relevant analyte in the *Sample solution* (µg/mL)
F = factor to convert mg to µg, 1000 µg/mg
W = weight of Black Cohosh taken to prepare the *Sample solution* (mg)

Calculate the percentage of triterpene glycosides in the portion of Black Cohosh taken by adding the percentages of the individual analytes.

Acceptance criteria: NLT 0.4% on the dried basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: The Black Cohosh rhizome is dark brown, longitudinally grooved, rough, strongly knotty, and somewhat curled and irregular. It is 15 cm long and up to 2.5 cm thick. The upper surface is covered with numerous round scars of the earlier stalks; laterally, it is clearly curled, and the lower surface is covered with thin, longitudinally grooved, dark brown, easily breakable roots. The fracture is horny and fibrous. The transverse cut shows a thin outer bark surrounding a ring of numerous pale, narrow wedges of vascular tissue alternating with dark medullary rays and a large central pith. Black Cohosh roots are dark brown, between 1 and 3 mm in diameter, brittle, nearly cylindrical or obtusely quadrangular, and longitudinally wrinkled. The fracture is short. The transverse cut shows a distinct cambium line separating a wide outer bark from a central region composed of three to six wedges of lignified xylem tissue united by their apices and separated by broad nonlignified medullary rays.

Microscopic: In a surface view, suberous epidermal cells are tabular with moderately thickened walls. The parenchymatous cortex is filled with starch. Xylem wedges are lignified and composed of numerous small vessels with bordered pits or reticulately thickened walls, thin-walled fibers, and xylem parenchyma. The parenchyma of the pith is unlignified. Medullary rays are filled with starch granules, which are spherical or polygonal and are mostly simple or two to three compounded but can be up to six compounded. Individual starch granules are between 3 and 15 µm in diameter, each with a somewhat central slit-shaped hilum.

- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩**:** NMT 2.0% of foreign organic matter, and NMT 5.0% of stem bases
- **ARTICLES OF BOTANICAL ORIGIN,** *Alcohol-Soluble Extractives, Method 2* ⟨561⟩**:** NLT 8.0%, using a mixture of alcohol and water (1:1) instead of alcohol
- **LOSS ON DRYING** ⟨731⟩
  Sample: 1.0 g of Black Cohosh
  Analysis: Dry the *Sample* at 105° for 2 h.
  Acceptance criteria: NMT 12.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 10.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 4.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in a well-closed, light-resistant container. Protect from moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article. Dosage forms prepared with this article should bear the following statement: Discontinue use and consult a healthcare practitioner if you have a liver disorder or develop symptoms of liver trouble, such as abdominal pain, dark urine, or jaundice.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Actein RS
  USP Powdered Black Cohosh Extract RS
  USP 23-*epi*-26-Deoxyactein RS

Powdered Black Cohosh

DEFINITION

Powdered Black Cohosh is Black Cohosh reduced to a powder or a very fine powder. It contains NLT 0.4% of triterpene glycosides, calculated as 23-*epi*-26-deoxyactein[1] ($C_{37}H_{56}O_{10}$) on the dried basis.

[1] 23-*epi*-26-Deoxyactein is sometimes referred to as 27-deoxyactein.

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IDENTIFICATION

• **A. Thin-Layer Chromatography**

Standard solution A: 100 mg/mL of USP Powdered Black Cohosh Extract RS in methanol

Standard solution B: 1 mg/mL each of USP Actein RS, USP 23-*epi*-26-Deoxyactein RS, and isoferulic acid in methanol

Sample solution: Transfer 5 g of Powdered Black Cohosh to a screw-cap centrifuge tube, add 10 mL of a mixture of alcohol and water (7:3), and heat on a steam bath for 10 min. Centrifuge, and use the clear supernatant.

Adsorbent: Chromatographic silica gel mixture with an average particle size of 10–15 µm (TLC plates)

Application volume: 10 µL

Developing solvent system: Use the upper phase of a mixture of butyl alcohol, glacial acetic acid, and water (5:1:4).

Derivatization reagent: Methanol, glacial acetic acid, sulfuric acid, and *p*-anisaldehyde (85: 10: 5: 0.5). [Note—Store in a refrigerator. The reagent is colorless; discard if color appears.]

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatograms until the solvent front has moved about 15 cm, and dry the plate in a stream of air. Examine the plate under UV light at 365 nm. Treat the plate with *Derivatization reagent*, heat at 100° for 5 min, and examine in white light.

Acceptance criteria: Under UV light at 365 nm, the chromatogram of the *Sample solution* exhibits main zones similar in position and color to the main zones of *Standard solution A*. In the upper third of the plate, the *Sample solution* exhibits a blue fluorescent zone at the level of the zone due to isoferulic acid of *Standard solution B*. Under white light, the *Sample solution* exhibits main zones similar in position and color to the main zones of *Standard solution A*. *Standard solution B* exhibits red-violet zones due to actein and 23-*epi*-26-deoxyactein. The *Sample solution* exhibits several greenish-brown spots in the lower third of the plate and several violet zones above; two of these violet zones occur at R_F values similar to those due to actein and 23-*epi*-26-deoxyactein of *Standard solution B*.

• **B. HPTLC for Articles of Botanical Origin** ⟨203⟩

Standard solution A: 0.5 mL of *Standard solution A* prepared in *Identification* test *A*, diluted with methanol to 2 mL

Standard solution B: 1.0 mL of *Standard solution B* prepared in *Identification* test *A*, diluted with methanol to 5 mL

Sample solution: Transfer 0.5 g of Powdered Black Cohosh to a screw-cap tube, add 5 mL of methanol, sonicate for 10 min, and filter into a 10-mL volumetric flask. Wash the residue on the filter paper four times, using 1 mL of methanol for each washing; add the washings to the volumetric flask; and dilute with methanol to volume.

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)

Application volume: 2 µL as 8-mm bands

Developing solvent system: Toluene, ethyl formate, and formic acid (5:3:2)

Derivatization reagent: Proceed as directed for *Identification* test *A*.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatograms until the solvent front has moved about two-thirds of the length of the plate, and dry the plate with in a current of air. Treat the plate with *Derivatization reagent*, heat at 100° for 5 min, and examine in white light.

Acceptance criteria: The *Sample solution* exhibits main zones similar in position and color to the main zones of *Standard solution A*. *Standard solution B* exhibits red-violet zones due to actein and 23-*epi*-26-deoxyactein at R_F values of about 0.5 and 0.4, respectively. The *Sample solution* exhibits zones similar in color and R_F values to those due to actein and 23-*epi*-26-deoxyactein of *Standard solution B*.

• **C.** The *Sample solution* exhibits peaks for cimiracemoside A, 26-deoxycimicifugoside, (26*S*)-actein, 23-*epi*-26-deoxyactein, cimigenol–arabinoside, and cimigenol–xyloside at retention times corresponding to those compounds in the *Standard solution*, as obtained in the test for *Content of Triterpene Glycosides*. The ratio of the peak areas of cimigenol–arabinoside to cimigenol–xyloside is NLT 0.4 (distinction from *Cimicifuga foetida*).

COMPOSITION

• **Content of Triterpene Glycosides**

Standard solution: Dissolve a quantity of USP Powdered Black Cohosh Extract RS in methanol with shaking for 1 min, and dilute with methanol to obtain a solution having a known concentration of 30 mg/mL. Pass through a membrane filter of 0.45-µm or finer pore size.

23-*epi*-26-Deoxyactein standard solutions: Dissolve USP 23-*epi*-26-Deoxyactein RS in methanol with shaking for 1 min. Dilute quantitatively, and stepwise if necessary, to obtain solutions having concentrations of 500, 100, 50, 25, and 12.5 µg/mL. Pass through a membrane filter of 0.45-µm or finer pore size.

System suitability solution: 0.1 mg/mL each of USP Actein RS and USP 23-*epi*-26-Deoxyactein RS in methanol

Sample solution: Accurately weigh about 750 mg of Powdered Black Cohosh, and place in a 20-mL PTFE-capped centrifuge tube. Add 15 mL of methanol, sonicate for 30 min, centrifuge, and retain the supernatant. Repeat the extraction twice. Evaporate the combined extracts under vacuum at 45°–50°. Dissolve the residue in methanol, and quantitatively transfer to a 10-mL volumetric flask. Dilute with methanol to volume, and pass through a membrane filter of 0.45-µm or finer pore size.

Solution A: 0.05% Trifluoroacetic acid in water

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Water (%)	Solution A (%)	Solution B (%)
0	0	80	20
8	0	80	20
15	68	0	32
55	36	0	64
65	5	0	95
70	5	0	95
85	0	80	20

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Evaporative light-scattering

[Note—The *Detector* is set up according to the manufacturer's instructions in order to achieve a signal-to-noise ratio of NLT 10 for the 12.5-µg/mL *23-epi-26-Deoxyactein standard solution*.]

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 35°

Flow rate: 1.6 mL/min

DS Monographs

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Injection volume: 20 µL

System suitability

Samples: *System suitability solution*, *Standard solution*, and 100-µg/mL *23-epi-26-Deoxyactein standard solution*

Suitability requirements

Chromatogram similarity: The chromatogram of the *Standard solution* is similar to the reference chromatogram provided with the lot of USP Powdered Black Cohosh Extract RS being used.

Resolution: NLT 1.0 between the (26*S*)-actein and the 23-*epi*-26-deoxyactein peaks, *System suitability solution*

Tailing factor: NMT 2.0 for the 23-*epi*-26-deoxyactein peak, 100-µg/mL *23-epi-26-Deoxyactein standard solution*

Relative standard deviation: NMT 2.0% of the logarithm of the area responses for replicate injections, 100-µg/mL *23-epi-26-Deoxyactein standard solution*

Analysis

Samples: *System suitability solution*, *Standard solution*, *23-epi-26-Deoxyactein standard solutions*, and *Sample solution*

Using the chromatogram of the *Standard solution* and the reference chromatogram provided with the lot of USP Powdered Black Cohosh Extract RS being used, identify the retention times of the peaks corresponding to the triterpene glycosides. The approximate relative retention times of the triterpene glycosides are provided in *Table 2*.

Table 2

Name	Relative Retention Time
Cimicifugoside H-1	0.61
Cimiracemoside A	0.78
(26*R*)-Actein	0.94
26-Deoxycimicifugoside	0.96
(26*S*)-Actein	0.98
23-*epi*-26-Deoxyactein	1.00
Acetyl-shengmanol–xyloside	1.03
Cimigenol–arabinoside	1.08
Cimigenol–xyloside (cimicifugoside)	1.13
26-Deoxyactein	1.22
25-Acetyl-cimigenol–arabinoside	1.60
(24*S*)-25-Acetyl-cimigenol–xyloside	1.64
25-*O*-Methyl-cimigenol–arabinoside	1.90
25-*O*-Methyl-cimigenol–xyloside	1.93

Plot the logarithms of the peak areas against the logarithms of the concentrations, in µg/mL, of the *23-epi-26-Deoxyactein standard solution*, and establish the calibration curve by least-squares regression. The correlation coefficient for the regression line is NLT 0.995. From the plot, determine the concentration, *C*, in µg/mL, of the relevant analytes in the *Sample solution*.

Separately calculate the percentages of the individual triterpene glycosides in *Table 2* as 23-*epi*-26-deoxyactein ($C_{37}H_{56}O_{10}$) in the portion of Powdered Black Cohosh taken:

$$\text{Result} = (V \times C)/(F \times W) \times 100$$

V = volume of the *Sample solution* (mL)

C = concentration of the relevant analyte in the *Sample solution* (µg/mL)

F = factor to convert mg to µg, 1000 µg/mg

W = weight of Powdered Black Cohosh taken to prepare the *Sample solution* (mg)

Calculate the percentage of triterpene glycosides in the portion of Powdered Black Cohosh taken by adding the percentages of the individual analytes.

Acceptance criteria: NLT 0.4% on the dried basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^5 cfu/g; the total combined molds and yeasts count does not exceed 10^3 cfu/g; and the bile-tolerant Gram-negative bacteria count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: The material is a light to dark brown powder, is odorless or has a slight odor, and has an acrid or bitter taste.

Microscopic: It shows numerous starch granules with concentric striations, simple or compound. The individual granules are spherical or more or less polygonal and are between 3 and 15 µm in diameter, each with a somewhat central slit-shaped hilum. Vessels with bordered pits occur, as do lignified fibers. Reddish to brown fragments of suberized epidermis with more or less tabular cells occur.

- **ARTICLES OF BOTANICAL ORIGIN,** *Alcohol-Soluble Extractives, Method 2* ⟨561⟩**:** NLT 8.0%, using a mixture of alcohol and water (1:1) instead of alcohol
- **LOSS ON DRYING** ⟨731⟩

Sample: 1 g of Powdered Black Cohosh

Analysis: Dry the *Sample* at 105° for 2 h.

Acceptance criteria: NMT 12.0%

- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 10.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 4.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed, light-resistant containers, and protect from moisture.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant from which the article was derived. Dosage forms prepared with this article should bear the following statement: Discontinue use and consult a healthcare practitioner if you have a liver disorder or develop symptoms of liver trouble, such as abdominal pain, dark urine, or jaundice.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Actein RS

USP Powdered Black Cohosh Extract RS

USP 23-*epi*-26-Deoxyactein RS

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Powdered Black Cohosh Extract

DEFINITION

Powdered Black Cohosh Extract is prepared from Black Cohosh by extraction with hydroalcoholic mixtures or other suitable solvents. It contains NLT 90.0% and NMT 110.0% of the labeled amount of triterpene glycosides, calculated as 23-*epi*-26-deoxyactein ($C_{37}H_{56}O_{10}$) on the dried basis.

IDENTIFICATION

• **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution A: 100 mg/mL of USP Powdered Black Cohosh Extract RS in methanol

Standard solution B: 1 mg/mL each of USP Actein RS, USP 23-*epi*-26-Deoxyactein RS, and isoferulic acid in methanol

Sample solution: Shake a quantity of Powdered Extract, equivalent to 25 mg of triterpene glycosides, in 10 mL of methanol. Allow to stand for 15 min before use.

Adsorbent: Chromatographic silica gel mixture with an average particle size of 10–15 µm (TLC plates)

Application volume: 10 µL

Developing solvent system: Use the upper phase of a mixture of butyl alcohol, glacial acetic acid, and water (5:1:4).

Spray reagent: Methanol, glacial acetic acid, sulfuric acid, and *p*-anisaldehyde (85: 10: 5: 0.5). [NOTE—Store in a refrigerator. The reagent is colorless; discard if color appears.]

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatograms until the solvent front has moved about 15 cm, and dry the plate with the aid of a current of air.

Acceptance criteria: Examine the plate under UV light at 365 nm. The chromatogram of the *Sample solution* exhibits main zones similar in position and color to the main zones in the chromatogram of *Standard solution A*. In the upper third of the plate, the chromatogram of the *Sample solution* exhibits a blue fluorescent zone at the level of the zone due to isoferulic acid in the chromatogram of *Standard solution B*. Spray the plate with *Spray reagent*, heat at 100° for 5 min, and examine in daylight. The chromatogram of the *Sample solution* exhibits main zones similar in position and color to the main zones in the chromatogram of *Standard solution A*. The chromatogram of *Standard solution B* exhibits red-violet zones due to actein and 23-*epi*-26-deoxyactein. The chromatogram of the *Sample solution* exhibits several greenish-brown spots in the lower third of the plate and several violet zones above; two of these violet zones occur at R_F values similar to those due to actein and 23-*epi*-26-deoxyactein in the chromatogram of *Standard solution B*.

• **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution A: 0.5 mL of *Standard solution A* prepared in *Identification* test *A*, diluted with methanol to 2 mL

Standard solution B: 1.0 mL of *Standard solution B* prepared in *Identification* test *A*, diluted with methanol to 5 mL

Sample solution: Dilute 1 mL of the *Sample solution* prepared in *Identification* test *A* with methanol to 10 mL.

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)

Application volume: 2 µL as an 8-mm band

Developing solvent system: Toluene, ethyl formate, and formic acid (5:3:2)

Spray reagent: Proceed as directed for *Identification* test *A*.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatograms until the solvent front has moved about two-thirds of the length of the plate, and dry the plate with the aid of a current of air. Spray the plate with *Spray reagent*, heat at 100° for 5 min, and examine in daylight.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits main zones similar in position and color to the main zones in the chromatogram of *Standard solution A*. The chromatogram of *Standard solution B* exhibits red-violet zones due to actein and 23-*epi*-26-deoxyactein at R_F values of about 0.5 and 0.4, respectively. The chromatogram of the *Sample solution* exhibits zones similar in color and R_F values to those due to actein and 23-*epi*-26-deoxyactein in the chromatogram of *Standard solution B*.

• **C.** The chromatogram of the *Sample solution* exhibits peaks for cimiracemoside A, 26-deoxycimicifugoside, (26*S*) actein, 23-*epi*-26-deoxyactein, cimigenol–arabinoside, and cimigenol–xyloside at retention times corresponding to those compounds in the chromatogram of the *Standard solution*, as obtained in the test for *Content of Triterpene Glycosides*. The ratio of the peak areas of cimigenol–arabinoside to cimigenol–xyloside is NLT 0.4 (distinction from *Cimicifuga foetida*).

COMPOSITION

• **CONTENT OF TRITERPENE GLYCOSIDES**

Standard solution: Dissolve a quantity of USP Powdered Black Cohosh Extract RS in methanol with shaking for 1 min, and dilute with methanol to obtain a solution having a known concentration of 30 mg/mL. Pass through a membrane filter of 0.45-µm or finer pore size.

23- *epi*-26-Deoxyactein standard solutions: Dissolve USP 23-*epi*-26-Deoxyactein RS in methanol with shaking for 1 min. Dilute quantitatively, and stepwise if necessary, to obtain solutions having known concentrations of 500, 100, 50, 25, and 12.5 µg/mL. Pass through a membrane filter of 0.45-µm or finer pore size.

System suitability solution: 0.1 mg/mL each of USP Actein RS and USP 23-*epi*-26-Deoxyactein RS in methanol

Sample solution: Transfer a quantity of Powdered Extract, equivalent to 7.5 mg of triterpene glycosides, to a 10-mL volumetric flask, add 7 mL of methanol, and sonicate for 30 min. Dilute with methanol to volume. Centrifuge, or pass through a filter of 0.45-µm or finer pore size.

Solution A: 0.05% trifluoroacetic acid in water

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Water (%)	Solution A (%)	Solution B (%)
0	0	80	20
8	0	80	20
15	68	0	32
55	36	0	64
65	5	0	95
70	5	0	95
85	0	80	20

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

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Detector: Evaporative light-scattering
[NOTE—The detector is set up according to the manufacturer's instruction in order to achieve a signal-to-noise ratio of NLT 10 for the 12.5-µg/mL *23-epi-26-Deoxyactein standard solution*.]
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 35°
Flow rate: 1.6 mL/min
Injection size: 20 µL

System suitability
Samples: *System suitability solution*, *Standard solution*, and 100-µg/mL *23-epi-26-Deoxyactein standard solution*
Suitability requirements
Chromatogram similarity: The chromatogram of the *Standard solution* is similar to the Reference Chromatogram provided with the lot of USP Powdered Black Cohosh Extract RS being used.
Resolution: NLT 1.0 between the (26*S*)-actein and the 23-*epi*-26-deoxyactein peaks, *System suitability solution*
Tailing factor: NMT 2.0 for the 23-*epi*-26-deoxyactein peak, 100-µg/mL *23-epi-26-Deoxyactein standard solution*
Relative standard deviation: NMT 2.0% of the logarithm of the area response of the 23-*epi*-26-deoxyactein peak in repeated injections, 100-µg/mL *23-epi-26-Deoxyactein standard solution*

Analysis
Samples: *System suitability solution*, *Standard solution*, *23-epi-26-Deoxyactein standard solutions*, and *Sample solution*
Using the chromatogram of the *Standard solution* and the Reference Chromatogram provided with the lot of USP Powdered Black Cohosh Extract RS, identify the retention times of the peaks corresponding to the triterpene glycosides. The approximate relative retention times of the triterpene glycosides are provided in *Table 2*.

Table 2

Name	Relative Retention Time
Cimicifugoside H-1	0.61
Cimiracemoside A	0.78
(26*R*)-Actein	0.94
26-Deoxycimicifugoside	0.96
(26*S*)-Actein	0.98
23-*epi*-26-Deoxyactein	1.00
Acetyl-shengmanol–xyloside	1.03
Cimigenol–arabinoside	1.08
Cimigenol–xyloside (cimicifugoside)	1.13
26-Deoxyactein	1.22
25-Acetyl-cimigenol–arabinoside	1.60
(24*S*)-25-Acetyl-cimigenol–xyloside	1.64
25-*O*-Methyl-cimigenol–arabinoside	1.90
25-*O*-Methyl-cimigenol–xyloside	1.93

Plot the logarithms of the peak area responses versus the logarithms of the concentrations, in µg/mL, of the *23-epi-26-Deoxyactein standard solutions*, and determine the regression line using a least-squares analysis. The correlation coefficient for the regression line is NLT 0.995. From the graphs so obtained, determine the concentration, *C*, in µg/mL, of the relevant analyte in the *Sample solution*. Separately calculate the percentages of cimicifugoside H-1, cimiracemoside A, (26*R*)-actein, 26-deoxycimicifugoside, (26*S*)-actein, 23-*epi*-26-deoxyactein, acetyl-shengmanol–xyloside, cimigenol–arabinoside, cimigenol–xyloside (cimicifugoside), 26-deoxyactein, 25-acetyl-cimigenol–arabinoside, (24*S*)-25-acetyl-cimigenol–xyloside, 25-*O*-methyl-cimigenol–arabinoside, and 25-O-methyl-cimigenol–xyloside as 23-*epi*-26-deoxyactein ($C_{37}H_{56}O_{10}$) in the portion of Extract taken:

$$\text{Result} = (V \times C)/(F \times W) \times 100$$

V = volume of the *Sample solution* (mL)
C = concentration of the relevant analyte in the *Sample solution* (µg/mL)
F = factor to convert mg to µg, 1000 µg/mg
W = weight of the Powdered Extract taken to prepare the *Sample solution* (mg)

Calculate the percentage of the labeled amount of triterpene glycosides in the portion of Extract taken by adding all of the percentages calculated for individual analytes.
Acceptance criteria: 90.0%–110.0% on the dried basis

CONTAMINANTS
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*. The total bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **OTHER REQUIREMENTS:** It meets the requirements under *Botanical Extracts* ⟨565⟩, *Pesticide Residues*.

SPECIFIC TESTS
- **LOSS ON DRYING** ⟨731⟩**:** NMT 5.0%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store in a cool place.
- **LABELING:** It meets the requirements under *Botanical Extracts* ⟨565⟩. Label it to indicate the content of triterpene glycosides, in percentage, calculated as 23-*epi*-26-deoxyactein. Dosage forms prepared with this article should bear the following statement: "Discontinue use and consult a healthcare practitioner if you have a liver disorder or develop symptoms of liver trouble, such as abdominal pain, dark urine, or jaundice."
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Actein RS
USP Powdered Black Cohosh Extract RS
USP 23-*epi*-26-Deoxyactein RS

Black Cohosh Fluidextract

DEFINITION
Black Cohosh Fluidextract is prepared from Black Cohosh by extraction with hydroalcoholic mixtures or isopropanol–water mixtures. Each mL contains the extracted constituents of 1 g of the plant material. It contains NLT 90.0% and NMT 110.0% of the labeled amount of triterpene glycosides, calculated as 23-*epi*-26-deoxyactein ($C_{37}H_{56}O_{10}$).

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IDENTIFICATION

• **A. Thin-Layer Chromatographic Identification Test**

Standard solution A: 100 mg/mL of USP Powdered Black Cohosh Extract RS in methanol

Standard solution B: 1 mg/mL each of USP Actein RS, USP 23-*epi*-26-Deoxyactein RS, and isoferulic acid in methanol

Sample solution: Fluidextract

Adsorbent: Chromatographic silica gel mixture with an average particle size of 10–15 µm (TLC plates)

Application volume: 10 µL

Developing solvent system: Use the upper phase of a mixture of butyl alcohol, glacial acetic acid, and water (5:1:4).

Spray reagent: Methanol, glacial acetic acid, sulfuric acid, and *p*-anisaldehyde (85: 10: 5: 0.5). [Note—Store in a refrigerator. The reagent is colorless; discard if color appears.]

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatograms until the solvent front has moved about 15 cm, and dry the plate with the aid of a current of air.

Acceptance criteria: Examine the plate under UV light at 365 nm. The *Sample solution* exhibits main zones similar in position and color to the main zones of *Standard solution A*. In the upper third of the plate, the *Sample solution* exhibits a blue fluorescent zone at the level of the zone due to isoferulic acid of *Standard solution B*. Spray the plate with *Spray reagent*, heat at 100° for 5 min, and examine in daylight. The *Sample solution* exhibits main zones similar in position and color to the main zones of *Standard solution A*. *Standard solution B* exhibits red-violet zones due to actein and 23-*epi*-26-deoxyactein. The *Sample solution* exhibits several greenish-brown spots in the lower third of the plate and several violet zones above; two of these violet zones occur at R_F values similar to those due to actein and 23-*epi*-26-deoxyactein of *Standard solution B*.

• **B. Thin-Layer Chromatographic Identification Test**

Standard solution A: 0.5 mL of *Standard solution A* prepared in *Identification* test *A*, diluted with methanol to 2.0 mL

Standard solution B: 1.0 mL of *Standard solution B* prepared in *Identification* test *A*, diluted with methanol to 5.0 mL

Sample solution: Use the Fluidextract, diluting if necessary with a suitable solvent to obtain a concentration of 0.25 mg/mL of triterpene glycosides.

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)

Application volume: 2 µL as an 8-mm band

Developing solvent system: Toluene, ethyl formate, and formic acid (5:3:2)

Spray reagent: Proceed as directed for *Identification* test *A*.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop until the solvent front has moved two-thirds of the length of the plate, and dry the plate with the aid of a current of air. Spray the plate with *Spray reagent*, heat at 100° for 5 min, and examine in daylight.

Acceptance criteria: The *Sample solution* exhibits main zones similar in position and color to the main zones of *Standard solution A*. *Standard solution B* exhibits red-violet zones due to actein and 23-*epi*-26-deoxyactein at R_F values of about 0.5 and 0.4, respectively. The *Sample solution* exhibits zones similar in color and R_F values to those due to actein and 23-*epi*-26-deoxyactein of *Standard solution B*.

• **C.** The *Sample solution* exhibits peaks for cimiracemoside A, 26-deoxycimicifugoside, (26*S*)-actein, 23-*epi*-26-deoxyactein, cimigenol–arabinoside, and cimigenol–xyloside at retention times corresponding to those compounds in the *Standard solution*, as obtained in the test for *Content of Triterpene Glycosides*. The ratio of the peak areas of cimigenol–arabinoside to cimigenol–xyloside is NLT 0.4 (distinction from *Cimicifuga foetida*).

COMPOSITION

• **Content of Triterpene Glycosides**

Standard solution: Dissolve a quantity of USP Powdered Black Cohosh Extract RS in methanol with shaking for 1 min, and dilute with methanol to obtain a solution having a known concentration of 30 mg/mL. Pass through a membrane filter of 0.45-µm or finer pore size.

23- *epi*-26-Deoxyactein standard solutions: Dissolve USP 23-*epi*-26-Deoxyactein RS in methanol with shaking for 1 min. Dilute quantitatively, and stepwise if necessary, to obtain solutions having known concentrations of 500, 100, 50, 25, and 12.5 µg/mL. Pass through a membrane filter of 0.45-µm or finer pore size.

System suitability solution: 0.1 mg/mL each of USP Actein RS and USP 23-*epi*-26-Deoxyactein RS in methanol

Sample solution: Use the Fluidextract, diluting if necessary with methanol to obtain a concentration of about 0.75 mg/mL of triterpene glycosides. Centrifuge, or pass through a filter of 0.45-µm or finer pore size.

Solution A: 0.05% trifluoroacetic acid in water

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Water (%)	Solution A (%)	Solution B (%)
0	0	80	20
8	0	80	20
15	68	0	32
55	36	0	64
65	5	0	95
70	5	0	95
85	0	80	20

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Evaporative light-scattering

[Note—The detector is set up according to the manufacturer's instruction in order to achieve a signal-to-noise ratio of NLT 10 for the 12.5-µg/mL 23-*epi*-26-*Deoxyactein standard solution*.]

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 35°

Flow rate: 1.6 mL/min

Injection size: 20 µL

System suitability

Samples: *System suitability solution*, *Standard solution*, and 100-µg/mL *23-epi-26-Deoxyactein standard solution*

Suitability requirements

Chromatogram similarity: The chromatogram of the *Standard solution* is similar to the Reference Chromatogram provided with the lot of USP Powdered Black Cohosh Extract RS being used.

Resolution: NLT 1.0 between the (26*S*)-actein and the 23-*epi*-26-deoxyactein peaks, *System suitability solution*

Tailing factor: NMT 2.0 for the 23-*epi*-26-deoxyactein peak, 100-µg/mL *23-epi-26-Deoxyactein standard solution*

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Relative standard deviation: NMT 2.0% of the logarithm of the area response of the 23-*epi*-26-deoxyactein peak in repeated injections, 100-µg/mL *23-epi-26-Deoxyactein standard solution*

Analysis

Samples: *System suitability solution*, *Standard solution*, *23-epi-26-Deoxyactein standard solutions*, and *Sample solution*

Using the chromatogram of the *Standard solution* and the Reference Chromatogram provided with the lot of USP Powdered Black Cohosh Extract RS, identify the retention times of the peaks corresponding to the triterpene glycosides. The approximate relative retention times of the triterpene glycosides are provided in *Table 2*.

Table 2

Compound	Relative Retention Time
Cimicifugoside H-1	0.61
Cimiracemoside A	0.78
(26*R*)-Actein	0.94
26-Deoxycimicifugoside	0.96
(26*S*)-Actein	0.98
23-*epi*-26-Deoxyactein	1.00
Acetyl-shengmanol–xyloside	1.03
Cimigenol–arabinoside	1.08
Cimigenol–xyloside (cimicifugoside)	1.13
26-Deoxyactein	1.22
25-Acetyl-cimigenol–arabinoside	1.60
(24*S*)-25-Acetyl-cimigenol–xyloside	1.64
25-*O*-Methyl-cimigenol–arabinoside	1.90
25-*O*-Methyl-cimigenol–xyloside	1.93

Plot the logarithms of the peak area responses versus the logarithms of the concentrations, in µg/mL, of the *23-epi-26-Deoxyactein standard solutions*, and determine the regression line using a least-squares analysis. The correlation coefficient for the regression line is NLT 0.995. From the graphs so obtained, determine the concentration, *C*, in µg/mL, of the relevant analyte in the *Sample solution*. Separately calculate the concentrations, in µg/mL, of cimicifugoside H-1, cimiracemoside A, (26*R*)-actein, 26-deoxycimicifugoside, (26*S*)-actein, 23-*epi*-26-deoxyactein, acetyl-shengmanol–xyloside, cimigenol–arabinoside, cimigenol–xyloside (cimicifugoside), 26-deoxyactein, 25-acetyl-cimigenol–arabinoside, (24*S*)-25-acetyl-cimigenol– xyloside, 25-*O*-methyl-cimigenol–arabinoside, and 25-*O*-methyl-cimigenol–xyloside as 23-*epi*-26-deoxyactein ($C_{37}H_{56}O_{10}$) in the portion of Fluidextract taken:

$$\text{Result} = (D \times C/V)$$

D = dilution factor for the *Sample solution*, if applicable: final volume of *Sample solution*/volume of aliquot of Fluidextract taken (mL/mL)

C = concentration of the relevant analyte in the *Sample solution* (µg/mL)

V = volume of the Fluidextract taken to prepare the *Sample solution* (mL)

Calculate the percentage of the labeled amount of triterpene glycosides in the portion of Fluidextract taken:

$$\text{Result} = \Sigma C/L \times 100$$

ΣC = sum of concentrations of the individual triterpene glycosides (mg/mL)

L = labeled concentration of triterpene glycosides of the Fluidextract (mg/mL)

Acceptance criteria: 90.0%–110.0%

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **OTHER REQUIREMENTS:** It meets the requirements under *Botanical Extracts* ⟨565⟩, *Residual Solvents* and *Pesticide Residues*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store in a cool place.
- **LABELING:** It meets the requirements for *Labeling* under *Botanical Extracts* ⟨565⟩. Label it to indicate the content, in percentage, of triterpene glycosides, calculated as 23-*epi*-26-deoxyactein. Dosage forms prepared with this article should bear the following statement: Discontinue use and consult a healthcare practitioner if you have a liver disorder or develop symptoms of liver trouble, such as abdominal pain, dark urine, or jaundice.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Actein RS
  USP Powdered Black Cohosh Extract RS
  USP 23-*epi*-26-Deoxyactein RS

Black Cohosh Tablets

DEFINITION

Black Cohosh Tablets contain Powdered Black Cohosh Extract or Black Cohosh Fluidextract. Tablets contain NLT 90.0% and NMT 110.0% of the labeled amount of Powdered Extract or Fluidextract, represented by the content of triterpene glycosides, calculated as 23-*epi*-26-deoxyactein ($C_{37}H_{56}O_{10}$).

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩

Adsorbent: Chromatographic silica gel mixture with an average particle size of 10–15 µm (TLC plates)

Sample solution: 10 mL of the *Sample solution* prepared for *Identification* test *B*. Evaporate to dryness, and redissolve in 1 mL of methanol.

Standard solution A: 100 mg/mL of USP Powdered Black Cohosh Extract RS in methanol

Standard solution B: 1 mg/mL each of USP Actein RS, USP 23-*epi*-26-Deoxyactein RS, and isoferulic acid in methanol

Application volume: 10 µL

Developing solvent system: Use the upper phase of a mixture of butyl alcohol, glacial acetic acid, and water (5:1:4).

Spray reagent: Methanol, glacial acetic acid, sulfuric acid, and *p*-anisaldehyde (85: 10: 5: 0.5)

[NOTE—Store in a refrigerator. The reagent is colorless; discard if color appears.]

DS Monographs

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Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Develop until the solvent front has moved 15 cm, and dry the plate with the aid of a current of air. Examine the plate under UV light at a wavelength of 365 nm. Spray the plate with *Spray reagent*, heat at 100° for 5 min, and examine in daylight.
Acceptance criteria: The *Sample solution* exhibits main zones similar in position and color to the main zones of *Standard solution A*. Examined under UV light, the *Sample solution* exhibits a blue fluorescent zone at the level of the zone due to isoferulic acid in *Standard solution B*, in the upper third of the plate. Examined after treatment with *Spray reagent*, the *Sample solution* exhibits several greenish-brown spots in the lower third of the plate and several violet zones above; two of these violet zones occur at R_F values similar to those due to actein and 23-*epi*-26-deoxyactein in *Standard solution B*.

• **B. Thin-Layer Chromatographic Identification Test** ⟨201⟩
Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)
Sample solution: Transfer the equivalent of the labeled amount of Powdered Extract or Fluidextract, containing 25 mg of triterpene glycosides from a portion of powdered Tablets, to 25 mL of water; shake to disperse; and sonicate for 10 min. Add 75 mL of methanol, and sonicate for 10 min. Allow to stand for 15 min, and use the clear supernatant.
Standard solution A: Methanol and *Standard solution A* prepared in *Identification* test *A* (3:1)
Standard solution B: Methanol and *Standard solution B* prepared in *Identification* test *A* (4:1)
Application volume: 2 µL as an 8-mm band
Developing solvent system: Toluene, ethyl formate, and formic acid (5:3:2)
Spray reagent: Methanol, glacial acetic acid, sulfuric acid, and *p*-anisaldehyde (85: 10: 5: 0.5)
[Note—Store in a refrigerator. The reagent is colorless; discard if color appears.]
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Develop until the solvent front has moved two-thirds of the length of the plate, and dry the plate with the aid of a current of air. Spray the plate with *Spray reagent*, heat at 100° for 5 min, and examine in daylight.
Acceptance criteria: The *Sample solution* exhibits main zones similar in position and color to the main zones of *Standard solution A*, two of which are red-violet zones at R_F values of 0.5 and 0.4, similar in color and R_F values to those due to actein and 23-*epi*-26-deoxyactein in *Standard solution B*.

• **C.** The *Sample solution* exhibits peaks for cimiracemoside A, 26-deoxycimicifugoside, (26*S*) actein, 23-*epi*-26-deoxyactein, cimigenol–arabinoside, and cimigenol–xyloside at retention times corresponding to those compounds in the *Standard solution*, as obtained in the test for *Content of Triterpene Glycosides*. The ratio of the peak areas of cimigenol–arabinoside to cimigenol–xyloside is NLT 0.4 (distinction from *Cimicifuga foetida*).

STRENGTH

• **Content of Triterpene Glycosides**
Solution A: Filtered and degassed 0.05% trifluoroacetic acid in water
Solution B: Filtered and degassed acetonitrile
Mobile phase: See the gradient table below.

Time (min)	Water (%)	Solution A (%)	Solution B (%)
0	0	80	20
8	0	80	20
15	68	0	32
55	36	0	64
65	5	0	95
70	5	0	95
85	0	80	20

System suitability solution: 0.1 mg/mL each of USP Actein RS and USP 23-*epi*-26-Deoxyactein RS in methanol
Standard solution: Dissolve a quantity of USP Powdered Black Cohosh Extract RS in methanol with shaking for 1 min, and dilute with methanol to obtain a solution having a known concentration of 30 mg/mL. Pass through a membrane filter having a 0.45-µm or finer porosity.
23-*epi*-26-Deoxyactein standard solutions: Dissolve USP 23-*epi*-26-Deoxyactein RS in methanol with shaking for 1 min. Dilute quantitatively, and stepwise if necessary, to obtain solutions having known concentrations of 500, 100, 50, 25, and 12.5 µg/mL. Pass through a membrane filter having a 0.45-µm or finer porosity.
Sample solution: Weigh NLT 20 Tablets, and finely powder. Transfer a quantity of the powder, equivalent to 8 mg of triterpene glycosides, to a suitable polytef-capped centrifuge tube. Add 3 mL of water, shake to disperse, and sonicate for 10 min at 60°. Add 3 mL of methanol, and sonicate for 10 min. Centrifuge, and transfer the clear supernatant to a 10-mL volumetric flask. Wash the residue twice with 1.5 mL of a mixture of methanol and water (1:1), and transfer the washings to the volumetric flask. Dilute with a mixture of methanol and water (1:1) to volume, and pass through a membrane filter having a 0.45-µm or finer porosity.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Evaporative light-scattering
[Note—Detector is set up according to the manufacturer's instruction in order to achieve a signal-to-noise ratio of NLT 10 for the 12.5 µg/mL *23-epi-26-Deoxyactein standard solution*.]
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 35°
Flow rate: 1.6 mL/min
Injection size: 20 µL
System suitability
Samples: *System suitability solution* and 100 µg/mL of *23-epi-26-Deoxyactein standard solution*
Suitability requirements
Chromatographic profile: The chromatogram of the *Standard solution* is similar to the Reference Chromatogram provided with the lot of USP Powdered Black Cohosh Extract RS.
Resolution: NLT 1.0 between the (26*S*)-actein and the 23-*epi*-26-deoxyactein peaks, *System suitability solution*
Tailing factor: NMT 2.0 for the 23-*epi*-26-deoxyactein peak, 100 µg/mL *23-epi-26-Deoxyactein standard solution*
Relative standard deviation: NMT 2.0% for the logarithm of the area responses for replicate injections, 100 µg/mL *23-epi-26-Deoxyactein standard solution*

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Analysis

Samples: *System suitability solution*, *Standard solution*, *23-epi-26-Deoxyactein standard solutions*, and *Sample solution*

Using the chromatogram of the *Standard solution* and the Reference Chromatogram provided with the lot of USP Powdered Black Cohosh Extract RS, identify the retention times of the peaks corresponding to the triterpene glycosides. The approximate relative retention times of the triterpene glycosides are provided in the following table.

Name	Relative Retention Time
Cimicifugoside H-1	0.61
Cimiracemoside A	0.78
(26*R*)-Actein	0.94
26-Deoxycimicifugoside	0.96
(26*S*)-Actein	0.98
23-*epi*-26-Deoxyactein	1.00
Acetyl-shengmanol–xyloside	1.03
Cimigenol–arabinoside	1.08
Cimigenol–xyloside (cimicifugoside)	1.13
26-Deoxyactein	1.22
25-Acetyl-cimigenol–arabinoside	1.60
(24*S*)-25-Acetyl-cimigenol–xyloside	1.64
25-*O*-Methyl-cimigenol–arabinoside	1.90
25-*O*-Methyl-cimigenol–xyloside	1.93

Plot the logarithms of the peak area responses versus the logarithms of the concentrations, in µg/mL, of the *23-epi-26-Deoxyactein standard solutions*, and determine the regression line using a least-squares analysis. The correlation coefficient for the regression line is NLT 0.995. From the graphs so obtained, determine the concentration, C, in µg/mL, of the relevant analyte in the *Sample solution*.

Calculate the quantity, in mg, of triterpene glycosides in the portion of Tablets taken:

$$\text{Result} = C_T/100$$

C_T = sum of the concentrations C, in µg/mL, of all the relevant triterpene glycosides, calculated as 23-*epi*-26-deoxyactein

Calculate the percentage of the labeled amount of Extract in the portion of Tablets taken:

$$\text{Result} = (A_{WT}/W) \times (100/L_E) \times (100/L) \times C_{TT}$$

A_{WT} = average weight of Tablets
W = weight of sample
L_E = labeled content, as percentage, of triterpenes in the Extract used to prepare the Tablets
L = labeled amount of Extract per Tablet
C_{TT} = content, in mg, of triterpenes in the sample

Acceptance criteria: 90.0%–110.0% of the labeled amount of Powdered Extract or Fluidextract, represented by the content of triterpene glycosides

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** ⟨2040⟩**:** Meet the requirements for *Disintegration*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩**:** Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS—NUTRITIONAL AND DIETARY SUPPLEMENTS** ⟨2021⟩**:** The total bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **MICROBIAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS—NUTRITIONAL AND DIETARY SUPPLEMENTS** ⟨2022⟩**:** Tablets meet the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the article from which the Tablets were prepared. The label also indicates the amount, in mg/Tablet, of Powdered Extract or Fluidextract; the solvents used to prepare the Powdered Extract or Fluidextract; and the ratio of starting crude plant material to Powdered Extract or Fluidextract. Label it to indicate the content, in percentage, of triterpene glycosides as 23-*epi*-26-deoxyactein in the Powdered Extract or Fluidextract used to prepare the Tablets.

  The label bears the following statement: Discontinue use and consult a healthcare practitioner if you have a liver disorder or develop symptoms of liver trouble, such as abdominal pain, dark urine, or jaundice.
- **USP REFERENCE STANDARDS** ⟨11⟩

  USP Actein RS
  USP Powdered Black Cohosh Extract RS
  USP 23-*epi*-26-Deoxyactein RS

Black Pepper

DEFINITION

Black Pepper consists of the dried fully developed unripe fruits of *Piper nigrum* L. (Fam. Piperaceae). It contains NLT 2.5% of piperine, calculated on the dried basis.

IDENTIFICATION

- **A.** Black Pepper meets the requirements under *Specific Tests, Botanical Characteristics*.
- **B. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 0.9 mg/mL of USP Piperine RS in methanol

Standard solution B: 2.0 mg/mL of borneol in methanol

Standard solution C: 5 mg/mL of USP Powdered Black Pepper Extract RS in methanol. Sonicate for about 10 min, centrifuge, and use the supernatant.

Sample solution: Sonicate for 10 min about 0.5 g of Black Pepper, finely powdered, in 5 mL of methanol, centrifuge, and use the supernatant.

Chromatographic system

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)

Application volume: 15 µL of *Standard solution C*, 7 µL of the *Sample solution*, and 3 µL of the *Standard solution A* and *Standard solution B*, as bands

Developing solvent system: Hexanes and ethyl acetate (5:3)

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Derivatization reagent: A mixture of 17 mL of ice-cooled methanol, 2 mL of acetic acid, 1 mL of sulfuric acid, and 0.1 mL of anisaldehyde, mixed in this order

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Apply the *Samples* as bands. Use a saturated chamber, and condition the plate to a relative humidity of about 33% using a suitable device. Develop until the solvent front has moved up about 7 cm from the lower edge of the plate. Remove the plate from the chamber, dry, and examine under UV light at 254 nm. Treat with the *Derivatization reagent*, heat for 5 min at 100°, and examine under white light.

Acceptance criteria: Under UV 254 nm, the chromatogram of the *Sample solution* exhibits an intense quenching band at R_F of about 0.15 corresponding to the piperine band in the chromatogram of *Standard solution A*, a quenching band at R_F of about 0.02, and three quenching bands located between R_F of about 0.3 and 0.5. Under white light, the derivatized chromatogram of the *Sample solution* exhibits main bands similar in position and color to the main bands in the chromatogram of *Standard solution C*. These bands include a dark green band of the same color and R_F as the piperine band in *Standard solution A* (R_F of about 0.15), a weak violet band at R_F of about 0.47 below the position of the band due to borneol in *Standard solution B*, and a greenish band in the lower part of the chromatogram at R_F of about 0.07. Other minor bands may be observed in the *Sample solution* and *Standard solution C* chromatograms. No blue bands are detected in the chromatogram of the *Sample solution* at R_F of about 0.10 and 0.58 (distinction from long pepper).

• C. HPLC

Analysis: Proceed as directed in the test for *Content of Piperine*.

Acceptance criteria: The chromatogram of the *Sample solution* obtained at 343 nm exhibits a major peak at the retention time corresponding to piperine. Identify other piperamide peaks in the *Sample solution* chromatogram by comparison with the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Black Pepper Extract RS being used. The *Sample solution* chromatogram shows an additional peak corresponding to piperyline. The chromatogram of the *Sample solution* obtained at 270 nm does not exhibit a peak due to (2*E*,4*E*)-*N*-isobutyldecadienamide at a relative retention time of 1.14 to the piperine peak (distinction from long pepper).

COMPOSITION

• CONTENT OF PIPERINE

Solution A: Dissolve 0.14 g of anhydrous potassium dihydrogen phosphate in 900 mL of water, and add 0.5 mL of phosphoric acid. Dilute with water to 1000 mL, mix, filter, and degas.

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	95	5
18	55	45
25	20	80
28	20	80
35	55	45
40	95	5
45	95	5

[NOTE—Proceed under subdued light or using low-actinic glassware.]

Standard solution A: 0.1 mg/mL of USP Piperine RS in methanol

Standard solution B: Sonicate a portion of USP Powdered Black Pepper Extract RS in methanol to obtain a solution having a concentration of about 0.5 mg/mL. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.

Sample solution: Transfer about 2.0 g of Black Pepper, finely powdered and accurately weighed, to a 250-mL flask fitted with a reflux condenser. Add 50 mL of methanol, reflux on a water bath for about 20 min, allow to settle, and decant the supernatant. Repeat until the last extract is colorless. Combine the extracts, concentrate under vacuum, and adjust the volume to 100 mL using methanol. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 343 nm and 270 nm

Column: 4.6-mm × 25-cm; 5-µm, 100 Å packing L1

Flow rate: 1.5 mL/min

Injection volume: 20 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram obtained from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Black Pepper Extract RS being used.

Tailing factor: NMT 1.5 for the piperine peak, *Standard solution A*

Relative standard deviation: NMT 2.5% determined from the piperine peak in replicate injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

[NOTE—*Standard solution A*, *Standard solution B*, and the *Sample solution* are stable for 6 h at room temperature.]

Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Black Pepper Extract RS being used, identify the retention times of the peaks corresponding to piperine and piperyline in the *Sample solution* chromatogram.

Calculate the percentage of piperine in the portion of Black Pepper taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of piperine from the *Sample solution* chromatogram at 343 nm

r_S = peak area of piperine from the *Standard solution A* chromatogram at 343 nm

C_S = concentration of piperine in *Standard solution A* (mg/mL)

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V = final volume of the *Sample solution* (mL)
W = weight of Black Pepper used to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 2.5% on the dried basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacterial count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: Fruit is an indehiscent, one-seeded berry, globose, ovoid to oblong, 3.5–6 mm in diameter, hard; surface is greyish-black to brownish-black, rough, with raised reticulate wrinkles, shows remains of sessile stigma on the tip and a basal scar showing point of attachment to the axis; characteristic aromatic odor; characteristic pungent taste; seed white and hollow.

Microscopic

Transverse cut: Circular in outline with corrugated margin; shows outer narrow brownish pericarp; a seed coat encircling the wide central whitish perisperm, hollow in center; a narrow vertically running channel connecting the hollow center of the fruit to a small endosperm adherent to the remains of the stigma; a small embryo is present in the endosperm; a conical short projection at the base showing point of attachment to the axis.

Transverse section: Shows a well-differentiated pericarp, testa and perisperm; pericarp consists of a layer of epicarp, a wide mesocarp, and a layer of endocarp; the epicarp layer is covered with thick cuticle containing a few stomata and small prisms of calcium oxalate; mesocarp composed of 2–3 layers of parenchyma cells showing groups of rectangular to circular lignified sclereids, a broad zone (12–15 layers) of tangentially running parenchyma cells containing starch grains and showing isolated oval oil cells, a broad zone (10–15 layers) of compactly arranged parenchyma cells smaller than those of the outer zone and showing groups of fibrovascular bundles, 1–2 layers of tangentially running oil cells, and 2–3 layers of thick-walled parenchyma cells; endocarp is composed of one layer of three-sided thickened pitted stone cells (beaker-shape cells); testa is composed of one layer of cells filled with brown pigments; perisperm is very wide, composed of cells full of starch grains, some aleurone grains, and oil cells.

- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩**:** NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Alcohol-Soluble Extractives, Method 1* ⟨561⟩**:** NLT 10.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Water-Soluble Extractives, Method 1* ⟨561⟩**:** NLT 9.0%
- **LOSS ON DRYING** ⟨731⟩

Sample: 1.0 g of finely powdered Black Pepper
Analysis: Dry the *Sample* at 105° for 2 h.
Acceptance criteria: NMT 12.0%

- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩

Sample: 1.0 g of finely powdered Black Pepper
Acceptance criteria: NMT 5.0%

- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩

Sample: 1.0 g of finely powdered Black Pepper
Acceptance criteria: NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Piperine RS
USP Powdered Black Pepper Extract RS

Powdered Black Pepper

DEFINITION

Powdered Black Pepper is Black Pepper reduced to powder or very fine powder. It contains NLT 2.5% of piperine, calculated on the dried basis.

IDENTIFICATION

- **A.** Powdered Black Pepper meets the requirements under *Specific Tests, Botanical Characteristics*.
- **B. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 0.9 mg/mL of USP Piperine RS in methanol
Standard solution B: 2.0 mg/mL of borneol in methanol
Standard solution C: 5 mg/mL of USP Powdered Black Pepper Extract RS in methanol. Sonicate for about 10 min, centrifuge, and use the supernatant.
Sample solution: Sonicate for 10 min about 0.5 g of Powdered Black Pepper in 5 mL of methanol, centrifuge, and use the supernatant.
Chromatographic system
Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)
Application volume: 15 µL of *Standard solution C*, 7 µL of the *Sample solution*, and 3 µL of *Standard solution A* and *Standard solution B*, as bands, 8 mm
Developing solvent system: Hexanes and ethyl acetate (5:3)
Derivatization reagent: A mixture of 17 mL of ice-cooled methanol, 2 mL of acetic acid, 1 mL of sulfuric acid, and 0.1 mL of anisaldehyde, mixed in this order
Analysis
Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*
Apply the *Samples* as bands. Use a saturated chamber, and condition the plate to a relative humidity of about 33% using a suitable device. Develop until the solvent front has moved up about 7 cm from the lower edge of the plate. Remove the plate from the chamber, dry, and examine under UV light at 254 nm. Treat with the *Derivatization reagent*, heat for 5 min at 100°, and examine under white light.
Acceptance criteria: Under UV 254 nm, the chromatogram of the *Sample solution* exhibits an intense quenching band at R_F of about 0.15 corresponding to the piperine band in the chromatogram of *Standard solution A*, a quenching band at R_F of about 0.02, and three quenching bands of similar intensity equally spaced located between R_F of about 0.3 and 0.5. Under white light, the derivatized chromatogram of the *Sample solution* exhibits main bands similar in position and color to the main bands in the chromatogram of *Standard solution* C. These bands

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include a dark green band of the same color and R_F as the piperine band in *Standard solution A* (R_F of about 0.15), a weak violet band at R_F of about 0.47 below the position of the band due to borneol in *Standard solution B*, and a greenish band in the lower part of the chromatogram at R_F of about 0.07. Other minor bands may be observed in the *Sample solution* and *Standard solution C* chromatograms. No blue bands are detected in the chromatogram of the *Sample solution* at R_F of about 0.10 and 0.58 (distinction from long pepper).

• **C. HPLC**

Analysis: Proceed as directed in the test for *Content of Piperine*.

Acceptance criteria: The chromatogram of the *Sample solution* obtained at 343 nm exhibits a major peak at the retention time corresponding to piperine. Identify other piperamide peaks in the *Sample solution* chromatogram by comparison with the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Black Pepper Extract RS being used. The *Sample solution* chromatogram shows an additional peak corresponding to piperyline. The chromatogram of the *Sample solution* obtained at 270 nm does not exhibit a peak due to (2*E*,4*E*)-*N*-isobutyldecadienamide at a relative retention time of 1.14 to the piperine peak (distinction from long pepper).

COMPOSITION

• **CONTENT OF PIPERINE**

Solution A: Dissolve 0.14 g of anhydrous potassium dihydrogen phosphate in 900 mL of water, and add 0.5 mL of phosphoric acid. Dilute with water to 1000 mL, mix, filter, and degas.

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	95	5
18	55	45
25	20	80
28	20	80
35	55	45
40	95	5
45	95	5

[NOTE—Proceed under subdued light or using low-actinic glassware.]

Standard solution A: 0.1 mg/mL of USP Piperine RS in methanol

Standard solution B: Sonicate a portion of USP Powdered Black Pepper Extract RS in methanol to obtain a solution having a concentration of about 0.5 mg/mL. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.

Sample solution: Transfer about 2.0 g of Powdered Black Pepper, accurately weighed, to a 250-mL flask fitted with a reflux condenser. Add 50 mL of methanol, reflux on a water bath for about 20 min, allow to settle, and decant the supernatant. Repeat until the last extract is colorless. Combine the extracts, concentrate under vacuum, and adjust the volume to 100 mL using methanol. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 343 nm and 270 nm

Column: 4.6-mm × 25-cm; 5-µm, 100 Å packing L1

Flow rate: 1.5 mL/min

Injection volume: 20 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram obtained from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Black Pepper Extract RS being used.

Tailing factor: NMT 1.5 for the piperine peak, *Standard solution A*

Relative standard deviation: NMT 2.5% determined from the piperine peak in replicate injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

[NOTE—*Standard solution A*, *Standard solution B*, and the *Sample solution* are stable for 6 h at room temperature.]

Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Black Pepper Extract RS being used, identify the retention times of the peaks corresponding to piperine and piperyline in the *Sample solution* chromatogram.

Calculate the percentage of piperine in the portion of Powdered Black Pepper taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of piperine from the *Sample solution* chromatogram at 343 nm

r_S = peak area of piperine from the *Standard solution A* chromatogram at 343 nm

C_S = concentration of piperine in *Standard solution A* (mg/mL)

V = final volume of the *Sample solution* (mL)

W = weight of Powdered Black Pepper used to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 2.5% on the dried basis

CONTAMINANTS

• **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements

• **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements

• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacterial count does not exceed 10^3 cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

• **BOTANICAL CHARACTERISTICS**

Macroscopic: Blackish-grey powder; characteristic aromatic odor; characteristic pungent taste

Microscopic: Fragments of polygonal epicarp cells, some containing small prisms of calcium oxalate; parenchyma cells containing starch grains; oil cells; lignified sclereids; three-sided thickened pitted stone cells (beaker-shape cells)

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both in surface and side view; yellowish-brown polygonal cells of the testa; fragments of spiral vessels; parenchyma cells full of starch grains; aleurone grains; oil drops; and starch grains

- **ARTICLES OF BOTANICAL ORIGIN,** *Alcohol-Soluble Extractives, Method 1* ⟨561⟩: NLT 10.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Water-Soluble Extractives, Method 1* ⟨561⟩: NLT 9.0%
- **LOSS ON DRYING** ⟨731⟩
  Sample: 1.0 g of Powdered Black Pepper
  Analysis: Dry the *Sample* at 105° for 2 h.
  Acceptance criteria: NMT 12.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩
  Sample: 1.0 g of Powdered Black Pepper
  Acceptance criteria: NMT 5.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩
  Sample: 1.0 g of Powdered Black Pepper
  Acceptance criteria: NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Piperine RS
  USP Powdered Black Pepper Extract RS

Powdered Black Pepper Extract

DEFINITION

Powdered Black Pepper Extract is prepared from Black Pepper using suitable solvents such as ethyl acetate, methanol, or a mixture of these solvents. The ratio of plant material to extract is about 15:1. It contains NLT 90.0% and NMT 110.0% of the labeled amount of piperine, calculated on the dried basis. It may contain suitable added substances as carriers.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHY**
  Standard solution A: 0.9 mg/mL of USP Piperine RS in methanol
  Standard solution B: 2.0 mg/mL of borneol in methanol
  Standard solution C: 5 mg/mL of USP Powdered Black Pepper Extract RS in methanol. Sonicate for about 10 min, centrifuge, and use the supernatant.
  Sample solution: Sonicate for about 10 min an amount of Powdered Extract, equivalent to about 10 mg of piperine, in 10 mL of methanol, centrifuge, and use the supernatant.
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
  Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)
  Application volume: 15 µL of *Standard solution C*, 7 µL of the *Sample solution*, and 3 µL of *Standard solution A* and *Standard solution B*, as bands, 8 mm
  Developing solvent system: A mixture of hexanes and ethyl acetate (5:3)
  Derivatization reagent: A mixture of 17 mL of ice-cooled methanol, 2 mL of acetic acid, 1 mL of sulfuric acid, and 0.1 mL of anisaldehyde mixed in this order.
  Analysis
  Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

  Apply the *Samples* as bands to a suitable high performance thin-layer chromatographic plate (see *Chromatography* ⟨621⟩). Use a saturated chamber, and condition the plate to a relative humidity of about 33% using a suitable device. Develop the chromatograms until the solvent front has moved up about 7 cm from the lower edge of the plate. Remove the plate from the chamber, dry, and examine under UV light at 254 nm. Derivatize with the *Derivatization reagent*, heat for 5 min at 100°, and examine under visible light.
  Acceptance criteria: Under UV 254 nm, the chromatogram of the *Sample solution* exhibits an intense quenching band at R_F of about 0.15 corresponding in R_F to the piperine band in the chromatogram of *Standard solution A*, a quenching band at R_F of about 0.02, and three quenching bands of similar intensity equally spaced located between R_F of about 0.3 and 0.5. Under visible light and after derivatization, the chromatogram of the *Sample solution* exhibits main bands similar in position and color to the main bands in the chromatogram of *Standard solution C*. These bands include a dark green band of the same color and R_F as the piperine band in *Standard solution A* (R_F of about 0.15), a weak violet band at R_F of about 0.47 below the position of the band due to borneol in *Standard solution B*, and a greenish band in the lower part of the chromatogram at R_F of about 0.07. Other minor bands may be observed in the *Sample solution* and *Standard solution C* chromatograms. No blue bands are detected in the chromatogram of the *Sample solution* at R_F of about 0.10 and 0.58 (distinction from Long Pepper).
- **B. HPLC**
  Analysis: Proceed as directed in the test for *Content of Piperine*.
  Acceptance criteria: The chromatogram of the *Sample solution* obtained at 343 nm exhibits a major peak at the retention time corresponding to piperine. Identify other piperamide peaks in the *Sample solution* chromatogram by comparison with the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Black Pepper Extract RS being used. The *Sample solution* chromatogram shows an additional peak corresponding to piperyline. The chromatogram of the *Sample solution* obtained at 270 nm does not exhibit a peak due to (2*E*,4*E*)-*N*-isobutyldecadienamide at a relative retention time of 1.14 to the piperine peak (distinction from Long Pepper).

COMPOSITION

- **CONTENT OF PIPERINE**
  Solution A: Dissolve 0.14 g of anhydrous potassium dihydrogen phosphate in 900 mL of water, and add 0.5 mL of phosphoric acid. Dilute with water to 1000 mL, mix, filter, and degas.
  Solution B: Acetonitrile
  Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	95	5
18	55	45
25	20	80
28	20	80
35	55	45
40	95	5

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Table 1 *(continued)*

Time (min)	Solution A (%)	Solution B (%)
45	95	5

[NOTE—Proceed under subdued light or using low-actinic glassware.]

Standard solution A: 0.1 mg/mL of USP Piperine RS in methanol

Standard solution B: Sonicate a portion of USP Powdered Black Pepper Extract RS in methanol to obtain a solution having a concentration of about 0.5 mg/mL. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.

Sample solution: Transfer an accurately weighed amount of Powdered Extract, equivalent to about 25 mg of piperine, to a 25-mL volumetric flask, add 15 mL of methanol, and sonicate for 10 min. Cool to room temperature, dilute with methanol to volume, and mix. Dilute the obtained solution in methanol (1:10). Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 343 nm and 270 nm
Column: 4.6-mm × 25-cm; 5-µm, 100 Å packing L1
Flow rate: 1.5 mL/min
Injection volume: 20 µL

System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram obtained from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Black Pepper Extract RS being used.
Tailing factor: NMT 1.5 for the piperine peak, *Standard solution A*
Relative standard deviation: NMT 2.5% determined from the piperine peak in repeated injections, *Standard solution A*

Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
[NOTE—*Standard solution A*, *Standard solution B*, and the *Sample solution* are stable for 6 hours at room temperature.]
Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Black Pepper Extract RS being used, identify the retention time of the peaks corresponding to piperine and piperyline in the *Sample solution* chromatogram.
Calculate the percentage of piperine in the portion of Powdered Extract taken:

$$P = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area for piperine from the *Sample solution* chromatogram at 343 nm
r_S = peak area for piperine from the *Standard solution A* chromatogram at 343 nm
C_S = concentration of piperine in *Standard solution A* (mg/mL)
C_U = concentration of Powdered Extract in the *Sample solution* (mg/mL)

Calculate the percentage of the labeled amount of piperine in the portion of Powdered Extract taken:

$$\text{Result} = (P/L) \times 100$$

P = content of piperine as determined above (%)
L = labeled amount of piperine (%)

Acceptance criteria: 90%–110% on the dried basis

CONTAMINANTS

Change to read:

- **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020)
  : Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS
- **LOSS ON DRYING** ⟨731⟩
  Sample: 1.0 g of Powdered Extract
  Analysis: Dry at 105° for 2 h.
  Acceptance criteria: NMT 7.0%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived. It meets other labeling requirements in *Botanical Extracts* ⟨565⟩.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Piperine RS
  USP Powdered Black Pepper Extract RS

Borage Seed Oil

[84012-16-8].

DEFINITION
Borage Seed Oil is derived from seeds of *Borago officinalis* L. The oil is extracted by cold press or supercritical fluid extraction and then refined. A suitable antioxidant may be added.

IDENTIFICATION
- **A.** It meets the requirements in *Specific Tests* for *Fats and Fixed Oils* ⟨401⟩, *Fatty Acid Composition*.
- **B. IDENTIFICATION OF FIXED OILS BY THIN-LAYER CHROMATOGRAPHY** ⟨202⟩**:** The R_F values of the principal spots of the *Sample solution* correspond to those of the *Standard solution*.

SPECIFIC TESTS
- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩**:** NMT 1.0
- **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩**:** NMT 5.0
- **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩**:** 184–194
- **FATS AND FIXED OILS,** *Unsaponifiable Matter* ⟨401⟩**:** NMT 2.0%
- **FATS AND FIXED OILS,** *Fatty Acid Composition* ⟨401⟩**:** Borage Seed Oil exhibits the composition profile of fatty acids in *Table 1*.

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Table 1

Fatty Acid	Shorthand Notation	Percentage (%)
Palmitic acid	16:0	8.0–11.0
Stearic acid	18:0	2.0–5.0
Oleic acid	18:1	14.0–19.0
Gamma-linolenic acid	18:3	18.0–24.0
Linoleic acid	18:2	34.0–42.0
Arachidic acid	20:0	NMT 0.5
Gadoleic acid	20:1	2.0–6.0
Behenic acid	22:0	NMT 0.8
Erucic acid	22:1	NMT 5.0
Nervonic acid	24:1	NMT 4.5

• **Refractive Index** ⟨831⟩: 1.474–1.478 at 20°

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Preserve in tight containers, preferably under an atmosphere of an inert gas, protected from light.

• **Labeling:** Label it to indicate the name and quantity of any added antioxidants. Where Borage Seed Oil is intended for use in the manufacture of dosage forms, it is so labeled.

Borage Seed Oil Capsules

DEFINITION

Borage Seed Oil Capsules are prepared with Borage Oil derived from seeds of *Borago officinalis* L. by cold pressing or supercritical fluid extraction and contain NLT 95.0% of the labeled amounts of γ-linolenic (C18:3 n–6), linoleic (C18:2 n–6), and oleic (C18:1 n–9) acids.

IDENTIFICATION

• **A. Fatty Acid Profile**

System suitability solution and **Chromatographic system:** Proceed as directed in *Strength*.

Sample solution: Proceed as directed for the *Sample solution* in *Strength*, beginning with "Transfer 80 mg" without the addition of the *Internal standard*.

Analysis: Identify the specified fatty acid methyl ester peaks by comparing them to the reference chromatogram provided with the lot of USP Borage Seed Oil RS being used. Determine the percentage of each constituent relative to the total integrated area.

Acceptance criteria: The *Sample solution* conforms to the fatty acid composition profile in *Table 1*.

Table 1

Fatty Acid	Shorthand Notation	Percentage (%)
Palmitic acid	16:0	8.0–11.0
Stearic acid	18:0	2.0–5.0
Oleic acid	18:1 n–9	14.0–19.0
γ-Linolenic acid	18:3 n–6	18.0–24.0
Linoleic acid	18:2 n–6	34.0–42.0
Arachidic acid	20:0	NMT 0.5
Gondoic acid	20:1 n–9	NMT 6.0

Table 1 *(continued)*

Fatty Acid	Shorthand Notation	Percentage (%)
Behenic acid	22:0	NMT 0.8
Erucic acid	22:1 n–9	NMT 5.0
Nervonic acid	24:1 n–9	NMT 4.5

STRENGTH

• **Content of γ-Linolenic, Linoleic, and Oleic Acids**

[Note—γ-Linolenic acid is quantitated against USP Methyl Linolenate RS in this procedure.]

0.5 N methanolic sodium hydroxide solution: Dissolve 2 g of sodium hydroxide in 100 mL of methanol.

Internal standard: Methyl pentadecanoic acid

Sample solution: Weigh NLT 10 Capsules. With a sharp blade, carefully slice open the Capsules, avoiding loss of shell material. Combine the Capsule contents in a suitable container, and mix well. Remove any adhering substance from the emptied Capsules by washing with several portions of diethyl ether, and discard the washings. Allow the empty Capsule shells to air-dry over a period of NMT 30 min, taking precautions to avoid uptake or loss of moisture. Weigh the empty Capsule shells, and calculate the average fill weight/Capsule (A_F). Transfer 80 mg of the accurately weighed combined Capsule contents directly into a tared 30-mL screw-top glass centrifuge tube. Re-tare, and accurately weigh about 40 mg of *Internal standard*. Add 2 mL of *0.5 N methanolic sodium hydroxide solution*, tightly cap, and transfer to a heating block or another appropriate heating device. Reflux the solution until fat globules disappear (usually 5–10 min). Add 2 mL of 0.14 g/mL boron trifluoride in methanol, cap, and reflux for 2 min. Add 4 mL of chromatographic *n*-heptane, cap, and reflux for 1 min. Cool, add about 8 mL of saturated sodium chloride solution, shake, and centrifuge to separate the layers. Dilute an aliquot of the upper (heptane) layer 1:8 with chromatographic *n*-heptane, and mix well.

System suitability solution: Using about 80 mg of USP Borage Seed Oil RS, proceed as directed for the *Sample solution*, beginning with "Transfer 80 mg" without the addition of the *Internal standard*.

Standard solution: Directly into a tared 30-mL screw-top glass centrifuge tube accurately weigh about 20 mg of USP Methyl Linolenate RS, 40 mg of USP Methyl Linoleate RS, and 20 mg of USP Methyl Oleate RS. Proceed as directed for the *Sample solution*, beginning with "Re-tare".

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.53-mm × 30-m fused silica capillary; coated with a 1.0-µm film of G16

Temperatures

Injection port: 220°

Detector: 260°

Column: See *Table 2*.

Table 2

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	0	70	2
70	5	240	5

Carrier gas: Helium

Linear velocity: 50 cm/s

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Split mode: Splitless
Injection volume: 1 µL
System suitability
Samples: *System suitability solution* and *Standard solution*
Suitability requirements
Chromatogram similarity: The *System suitability solution* chromatogram is similar to the reference chromatogram provided with the lot of USP Borage Seed Oil RS being used.
Resolution: NLT 1.5 between methyl oleate and methyl stearate, *System suitability solution*
Relative standard deviation: NMT 2% for the peak area ratios of analytes to internal standard, *Standard solution*
Analysis
Samples: *Sample solution* and *Standard solution*
[NOTE—The relative retention times for gamma methyl linolenate and alpha methyl linolenate are about 0.98 and 1.0, respectively.]
Identify the retention times of the relevant fatty acid methyl esters by comparing the peaks in the chromatogram of the *System suitability solution* with those in the reference chromatogram. Identify the locus for the internal standard peak by comparison of the chromatograms of the *Standard solution* and *System suitability solution*.
Calculate the content, in mg/g, of γ-linolenic, linoleic, and oleic acids in the portion of Capsules taken:

$$\text{Result} = (R_U/R_S) \times (A_U/A_S) \times (m_S/W) \times (M_{r1}/M_{r2})$$

R_U = peak area ratio of the relevant methyl ester to the internal standard from the *Sample solution*
R_S = peak area ratio of the relevant methyl ester to the internal standard from the *Standard solution*
A_U = weight of the *Internal standard* in the *Sample solution* (mg)
A_S = weight of the *Internal standard* in the *Standard solution* (mg)
m_S = weight of the relevant USP Methyl Ester RS in the *Standard solution* (mg)
W = sample weight used to prepare the *Sample solution* (g)
M_{r1} = molecular weight of the relevant fatty acid (g/mol)
M_{r2} = molecular weight of the relevant fatty acid methyl ester (g/mol)

Calculate the percentage of the labeled amounts of each (γ-linolenic, linoleic, and oleic) acid in the portion of Capsules taken:

$$\text{Result} = A \times A_F \times (100/L)$$

A = content of the relevant fatty acid in the portion of Capsule content taken (mg/g)
A_F = average fill weight (g)
L = labeled content of the relevant fatty acid (mg/Capsule)

Acceptance criteria: 95.0% of the labeled amounts of γ-linolenic, linoleic, and oleic acids

PERFORMANCE TESTS
- **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩**:** Meet the requirements in *Rupture Test for Soft Shell Capsules*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩ **:** Meet the requirements

SPECIFIC TESTS
- **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩**:** NMT 10.0

CONTAMINANTS
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 1×10^3 cfu/g, and the combined molds and yeasts count does not exceed 3×10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meet the requirements of the tests for absence of *Escherichia coli*

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tightly-closed, light-resistant containers.
- **LABELING:** The label states the article which the Capsules were prepared with and content of γ-linolenic, linoleic, and oleic acids in mg/Capsule.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Borage Seed Oil RS
USP Methyl Linoleate RS
USP Methyl Linolenate RS
USP Methyl Oleate RS

Boswellia serrata

DEFINITION
Boswellia serrata is the oleogum resin obtained by incision or produced by spontaneous exudation from the stem and branches of *Boswellia serrata* Roxb. (Fam. Burseraceae). It contains NLT 1.0% of the keto derivatives of β-boswellic acid, calculated on the dried basis as the sum of 11-keto-β-boswellic acid and 3-acetyl-11-keto-β-boswellic acid.

IDENTIFICATION
- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩
Standard solution: Treat a quantity of USP *Boswellia serrata* Extract RS with gentle heating in methanol to obtain a solution having a known concentration of 30 mg/mL, cool, centrifuge, and use the supernatant.
Sample solution: Use the *Sample solution*, prepared as directed in the test below for *Content of Keto-Derivatives of β-Boswellic Acids*, and concentrate to 10% of the volume.
Adsorbent: 0.25-mm layer of chromatographic silica gel
Developing solvent system: A mixture of hexane and ethyl acetate (6:4)
Derivatization reagent: Prepare a solution of 10% sulfuric acid in methanol. [NOTE—Prepare immediately before use.]
Application volume: 10 µL
Analysis
Samples: *Standard solution* and *Sample solution*
Apply the samples as bands to a suitable thin-layer chromatographic plate (see *Chromatography* ⟨621⟩). Use a saturated chamber. Develop until the solvent front has moved up about 90% of the plate. Remove, dry, and examine under UV light at 254 nm. Dip in the *Derivatization reagent*, heat for 5–10 min at 100°, and examine under white light.
Acceptance criteria: Under UV light at 254 nm, the *Sample solution* exhibits two main zones due to 11-keto-β-boswellic acid and 3-acetyl-11-keto-β-boswellic acid at R_F values of about 0.30 and 0.36, respectively, corresponding to zones from the *Standard solution*. Under white light, the *Sample solution* exhibits two additional zones due to β-boswellic acid and 3-acetyl-β-boswellic acid at R_F values of about 0.49 and 0.58, respectively, corresponding to zones from the *Standard solution*. Other, less intense zones are observed for the *Sample solution* and the *Standard solution*.

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• **B.** The 210-nm chromatogram of the *Sample solution*, in the test for *Content of Keto-Derivatives of β-Boswellic Acids*, exhibits peaks for 11-keto-β-boswellic acid, 3-acetyl-11-keto-β-boswellic acid, β-boswellic acid, and 3-acetyl-β-boswellic acid at retention times that correspond to those in the 210-nm chromatogram of *Standard solution B* and the 210-nm reference chromatogram provided with the USP *Boswellia serrata* Extract RS.

COMPOSITION

• **CONTENT OF KETO-DERIVATIVES OF β-BOSWELLIC ACIDS**

Standard solution A: Dissolve a quantity of USP 3-Acetyl-11-keto-β-Boswellic Acid RS in methanol to obtain a solution having a known concentration of 0.1 mg/mL.

Standard solution B: Treat a quantity of USP *Boswellia serrata* Extract RS with gentle heating in methanol to obtain a solution having a known concentration of 10 mg/mL. Before injection, pass through a filter of 0.45-µm pore size.

Sample solution: Transfer about 2.0 g of crushed *Boswellia serrata* to a round-bottom flask, and reflux in 50 mL of methanol on a water bath for 15 min, stirring magnetically. Repeat until the extract is colorless. Evaporate the combined extracts to about 50 mL, transfer to a 100-mL volumetric flask, and dilute with methanol to volume. Before injection, pass through a filter of 0.45-µm pore size, and discard the first few mL of the filtrate.

Mobile phase: Prepare a filtered and degassed mixture of acetonitrile, water, and glacial acetic acid (900: 100: 0.1). Make adjustments if necessary.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; packing L1
Flow rate: See *Table 1*.

Table 1

Time (min)	Flow Rate (mL/min)
0	1
5	1.5
10	2
30	2
32	1
45	1

Injection volume: 20 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
[NOTE—The relative retention times for 11-keto-β-boswellic acid and 3-acetyl-11-keto-β-boswellic acid are about 1.0 and 1.4, respectively.]
Suitability requirements: The chromatogram of *Standard solution B* is similar to the 254-nm reference chromatogram provided with the lot of USP *Boswellia serrata* Extract RS being used.
Relative standard deviation: NMT 2.0% for the 3-acetyl-11-keto-β-boswellic acid peak in replicate injections, *Standard solution A*
Tailing factor: NMT 1.5, 3-acetyl-11-keto-β-boswellic acid peak, *Standard solution A*
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP *Boswellia serrata* Extract RS being used, identify the retention times of the peaks of 11-keto-β-boswellic acid and 3-acetyl-11-keto-β-boswellic acid in the *Sample solution*.
Separately calculate the percentages of the two analytes in the portion of *Boswellia serrata* taken:

$$\text{Result} = (r_U/r_S) \times (C_S/W) \times 10F$$

r_U = peak area of each analyte from the *Sample solution*
r_S = peak area of 3-acetyl-11-keto-β-boswellic acid from *Standard solution A*
C_S = concentration of USP 3-Acetyl-11-keto-β-Boswellic Acid RS in *Standard solution A* (mg/mL)
W = weight of *Boswellia serrata* taken to prepare the *Sample solution* (g)
F = conversion factor for each analyte: 0.93 for 11-keto-β-boswellic acid and 1.0 for 3-acetyl-11-keto-β-boswellic acid

Acceptance criteria: Add the percentages calculated for 11-keto-β-boswellic acid and 3-acetyl-11-keto-β-boswellic acid; NLT 1.0% on the dried basis.

IMPURITIES

• **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
• **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩**:** NMT 2.0%
• **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements

SPECIFIC TESTS

• **BOTANICAL CHARACTERISTICS**
Macroscopic: It occurs as small ovoid tears, sometimes forming agglomerated masses up to 5 cm long and 2 cm thick; whitish to golden yellow; fracture is brittle, and fractured surface is waxy and translucent characteristic aromatic odor; aromatic, slightly mucilaginous taste.
• **LOSS ON DRYING** ⟨731⟩
Sample: 1.0 g of *Boswellia serrata*, finely powdered
Analysis: Dry the *Sample* at 105° for 2 h.
Acceptance criteria: NMT 12.0%
• **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩
Sample: 2.0 g of *Boswellia serrata*, finely powdered
Acceptance criteria: NMT 2.0%
• **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 0.5%
• **ARTICLES OF BOTANICAL ORIGIN,** *Alcohol-Soluble Extractives, Method 2* ⟨561⟩**:** NLT 56%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store in a cool place.
• **LABELING:** The label states the Latin binomial of the species of *Boswellia* from which the oleogum resin was obtained.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP 3-Acetyl-11-keto-β-Boswellic Acid RS
USP *Boswellia serrata* Extract RS

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Boswellia serrata Extract

DEFINITION

Boswellia serrata Extract is prepared from pulverized *Boswellia serrata*, using suitable solvents such as isopropanol, alcohol, methanol, hexanes, or mixtures of these solvents. The ratio of starting plant material to Extract is approximately 6:1. It contains NLT 90.0% and NMT 110.0% of the labeled amount of Extract, calculated, on the dried basis, as the sum of 11-keto-β-boswellic acid and 3-acetyl-11-keto-β-boswellic acid; it may contain suitable added substances.

IDENTIFICATION

• **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩

Standard solution: Treat a quantity of USP *Boswellia serrata* Extract RS with gentle heating in methanol to obtain a solution having a known concentration of 30 mg/mL, cool, centrifuge, and use the supernatant.

Sample solution: Treat a quantity of Extract with gentle heating in methanol to obtain a solution having a known concentration of 30 mg/mL, cool, centrifuge, and use the supernatant.

Adsorbent: 0.25-mm layer of chromatographic silica gel

Developing solvent system: A mixture of hexane and ethyl acetate (6:4)

Dipping reagent: Prepare a solution of 10% sulfuric acid in methanol. [NOTE—Prepare fresh immediately before use.]

Application volume: 10 µL

Analysis

Samples: *Standard solution* and *Sample solution*

Apply the *Samples* as bands to a suitable thin-layer chromatographic plate (see *Chromatography* ⟨621⟩). Use a saturated chamber. Develop until the solvent front has moved up about 90% of the plate. Remove, dry, and examine under UV light at 254 nm. Dip in the *Dipping reagent*, heat for 5–10 min at 100°, and examine under visible light.

Acceptance criteria: Under UV light at 254 nm, the *Sample solution* exhibits two main zones due to 11-keto-β-boswellic acid and 3-acetyl-11-keto-β-boswellic acid at R_F values of about 0.30 and 0.36, respectively, corresponding to zones from the *Standard solution*. Under visible light, the *Sample solution* exhibits two additional zones due to β-boswellic acid and 3-acetyl-β-boswellic acid at R_F values of about 0.49 and 0.58, respectively, corresponding to zones from the *Standard solution*. Other, less intense zones are observed for the *Sample solution* and the *Standard solution*.

• **B.** The 210-nm chromatogram of the *Sample solution*, in the test for *Content of Keto-Derivatives of β-Boswellic Acids*, exhibits peaks for 11-keto-β-boswellic acid, 3-acetyl-11-keto-β-boswellic acid, β-boswellic acid, and 3-acetyl-β-boswellic acid at retention times that correspond to those in the 210-nm chromatogram of *Standard solution B* and the 210-nm Reference Chromatogram provided with the USP *Boswellia serrata* Extract RS.

COMPOSITION

• **CONTENT OF KETO-DERIVATIVES OF β-BOSWELLIC ACIDS**

Standard solution A: Dissolve a quantity of USP 3-Acetyl-11-keto-β-Boswellic Acid RS in methanol to obtain a solution having a known concentration of 0.1 mg/mL.

Standard solution B: Treat a quantity of USP *Boswellia serrata* Extract RS with gentle heating in methanol to obtain a solution having a known concentration of 10 mg/mL. Before injection, pass through a filter of 0.45-µm pore size.

Sample solution: Treat a quantity of Extract with gentle heating in methanol to obtain a solution having a known concentration of 10 mg/mL. Before injection, pass through a filter of 0.45-µm pore size, and discard the first few mL of the filtrate.

Mobile phase: Prepare a filtered and degassed mixture of acetonitrile, water, and glacial acetic acid (900: 100: 0.1). Make adjustments if necessary.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: See the gradient table below.

Time (min)	Flow Rate (mL/min)
0	1
5	1.5
10	2
30	2
32	1
45	1

Injection size: 20 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

[NOTE—The relative retention times for 11-keto-β-boswellic acid and 3-acetyl-11-keto-β-boswellic acid are about 1.0 and 1.4, respectively.]

Suitability requirements: The chromatogram of *Standard solution B* is similar to the 254-nm Reference Chromatogram provided with the USP *Boswellia serrata* Extract RS.

Tailing factor: NMT 1.5, 3-acetyl-11-keto-β-boswellic acid peak, *Standard solution A*

Relative standard deviation: NMT 2.0% of the 3-acetyl-11-keto-β-boswellic acid peak response for replicate injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatogram of *Standard solution B* and the 254-nm Reference Chromatogram provided with the lot of USP *Boswellia serrata* Extract RS, identify the retention times of the peaks of 11-keto-β-boswellic acid and 3-acetyl-11-keto-β-boswellic acid in the *Sample solution* chromatogram.

Separately calculate the percentages of 11-keto-β-boswellic acid and 3-acetyl-11-keto-β-boswellic acid in the portion of Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S V/W) \times 100F$$

r_U = peak area of each analyte from the *Sample solution*

r_S = peak area of 3-acetyl-11-keto-β-boswellic acid from *Standard solution A*

C_S = concentration of USP 3-Acetyl-11-keto-β-Boswellic Acid RS in *Standard solution A* (mg/mL)

V = final volume of the *Sample solution* (mL)

W = weight of Extract taken to prepare the *Sample solution* (mg)

F = conversion factor for each analyte (0.93 for 11-keto-β-boswellic acid and 1.0 for 3-acetyl-11-keto-β-boswellic acid)

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Acceptance criteria: Add the percentages of the two analytes. It contains NLT 90.0% and NMT 110.0% of the labeled amount of Extract, calculated on the dried basis, as the sum of 11-keto-β-boswellic acid and 3-acetyl-11-keto-β-boswellic acid.

IMPURITIES

Change to read:

ORGANIC IMPURITIES

- ▲**Botanical Extracts** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020)
: Meets the requirements

SPECIFIC TESTS

- **LOSS ON DRYING** ⟨731⟩**:** Dry 1.0 g of Extract at 105° for 2 h: it loses NMT 5.0% of its weight.
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store in a cool place.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. It meets other labeling requirements under *Botanical Extracts* ⟨565⟩.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP 3-Acetyl-11-keto-β-Boswellic Acid RS
USP *Boswellia serrata* Extract RS

Calcifediol—*see Calcifediol General Monographs*

Calcifediol Capsules—*see Calcifediol Capsules General Monographs*

Calcium Ascorbate—*see Calcium Ascorbate General Monographs*

Calcium Carbonate
—*see Calcium Carbonate General Monographs*

Calcium Carbonate Oral Suspension—*see Calcium Carbonate Oral Suspension General Monographs*

Calcium Carbonate Tablets—*see Calcium Carbonate Tablets General Monographs*

Calcium Citrate—*see Calcium Citrate General Monographs*

Calcium Citrate Tablets

DEFINITION

Calcium Citrate Tablets contain NLT 90.0% and NMT 110.0% of the labeled amount of calcium (Ca).

IDENTIFICATION

- **A.** The *Sample solution* from the test for *Strength* produces line emissions or absorptions at the characteristic wavelengths for calcium.
- **B. IDENTIFICATION TESTS—GENERAL,** *Calcium* ⟨191⟩and*Citrate* ⟨191⟩
Analysis: Grind a Tablet to a fine powder in a mortar. Transfer the powder to a centrifuge tube, add 2–5 mL of water, sonicate for 1 min, shake, and centrifuge.
Acceptance criteria: The supernatant meets the requirements of the tests.

STRENGTH

[NOTE—A standard stock solution is commercially available at different calcium concentrations. Necessary volumetric adjustment can be made in the *Standard solution*. Concentrations of the *Standard solution* and the *Sample solution* may be modified to fit the linear or working range of the instrument.]

- **CONTENT OF CALCIUM,** ***Procedure 1***

Standard stock solution: Weigh about 1.001 g of calcium carbonate, previously dried at 300° for 3 h and cooled in a desiccator for 2 h, and dissolve in 25 mL of 1 N hydrochloric acid. Boil to expel carbon dioxide, and dilute with water to 100 mL to obtain a solution having a known concentration of about 4000 µg/mL of calcium.

Standard solution: To a 200-mL volumetric flask add 100 mL of water and 4 mL of nitric acid, and mix thoroughly. Pipet 25.0 mL of the *Standard stock solution* into the volumetric flask, and dilute with water to volume to obtain a solution having a known concentration of about 500 µg/mL of calcium.

Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer a weighed portion of the powdered Tablets, equivalent to about 0.1 g of calcium, to a 50-mL flask. Add 4 mL of nitric acid, and heat the solution to boil gently, during which fuming evolves. Boil the solution for an additional 30 min with constant swirling, during which no fuming should be observed. Cool the solution to room temperature, quantitatively transfer all of the solution to a 200-mL volumetric flask, dilute with water to volume, mix, and filter.

Instrumental conditions

(See *Plasma Spectrochemistry* ⟨730⟩.)

Mode: ICP–AES

Analytical wavelength: 317.93 nm. [NOTE—The operating conditions may be developed and optimized based on the manufacturer's recommendation. A typical setting includes radio frequency (RF) power of about 1300 watts, argon torch flow of about 15 L/min, argon auxiliary flow of about 0.2 L/min, and a nebulizer flow rate of about 0.8 L/min.]

Analysis: Determine the emission of the *Standard solution*, the *Sample solution*, and a 2% nitric acid solution as the blank at the wavelength indicated above.

Calculate the percentage of the labeled amount of calcium (Ca) in the portion of Tablets taken:

DS Monographs

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$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of calcium from the *Sample solution*
r_S = peak response of calcium from the *Standard solution*
C_S = concentration of calcium in the *Standard solution* (µg/mL)
C_U = nominal concentration of calcium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–110.0%

- **CONTENT OF CALCIUM, *Procedure 2***

Lanthanum chloride solution: 267 mg/mL of lanthanum chloride heptahydrate in 0.125 N hydrochloric acid

Calcium standard solution: Dissolve 1.001 g of calcium carbonate, previously dried at 300° for 3 h and cooled in a desiccator for 2 h, in 25 mL of 1 N hydrochloric acid. Boil to expel carbon dioxide, and dilute with water to 1000 mL to obtain a concentration of 400 µg/mL of calcium.

Standard stock solution: 100 µg/mL of calcium from *Calcium standard solution* in 0.125 N hydrochloric acid

Standard solutions: Into separate 100-mL volumetric flasks pipet 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, and dilute with water to volume to obtain concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 µg/mL of calcium.

Sample solution: [NOTE—Finely powder NLT 20 Tablets.] Transfer an equivalent to 5 Tablets from powdered Tablets to a porcelain crucible. Heat the crucible in a muffle furnace maintained at 550° for 6–12 h, and cool. Add 60 mL of hydrochloric acid, and boil gently on a hot plate or steam bath for 30 min, intermittently rinsing the inner surface of the crucible with 6 N hydrochloric acid. Cool, and quantitatively transfer the contents of the crucible to a 100-mL volumetric flask. Rinse the crucible with small portions of 6 N hydrochloric acid, and add the rinsings to the flask. Dilute with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute this solution quantitatively, with 0.125 N hydrochloric acid to obtain a concentration of 2 µg/mL of calcium, adding 1 mL of *Lanthanum chloride solution* per 100 mL of the final volume.

Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Analytical wavelength: Calcium emission line at 422.7 nm
Lamp: Calcium hollow-cathode
Flame: Nitrous oxide–acetylene
Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution* per 100 mL

Analysis
Samples: *Standard solutions* and the *Sample solution*
Determine the absorbances of the solutions, using the *Blank*. From a linear regression equation, calculated using the absorbance of the *Standard solutions* versus concentrations, determine the concentration, *C*, in µg/mL of calcium in the *Sample solution*.
Calculate the percentage of the labeled amount of calcium (Ca) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = determined concentration of calcium in the *Sample solution*
C_U = nominal concentration of calcium in the *Sample solution*

Acceptance criteria: 90.0%–110.0%

CONTAMINANTS
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic microbial count does not exceed 1000 cfu/g. The total combined yeasts and molds count does not exceed 100 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩: Meet the requirements of the test for absence of *Escherichia coli*

PERFORMANCE TESTS
- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** ⟨2040⟩: Meet the requirements for *Disintegration*, 15 min
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩: Meet the requirements

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **LABELING:** The label states the quantity of calcium in terms of mg/Tablet.

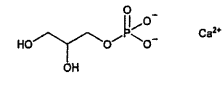
Calcium Glubionate Syrup—*see Calcium Glubionate Syrup General Monographs*

Calcium Gluceptate—*see Calcium Gluceptate General Monographs*

Calcium Gluconate—*see Calcium Gluconate General Monographs*

Calcium Gluconate Tablets—*see Calcium Gluconate Tablets General Monographs*

Calcium Glycerophosphate



$C_3H_7CaO_6P$ 210.14
1,2,3-Propanetriol, mono(dihydrogen phosphate) calcium salt (1:1);
Calcium glycerophosphate [27214-00-2].

DEFINITION
Calcium Glycerophosphate is a mixture, in variable proportions, of calcium (*RS*)-2,3-dihydroxypropyl phosphate and calcium 2-hydroxy-1-(hydroxymethyl)ethyl phosphate, which may be hydrated. Calcium Glycerophosphate contains NLT 18.6% and NMT 19.4% of calcium (Ca), calculated on the dried basis.

IDENTIFICATION
- **A.**

Analysis: Ignite 0.1 g in a crucible. Take up the residue with 5 mL of nitric acid, heat on a water bath for 1 min, and filter. Mix 1 mL of the filtrate with 2 mL of ammonium molybdate TS.

USP Monographs

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Acceptance criteria: A yellow color develops.

• **B.**

Analysis: Dissolve 20 mg of the substance being examined in 5 mL of 5 M acetic acid, and add 0.5 mL of potassium ferrocyanide solution (53 mg/mL). The resulting solution remains clear. To the clear solution, add 50 mg of ammonium chloride.

Acceptance criteria: A white crystalline precipitate is produced.

ASSAY

• **PROCEDURE**

Sample: 200 mg

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Direct titration

Titrant: 0.1 M edetate disodium VS

Endpoint detection: Colorimetric

Blank: 300 mL of water. Add 6 mL of 10 M sodium hydroxide and 15 mg of calconcarboxylic acid triturate.

Analysis: Dissolve the *Sample* in 300 mL of water, add 6 mL of 10 M sodium hydroxide and 15 mg of calconcarboxylic acid triturate. Titrate with *Titrant* until the solution is a distinct blue color.

Calculate the percentage of calcium (Ca) in the portion of Calcium Glycerophosphate taken:

$$\text{Result} = [(V - B) \times M \times F \times 100]/W$$

V = *Sample* titrant volume (mL)
B = *Blank* titrant volume (mL)
M = titrant molarity (mM/mL)
F = equivalency factor, 40.08 mg/mM
W = weight of the *Sample* (mg)

Acceptance criteria: 18.6%–19.4% on the dried basis

IMPURITIES

• **LEAD** ⟨251⟩**:** NMT 4 ppm

• **IRON** ⟨241⟩

Standard solution: Dilute 1 volume of *Standard Iron Solution*, prepared as directed in the chapter, with water to 10 volumes (1 µg/mL).

Analysis: Dissolve 0.20 g in 10 mL of water. Add 2 mL of a 20% (w/v) solution of citric acid, 0.1 mL of thioglycolic acid, and mix. Make alkaline with 10 M ammonia, dilute with water to 20 mL, and allow to stand for 5 min. Any pink color produced is not more intense than that obtained by treating 4 mL of the *Standard solution* in the same manner.

Acceptance criteria: NMT 20 ppm

• **LIMIT OF CHLORIDE**

Standard solution: 8.24 µg/mL of sodium chloride in water

Sample solution: Dissolve 125 mg in a 10-mL mixture of 5 M acetic acid and water (2:8), and dilute with water to 15 mL.

Analysis: To the *Sample solution* add 1 mL of 2 M nitric acid, then add 1 mL of a silver nitrate solution (17 mg/mL), and allow to stand for 5 min protected from light. To 10 mL of the *Standard solution* add 5 mL of water, 1 mL of 2 M nitric acid, and 1 mL of silver nitrate solution (17 mg/mL), and allow to stand for 5 min protected from light. When viewed against a dark background, the *Sample solution* is not more turbid than the *Standard solution*.

Acceptance criteria: NMT 0.04%

• **LIMIT OF SULFATE**

Standard solution: 36.2 µg/mL of potassium sulfate in water

Sample solution: Use the *Sample solution* prepared as directed in the test for *Appearance of Solution*.

Analysis: To 15 mL of the *Standard solution* add 0.5 mL of 5 M acetic acid and 1 mL of barium chloride solution (250 mg/mL). Repeat, using 15 mL of the *Sample solution*. Allow the solutions to stand for 5 min protected from light. When viewed against a dark background, the *Sample solution* is not more turbid than the *Standard solution*.

Acceptance criteria: NMT 0.2%

• **LIMIT OF ARSENIC**

Standard stock solution: In a 250-mL volumetric flask dissolve 330 mg of arsenic trioxide in 5 mL of 2 N sodium hydroxide, and dilute with water to volume. Transfer 1 mL of this solution to a 100-mL volumetric flask, and dilute with water to volume.

Standard solution: Transfer 1 mL of *Standard stock solution* into a 10-mL volumetric flask, and dilute with water volume.

Tin(II) chloride solution: Heat 20 g of tin with 85 mL of hydrochloric acid until no more hydrogen is released. Allow to cool. Dilute 1 volume of this solution with 10 volumes of dilute hydrochloric acid (200 mg/mL of hydrochloric acid in water).

Sample solution: Transfer 330 mg of Calcium Glycerophosphate to a 25.0-mL volumetric flask, and dilute with water to volume.

Apparatus: Prepare a 100-mL side-arm conical flask containing a magnetic stirring bar. Attach to the conical flask a ground-glass stopper through which passes a glass tube 20-cm long, with an internal diameter of 5 mm. The lower end of the tube is inside the conical flask and has been drawn to a tip with an internal diameter of 1 mm. An orifice 3 mm in diameter is 15 mm from the tip and at least 3 mm below the lower surface of the stopper. The upper end of the tube has a flat ground surface at a right angle to the axis of the tube. A second glass tube of the same internal diameter and 30 mm long, with a similar flat ground surface, is placed in contact with the ground surface of the first tube and is held in position by a clamp and springs. Into the lower tube, insert 55 mg of loosely packed lead acetate cotton. Place a disk of mercuric bromide paper between the flat surfaces of the tubes.

Analysis: Before placing the tube assembly into the flask, transfer the *Sample solution* to the flask, and add 15.0 mL of hydrochloric acid, 0.1 mL of *Tin(II) chloride solution*, and 5 mL of potassium iodide TS. Allow to stand for 15 min, and add 5 g of activated zinc. Assemble the apparatus immediately, and immerse the flask in a water bath at a temperature such that a uniform evolution of gas is maintained. After not less than 2 h, examine the stain produced on the mercuric bromide paper. Perform the same procedure using 1.0 mL of the *Standard solution*. The stain produced on the mercuric bromide paper by the *Sample solution* is not more intense than that produced by the *Standard solution*.

Acceptance criteria: NMT 3 ppm

• **LIMIT OF PHOSPHATES**

Sulfomolybdic solution: Dissolve with heating 2.5 g of ammonium molybdate in 20 mL of water. Dilute 28 mL of sulfuric acid with 50 mL of water, and cool. Mix the two solutions, and dilute with water to 100 mL.

Tin(II) chloride solution: Prepare as directed in the test for *Limit of Arsenic*.

Standard stock solution: 14.3 µg/mL of monobasic potassium phosphate in water.

Standard solution: Transfer 1.0 mL of *Standard stock solution* to a 100-mL volumetric flask, and dilute with water to volume.

Sample solution: Transfer 2.5 mL of the *Sample solution* from the test for *Appearance of Solution* to a 100-mL volumetric flask, and dilute with water to volume.

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Analysis: To 100 mL of the *Sample solution* add 4 mL of *Sulfomolybdic solution*, mix, and add 0.1 mL of *Tin(II) chloride solution*. To 100 mL of the *Standard solution*, add 4 mL of *Sulfomolybdic solution*, mix, and add 0.1 mL of *Tin(II) chloride solution*. Allow the preparations to stand for 10 min, then examine 20 mL of each preparation. Any color produced by the *Sample solution* is not more intense than that produced by the *Standard solution*.

Acceptance criteria: NMT 0.04%

- **CITRIC ACID**

Mercury(II) sulfate solution: 1 g of mercuric oxide in 20 mL of water and 4 mL of sulfuric acid

Analysis: Mix 5 g of Calcium Glycerophosphate with 20 mL of carbon dioxide-free water, and filter. To the filtrate add 0.15 mL of sulfuric acid, and filter. To the filtrate add 5 mL of *Mercury(II) sulfate solution*, and heat to boiling. Add 0.5 mL of 0.2 M potassium permanganate, and heat to boiling.

Acceptance criteria: No precipitate is formed.

- **GLYCEROL AND ALCOHOL-SOLUBLE SUBSTANCES**

Analysis: Mix 1 g with 25 mL of alcohol, and shake for 1 min. Filter, evaporate the filtrate to dryness on a water bath, and dry the residue at 70° for 1 h.

Acceptance criteria: The residue weighs NMT 5 mg (0.5%).

SPECIFIC TESTS

- **APPEARANCE OF SOLUTION**

Opalescent suspension: Dissolve 1g of hydrazine sulfate in 100 mL of water and allow to stand for 4–6 h. Add 25 mL of this solution to 25 mL of a solution containing 100mg/mL of methenamine in water, and allow to stand for 24 h.

Primary reference suspension: Dilute 15.0 mL of the *Opalescent suspension* with water to 1000 mL. [NOTE—This suspension is freshly prepared and may be stored for NMT 24 h.]

Reference suspension: *Primary reference suspension* and water (30:70). [NOTE—Shake before using.]

Sample solution: Dissolve 1.5 g at room temperature in 150 mL of carbon dioxide-free water.

Analysis: Compare the opalescence of equal volumes of the *Sample solution* and the *Reference suspension*.

Acceptance criteria: The *Sample solution* is not more opalescent than the *Reference suspension*.

- **ACIDITY OR ALKALINITY**

Analysis: Dissolve 1 g of Calcium Glycerophosphate in 100 mL of water. Add 0.1 mL of 1.0% (w/v) phenolphthalein solution.

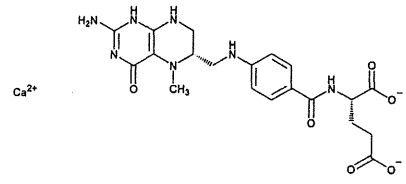
Acceptance criteria: NMT 0.5 mL of 0.1 M sodium hydroxide or 0.1 M hydrochloric acid is required to change the color of the indicator.

- **LOSS ON DRYING** ⟨731⟩: Dry a sample at 150° for 4 h: it loses NMT 12.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at controlled room temperature.

Calcium L-5-Methyltetrahydrofolate



$C_{20}H_{23}CaN_7O_6 \cdot xH_2O$

$C_{20}H_{23}CaN_7O_6$ (anhydrous) 497.52

N-[4-[[(2-Amino-1,4,5,6,7,8-hexahydro-5-methyl-4-oxo-(6*S*)-pteridinyl)methyl]amino]benzoyl]-L-glutamic acid, calcium salt (1:1);
N-{4-[[((6*S*)-2-Amino-1,4,5,6,7,8-hexahydro-5-methyl-4-oxo-6-pteridinyl)methyl]amino]-benzoyl}-L-glutamic acid, calcium salt (1:1) [151533-22-1].

DEFINITION

Calcium L-5-Methyltetrahydrofolate contains NLT 95.0% and NMT 102.0% of calcium 5-methyltetrahydrofolate ($C_{20}H_{23}CaN_7O_6$), the sum of the L- and D-diastereoisomers, calculated on the anhydrous and solvent-free basis, of which NMT 1.0% corresponds to calcium D-5-methyltetrahydrofolate.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

[NOTE—If the spectra obtained show differences, dissolve the substance to be examined and the USP Calcium DL-5-Methyltetrahydrofolate RS separately in the minimum quantity of water, and add dropwise sufficient acetone to produce a precipitate. Allow to stand for 15 min, centrifuge to collect the precipitate, wash the precipitate twice with a minimum quantity of acetone, and dry. Record new spectra using the residues.]

- **B. IDENTIFICATION TESTS—GENERAL,** *Calcium* ⟨191⟩: A 5-mg/mL solution meets the requirements.
- **C. HPLC:** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*. It complies with the acceptance criteria of the test for *Enantiomeric Purity*.

ASSAY

- **PROCEDURE**

Buffer: 7.8 g/L of sodium dihydrogen phosphate dihydrate in water

Solution A: Adjust the *Buffer* with 32% (w/v) sodium hydroxide solution to a pH of 6.5.

Solution B: Methanol and *Buffer* (35:65). Adjust with 32% (w/v) sodium hydroxide solution to a pH of 8.0.

Mobile phase: Gradient elution. See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
14	45	55
17	0	100
24	0	100
24.01	100	0

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Table 1 *(continued)*

Time (min)	Solution A (%)	Solution B (%)
33	100	0

[NOTE—After analysis the column should be flushed and stored in a mixture of methanol and water (85:15).]

System suitability solution: Transfer 25 mg of USP Folic Acid RS and 25 mg of USP 4-Aminobenzoylglutamic Acid RS to a 100-mL volumetric flask. Add about 15 mg each of sodium hydrogen carbonate and sodium carbonate to the flask, add sufficient water, sonicate to dissolve, and dilute with water to volume. Transfer 1.0 mL of this solution to a second 100-mL volumetric flask containing 50 mg of USP Calcium DL-5-Methyltetrahydrofolate RS, dissolve, and dilute with water to volume.

[NOTE—The following Standard and Sample solutions must be injected immediately after preparation and injected only once.]

Standard solution: 0.5 mg/mL of USP Calcium DL-5-Methyltetrahydrofolate RS in water

Sample solution: 0.5 mg/mL of Calcium L-5-Methyltetrahydrofolate in water

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 32°

Flow rate: 1.1 mL/min

Injection volume: 10 µL

System suitability

Samples: *System suitability solution* and *Standard solution*

[NOTE—For the *System suitability solution* the relative retention times of the component peaks are listed in *Table 2*. The L- and D-isomers of 5-methyltetrahydrofolate co-elute as a single peak. The 4a-hydroxy-5-methyltetrahydrofolic acid, 5-methyltetrahydropteroic acid, and dimethyltetrahydrofolic acid are included as minor components in USP Calcium DL-5-Methyltetrahydrofolate RS.]

Suitability requirements

Resolution: *System suitability solution*

NLT 6 between 4-aminobenzoylglutamic acid and 4a-hydroxy-5-methyltetrahydrofolic acid

NLT 8 between folic acid and 5-methyltetrahydrofolic acid

NLT 15 between 5-methyltetrahydrofolic acid and dimethyltetrahydrofolic acid

Relative standard deviation: Prepare three separate *Standard solutions*, and inject each immediately and only one time. NMT 2.0%; peak response factor from three injections

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of calcium 5-methyltetrahydrofolate ($C_{20}H_{23}CaN_7O_6$), the sum of the L- and D-diastereoisomers, in the portion of Calcium L-5-Methyltetrahydrofolate taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Calcium DL-5-Methyltetrahydrofolate RS in the *Standard solution* (mg/mL)

C_U = concentration of Calcium L-5-Methyltetrahydrofolate in the *Sample solution* (mg/mL)

Acceptance criteria: 95.0%–102.0% on the anhydrous and solvent-free basis

IMPURITIES

• **CHLORIDE**

Sample: 300 mg

Blank: Mix 1 mL of nitric acid with 75 mL of water.

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Direct titration

Titrant: 0.005 M silver nitrate VS

Endpoint detection: Potentiometric

Analysis: Dissolve the *Sample* in 75 mL of water (heat to maximum of 40°), add 1 mL of nitric acid, and titrate with the *Titrant*. Perform a *Blank* determination, and make any necessary correction.

Calculate the percentage of chloride (Cl) in the *Sample* taken:

$$\text{Result} = [(V_S - V_B) \times M \times F/W] \times 100$$

V_S = volume of *Titrant* consumed by the *Sample* (mL)

V_B = volume of *Titrant* consumed by the *Blank* (mL)

M = actual molarity of the *Titrant* (mmol/mL)

F = equivalency factor, 35.45 mg/mmol

W = *Sample* weight (mg)

Acceptance criteria: NMT 0.5%

• **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩

Acceptance criteria

Boron: NMT 50 µg/g

Platinum: NMT 10 µg/g

Arsenic: NMT 1.5 µg/g

Cadmium: NMT 0.5 µg/g

Lead: NMT 1.0 µg/g

Mercury: NMT 1.5 µg/g

• **RESIDUAL SOLVENTS** ⟨467⟩

Acceptance criteria

Ethanol: NMT 0.5%

2-Propanol: NMT 0.5%

[NOTE—For acceptance criteria for any other residual solvents, see *Residual Solvents* ⟨467⟩.]

• **RELATED COMPOUNDS**

Solution A, Solution B, Mobile phase, System suitability solution, Standard solution, Sample solution, Chromatographic system, System suitability, and **Suitability requirements:** Proceed as directed in the *Assay*.

Analysis

Samples: *Standard solution* and *Sample solution*

[NOTE—The impurities are listed in *Table 2*.]

Calculate the percentage of each impurity, as free acid, in the portion of Calcium L-5-Methyltetrahydrofolate taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak response of the corresponding impurity from the *Sample solution*

r_S = peak response of the principal peak from the *Standard solution*

C_S = concentration of USP Calcium DL-5-Methyltetrahydrofolate RS in the *Standard solution* (mg/mL)

C_U = concentration of Calcium L-5-Methyltetrahydrofolate in the *Sample solution* (mg/mL)

F = relative response factor for the corresponding impurity peak (see *Table 2*)

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M_{r1} = molecular weight of L-5-methyltetrahydrofolic acid, 459.46
M_{r2} = molecular weight of calcium L-5-methyltetra hydrofolate, 497.52

Acceptance criteria
[NOTE—Disregard any impurity peak less than 0.05%.]
Individual impurities: See *Table 2*.

Table 2

Name	Relative Retention Time	Relative Response Factor	Acceptance Criteria, NMT (%)
4-Aminobenzoylglutamic acid[a]	0.29	0.91	0.5
4a-Hydroxy-5-methyltetrahydrofolic acid[b]	0.37	1.09	1.0
(6*R*)-Mefox[c, d]	0.49	1.05	—
(6*S*)-Mefox[c, d]	0.50	1.05	1.0 (sum of 6*R* and 6*S*)
Tetrahydrofolic acid[e]	0.65	1.00[k]	0.5
7,8-Dihydrofolic acid[f]	0.83	0.95	0.5
Folic acid[g]	0.85	0.83	0.5
5,10-Methylenetetrahydrofolic acid[h]	0.88	1.00[k]	0.5
5-Methyltetrahydropteroic acid[i]	1.10	0.67	0.5
Dimethyltetrahydrofolic acid[j]	1.25	1.00[k]	0.15
Total impurities	—	—	2.5

[a] *N*-(4-Aminobenzoyl)-L-glutamic acid.
[b] *N*-[4-({[(6*S*)-2-Amino-4a-hydroxy-5-methyl-4-oxo-1,4,4a,5,6,7,8,8a-octahydropteridin-6-yl]methyl}amino)benzoyl]-L-glutamic acid.
[c] 2-Amino-8-methyl-4,9-dioxo-7-methyl-*p*-aminobenzoyl-glutamate-6,7,8,9-tetrahydro-4*H*-pyrazino-(1,2-*a*)-*s*-triazine.
[d] Report the impurity Mefox as the sum of 6*S*- and 6*R*-Mefox.
[e] *N*-[4-({[(*S*)-2-Amino-4-oxo-1,4,5,6,7,8-hexahydropteridin-6-yl]methyl}amino)benzoyl]-L-glutamic acid.
[f] *N*-(4-{[(2-Amino-4-oxo-1,4,7,8-tetrahydropteridin-6-yl)methyl]amino}benzoyl)-L-glutamic acid.
[g] *N*-(4-{[(2-Amino-4-oxo-1,4-dihydropteridin-6-yl)methyl]amino}benzoyl)-L-glutamic acid.
[h] *N*-(4-(3-Amino-1-oxo-5,6,6a,7-tetrahydroimidazo[1,5-*f*]pteridin-8(1*H*,4*H*,9*H*)-yl)benzyl)-L-glutamic acid.
[i] (*S*)-4-{[(2-Amino-5-methyl-4-oxo-1,4,5,6,7,8-hexahydropteridin-6-yl)methyl]amino}benzoic acid.
[j] *N*-[4-({[(*S*)-5-Methyl-2-(methylamino)-4-oxo-1,4,5,6,7,8-hexahydropteridin-6-yl]methyl}amino)benzoyl]-L-glutamic acid.
[k] Estimated factor.

• **ENANTIOMERIC PURITY**
Buffer: 4.54 g/L of sodium dihydrogen phosphate dihydrate in water
Mobile phase: Acetonitrile and *Buffer* (3:97). Adjust with 32% (w/v) sodium hydroxide to a pH of 6.8.
Standard solution: 0.5 mg/mL of USP Calcium DL-5-Methyltetrahydrofolate RS in water
Sample solution: 0.5 mg/mL of Calcium L-5-Methyltetrahydrofolate in water
System suitability solution: Transfer 1.0 mL of *Standard solution* to a 50-mL volumetric flask, and dilute with *Sample solution* to volume.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 280 nm
Column: 4.0-mm × 15-cm; 5-µm packing L79
Column temperature: 40°
Flow rate: 1.0 mL/min
Injection volume: 10 µL
System suitability
Sample: *System suitability solution*
[NOTE—The relative retention times of L-5-methyltetrahydrofolate and D-5-methyltetrahydrofolate are about 1 and 1.5, respectively.]
Suitability requirements
Resolution: NLT 1.5 between L-5-methyltetrahydrofolate and D-5-methyltetrahydrofolate
Analysis
Sample: *Sample solution*
Calculate the percentage of D-5-methyltetrahydrofolate in the portion of Calcium L-5-Methyltetrahydrofolate taken:

$$\text{Result} = [(r_D/(r_D + r_L) \times 100]$$

r_D = peak response of D-5-methyltetrahydrofolate from the *Sample solution*
r_L = peak response of L-5-methyltetrahydrofolate from the *Sample solution*

Acceptance criteria: NMT 1.0% of D-5-methyltetra hydrofolate

SPECIFIC TESTS

• **CALCIUM**
Sample: 250 mg
Blank: 150 mL of water, 15 mL of 1 N sodium hydroxide, and 300 mg of hydroxy naphthol blue
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.05 M edetate disodium VS
Endpoint detection: Visual
Analysis: Dissolve the *Sample* in 150 mL of water, add 15 mL of 1 N sodium hydroxide and 300 mg of hydroxy naphthol blue, and titrate with the *Titrant* until the solution is deep blue in color. Perform a *Blank* determination, and make any necessary correction.
Calculate the percentage of calcium (Ca) in the *Sample* taken:

$$\text{Result} = [(V_S - V_B) \times M \times F/W] \times 100$$

V_S = volume of *Titrant* consumed by the *Sample* (mL)
V_B = volume of *Titrant* consumed by the *Blank* (mL)
M = actual molarity of the *Titrant* (mmol/mL)
F = equivalency factor, 40.08 mg/mmol
W = *Sample* weight (mg)

Acceptance criteria: 7.0%–8.5% on the anhydrous and solvent-free basis

• **WATER DETERMINATION,** *Method Ic* ⟨921⟩
Sample: Transfer 40 mg of Calcium L-5-Methyltetrahydrofolate to a 20-mL headspace vial, and cap tightly. Heat the vial in a suitable Karl Fischer oven at 250°.
Analysis: The released and evaporated water is transferred into the titration-cell in a stream of dry nitrogen at a flow rate of about 40 mL/min as directed in *Water Determination, Method Ic* ⟨921⟩.
Acceptance criteria: 6.0%–17.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Store in a tight container, in a cool and dry place.

DS Monographs

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• **USP REFERENCE STANDARDS** ⟨11⟩
USP 4-Aminobenzoylglutamic Acid RS
N-(4-Aminobenzoyl)-L-glutamic acid.
$C_{12}H_{14}N_2O_5$ 266.25
USP Calcium DL-5-Methyltetrahydrofolate RS
N[4-[[(2-Amino-1,4,5,6,7,8-hexahydro-5-methyl-4-oxo-6-pteridinyl)methyl]amino]benzoyl]-L-glutamic acid, calcium salt (1:1).
$C_{20}H_{23}CaN_7O_6$ 497.52
USP Folic Acid RS

Calcium L-5-Methyltetrahydrofolate Capsules

DEFINITION
Calcium L-5-Methyltetrahydrofolate Capsules contain NLT 90.0% and NMT 110.0% of the labeled amount of calcium L-5-methyltetrahydrofolate ($C_{20}H_{23}CaN_7O_6$).

IDENTIFICATION
• **A. HPLC:** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in *Strength*, and to the L-isomer of the *Standard solution* in the test for *Enantiomeric Purity*.

STRENGTH
• **PROCEDURE**
Antioxidant solution: 1.5% sodium sulfite in water
Buffer: 7.8 g/L of sodium dihydrogen phosphate dihydrate in water
Solution A: Adjust the *Buffer* with 32% (w/v) sodium hydroxide solution to a pH of 6.5.
Solution B: Methanol and *Buffer* (35:65). Adjust with 32% (w/v) sodium hydroxide solution to a pH of 8.0.
Mobile phase: Gradient elution. See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
14	45	55
17	0	100
24	0	100
24.01	100	0
33	100	0

System suitability solution: Transfer 25 mg of USP Folic Acid RS to a 100-mL volumetric flask. Add about 15 mg each of sodium hydrogen carbonate and sodium carbonate to the flask, add sufficient water, sonicate to dissolve, and dilute with water to volume. Transfer 1.0 mL of this solution to a second 100-mL volumetric flask containing 50 mg of USP Calcium D,L-5-Methyltetrahydrofolate RS, dissolve, and dilute with water to volume.
Standard solution: 0.1 mg/mL of USP Calcium D,L-5-Methyltetrahydrofolate RS in *Antioxidant solution*
Sample solution: Remove, as completely as possible, the contents of NLT 30 Capsules, and weigh accurately. Transfer a portion of the Capsule contents, nominally equivalent to 2.5 mg of calcium L-5-methyltetrahydrofolate, to a 25-mL volumetric flask. Add 20 mL of *Antioxidant solution* and sonicate for 20 min with occasional shaking, cool to room temperature, dilute with *Antioxidant solution* to volume, mix well, and filter.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 280 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 32°
Flow rate: 1.1 mL/min
Injection volume: 10 µL
System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—For the *System suitability solution* the relative retention times of folic acid and L- and D-isomers of 5-methyltetrahydrofolate, which co-elute as a single peak, are 0.85 and 1.0, respectively.]
Suitability requirements
Resolution: NLT 8 between folic acid and 5-methyltetrahydrofolic acid, *System suitability solution*
Relative standard deviation: NMT 2.0%, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of calcium L-5-methyltetrahydrofolate ($C_{20}H_{23}CaN_7O_6$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP Calcium D,L-5-Methyltetrahydrofolate RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of calcium L-5-methyltetrahydrofolate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–110.0%

IMPURITIES
• **ENANTIOMERIC PURITY**
Buffer: 4.54 g/L of sodium dihydrogen phosphate dihydrate in water
Mobile phase: Acetonitrile and *Buffer* (3:97). Adjust with 32% (w/v) sodium hydroxide to a pH of 6.8.
Standard solution: 0.4 mg/mL of USP Calcium D,L-5-Methyltetrahydrofolate RS in water
Sample solution: Filtered portion, equivalent to 0.4 mg/mL of calcium L-5-methyltetrahydrofolate, from the contents of NLT 30 Capsules, in water
System suitability solution: Transfer 0.2 mL of *Standard solution* to a 10-mL volumetric flask, and dilute with *Sample solution* to volume.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 280 nm
Column: 4.0-mm × 15-cm; 5-µm packing L79[1]
Column temperature: 40°
Flow rate: 1.0 mL/min
Injection volume: 10 µL
System suitability
Sample: *System suitability solution*
[NOTE—The relative retention times of L-5-methyltetrahydrofolate and D-

[1] A chiral-recognition protein, human serum albumin (HSA), chemically bonded to silica particle, about 5 µm in diameter. For example: Chromtech Chiral HSA, available at www.chromtech.com.

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5-methyltetrahydrofolate are about 1 and 1.5, respectively.]

Suitability requirements

Resolution: NLT 1.5 between L-5-methyltetrahydrofolate and D-5-methyltetrahydrofolate

Analysis

Sample: *Sample solution*

Calculate the percentage of D-5-methyltetrahydrofolate in the portion of calcium L-5-methyltetrahydrofolate taken:

$$\text{Result} = [r_D/(r_D + r_L)] \times 100$$

r_D = peak response of D-5-methyltetrahydrofolate from the *Sample solution*

r_L = peak response of L-5-methyltetrahydrofolate from the *Sample solution*

Acceptance criteria: NMT 1.0%

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩, *Disintegration*: Meet the requirements
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩ : Meet the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Store in a tight, light-resistant container, in a cool and dry place.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Calcium D,L-5-Methyltetrahydrofolate RS

USP Folic Acid RS

Calcium L-5-Methyltetrahydrofolate Tablets

DEFINITION

Calcium L-5-Methyltetrahydrofolate Tablets contain NLT 90.0% and NMT 110.0% of the labeled amount of calcium L-5-methyltetrahydrofolate ($C_{20}H_{23}CaN_7O_6$).

IDENTIFICATION

- **A. HPLC:** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in *Strength*, and to the L-isomer of the *Standard solution* in the test for *Enantiomeric Purity*.

STRENGTH

- **PROCEDURE**

Antioxidant solution: 1.5% sodium sulfite in water

Buffer: 7.8 g/L of sodium dihydrogen phosphate dihydrate in water

Solution A: Adjust the *Buffer* with 32% (w/v) sodium hydroxide solution to a pH of 6.5.

Solution B: Methanol and *Buffer* (35:65). Adjust with 32% (w/v) sodium hydroxide solution to a pH of 8.0.

Mobile phase: Gradient elution. See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
14	45	55
17	0	100
24	0	100

Table 1 *(continued)*

Time (min)	Solution A (%)	Solution B (%)
24.01	100	0
33	100	0

System suitability solution: Transfer 25 mg of USP Folic Acid RS to a 100-mL volumetric flask. Add about 15 mg each of sodium hydrogen carbonate and sodium carbonate to the flask, add sufficient water, sonicate to dissolve, and dilute with water to volume. Transfer 1.0 mL of this solution to a second 100-mL volumetric flask containing 50 mg of USP Calcium D,L-5-Methyltetrahydrofolate RS, dissolve, and dilute with water to volume.

Standard solution: 0.1 mg/mL of USP Calcium D,L-5-Methyltetrahydrofolate RS in *Antioxidant solution*

Sample solution: Transfer a portion from NLT 30 finely powdered Tablets, nominally equivalent to 2.5 mg of calcium L-5-methyltetrahydrofolate, to a 25-mL volumetric flask. Add 20 mL of *Antioxidant solution* and sonicate for 20 min with occasional shaking, cool to room temperature, dilute with *Antioxidant solution* to volume, mix well, and filter.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 32°

Flow rate: 1.1 mL/min

Injection volume: 10 µL

System suitability

Samples: *System suitability solution* and *Standard solution*

[NOTE—For the *System suitability solution* the relative retention times of folic acid and L- and D-isomers of 5-methyltetrahydrofolate, which co-elute as a single peak, are 0.85 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 8 between folic acid and 5-methyltetrahydrofolic acid, *System suitability solution*

Relative standard deviation: NMT 2.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of calcium L-5-methyltetrahydrofolate ($C_{20}H_{23}CaN_7O_6$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Calcium D,L-5-Methyltetrahydrofolate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of calcium L-5-methyltetrahydrofolate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–110.0%

IMPURITIES

- **ENANTIOMERIC PURITY**

Buffer: 4.54 g/L of sodium dihydrogen phosphate dihydrate in water

Mobile phase: Acetonitrile and *Buffer* (3:97). Adjust with 32% (w/v) sodium hydroxide to a pH of 6.8.

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Standard solution: 0.4 mg/mL of USP Calcium D,L-5-Methyltetrahydrofolate RS in water
Sample solution: Filtered portion, equivalent to 0.4 mg/mL of calcium L-5-methyltetrahydrofolate, from NLT 30 finely powdered Tablets, in water
System suitability solution: Transfer 0.2 mL of *Standard solution* to a 10-mL volumetric flask, and dilute with *Sample solution* to volume.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 280 nm
Column: 4.0-mm × 15-cm; 5-µm packing L79[1]
Column temperature: 40°
Flow rate: 1.0 mL/min
Injection volume: 10 µL
System suitability
Sample: *System suitability solution*
[NOTE—The relative retention times of L-5-methyltetrahydrofolate and D-5-methyltetrahydrofolate are about 1 and 1.5, respectively.]
Suitability requirements
Resolution: NLT 1.5 between L-5-methyltetrahydrofolate and D-5-methyltetrahydrofolate
Analysis
Sample: *Sample solution*
Calculate the percentage of D-5-methyltetrahydrofolate in the portion of calcium L-5-methyltetrahydrofolate taken:

$$\text{Result} = [r_D/(r_D + r_L)] \times 100$$

r_D = peak response of D-5-methyltetrahydrofolate from the *Sample solution*
r_L = peak response of L-5-methyltetrahydrofolate from the *Sample solution*

Acceptance criteria: NMT 1.0%

PERFORMANCE TESTS
- **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩, *Disintegration*: Meet the requirements
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩ : Meet the requirements

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Store in a tight, light-resistant container, in a cool and dry place.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Calcium D,L-5-Methyltetrahydrofolate RS
USP Folic Acid RS

Calcium Lactate—*see Calcium Lactate General Monographs*

Calcium Lactate Tablets—*see Calcium Lactate Tablets General Monographs*

[1] A chiral-recognition protein, human serum albumin (HSA), chemically bonded to silica particle, about 5 µm in diameter. For example: Chromtech Chiral HSA, available at www.chromtech.com.

Calcium Lactobionate—*see Calcium Lactobionate General Monographs*

Calcium Levulinate—*see Calcium Levulinate General Monographs*

Calcium Pantothenate—*see Calcium Pantothenate General Monographs*

Calcium Pantothenate Tablets—*see Calcium Pantothenate Tablets General Monographs*

Calcium Pantothenate, Racemic—*see Racemic Calcium Pantothenate General Monographs*

Calcium Phosphate, Tribasic
—*see Tribasic Calcium Phosphate NF Monographs*

Calcium with Vitamin D Tablets

DEFINITION
Calcium with Vitamin D Tablets contain NLT 90.0% and NMT 125.0% of the labeled amount of calcium (Ca), derived from substances generally recognized as safe, and NLT 90.0% and NMT 165.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$). They contain no other vitamins or minerals for which nutritional value is claimed. They may contain other labeled added substances or additional ingredients in amounts that are unobjectionable.

IDENTIFICATION

Add the following:

▲• **A.** Characteristic absorption of calcium as obtained in the tests in *Strength*▲ (USP 1-May-2020)

Add the following:

▲• **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Vitamin D (Cholecalciferol or Ergocalciferol), Method 2*.▲ (USP 1-May-2020)

STRENGTH

Add the following:

▲[NOTE—In the following assays, where more than one assay method is given for an individual ingredient, the requirements may be met by following any one of the specified methods, with the method used being stated

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in the labeling only if *Method 1* is not used.] ▲ (USP 1-May-2020)

- **CALCIUM**

[NOTE—A commercially available atomic absorption standard solution for calcium may be used where preparation of a *Calcium standard stock solution* is described in the following assay. Concentrations of the *Standard solutions* and the *Sample stock solution* may be modified to fit the linear or working range of the instrument.]

Lanthanum chloride solution: Dissolve 26.7 g of lanthanum chloride in 0.125 N hydrochloric acid to make 100 mL.

Calcium standard stock solution: Weigh 1.001 g of calcium carbonate, chelometric standard, previously dried at 300° for 3 h and cooled in a desiccator for 2 h. Dissolve in 25 mL of 1 N hydrochloric acid. Boil to expel carbon dioxide, and dilute with water to 1000 mL to obtain a solution with a concentration of 400 µg/mL of calcium.

Standard stock solution: 100 µg/mL of calcium from a volume of *Calcium standard stock solution* in 0.125 N hydrochloric acid

Standard solutions: Into separate 100-mL volumetric flasks separately pipet 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, dilute with water to volume, and obtain *Standard solutions* with concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 µg/mL, of calcium.

Sample stock solution: Weigh and finely powder NLT 20 Tablets. Transfer the equivalent to 500 mg of calcium, in 25 mL of hydrochloric acid, and heat for 30 min on a steam bath. Cool, dilute with water to 1000 mL, and filter.

Sample solution: Quantitatively dilute a volume of *Sample stock solution* with 0.125 N hydrochloric acid to obtain a concentration of 100 µg/mL of calcium. Transfer 2.0 mL of this solution to a 100-mL volumetric flask, add 1.0 mL of *Lanthanum chloride solution*, and dilute with water to volume.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometer

Lamp: Calcium hollow-cathode

Flame: Nitrous oxide–acetylene

Analytical wavelength: Calcium emission line, 422.7 nm

Blank: 1 mL of *Lanthanum chloride solution* per 100 mL of 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions, against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of calcium, and draw the straight line best fitting the 5 plotted points. From the graph, determine the concentration (C), in µg/mL, of calcium in the *Sample solution*.

Calculate the percentage of the labeled amount of calcium (Ca) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of calcium in the *Sample solution* (µg/mL)

C_U = nominal concentration of calcium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of calcium (Ca)

Change to read:

- **VITAMIN D (CHOLECALCIFEROL OR ERGOCALCIFEROL),** ▲***Method 1***

[NOTE—Where the use of vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant Reference Standard. ▲ (USP 1-May-2020) Use low-actinic glassware throughout this procedure.]

Mobile phase: *n-Hexane* and isopropyl alcohol (99:1)

Standard solution: 2 µg/mL of USP Ergocalciferol RS or USP Cholecalciferol RS in *n-hexane*

System suitability solution: Heat a volume of *Standard solution* at 60° for 1 h to partially isomerize vitamin D (ergocalciferol or cholecalciferol) to its corresponding precursor.

Sample solution: Weigh and grind NLT 20 Tablets. Transfer the equivalent to 20 µg of cholecalciferol or ergocalciferol to a container having a polytef-lined screw-cap. Add 8 mL of dimethyl sulfoxide and 12 mL of *n-hexane*, and shake for 45 min on a wrist-action shaker with tubes in a water bath maintained at 60°. Centrifuge for 10 min, withdraw the hexane layer by means of a pipet, and transfer to an evaporation flask. Add 12 mL of *n-hexane* to the dimethyl sulfoxide layer, mix on a vortex mixer for 5 min, and again withdraw the hexane layer by means of a pipet and add to the evaporation flask. Repeat this extraction with 3 additional 12-mL portions of *n-hexane*, adding the hexane extracts to the evaporation flask. Evaporate the combined hexane extracts under vacuum at room temperature to dryness. Dissolve in and dilute the residue in a volume of *n-hexane* to obtain a concentration of 2 µg/mL.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Column: 4.6-mm × 15-cm; 3-µm packing L8

Detector: UV 265 nm

Flow rate: 1 mL/min

Injection volume: 100 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 10 between the vitamin D form present and its corresponding precursor, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for vitamin D.

Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*

r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*

C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

F = correction factor to account for the average amount of previtamin D present in the *Sample solution*, 1.09

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Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

Add the following:

▲• **VITAMIN D (CHOLECALCIFEROL OR ERGOCALCIFEROL), *Method 2***

[NOTE—Where the use of a vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant Reference Standard. Use low-actinic glassware throughout this procedure.]

Diluent: Water, dimethylformamide, and isopropyl alcohol (5:10:35, v/v)

Mobile phase: Acetonitrile and water (70:30)

Cholecalciferol standard stock solution: 0.5 mg/mL of USP Cholecalciferol RS in isopropyl alcohol

Ergocalciferol standard stock solution: 0.5 mg/mL of USP Ergocalciferol RS in isopropyl alcohol

System suitability solution: Combine equal volumes of *Cholecalciferol standard stock solution* and *Ergocalciferol standard stock solution*. Dilute with *Diluent* to obtain a concentration of about 1 µg/mL each of cholecalciferol and ergocalciferol.

Standard solution: 1 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in *Diluent* from the *Cholecalciferol standard stock solution* or the *Ergocalciferol standard stock solution*, prepared accurately

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powder, nominally equivalent to 50 µg of cholecalciferol, into a 50-mL volumetric flask, add 5 mL of water, and sonicate for 10 min with intermittent shaking. Add 10 mL of dimethylformamide, sonicate for 15 min with intermittent shaking, cool to room temperature, and dilute with isopropyl alcohol to volume. Mix well and pass through a nylon filter of 0.45-µm pore size.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 15-cm; 2.7-µm packing L96

Column temperature: 40°

Flow rate: 1 mL/min

Injection volume: 20 µL

System suitability

Samples: *System suitability solution* and *Standard solution*

[NOTE—The relative retention times for cholecalciferol and ergocalciferol are 1.0 and 1.1, respectively.]

Suitability requirements

Resolution: NLT 1.5 between the cholecalciferol and ergocalciferol peaks, *System suitability solution*

Relative standard deviation: NMT 2.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*

r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*

C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% ▲(USP 1-May-2020)

PERFORMANCE TESTS

• **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩, *Dissolution*: Meet the requirements with respect to calcium

Medium: 0.1 N hydrochloric acid; 900 mL

Apparatus 2: 75 rpm

Time: 30 min

Analysis: Determine the amount of calcium (Ca) dissolved, using the procedure in the assay for *Calcium*, making any necessary volumetric adjustments.

Tolerances: NLT 75% of the labeled amount of calcium (Ca) is dissolved.

• **WEIGHT VARIATION** ⟨2091⟩: Meet the requirements

CONTAMINANTS

• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic microbial count does not exceed 3×10^3 cfu/g, and the total combined molds and yeasts count does not exceed 3×10^2 cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meet the requirements

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

• **LABELING:** The label states that the product is Calcium with Vitamin D Tablets. The label states also the quantities of calcium and vitamin D in terms of metric units/Tablet, and the salt form of calcium and the chemical form of vitamin D present in the Tablet.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Cholecalciferol RS

USP Ergocalciferol RS

Calcium and Vitamin D with Minerals Tablets

DEFINITION

Calcium and Vitamin D with Minerals Tablets contain vitamin D as Ergocalciferol (vitamin D_2) or Cholecalciferol (vitamin D_3), Calcium, and one or more minerals derived from substances generally recognized as safe, furnishing one or more of the following elements in ionizable form: copper, magnesium, manganese, and zinc. Tablets contain NLT 90.0% and NMT 165.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$), and NLT 90.0% and NMT 125.0% of the labeled amount of calcium (Ca), copper (Cu), magnesium (Mg), manganese (Mn), and zinc (Zn). Calcium and Vitamin D with Minerals Tablets may contain other labeled added substances that are generally recognized as safe, in amounts that are unobjectionable.

IDENTIFICATION

Add the following:

▲• **A.** Characteristic absorption of each mineral as obtained in the tests in *Strength*▲ (USP 1-May-2020)

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Add the following:

▲• **B.** The retention time of the vitamin D peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Vitamin D (Cholecalciferol or Ergocalciferol), Method 2*.▲ (USP 1-May-2020)

STRENGTH

Change to read:

▲[NOTE—In the following assays, where more than one assay method is given for an individual ingredient, the requirements may be met by following any one of the specified methods, with the method used being stated in the labeling only if *Method 1* is not used.]▲ (USP 1-May-2020)

Change to read:

• **VITAMIN D (CHOLECALCIFEROL OR ERGOCALCIFEROL),** ▲***Method 1***

[NOTE—Where the use of a vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant Reference Standard.▲ (USP 1-May-2020) Use low-actinic glassware throughout this procedure.]

Mobile phase: *n-Hexane* and isopropyl alcohol (99:1)

Standard solution: 2 µg/mL of USP Ergocalciferol RS or USP Cholecalciferol RS in *n-hexane*

System suitability solution: Heat a volume of *Standard solution* at 60° for 1 h to partially isomerize vitamin D (ergocalciferol or cholecalciferol) to its corresponding precursor.

Sample solution: Weigh and grind NLT 20 Tablets. Transfer the equivalent to 20 µg of cholecalciferol or ergocalciferol to a container having a polytef-lined screw-cap. Add 8 mL of dimethyl sulfoxide and 12 mL of *n-hexane*, and shake for 45 min on a wrist-action shaker with tubes in a water bath maintained at 60°. Centrifuge for 10 min, withdraw the hexane layer by means of a pipet, and transfer to an evaporation flask. Add 12 mL of *n-hexane* to the dimethyl sulfoxide layer, mix on a vortex mixer for 5 min, and again withdraw the hexane layer by means of a pipet and add to the evaporation flask. Repeat this extraction with 3 additional 12-mL portions of *n-hexane*, adding the hexane extracts to the evaporation flask. Evaporate the combined hexane extracts under vacuum at room temperature to dryness. Dissolve in and dilute the residue in a volume of *n-hexane* to obtain a concentration of 2 µg/mL.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 15-cm; 5-µm packing L8

Flow rate: 1 mL/min

Injection volume: 100 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 10 between the vitamin D form present and its corresponding precursor, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the responses for the vitamin D peaks.

Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak height for cholecalciferol or ergocalciferol from the *Sample solution*

r_S = peak height for cholecalciferol or ergocalciferol from the *Standard solution*

C_S = concentration of USP Ergocalciferol RS or USP Cholecalciferol RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of ergocalciferol or cholecalciferol in the *Sample solution* (µg/mL)

F = correction factor to account for the average amount of previtamin D present in the *Sample solution*, 1.09

Acceptance criteria: 90.0%–165.0% of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

Add the following:

▲• **VITAMIN D (CHOLECALCIFEROL OR ERGOCALCIFEROL),** ***Method 2***

[NOTE—Where the use of a vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant Reference Standard. Use low-actinic glassware throughout this procedure.]

Diluent: Water, dimethylformamide, and isopropyl alcohol (5:10:35, v/v)

Mobile phase: Acetonitrile and water (70:30)

Cholecalciferol standard stock solution: 0.5 mg/mL of USP Cholecalciferol RS in isopropyl alcohol

Ergocalciferol standard stock solution: 0.5 mg/mL of USP Ergocalciferol RS in isopropyl alcohol

System suitability solution: Combine equal volumes of *Cholecalciferol standard stock solution* and *Ergocalciferol standard stock solution*. Dilute with *Diluent* to obtain a concentration of about 1 µg/mL each of cholecalciferol and ergocalciferol.

Standard solution: 1 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in *Diluent* from the *Cholecalciferol standard stock solution* or *Ergocalciferol standard stock solution*, prepared accurately

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powder, nominally equivalent to 50 µg of cholecalciferol (or ergocalciferol), into a 50-mL volumetric flask, add 5 mL of water, and sonicate for 10 min with intermittent shaking. Add 10 mL of dimethylformamide, sonicate for 15 min with intermittent shaking, cool to room temperature, and dilute with isopropyl alcohol to volume. Mix well and pass through a nylon filter of 0.45-µm pore size.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 15-cm; 2.7-µm packing L96

Column temperature: 40°

Flow rate: 1 mL/min

Injection volume: 20 µL

System suitability

Samples: *System suitability solution* and *Standard solution*

[NOTE—The relative retention times for cholecalciferol and ergocalciferol are 1.0 and 1.1, respectively.]

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Suitability requirements
Resolution: NLT 1.5 between the cholecalciferol and ergocalciferol peaks, *System suitability solution*
Relative standard deviation: NMT 2.0%, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*
r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*
C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% ▲(USP 1-May-2020)

• **CALCIUM, *Method 1***
[NOTE—A commercially available atomic absorption standard solution for calcium may be used where preparation of a *Calcium standard stock solution* is described in the following assay. Concentrations of the *Standard solutions* and the *Sample stock solution* may be modified to fit the linear or working range of the instrument.]
Lanthanum chloride solution: 267 mg/mL of lanthanum chloride heptahydrate in 0.125 N hydrochloric acid
Calcium standard stock solution: 400 µg/mL of calcium. Dissolve 1.001 g of calcium carbonate, previously dried at 300° for 3 h and cooled in a desiccator for 2 h. Dissolve in 25 mL of 1 N hydrochloric acid. Boil to expel carbon dioxide, and dilute with water to 1000 mL.
Standard stock solution: 100 µg/mL of calcium from *Calcium standard stock solution* diluted with 0.125 N hydrochloric acid
Standard solutions: Into separate 100-mL volumetric flasks pipet 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, and dilute with water to volume to obtain *Standard solutions* with concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 µg/mL of calcium.
Sample stock solution: Weigh and finely powder NLT 20 Tablets. Mix a portion of the powder equivalent to a nominal amount of 500 mg of calcium with 25 mL of concentrated hydrochloric acid, and heat for 30 min on a steam bath. Cool, dilute with water to 1000 mL, and filter.
Sample solution: Quantitatively dilute a volume of the *Sample stock solution* with 0.125 N hydrochloric acid to obtain a nominal concentration of 100 µg/mL of calcium. Transfer 2.0 mL of this solution to a 100-mL volumetric flask, add 1.0 mL of *Lanthanum chloride solution*, and dilute with water to volume.
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Lamp: Calcium hollow-cathode
Flame: Nitrous oxide–acetylene
Analytical wavelength: Calcium emission line, 422.7 nm
Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution* per 100 mL
Analysis
Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus concentration, in µg/mL, of calcium, and draw the straight line best fitting the 5 plotted points. From the graph, determine the concentration (C), in µg/mL, of calcium in the *Sample solution*.
Calculate the percentage of the labeled amount of calcium (Ca) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of calcium in the *Sample solution* (µg/mL)
C_U = nominal concentration of calcium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of calcium (Ca)

• **COPPER, *Method 1***
Copper standard solution: Dissolve 1.00 g of copper foil in a minimum volume of a 50% (v/v) solution of nitric acid solution, and dilute with a 1% (v/v) solution of nitric acid to 1000 mL. This solution contains 1000 µg/mL of copper.
Standard stock solution: 100 µg/mL of copper from *Copper standard solution* diluted with 0.125 N hydrochloric acid
Standard solutions: To separate 200-mL volumetric flasks transfer 1.0, 2.0, 4.0, 6.0, and 8.0 mL of the *Standard stock solution*. Dilute with water to volume to obtain concentrations of 0.5, 1.0, 2.0, 3.0, and 4.0 µg/mL of copper.
Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer the equivalent of 5 mg of copper from powdered Tablets to a porcelain crucible. Heat for 6–12 h in a muffle furnace maintained at 550°, and cool. Add 15 mL of hydrochloric acid, and boil gently on a hot plate or a steam bath for 30 min, intermittently rinsing the inner surface of the crucible with 6 N hydrochloric acid. Cool, and quantitatively transfer the contents of the crucible to a 100-mL volumetric flask, rinsing the crucible with portions of 6 N hydrochloric acid. Dilute the contents of the flask with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute the filtrate quantitatively with 0.125 N hydrochloric acid to obtain a concentration of 2 µg/mL of copper.
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Lamp: Copper hollow-cathode
Flame: Air–acetylene
Analytical wavelength: Copper emission line, 324.7 nm
Blank: 0.125 N hydrochloric acid
Analysis
Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus concentration, in µg/mL, of copper, and draw the straight line best fitting the 5 plotted points. From the graph, determine the concentration (C), in µg/mL, of copper in the *Sample solution*.
Calculate the percentage of the labeled amount of copper (Cu) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of copper in the *Sample solution* (µg/mL)
C_U = nominal concentration of copper in the *Sample solution* (µg/mL)

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Acceptance criteria: 90.0%–125.0% of the labeled amount of copper (Cu)

• **MAGNESIUM, *Method 1***

Lanthanum chloride solution: 267 mg/mL of lanthanum chloride heptahydrate in 0.125 N hydrochloric acid

Magnesium standard stock solution: Transfer 1.00 g of magnesium to a 1000-mL volumetric flask, dissolve in 50 mL of 6 N hydrochloric acid, dilute with water to volume, and mix to obtain a solution having a known concentration of 1000 µg/mL.

Standard stock solution: 20 µg/mL of magnesium from *Magnesium standard stock solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 2.5, and 3.0 mL of *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, and dilute with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.2, 0.3, 0.4, 0.5, and 0.6 µg/mL of magnesium.

Sample solution: Finely powder NLT 20 Tablets. Transfer the equivalent of 200 mg of magnesium to a porcelain crucible. Heat for 6–12 h in a muffle furnace maintained at 550°, and cool. Add 15 mL of hydrochloric acid, and boil gently on a hot plate or a steam bath for 30 min, intermittently rinsing the inner surface of the crucible with 6 N hydrochloric acid. Cool, and quantitatively transfer the contents of the crucible to a 100-mL volumetric flask, rinsing the crucible with portions of 6 N hydrochloric acid. Dilute the contents of the flask with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute the filtrate quantitatively with 0.125 N hydrochloric acid to obtain a concentration of 0.4 µg/mL of magnesium, adding 1 mL of *Lanthanum chloride solution* per 100 mL of the final volume.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Lamp: Magnesium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Magnesium emission line, 285.2 nm

Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution* per 100 mL

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus concentration, in µg/mL, of magnesium, and draw the straight line best fitting the 5 plotted points. From the graph, determine the concentration (C), in µg/mL, of magnesium in the *Sample solution*.

Calculate the percentage of the labeled amount of magnesium (Mg) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of magnesium in the *Sample solution* (µg/mL)

C_U = nominal concentration of magnesium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of magnesium (Mg)

• **MANGANESE, *Method 1***

Manganese standard stock solution: Transfer 1.00 g of manganese, weighed, to a 1000-mL volumetric flask. Dissolve in 20 mL of nitric acid, dilute with 6 N hydrochloric acid to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of manganese.

Standard stock solution: 50 µg/mL of manganese from *Manganese standard stock solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Standard stock solution*. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions with concentrations of 0.5, 0.75, 1.0, 1.5, and 2.0 µg/mL of manganese.

Sample solution: Finely powder NLT 20 Tablets. Transfer the equivalent of 9 mg of manganese from powdered Tablets to a porcelain crucible. Heat for 6–12 h in a muffle furnace maintained at 550°, and cool. Add 15 mL of hydrochloric acid, and boil gently on a hot plate or a steam bath for 30 min, intermittently rinsing the inner surface of the crucible with 6 N hydrochloric acid. Cool, and quantitatively transfer the contents of the crucible to a 100-mL volumetric flask, rinsing the crucible with portions of 6 N hydrochloric acid. Dilute the contents of the flask with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute the filtrate quantitatively with 0.125 N hydrochloric acid to obtain a concentration of 1 µg/mL of manganese.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Lamp: Manganese hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Manganese emission line, 279.5 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of manganese, and draw the straight line best fitting the 5 plotted points. From the graph, determine the concentration (C), in mg/mL, of manganese in the *Sample solution*.

Calculate the percentage of the labeled amount of manganese (Mn) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of manganese in the *Sample solution* (µg/mL)

C_U = nominal concentration of manganese in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of manganese

• **ZINC, *Method 1***

Zinc standard stock solution: 1000 µg/mL of zinc from zinc oxide dissolved in 5 M hydrochloric acid (3.89 mg/mL), and diluted with water to final volume. [NOTE—Dissolve in 5 M hydrochloric acid by warming, if necessary, cool, and then dilute to final volume.]

Standard stock solution: 50 µg/mL of zinc from *Zinc standard stock solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Transfer 1.0, 2.0, 3.0, 4.0, and 5.0 mL of *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of zinc.

Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer the equivalent of 40 mg of zinc from powdered Tablets to a porcelain crucible. Heat for 6–12 h in a muffle furnace maintained at 550°, and cool. Add 15 mL of

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hydrochloric acid, and boil gently on a hot plate or a steam bath for 30 min, intermittently rinsing the inner surface of the crucible with 6 N hydrochloric acid. Cool, and quantitatively transfer the contents of the crucible to a 100-mL volumetric flask, rinsing the crucible with portions of 6 N hydrochloric acid. Dilute the contents of the flask with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute the filtrate quantitatively with 0.125 N hydrochloric acid to obtain a concentration of 2 µg/mL of zinc.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Lamp: Zinc hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Zinc emission line, 213.8 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus concentration, in µg/mL, of zinc, and draw the straight line best fitting the 5 plotted points. From the graph, determine the concentration (C), in µg/mL, of zinc in the *Sample solution*.

Calculate the percentage of the labeled amount of zinc (Zn) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of zinc in the *Sample solution* (µg/mL)

C_U = nominal concentration of zinc in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of zinc (Zn)

• **CALCIUM, COPPER, MAGNESIUM, MANGANESE, AND ZINC, *Method 2***

Stock aqua regia solution: Prepare a mixture of hydrochloric acid and nitric acid (3:1) by adding the nitric acid to the hydrochloric acid. [NOTE—Periodically vent the solution in an appropriate fume hood.]

Diluent: Prepare a mixture of *Stock aqua regia solution* and water (1:9) by adding 1 volume of *Stock aqua regia solution* to 2 volumes of water. Dilute with additional water to volume, and mix well.

System suitability solution: Prepare a mixture of 1000 mg/L of yttrium in 5% (v/v) nitric acid solution and 1000 mg/L of scandium in 5% (v/v) nitric acid solution with *Diluent* (1:1:198), and mix.

Standard stock solution (Ca, Cu, Mg, Mn, and Zn): [NOTE—It is only necessary to include the minerals of interest in the solution.] Using commercially available element standard (single- or multi-element) solutions in 5% (v/v) nitric acid solution, pipet the appropriate amount of element standard solution into a volumetric flask, and dilute with 5% (v/v) nitric acid solution to obtain a solution having final concentrations of about 1000 mg/L of calcium, 100 mg/L of copper, 500 mg/L of magnesium, 100 mg/L of manganese, and 250 mg/L of zinc.

Standard solutions: Prepare a mixture of *Standard stock solution* in *Diluent* to prepare a 6-point calibration curve to bracket the concentration range of each mineral of interest.

Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer a portion equal to the average Tablet weight to a 250-mL volumetric flask. Slowly add 25 mL of *Stock aqua regia solution* in 5-mL increments, followed by mixing. [NOTE—If the sample contains a carbonate, bubbling will occur. Wait until bubbling ends to proceed.] Bring the solution to a boil on a hot plate. Continue to heat gently until fumes cease (about 1 h). Remove from heat, cool, and dilute with water to volume. Pass about 30 mL into a centrifuge tube using a 5-µm pore size nylon syringe filter. If necessary, make any further dilutions using the *Diluent*.

Instrumental conditions

(See *Plasma Spectrochemistry* ⟨730⟩.)

Mode: Inductively coupled plasma spectrometry, using a spectrometer set to measure the emission of each mineral of interest at about the corresponding wavelength. [NOTE—The operating conditions may be developed and optimized based on the manufacturer's recommendation. The wavelengths selected should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision.]

System suitability

Sample: *System suitability solution*

[NOTE—Analyze the *System suitability solution* and obtain the response as directed for *Analysis*.]

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the emission of each mineral of interest in the *Standard solutions* and *Sample solution* with an inductively coupled plasma system using the *Diluent* as the blank. Plot the emission of the *Standard solutions* versus concentration, in mg/L, of the minerals of interest, and draw the straight line best fitting the plotted points. From the graph, determine the concentration (C), in mg/L, for each mineral of interest in the *Sample solution*.

Calculate the percentage of the labeled amount for each mineral:

$$\text{Result} = C \times (V/W) \times F \times (W_T/L) \times 100$$

C = measured concentration of the relevant element in the *Sample solution* (mg/L)

V = volume of the *Sample solution* (L)

W = sample weight (mg)

F = dilution factor of the *Sample solution*

W_T = average weight (mg/Tablet)

L = labeled amount of the relevant element (mg/Tablet)

Acceptance criteria: NLT 90.0%–125.0% of the labeled amount of calcium (Ca), copper (Cu), magnesium (Mg), manganese (Mn), and zinc (Zn)

PERFORMANCE TESTS

• **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩, *Dissolution*: Meet the requirements with respect to calcium

Medium: 0.1 N hydrochloric acid; 900 mL

Apparatus 2: 75 rpm

Time: 30 min

Analysis: Determine the amount of calcium (Ca) dissolved, using the procedure in the assay for *Calcium*, making any necessary volumetric adjustments.

Tolerances: NLT 75% of the labeled amount of Ca is dissolved.

• **WEIGHT VARIATION** ⟨2091⟩: Meet the requirements

SPECIFIC TESTS

• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic microbial count does not exceed 3×10^3 cfu/g, and the total combined molds and yeasts count does not exceed 3×10^2 cfu/g.

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• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meet the requirements

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

Change to read:

• **LABELING:** The label states that the product is *Calcium and Vitamin D with Minerals Tablets*. The label also states the quantities of each mineral and vitamin D ▲in terms of metric units/Tablet,▲ (USP 1-May-2020) the salt form of the mineral used as the source of each element present, and the chemical form of vitamin D present in the dosage unit.

• **USP REFERENCE STANDARDS** ⟨11⟩
USP Cholecalciferol RS
USP Ergocalciferol RS

Anhydrous Dibasic Calcium Phosphate

—see Dibasic Calcium Phosphate Dihydrate General Monographs

Dibasic Calcium Phosphate Dihydrate

—see Dibasic Calcium Phosphate Dihydrate General Monographs

Calcium Phosphate, Dibasic Tablets

—see Dibasic Calcium Phosphate Tablets General Monographs

Add the following:

▲Carotenes

DEFINITION

Carotenes consist mainly of beta carotene and alpha carotene that are obtained from palm oil, carrots, or other edible sources. They contain NLT 90% and NMT 110% of the labeled amount of total carotenes on the anhydrous basis. They contain NLT 90% and NMT 110% of the labeled amount each of beta carotene and alpha carotene on the anhydrous basis. The content of alpha carotene and beta carotene is NLT 25% and NLT 60%, respectively, of total carotenes on the anhydrous basis. Carotenes may be standardized with edible oils.

IDENTIFICATION

• **A.**
Sample solution: Transfer 5.0 mL of the *Sample stock solution* from the test for *Content of Total Carotenes* into a 100-mL volumetric flask, and dilute with cyclohexane to volume. Pass the solution through a membrane filter of 0.45-µm pore size.
Analysis: Record the UV-Vis spectrum from 300 to 600 nm.
Acceptance criteria: The *Sample solution* exhibits a shoulder at about 427 nm, an absorption maximum at about 440–457 nm, and another absorption maximum at about 470–486 nm.

• **B.** The chromatogram of the *Sample solution* obtained in the test for *Content of Alpha and Beta Carotenes* exhibits two main peaks due to alpha carotene and beta carotene. These two peaks correspond to those of the *System suitability solution*, with beta carotene being the most abundant, followed by alpha carotene. Additional minor peaks corresponding to 13-*cis*-beta carotene, *cis*-alpha carotene, and 9-*cis*-beta carotene may be observed.

COMPOSITION

• **CONTENT OF TOTAL CAROTENES**
[NOTE—Use low-actinic glassware.]
Sample stock solution: Warm the Carotenes to 60°–65° and mix to homogenize. Transfer an amount of Carotenes, equivalent to 5 mg of total carotenes to a 50-mL volumetric flask, dissolve, and dilute with tetrahydrofuran to volume.
Sample solution: Transfer 3.0 mL of the *Sample stock solution* into a 100-mL volumetric flask, and dilute with cyclohexane to volume.
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Analytical wavelength: Use the maximum absorbance between 440 and 457 nm.
Cell path: 1 cm
Blank: Cyclohexane
Analysis
Sample: *Sample solution*
Calculate the percentage of the labeled amount of total carotenes as beta carotene in the portion of sample taken:

$$\text{Result} = A_U/(F \times C_U)$$

A_U = absorbance of the *Sample solution*
F = 2500, coefficient of extinction ($E^{1\%}$) of pure all-*trans*-beta carotene in cyclohexane (100 mL · g^{-1} · cm^{-1})
C_U = nominal concentration of total carotenes in the *Sample solution* (g/mL)

Acceptance criteria: 90.0%–110% on the anhydrous basis

• **CONTENT OF ALPHA AND BETA CAROTENES**
[NOTE—Use low-actinic glassware.]
Solution A: Water, methanol, and *tert*-butyl methyl ether (4:81:15)
Solution B: Water, methanol, and *tert*-butyl methyl ether (4:15:81)
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
30	0	100
40	100	0
50	100	0

System suitability solution: Warm the USP Carotenes RS to 60°–65° and mix to homogenize. Transfer an amount of standard, equivalent to 5 mg of total carotenes, to a 50-mL volumetric flask, dissolve, and dilute with tetrahydrofuran to volume. Transfer 3.0 mL of the resultant solution to a 50-mL volumetric flask and dilute with cyclohexane to volume.
Standard stock solution: 60 µg/mL of USP Beta Carotene RS in tetrahydrofuran
Standard solution A: Transfer 5.0 mL of the *Standard stock solution* into a 100-mL volumetric flask, and dilute with

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cyclohexane to volume. The concentration of beta carotene in this solution will be determined by the spectrophotometric procedure using *Standard solution B* as follows.

Standard solution B: Transfer 5.0 mL of the *Standard stock solution* into a 100-mL volumetric flask, and dilute with cyclohexane to volume. Prepare in triplicate.

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Analytical wavelength: Use the maximum absorbance between 440 and 457 nm.

Cell path: 1 cm

Blank: Cyclohexane

Analysis

Sample: *Standard solution B*

Calculate the concentration of total beta carotene, in mg/mL, in *Standard solution B*:

$$\text{Result} = A/F$$

A = average absorbance of the three preparations of *Standard solution B*

F = absorptivity of pure beta carotene in cyclohexane, 250

Sample stock solution: Warm the Carotenes to 60°–65° and mix to homogenize. Transfer an amount of Carotenes, equivalent to 5 mg of total carotenes, to a 50-mL volumetric flask, dissolve, and dilute with tetrahydrofuran to volume.

Sample solution: Transfer 5.0 mL of the *Sample stock solution* into a 100-mL volumetric flask, and dilute with cyclohexane to volume.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV. Use the maximum absorbance between 440 and 457 nm.

Column: 4.6-mm × 25-cm; 5-µm packing L62

Column temperature: 30°

Flow rate: 1.0 mL/min

Injection volume: 20 µL

System suitability

Samples: *System suitability solution* and *Standard solution A*

Suitability requirements

Chromatogram similarity: The chromatogram from the *System suitability solution* is similar to the reference chromatogram provided with the lot of USP Carotenes RS being used. The approximate relative retention times of the components in the *System suitability solution* are listed in *Table 2*.

Table 2

Name	Relative Retention Time	Correction Factor[a]
15-*cis*-Beta carotene	0.88	1.4
13-*cis*-Beta carotene	0.91	1.2
All-*trans*-alpha carotene	0.93	1.05
Cis-alpha carotene	0.96	1.05
All-*trans*-beta carotene	1.00	1.0
9-*cis*-Beta carotene	1.05	1.0
Gamma carotene	1.28	1.0
Lycopene	1.58	1.0

[a] Determined at 445 nm.

Resolution: NLT 1.5 between *cis*-alpha carotene peak and all-*trans*-beta carotene peak, *System suitability solution*

Relative standard deviation: NMT 2.0%, *Standard solution A*

Analysis

Samples: *Standard solution A* and *Sample solution*

Calculate the percentage of the labeled amount of alpha carotene in the portion of Carotenes taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = sum of the peak area responses of all alpha carotenes (*cis*- and *trans*-isomers) from the *Sample solution*

r_S = peak area of beta carotene from *Standard solution A*

C_S = concentration of beta carotene in *Standard solution A* as determined by spectrophotometric procedure (mg/mL)

C_U = nominal concentration of alpha carotene in the *Sample solution* (mg/mL)

F = correction factor, 1.05 (see *Table 2*)

Calculate the percentage of the labeled amount of beta carotene in the portion of Carotenes taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = [(peak areas of all-*trans*-beta carotene) + (peak area of 9-*cis*-beta carotene) + (peak area of 13-*cis*-beta carotene × 1.2) + (peak area of 15-*cis*-beta carotene × 1.4)] from the *Sample solution*

r_S = peak area of beta carotene from *Standard solution A*

C_S = concentration of beta carotene in *Standard solution A* as determined by spectrophotometric procedure (mg/mL)

C_U = nominal concentration of beta carotene in the *Sample solution* (mg/mL)

Acceptance criteria

Alpha carotene: 90.0%–110% on the anhydrous basis

Beta carotene: 90.0%–110% on the anhydrous basis

Content of alpha carotene and beta carotene in total carotenes: NLT 25% for alpha carotene and NLT 60% for beta carotene on the anhydrous basis

SPECIFIC TESTS

• Limit of Gamma Carotene and Lycopene

Mobile phase, System suitability solution, Sample solution, and **Chromatographic system:** Proceed as directed in the test for *Content of Alpha and Beta Carotenes*.

Analysis

Sample: *Sample solution*

Separately calculate the percentage of gamma carotene and lycopene in the portion of Carotenes taken:

$$\text{Result} = (r_U/r_T) \times 100$$

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r_U = peak area of gamma carotene or lycopene from the *Sample solution*

r_T = [(peak areas of all-*trans*-alpha carotene × 1.05) + (peak area of *cis*-alpha carotene × 1.05) + (peak areas of all-*trans*-beta carotene) + (peak area of 9-*cis*-beta carotene) + (peak area of 13-*cis*-beta carotene × 1.2) + (peak area of 15-*cis*-beta carotene × 1.4) + (peak area of gamma carotene and lycopene)] from the *Sample solution*

Acceptance criteria

Gamma carotene: NMT 1.0% on the anhydrous basis

Lycopene: NMT 1.0% on the anhydrous basis

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total bacterial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species, Test for Absence of Escherichia coli,* and *Test for Absence of Staphylococcus aureus***:** Meet the requirements.
- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Peroxide Value***:** NMT 10.0
- **WATER DETERMINATION** ⟨921⟩, *Method I, Method Ia***:** NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers. Store in a cool place.
- **LABELING:** The label states the content of total carotenes, alpha carotene, and beta carotene, in mg/g.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Carotenes RS

USP Beta Carotene RS▲ 2S (USP41)

Cat's Claw

DEFINITION

Cat's Claw consists of the inner bark of the stems of *Uncaria tomentosa* (Willd.) DC. (*Rubiaceae*). It contains NLT 0.3% of pentacyclic oxindole alkaloids as isopteropodine, calculated on the dried basis, as the sum of speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine, and isopteropodine.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩

Standard solution: 100 mg of USP Powdered Cat's Claw Extract RS in 2 mL of methanol. Sonicate for 5 min, shaking occasionally, heat in a water bath at 60° for 15 min, cool, and centrifuge.

Sample solution: 5 g of powdered Cat's Claw in 10 mL of methanol. Sonicate for 5 min, shaking occasionally. Heat the mixture in a water bath at 60° for 15 min, cool, and filter.

Adsorbent: Chromatographic silica gel mixture with an average particle size of 10–15 µm (TLC plates)

Application volume: 20 µL, as bands that are 1 cm in length

Developing solvent system: Ethyl acetate and hexane (95:5)

Derivatization reagent A: Dissolve 0.85 g of basic bismuth nitrate in 10 mL of glacial acetic acid and 40 mL of water by heating. Filter if necessary (Solution A). Dissolve 8 g of potassium iodide in 30 mL of water (Solution B). Mix *Solution A* and *Solution B* (1:1) to obtain a stock solution. Dilute 1 mL of the stock solution with 2 mL of glacial acetic acid and 10 mL of water. [NOTE—Use freshly mixed *Solution A* and *Solution B*.]

Derivatization reagent B: Use a 10% solution of sodium nitrite in water.

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the chromatogram to a length of NLT 12 cm, and dry the plate in a current of air.

Acceptance criteria: Examine the plate under short-wave UV light (254 nm). The *Sample solution* chromatogram shows multiple zones that correspond in R_F values to those observed in the *Standard solution* chromatogram. Other zones of varying intensities may be observed in the *Sample solution*. Treat the plate with *Derivatization reagent A* followed by *Derivatization reagent B*, and examine the plate under white light. The *Sample solution* chromatogram shows multiple orange-brown zones that correspond in color and R_F values to those observed in the *Standard solution* chromatogram. Other colored zones of varying intensities may be observed in the *Sample solution*.

- **B.** The *Sample solution* exhibits peaks for speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine, and isopteropodine at retention times that correspond to those in *Standard solution A*, as obtained in the test for *Content of Pentacyclic Oxindole Alkaloids and Limit of Tetracyclic Oxindoles*.

COMPOSITION

- **CONTENT OF PENTACYCLIC OXINDOLE ALKALOIDS AND LIMIT OF TETRACYCLIC OXINDOLES**

Standard solution A: Dissolve an accurately weighed quantity of USP Powdered Cat's Claw Extract RS in methanol, shaking for 1 min. Dilute with methanol to obtain a solution having a known concentration of about 0.5 mg of the labeled amount of total oxindole alkaloids per mL. Pass through a filter of 0.45-µm or finer pore size.

Standard solution B: 0.1 mg/mL of USP Isopteropodine RS in methanol. Pass through a nylon filter of 0.45-µm or finer pore size.

Sample solution: Accurately weigh approximately 750 mg of ground Cat's Claw, and place in a 10-mL centrifuge tube. Sonicate with 2.5 mL of methanol for 10 min. Centrifuge, and transfer this solution to a 10-mL volumetric flask. Repeat the extraction three additional times combining the extracts in the 10-mL volumetric flask, and dilute with methanol to volume. Transfer about 3 mL of the solution to a test tube containing 300 mg of polyamide powder, and shake for 1 min. Pass through a nylon filter of 0.45-µm or finer pore size, discarding the first part of the filtrate.

Solution A: Prepare a filtered and degassed 10 mM pH 7.0 phosphate buffer by mixing 6 mL of 1 N sodium hydroxide, 10 mL of 1 M monobasic potassium phosphate, and sufficient water to make 1000 mL. Adjust to a pH of 7.0 ± 0.1 by adding more of either solution.

Solution B: Acetonitrile

Solution C: Methanol and glacial acetic acid (99:1)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)	Solution C (%)
0	65	35	0
17	65	35	0
25	50	50	0
30	50	50	0
31	0	0	100

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Table 1 *(continued)*

Time (min)	Solution A (%)	Solution B (%)	Solution C (%)
36	0	0	100
39	65	35	0
49	65	35	0

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 245 nm
Column: 4.6-mm × 10-cm; end-capped 3-µm packing L1
Flow rate: 0.75 mL/min
Injection volume: 10 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram obtained using *Standard solution A* is similar to the reference chromatogram provided with the USP Powdered Cat's Claw Extract RS.
Tailing factor: NMT 2.0 for the isopteropodine peak, *Standard solution B*
Relative standard deviation: NMT 2.0% for the isopteropodine peak in repeated injections, *Standard solution B*
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Measure the areas of the analyte peaks. Identify the retention times of the peaks corresponding to speciophylline, uncarine F, mitraphylline, isomitraphylline, rhynchophylline, isorhynchophylline, pteropodine, and isopteropodine by comparison of the chromatogram of *Standard solution A* with the reference chromatogram provided with the lot of the USP Powdered Cat's Claw Extract RS used.
Separately calculate the percentages of speciophylline, uncarine F, mitraphylline, isomitraphylline, rhynchophylline, isorhynchophylline, pteropodine, and isopteropodine, as isopteropodine, in the portion of Cat's Claw taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response for each relevant alkaloid from the *Sample solution*
r_S = peak response for isopteropodine from *Standard solution B*
C_S = concentration of USP Isopteropodine RS in *Standard solution B* (mg/mL)
C_U = concentration of Cat's Claw in the *Sample solution* (mg/mL)

Calculate the content of pentacyclic oxindole alkaloids by adding the percentages of speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine, and isopteropodine.
Calculate the content of tetracyclic oxindole alkaloids by adding the individual percentages of rhynchophylline and isorhynchophylline.
Acceptance criteria
Pentacyclic oxindole alkaloids: NLT 0.3%
Tetracyclic oxindole alkaloids: NMT 0.05%

CONTAMINANTS

- **Articles of Botanical Origin,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
- **Articles of Botanical Origin,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements
- **Microbial Enumeration Tests** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^5/g; the total combined molds and yeasts count does not exceed 10^3/g; and the bile-tolerant Gram-negative bacteria do not exceed 10^3/g.
- **Absence of Specified Microorganisms** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

- **Botanical Characteristics**

[Note—The Pharmacopeial article is constituted only by the stem inner bark of *U. tomentosa* (Willd.) DC. Descriptions of other parts of the plant are given to aid in the collection of the right species. Compliance should be determined using the entire monograph and not only the botanical description.]

Macroscopic: Cat's Claw is a woody vine with a main stem up to 20 cm in diameter and up to 30 m long. The branches are obtusely quadrangular and generally puberulous. Stipules in the buds are densely tomentose in the upper side (different from *U. guianensis* in which the stipules are glabrous) with the hairs, often with curved tips, meshed together and with the longer hairs of the leaf helping to connect the pair of stipules along the margins, but split when older (different from *U. guianensis* in which the stipules separate early in the bud development). Thorns are straight to sickle-shaped, not spirally twisted (different from *U. guianensis*), very pungent and woody, from 8 to 20 mm long and from 3 to 6 mm wide. When recently cut, the color of the inner bark can be whitish gray, yellowish brown, or dark red, with longitudinal fissures and persistent rhytidome. The internal part has a slightly dusty fibrous and laminar texture with a characteristic ferruginous dust and an extremely astringent taste. The terminal branches have a quadrangular section and yellowish green internal medulla.
Microscopic: The periderm with cork (phellem) is constituted by 6–8 rows of cells having walls evenly thickened, a compressed phellogen and a phelloderm with 1–7 rows of sclereids. [Note—The periderm and phellogen should be absent in the Pharmacopeial article.] The secondary cortex with concentric rings of fibers are separated by rings of parenchyma; rings of fibers are frequently interrupted by radial rows of parenchyma cells (predominately 1 cell broad) or narrow medullary rays (few cells broad), forming rectangular bundles of fibers in a regular network. In longitudinal view, the fibers appear with numerous conspicuous pits; calcium oxalate microcrystals (sand-like) are abundant in the parenchyma, but usually absent as large polyhedral crystals or in the form of styloids with bifurcated endings, the latter forms typically present in the parenchyma of *U. guianensis*; a brown substance is dispersed in parenchyma cells; starch is abundant, granules are solitary (circular in outline, up to 10 µm in diameter) or compound (2–3 components up to 15 µm in diameter).

- **Articles of Botanical Origin,** *Foreign Organic Matter* ⟨561⟩**:** NMT 2.0%
- **Loss on Drying** ⟨731⟩
Sample: 1.0 g of Cat's Claw, finely powdered
Analysis: Dry the *Sample* at 105° for 2 h.
Acceptance criteria: NMT 7.0%
- **Articles of Botanical Origin,** *Total Ash* ⟨561⟩**:** NMT 8.0%
- **Articles of Botanical Origin,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 2.0%

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in tight, light-resistant containers, and store at room temperature.

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- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Isopteropodine RS
  USP Powdered Cat's Claw Extract RS

Powdered Cat's Claw

DEFINITION

Powdered Cat's Claw is Cat's Claw reduced to a powder or very fine powder. It contains NLT 0.3% of pentacyclic oxindole alkaloids as isopteropodine, calculated on the dried basis, as the sum of speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine, and isopteropodine.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩

Standard solution: 100 mg of USP Powdered Cat's Claw Extract RS in 2 mL of methanol. Sonicate for 5 min, shaking occasionally, heat in a water bath at 60° for 15 min, cool, and centrifuge.

Sample solution: 5 g of Powdered Cat's Claw in 10 mL of methanol. Sonicate for 5 min, shaking occasionally. Heat the mixture in a water bath at 60° for 15 min, cool, and filter.

Adsorbent: Chromatographic silica gel mixture with an average particle size of 10–15 µm (TLC plates)

Application volume: 20 µL, as bands that are 1 cm in length

Developing solvent system: Ethyl acetate and hexane (95:5)

Derivatization reagent A: Dissolve 0.85 g of basic bismuth nitrate in 10 mL of glacial acetic acid and 40 mL of water by heating. Filter if necessary (Solution A). Dissolve 8 g of potassium iodide in 30 mL of water (Solution B). Mix *Solution A* and *Solution B* (1:1) to obtain a stock solution. Dilute 1 mL of the stock solution with 2 mL of glacial acetic acid and 10 mL of water. [NOTE—Use freshly mixed *Solution A* and *Solution B*.]

Derivatization reagent B: Use a 10% solution of sodium nitrite in water.

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the chromatogram to a length of NLT 12 cm, and dry the plate in a current of air.

Acceptance criteria: Examine the plate under short-wave UV light (254 nm). The *Sample solution* chromatogram shows multiple zones that correspond in R_F values to those observed in the *Standard solution* chromatogram. Other zones of varying intensities may be observed in the *Sample solution*. Treat the plate with *Derivatization reagent A* followed by *Derivatization reagent B*, and examine the plate under white light. The *Sample solution* chromatogram shows multiple orange-brown zones that correspond in color and R_F values to those observed in the *Standard solution* chromatogram. Other colored zones of varying intensities may be observed in the *Sample solution*.

- **B.** The *Sample solution* exhibits peaks for speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine, and isopteropodine at retention times that correspond to those in *Standard solution A*, as obtained in the test for *Content of Pentacyclic Oxindole Alkaloids and Limit of Tetracyclic Oxindoles*.

COMPOSITION

- **CONTENT OF PENTACYCLIC OXINDOLE ALKALOIDS AND LIMIT OF TETRACYCLIC OXINDOLES**

Standard solution A: Dissolve an accurately weighed quantity of USP Powdered Cat's Claw Extract RS in methanol, shaking for 1 min. Dilute with methanol to obtain a solution having a known concentration of about 0.5 mg of the labeled amount of total oxindole alkaloids per mL. Pass through a filter of 0.45-µm or finer pore size.

Standard solution B: 0.1 mg/mL of USP Isopteropodine RS in methanol. Pass through a nylon filter of 0.45-µm or finer pore size.

Sample solution: Accurately weigh approximately 750 mg of Powdered Cat's Claw, and place in a 10-mL centrifuge tube. Sonicate with 2.5 mL of methanol for 10 min. Centrifuge, and transfer this solution to a 10-mL volumetric flask. Repeat the extraction three additional times combining the extracts in the 10-mL volumetric flask, and dilute with methanol to volume. Transfer about 3 mL of the solution to a test tube containing 300 mg of polyamide powder, and shake for 1 min. Pass through a nylon filter of 0.45-µm or finer pore size, discarding the first part of the filtrate.

Solution A: Prepare a filtered and degassed 10 mM pH 7.0 phosphate buffer by mixing 6 mL of 1 N sodium hydroxide, 10 mL of 1 M monobasic potassium phosphate, and sufficient water to make 1000 mL. Adjust to a pH of 7.0 ± 0.1 by adding more of either solution.

Solution B: Acetonitrile

Solution C: Methanol and glacial acetic acid (99:1)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)	Solution C (%)
0	65	35	0
17	65	35	0
25	50	50	0
30	50	50	0
31	0	0	100
36	0	0	100
39	65	35	0
49	65	35	0

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 245 nm

Column: 4.6-mm × 10-cm; end-capped 3-µm packing L1

Flow rate: 0.75 mL/min

Injection volume: 10 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram obtained using *Standard solution A* is similar to the reference chromatogram provided with the USP Powdered Cat's Claw Extract RS.

Tailing factor: NMT 2.0 for the isopteropodine peak, *Standard solution B*

Relative standard deviation: NMT 2.0% for the isopteropodine peak in repeated injections, *Standard solution B*

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Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Measure the areas of the analyte peaks. Identify the retention times of the peaks corresponding to speciophylline, uncarine F, mitraphylline, isomitraphylline, rhynchophylline, isorhynchophylline, pteropodine, and isopteropodine by comparison of the chromatogram of *Standard solution A* with the reference chromatogram provided with the lot of the USP Powdered Cat's Claw Extract RS used.
Separately calculate the percentages of speciophylline, uncarine F, mitraphylline, isomitraphylline, rhynchophylline, isorhynchophylline, pteropodine, and isopteropodine, as isopteropodine, in the portion of Powdered Cat's Claw taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response for each relevant alkaloid from the *Sample solution*
r_S = peak response for isopteropodine from *Standard solution B*
C_S = concentration of USP Isopteropodine RS in *Standard solution B* (mg/mL)
C_U = concentration of Powdered Cat's Claw in the *Sample solution* (mg/mL)

Calculate the content of pentacyclic oxindole alkaloids by adding the percentages of speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine, and isopteropodine.
Calculate the content of tetracyclic oxindole alkaloids by adding the individual percentages of rhynchophylline and isorhynchophylline.
Acceptance criteria
Pentacyclic oxindole alkaloids: NLT 0.3%
Tetracyclic oxindole alkaloids: NMT 0.05%

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^5/g; the total combined molds and yeasts count does not exceed 10^3/g; and the bile-tolerant Gram-negative bacteria do not exceed 10^3/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS:** Presence of fragments of cork and suberized cells, with cell walls evenly thickened; presence of phelloderm sclereids; fragments of fibers that are crossed by vascular rays darkened due to the presence of sand-like calcium oxalate microcrystals; solitary or two- to three-compound starch grains up to 15 µm in diameter; absence of styloids, typically present in *U. guianensis*
- **LOSS ON DRYING** ⟨731⟩
Sample: 1.0 g of Powdered Cat's Claw
Analysis: Dry the *Sample* at 105° for 2 h.
Acceptance criteria: NMT 7.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 8.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 2.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Isopteropodine RS
USP Powdered Cat's Claw Extract RS

Powdered Cat's Claw Extract

DEFINITION

Powdered Cat's Claw Extract is prepared from Cat's Claw by extraction with hydroalcoholic mixtures or other suitable solvents. The ratio of plant material to extract is between 4:1 to 6:1. It contains NLT 90.0% and NMT 110.0% of the labeled amount of pentacyclic oxindole alkaloids as isopteropodine, calculated on the dried basis, as the sum of speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine, and isopteropodine. It may contain suitable added substances.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**
Standard solution: 100 mg of USP Powdered Cat's Claw Extract RS in 2 mL of methanol. Sonicate for 5 min, shaking occasionally, heat in a water bath at 60° for 15 min, cool, and centrifuge.
Sample solution: Shake a quantity of Powdered Extract, equivalent to about 25 mg of the labeled amount of pentacyclic oxindole alkaloids, in 20 mL of methanol. Allow to stand for 15 min before use.
Adsorbent: Chromatographic silica gel mixture with an average particle size of 10–15 µm (TLC plates)
Application volume: 20 µL, as bands that are 1 cm in length
Developing solvent system: Ethyl acetate and hexane (95:5)
Spray reagent A: Dissolve 0.85 g of basic bismuth nitrate in 10 mL of glacial acetic acid and 40 mL of water by heating. Filter if necessary (Solution A). Dissolve 8 g of potassium iodide in 30 mL of water (Solution B). Mix *Solution A* and *Solution B* (1:1) to obtain a stock solution. Dilute 1 mL of the stock solution with 2 mL of glacial acetic acid and 10 mL of water. [NOTE—Use freshly mixed *Solution A* and *Solution B*.]
Spray reagent B: Use a 10% solution of sodium nitrite in water.
Analysis
Samples: *Standard solution* and *Sample solution*
Develop the chromatogram to a length of NLT 12 cm, and dry the plate in a current of air.
Acceptance criteria: Examine the plate under short UV light. The *Sample solution* chromatogram shows multiple zones that correspond in R_F values to those observed from the *Standard solution* chromatogram. Other zones of varying intensities may be observed in the *Sample solution*. Spray the plate with *Spray reagent A* followed by *Spray reagent B*, and examine the plate under daylight. The *Sample solution* chromatogram shows multiple orange-brown zones that correspond in color and R_F values to those observed in the *Standard solution* chromatogram. Other colored zones of varying intensities may be observed in the *Sample solution*.
- **B.** The chromatogram of the *Sample solution* exhibits peaks for speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine, and isopteropodine at

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retention times that correspond to those of *Standard solution A*, as obtained in the test for *Content of Pentacyclic Oxindole Alkaloids* and *Limit of Tetracyclic Oxindole Alkaloids*. The sum of the peak areas for the tetracyclic oxindole alkaloids rhynchophylline and isorhynchophylline is less than 25% of the total peak areas detected for pentacyclic oxindole alkaloids.

COMPOSITION

• **CONTENT OF PENTACYCLIC OXINDOLE ALKALOIDS AND LIMIT OF TETRACYCLIC OXINDOLES**

Standard solution A: Dissolve an accurately weighed quantity of USP Powdered Cat's Claw Extract RS in methanol, shaking for 1 min. Dilute with methanol to obtain a solution having a known concentration of about 0.5 mg of the labeled amount of total oxindole alkaloids per mL. Pass through a filter of 0.45-µm or finer pore size.

Standard solution B: 0.1 mg/mL of USP Isopteropodine RS in methanol. Pass through a nylon filter of 0.45-µm or finer pore size.

Sample solution: Transfer an accurately weighed quantity of Powdered Extract, equivalent to about 5 mg of the labeled content of pentacyclic oxindole alkaloids, to a 10-mL centrifuge tube. Add 2.5 mL of methanol, and sonicate for 10 min. Centrifuge, and transfer the supernatant to a 10-mL volumetric flask. Repeat the extraction three additional times combining the extracts in the 10-mL volumetric flask, and dilute with methanol to volume. Transfer about 3 mL of the solution to a test tube containing 300 mg of polyamide powder, and shake for 1 min. Pass through a nylon filter of 0.45-µm or finer pore size, discarding the first part of the filtrate.

Solution A: Prepare a filtered and degassed 10 mM pH 7.0 phosphate buffer by mixing 6 mL of 1 N sodium hydroxide, 10 mL of 1 M monobasic potassium phosphate, and sufficient water to make 1000 mL. Adjust to a pH of 7.0 ± 0.1 by adding more of either solution.

Solution B: Acetonitrile

Solution C: Methanol and glacial acetic acid (99:1)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)	Solution C (%)
0	65	35	0
17	65	35	0
25	50	50	0
30	50	50	0
31	0	0	100
36	0	0	100
39	65	35	0
49	65	35	0

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 245 nm

Column: 4.6-mm × 10-cm; end-capped 3-µm packing L1

Flow rate: 0.75 mL/min

Injection size: 10 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram obtained using *Standard solution A* is similar to the Reference Chromatogram provided with the USP Powdered Cat's Claw Extract RS being used.

Tailing factor: NMT 2.0 for the isopteropodine peak, *Standard solution B*

Relative standard deviation: NMT 2.0% for the isopteropodine peak in repeated injections, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Measure the areas of the analyte peaks. Identify the retention times of the peaks corresponding to speciophylline, uncarine F, mitraphylline, isomitraphylline, rhynchophylline, isorhynchophylline, pteropodine, and isopteropodine by comparison of the chromatogram of *Standard solution A* with the Reference Chromatogram provided with the lot of the USP Powdered Cat's Claw Extract RS used.

Separately calculate the percentages of speciophylline, uncarine F, mitraphylline, isomitraphylline, rhynchophylline, isorhynchophylline, pteropodine, and isopteropodine, as isopteropodine, in the portion of Powdered Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response for each relevant alkaloid from the *Sample solution*

r_S = peak response for isopteropodine from *Standard solution B*

C_S = concentration of USP Isopteropodine RS in *Standard solution B* (mg/mL)

C_U = concentration of Powdered Extract in the *Sample solution* (mg/mL)

Calculate the percentage of the labeled amount of pentacyclic oxindole alkaloids in the Powdered Extract:

$$\text{Result} = (\Sigma PA/L) \times 100$$

ΣPA = sum of percentages of speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine, and isopteropodine (%)

L = labeled amount of pentacyclic alkaloids as isopteropodine (%)

Calculate the content of tetracyclic oxindole alkaloids by adding the individual percentages of rhynchophylline and isorhynchophylline.

Acceptance criteria

Pentacyclic oxindole alkaloids: 90.0%–110.0% of the labeled amount on the dried basis

Tetracyclic oxindole alkaloids: NMT 25% of the labeled amount of pentacyclic oxindole alkaloids on the dried basis

CONTAMINANTS

• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*. The total aerobic microbial count does not exceed 10^4/g, and the total combined molds and yeasts count does not exceed 10^3/g.

• **OTHER REQUIREMENTS:** It meets the requirements for *Botanical Extracts* ⟨565⟩, *Residual Solvents* and *Pesticide Residues*.

SPECIFIC TESTS

• **LOSS ON DRYING** ⟨731⟩**:** Dry 1 g at 105° for 2 h: it loses NMT 10.0% of its weight.

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ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. The label also indicates the content of pentacyclic oxindole alkaloids, the extracting solvent or solvent mixture used for preparation, and the ratio of the starting crude plant material to Powdered Extract. It meets the requirements for *Botanical Extracts* ⟨565⟩, *Labeling*.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Isopteropodine RS
  USP Powdered Cat's Claw Extract RS

Cat's Claw Capsules

DEFINITION

Cat's Claw Capsules contain Powdered Cat's Claw Extract. Capsules contain NLT 90.0% and NMT 110.0% of the labeled amount of Powdered Extract, calculated as pentacyclic oxindole alkaloids.

IDENTIFICATION

- The *Sample solution* chromatogram exhibits peaks for speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine, and isopteropodine at retention times that correspond to those in *Standard solution A*, as obtained in the test for *Content of Pentacyclic Oxindole Alkaloids and Limit of Tetracyclic Oxindole Alkaloids*. The content of tetracyclic oxindole alkaloids, calculated as the sum of rhynchophylline and isorhynchophylline, is NMT 25% of the labeled amount of pentacyclic oxindole alkaloids.

STRENGTH

- **CONTENT OF PENTACYCLIC OXINDOLE ALKALOIDS AND LIMIT OF TETRACYCLIC OXINDOLE ALKALOIDS**

Solution A: Prepare a 10 mM pH 7.0 phosphate buffer by mixing 1 N sodium hydroxide, 1 M monobasic potassium phosphate, and water (3:5:492), and adjust to a pH of 7.0 ± 0.1 by adding more of either solution.
Solution B: Acetonitrile
Solution C: Methanol and glacial acetic acid (99:1)
Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)	Solution C (%)
0	65	35	0
17	65	35	0
25	50	50	0
30	50	50	0
31	0	0	100
36	0	0	100
39	65	35	0
49	65	35	0

Standard solution A: Dissolve an accurately weighed quantity of USP Powdered Cat's Claw Extract RS in methanol, shake for 1 min, and dilute with methanol to obtain a solution having a known concentration of about 0.5 mg/mL of the labeled amount of total oxindole alkaloids. Pass through a filter of 0.45-µm or finer pore size.

Standard solution B: 0.1 mg/mL of USP Isopteropodine RS in methanol. Pass through a nylon filter of 0.45-µm or finer pore size.

Sample solution: Accurately weigh the contents of not fewer than 20 Capsules and pulverize. Transfer an accurately weighed quantity of the powder, equivalent to 20 mg of the labeled amount of pentacyclic oxindole alkaloids, to a 50-mL centrifuge tube. Sonicate with 10 mL of methanol for 10 min. Centrifuge and transfer this solution to a 50-mL volumetric flask. Repeat the above extraction three more times, combining the extracts in the 50-mL volumetric flask, and dilute with methanol to volume. Transfer 3 mL of the solution to a test tube containing 300 mg of polyamide powder, and shake for 1 min. Pass through a nylon filter of 0.45-µm or finer pore size, and discard the first part of the filtrate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 245 nm
Column: 4.6-mm × 10-cm; endcapped 3-µm packing L1
Flow rate: 0.75 mL/min
Injection size: 10 µL

System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram obtained using *Standard solution A* is similar to the Reference Chromatogram provided with the USP Powdered Cat's Claw Extract RS being used.
Tailing factor: NMT 2.0 for the isopteropodine peak, *Standard solution B*
Relative standard deviation: NMT 2.0% from the isopteropodine peak in repeated injections, *Standard solution B*

Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Measure the areas of the analyte peaks. Identify the retention times of the peaks corresponding to speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine, isopteropodine, rhynchophylline, and isorhynchophylline by comparison of the chromatogram of *Standard solution A* with the Reference Chromatogram provided with the lot of the USP Powdered Cat's Claw Extract RS being used.
Calculate the content, in mg, of speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine, and isopteropodine, as isopteropodine, in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times C_S \times V$$

r_U = peak response for each relevant alkaloid from the *Sample solution*
r_S = peak response for isopteropodine from *Standard solution B*
C_S = concentration of USP Isopteropodine RS in *Standard solution B* (mg/mL)
V = final dilution volume of the *Sample solution* (mL)

Calculate the content, in mg, of total pentacyclic oxindole alkaloids (C_C) in the portion of Capsules taken by adding the individual contents of speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine, and isopteropodine.
Calculate the percentage of Powdered Cat's Claw Extract with respect to the label claim:

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$$\text{Result} = C_C \times (A_{WC}/W) \times (100/L_E) \times (100/L)$$

C_C = content of total pentacyclic oxindole alkaloids in the portion of Capsules taken (mg)
A_{WC} = average weight of Capsules contents (mg/Capsule)
W = weight of the portion of Capsules taken (mg)
L_E = content of total pentacyclic oxindole alkaloids, mg, in 100 mg of the Extract used to prepare the Capsules
L = amount of Extract per Capsule according to label claim (mg/Capsule)

Calculate the percentage of tetracyclic oxindole alkaloids with respect to the content of pentacyclic oxindole alkaloids in the portion of Capsules taken:

$$\text{Result} = (r_T/r_P) \times 100$$

r_T = sum of peak responses for rhynchophylline and isorhynchophylline in the chromatogram of the *Sample solution*
r_P = sum of peak responses for speciophylline, uncarine F, mitraphylline, isomitraphylline, petoropodine and isoperopodine in the chromatogram of the *Sample solution*

Acceptance criteria: 90.0%–110.0% of the labeled amount of Powdered Extract calculated as pentacyclic oxindole alkaloids; and NMT 25% of tetracyclic oxindole alkaloids with respect to the labeled amount of pentacyclic oxindole alkaloids is found.

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** ⟨2040⟩**:** Meets the requirements for *Disintegration*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩**:** Meets the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the article from which the Capsules were prepared. If prepared with Extract, the label also indicates the quantity, in mg, of Extract per Capsule and the content, in mg, of pentacyclic oxindole alkaloids per 100 mg of Powdered Extract.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Isopteropodine RS
  USP Powdered Cat's Claw Extract RS

Cat's Claw Tablets

DEFINITION

Cat's Claw Tablets contain Powdered Cat's Claw Extract. Tablets contain NLT 90.0% and NMT 110.0% of the labeled amount of Powdered Extract, calculated as pentacyclic oxindole alkaloids.

IDENTIFICATION

- The *Sample solution* chromatogram exhibits peaks for speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine, and isopteropodine at retention times that correspond to those in *Standard solution A*, as obtained in the test for *Content of Pentacyclic Oxindole Alkaloids and Limit of Tetracyclic Oxindole Alkaloids*. The content of tetracyclic oxindole alkaloids, calculated as the sum of rhynchophylline and isorhynchophylline, is NMT 25% of the labeled amount of pentacyclic oxindole alkaloids.

STRENGTH

- **CONTENT OF PENTACYCLIC OXINDOLE ALKALOIDS AND LIMIT OF TETRACYCLIC OXINDOLE ALKALOIDS**

Solution A: Prepare a 10 mM pH 7.0 phosphate buffer by mixing 1 N sodium hydroxide, 1 M monobasic potassium phosphate, and water (3:5:492), and adjust to a pH of 7.0 ± 0.1 by adding more of either solution.
Solution B: Acetonitrile
Solution C: Methanol and glacial acetic acid (99:1)
Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)	Solution C (%)
0	65	35	0
17	65	35	0
25	50	50	0
30	50	50	0
31	0	0	100
36	0	0	100
39	65	35	0
49	65	35	0

Standard solution A: Dissolve an accurately weighed quantity of USP Powdered Cat's Claw Extract RS in methanol, shake for 1 min, and dilute with methanol to obtain a solution having a known concentration of about 0.5 mg/mL of the labeled amount of total oxindole alkaloids. Pass through a filter of 0.45-µm or finer pore size.
Standard solution B: 0.1 mg/mL of USP Isopteropodine RS in methanol. Pass through a nylon filter of 0.45-µm or finer pore size.
Sample solution: Accurately weigh not fewer than 20 Tablets and pulverize. Transfer an accurately weighed quantity of the powder, equivalent to 20 mg of the labeled amount of pentacyclic oxindole alkaloids, to a 50-mL centrifuge tube. Sonicate with 10 mL of methanol for 10 min. Centrifuge and transfer this solution to a 50-mL volumetric flask. Repeat the above extraction three more times, combining the extracts in the 50-mL volumetric flask, and dilute with methanol to volume. Transfer 3 mL of the solution to a test tube containing 300 mg of polyamide powder, and shake for 1 min. Pass through a nylon filter of 0.45-µm or finer pore size, and discard the first part of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 245 nm
Column: 4.6-mm × 10-cm; endcapped 3-µm packing L1
Flow rate: 0.75 mL/min
Injection size: 10 µL

DS Monographs

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System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram obtained using *Standard solution A* is similar to the Reference Chromatogram provided with the USP Powdered Cat's Claw Extract RS being used.
Tailing factor: NMT 2.0 for the isopteropodine peak, *Standard solution B*
Relative standard deviation: NMT 2.0% from the isopteropodine peak in repeated injections, *Standard solution B*
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Measure the areas of the analyte peaks. Identify the retention times of the peaks corresponding to speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine, isopteropodine, rhynchophylline, and isorhynchophylline by comparison of the chromatogram of *Standard solution A* with the Reference Chromatogram provided with the lot of the USP Powdered Cat's Claw Extract RS being used.
Calculate the content, in mg, of speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine, and isopteropodine, as isopteropodine, in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times C_S \times V$$

r_U = peak response for each relevant alkaloid from the *Sample solution*
r_S = peak response for isopteropodine from *Standard solution B*
C_S = concentration of USP Isopteropodine RS in *Standard solution B* (mg/mL)
V = final dilution volume of the *Sample solution* (mL)

Calculate the content, in mg, of total pentacyclic oxindole alkaloids (C_T) in the portion of Tablets taken by adding the individual contents of speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine, and isopteropodine.
Calculate the percentage of Powdered Cat's Claw Extract with respect to the label claim:

$$\text{Result} = C_T \times (A_{WT}/W) \times (100/L_E) \times (100/L)$$

C_T = content of total pentacyclic oxindole alkaloids in the portion of Tablets taken (mg)
A_{WT} = average weight of Tablets (mg/Tablet)
W = weight of the portion of Tablets taken (mg)
L_E = content of total pentacyclic oxindole alkaloids, mg, in 100 mg of the Extract used to prepare the Tablets
L = amount of Extract per Tablet according to label claim (mg/Tablet)

Calculate the percentage of tetracyclic oxindole alkaloids with respect to the content of pentacyclic oxindole alkaloids in the portion of Tablets taken:

$$\text{Result} = (r_T/r_P) \times 100$$

r_T = sum of peak responses for rhynchophylline and isorhynchophylline in the chromatogram of the *Sample solution*
r_P = sum of peak responses for speciophylline, uncarine F, mitraphylline, isomitraphylline, petoropodine and isoperopodine in the chromatogram of the *Sample solution*

Acceptance criteria: 90.0%–110.0% of the labeled amount of Powdered Extract calculated as pentacyclic oxindole alkaloids; and NMT 25% of tetracyclic oxindole alkaloids with respect to the labeled amount of pentacyclic oxindole alkaloids is found.

PERFORMANCE TESTS
- **Disintegration and Dissolution of Dietary Supplements** ⟨2040⟩: Meets the requirements for *Disintegration*
- **Weight Variation of Dietary Supplements** ⟨2091⟩: Meets the requirements

CONTAMINANTS
- **Microbial Enumeration Tests** ⟨2021⟩: The total aerobic microbial count does not exceed 10^4 cfu/g. The total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **Absence of Specified Microorganisms** ⟨2022⟩: Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

ADDITIONAL REQUIREMENTS
- **Packaging and Storage:** Preserve in tight, light-resistant containers, and store at room temperature.
- **Labeling:** The label states the Latin binomial and, following the official name, the article from which Tablets were prepared. The label also indicates the quantity of Powdered Extract per Tablet, in mg. Label Tablets to indicate the content, in mg, of pentacyclic oxindole alkaloids per 100 mg of Powdered Extract.
- **USP Reference Standards** ⟨11⟩
  USP Isopteropodine RS
  USP Powdered Cat's Claw Extract RS

Centella asiatica

DEFINITION
Centella asiatica consists of the dried aerial parts of *Centella asiatica* (L.) Urb. [Syn: *Hydrocotyle asiatica* L.] (Fam. Apiaceae). It is also known in commerce as gotu kola. It contains NLT 2.0% of triterpene derivatives, calculated on the dried basis.

IDENTIFICATION
- **A.** *Centella asiatica* meets the requirements for *Specific Tests, Botanical Characteristics*.
- **B. Thin-Layer Chromatography**
  Standard solution A: 0.5 mg/mL of USP Asiaticoside RS in methanol
  Standard solution B: 10 mg/mL of USP Powdered Centella asiatica Extract RS in methanol. Sonicate for about 10 min, centrifuge, and use the supernatant.
  Sample solution: About 0.5 g of *Centella asiatica*, finely powdered, in 5 mL of methanol. Sonicate for 10 min, centrifuge, and use the supernatant.
  Adsorbent: Chromatographic silica gel with an average particle size of 10–15 µm (TLC plates) or with an average particle size of 5 µm (HPTLC plates)
  Application volume: 10 µL (TLC plates) or 4 µL (HPTLC plates)
  Developing solvent system: Methylene chloride, methanol, and water (14:6:1)
  Derivatization reagent: A solution of 10% sulfuric acid in methanol. [Note—Prepare fresh.]

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Analysis

Samples: *Standard solution A, Standard solution B,* and *Sample solution*

Apply the *Samples* as bands. Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about three-fourths of the plate. Remove the plate from the chamber, dry, treat with *Derivatization reagent*, heat for 3 min at 120°, and examine under white light.

Acceptance criteria: The *Sample solution* chromatogram exhibits a violet band in the lower third of the plate due to asiaticoside, corresponding in color and R_F to that in *Standard solution A*; a violet band due to madecassoside at an R_F lower than that of asiaticoside; and two additional violet bands in the upper third of the plate due to asiatic acid and madecassic acid. Bands detected in the *Sample solution* correspond in position and color to bands in *Standard solution B*. Other minor bands may be observed in the *Sample solution* and *Standard solution B*.

• **C. HPLC:** The *Sample solution* chromatogram from the test for *Content of Triterpene Derivatives* shows a peak at the retention time corresponding to that of asiaticoside in *Standard solution A*. Identify other triterpene derivative peaks in the *Sample solution* by comparison with the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Centella asiatica Extract RS being used. The *Sample solution* shows additional peaks corresponding to madecassoside and asiaticoside B (these two peaks may co-elute), madecassic acid, terminolic acid, and asiatic acid.

COMPOSITION

• **CONTENT OF TRITERPENE DERIVATIVES**

Solution A: Dilute 3 mL of phosphoric acid with water to 1000 mL, mix, filter, and degas.

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	78	22
65	45	55
66	5	95
75	5	95
76	78	22
85	78	22

Standard solution A: 0.05 mg/mL of USP Asiaticoside RS in methanol

Standard solution B: Sonicate a portion of USP Powdered Centella asiatica Extract RS in methanol to obtain a solution with a concentration of about 5.0 mg/mL. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.

Sample stock solution: Transfer about 1.0 g of *Centella asiatica*, finely powdered and accurately weighed, to a Soxhlet apparatus. Add 100 mL of methanol, extract for 8 h, cool, and dilute with methanol to 100 mL. Pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate. [NOTE—Use a thimble of a suitable size such that the volume of methanol used in the Soxhlet extraction is at least twice the volume of the thimble.]

Sample solution: Dilute 5.0 mL of *Sample stock solution* with methanol to 10.0 mL.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 200 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Flow rate: 1.0 mL/min

Injection volume: 10 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Centella asiatica Extract RS being used.

Tailing factor: Between 0.8 and 2.0 for the asiaticoside peak, *Standard solution A*

Resolution: NLT 1.5 between the madecassic acid and terminolic acid peaks, *Standard solution B*

Relative standard deviation: NMT 2.0% determined from the asiaticoside peak in replicate injections, *Standard solution A*

Analysis

Samples: *Standard solution A, Standard solution B,* and *Sample solution*. [NOTE—*Standard solution A, Standard solution B,* and the *Sample solution* are stable for 48 h at room temperature.]

Using the chromatograms of *Standard solution A, Standard solution B,* and the reference chromatogram provided with the lot of USP Powdered Centella asiatica Extract RS being used, identify the retention times of the peaks corresponding to different triterpene derivatives. The approximate relative retention times of the different triterpene derivatives are provided in *Table 2*.

Table 2

Analyte	Approximate Relative Retention Time
Madecassoside	0.71
Asiaticoside B	0.72
Asiaticoside	1.00
Madecassic acid	2.40
Terminolic acid	2.44
Asiatic acid	3.12

Separately calculate the percentages of the sum of madecassoside and asiaticoside B (these two peaks may co-elute), asiaticoside, the sum of madecassic acid and terminolic acid, and asiatic acid in the portion of *Centella asiatica* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times F \times 100$$

r_U = peak areas of the triterpene derivatives from the *Sample solution*

r_S = peak area of asiaticoside from *Standard solution A*

C_S = concentration of USP Asiaticoside RS in *Standard solution A* (mg/mL)

V = final volume of *Sample stock solution* (mL)

W = weight of *Centella asiatica* used to prepare *Sample stock solution* (mg)

D = dilution factor to prepare the *Sample solution* from the *Sample stock solution*

F = conversion factors for analytes: 1.00 for asiaticoside, 1.017 for the sum of madecassoside and asiaticoside B, 0.526 for the sum of

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madecassic acid and terminolic acid, and 0.509 for asiatic acid

Acceptance criteria: Add the percentages of different triterpene derivatives: NLT 2.0% on the dried basis.

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined yeasts and molds count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: Stem is slender, yellowish-brown, with long internodes, rooting at nodes; leaves are grayish-green, simple, alternate or grouped together at the nodes, reniform or oblong-elliptic, have palmate venation, usually with 7 veins, apex obtuse, margin crenate, base cordate, variable in size, 1–4 cm long, 2–4 cm and sometimes up to 7 cm wide, young leaves show a few trichomes on the lower surface and adult leaves are glabrous; petioles are long, grooved, base wider and sheathing; the inflorescence, if present, is a single umbel and consists of 3 flowers, rarely 2 or 4; the flowers are very small (about 2 mm), pentamerous, and have an inferior ovary; the fruit, brownish-gray, orbicular cremocarp, up to 5 mm long, is very flattened laterally and has 7–9 prominent curved ridges. Pharmacopeial article is green to yellowish-green masses composed mostly of leaves and stem fragments; odor slight; taste slightly bitter to sweet.

Microscopic

Transverse section of stems: Epidermal layer, subrounded or subsquare cells; 2–4 layers of collenchyma cells; 6–8 layers of thin-walled parenchyma cells with intercellular spaces; 6–7 collateral vascular bundles, xylem vessels radially arranged, slightly lignified fiber groups occurring outside the phloem; pith large, composed of thin-walled parenchyma cells; secretory canals, composed of 5–7 secretory cells, observed in cortex and medullary rays

Transverse section of leaves: Upper and lower epidermis; mesophyll composed of parenchyma cells, some contain crystals of calcium oxalate; 2–3 layers of collenchyma present in the midrib region next to both epidermal layers; vascular bundles in the center with xylem on the ventral side and phloem on the dorsal side. Transverse section of petioles has a U shape, showing an upper and a lower epidermis, followed by 2–3 layers of collenchyma next to both epidermal layers; a broad parenchymatous zone, some cells contain crystals of calcium oxalate; 7 vascular bundles forming a U-shape in the parenchymatous zone, the two present in the projecting arms being less developed.

- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩**:** NMT 7.0%, of which NMT 5.0% are of underground organs and NMT 2% are of other foreign matter
- **LOSS ON DRYING** ⟨731⟩

Sample: 1.0 g of finely powdered *Centella asiatica*
Analysis: Dry the *Sample* at 105° for 2 h.
Acceptance criteria: NMT 12.0%

- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩

Sample: 1.0 g of finely powdered *Centella asiatica*
Acceptance criteria: NMT 12%

- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 3.5%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article. The label states that this article is exempted from the requirements of the *Labeling* ⟨7⟩, *Labels and Labeling for Products and Other Categories, Botanicals,* with respect to the pregnancy and lactation statement.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Asiaticoside RS
USP Powdered Centella asiatica Extract RS

Powdered *Centella asiatica*

DEFINITION

Powdered *Centella asiatica* is *Centella asiatica* reduced to a powder or very fine powder. It contains NLT 2.0% of triterpene derivatives, calculated on the dried basis.

IDENTIFICATION

- **A.** Powdered *Centella asiatica* meets the requirements for *Specific Tests, Botanical Characteristics.*
- **B. THIN-LAYER CHROMATOGRAPHY**

Standard solution A: 0.5 mg/mL of USP Asiaticoside RS in methanol
Standard solution B: 10 mg/mL of USP Powdered Centella asiatica Extract RS in methanol. Sonicate for about 10 min, centrifuge, and use the supernatant.
Sample solution: About 0.5 g of Powdered *Centella asiatica* in 5 mL of methanol. Sonicate for 10 min, centrifuge, and use the supernatant.
Adsorbent: Chromatographic silica gel with an average particle size of 10–15 µm (TLC plates) or with an average particle size of 5 µm (HPTLC plates)
Application volume: 10 µL (TLC plates) or 4 µL (HPTLC plates)
Developing solvent system: Methylene chloride, methanol, and water (14:6:1)
Derivatization reagent: A solution of 10% sulfuric acid in methanol. [NOTE—Prepare fresh.]
Analysis
Samples: *Standard solution A, Standard solution B,* and *Sample solution*
Apply the *Samples* as bands. Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about three-fourths of the plate. Remove the plate from the chamber, dry, treat with *Derivatization reagent,* heat for 3 min at 120°, and examine under white light.
Acceptance criteria: The *Sample solution* chromatogram exhibits a violet band in the lower third of the plate due to asiaticoside, corresponding in color and R_F to that in *Standard solution A;* a violet band due to madecassoside at an R_F lower than that of asiaticoside; and two additional violet bands in the upper third of the plate due to asiatic acid and madecassic acid. Bands detected in the *Sample solution* correspond in position and color to bands in *Standard solution B.* Other minor bands may be observed in the *Sample solution* and *Standard solution B.*

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• **C. HPLC:** The *Sample solution* chromatogram from the test for *Content of Triterpene Derivatives* shows a peak at the retention time corresponding to that of asiaticoside in *Standard solution A*. Identify other triterpene derivative peaks in the *Sample solution* by comparison with the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Centella asiatica Extract RS being used. The *Sample solution* shows additional peaks corresponding to madecassoside and asiaticoside B (these two peaks may co-elute), madecassic acid, terminolic acid, and asiatic acid.

COMPOSITION

• **CONTENT OF TRITERPENE DERIVATIVES**

Solution A: Dilute 3 mL of phosphoric acid with water to 1000 mL, mix, filter, and degas.

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	78	22
65	45	55
66	5	95
75	5	95
76	78	22
85	78	22

Standard solution A: 0.05 mg/mL of USP Asiaticoside RS in methanol

Standard solution B: Sonicate a portion of USP Powdered Centella asiatica Extract RS in methanol to obtain a solution with a concentration of about 5.0 mg/mL. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.

Sample stock solution: Transfer about 1.0 g of Powdered *Centella asiatica*, accurately weighed, to a Soxhlet apparatus. Add 100 mL of methanol, extract for 8 h, cool, and dilute with methanol to 100 mL. Pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate. [NOTE—Use a thimble of a suitable size such that the volume of methanol used in the Soxhlet extraction is at least twice the volume of the thimble.]

Sample solution: Dilute 5.0 mL of *Sample stock solution* with methanol to 10.0 mL.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 200 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Flow rate: 1.0 mL/min

Injection volume: 10 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Centella asiatica Extract RS being used.

Tailing factor: Between 0.8 and 2.0 for the asiaticoside peak, *Standard solution A*

Resolution: NLT 1.5 between the madecassic acid and terminolic acid peaks, *Standard solution B*

Relative standard deviation: NMT 2.0% determined from the asiaticoside peak in replicate injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*. [NOTE—*Standard solution A*, *Standard solution B*, and *Sample solution* are stable for 48 h at room temperature.]

Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Centella asiatica Extract RS being used, identify the retention times of the peaks corresponding to different triterpene derivatives. The approximate relative retention times of the different triterpene derivatives are provided in *Table 2*.

Table 2

Analyte	Approximate Relative Retention Time
Madecassoside	0.71
Asiaticoside B	0.72
Asiaticoside	1.00
Madecassic acid	2.40
Terminolic acid	2.44
Asiatic acid	3.12

Separately calculate the percentages of the sum of madecassoside and asiaticoside B (these two peaks may co-elute), asiaticoside, the sum of madecassic acid and terminolic acid, and asiatic acid in the portion of Powdered *Centella asiatica* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times F \times 100$$

r_U = peak areas of the triterpene derivatives from the *Sample solution*

r_S = peak area of asiaticoside from *Standard solution A*

C_S = concentration of USP Asiaticoside RS in *Standard solution A* (mg/mL)

V = final volume of *Sample stock solution* (mL)

W = weight of Powdered *Centella asiatica* used to prepare the *Sample stock solution* (mg)

D = dilution factor to prepare the *Sample solution* from the *Sample stock solution*

F = conversion factors for analytes: 1.00 for asiaticoside, 1.017 for the sum of madecassoside and asiaticoside B, 0.526 for the sum of madecassic acid and terminolic acid, and 0.509 for asiatic acid

Acceptance criteria: Add the percentages of different triterpene derivatives: NLT 2.0% on the dried basis.

CONTAMINANTS

• **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements

• **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements

• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined yeast and mold count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria count does not exceed 10^3 cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

DS Monographs

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SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS:** Greenish-gray to greenish-brown in color; odor slight; taste slightly bitter to sweet; under a microscope, factions of epidermal cells of the leaves with irregular striated cuticle, showing anisocytic, some paracytic, and rarely anomocytic stomata; epidermal cells of young leaves showing unicellular, occasionally multicellular, non-glandular trichomes; secretory canals composed of 5–7 secretory cells; parenchyma cells, some showing prisms or rosettes of calcium oxalate; bundles of narrow septate fibers from the stem; spiral vessels; fragments of the fruits, layers of wide cells in a parquetry arrangement, annular vessels, parenchyma cells containing simple or compound starch granules
- **LOSS ON DRYING** ⟨731⟩
  Sample: 1.0 g of Powdered *Centella asiatica*
  Analysis: Dry the *Sample* at 105° for 2 h.
  Acceptance criteria: NMT 12.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩
  Sample: 1.0 g of Powdered *Centella asiatica*
  Analysis: NMT 12%
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 3.5%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article. The label states that this article is exempted from the requirements of the *Labeling* ⟨7⟩, *Labels and Labeling for Products and Other Categories, Botanicals,* with respect to the pregnancy and lactation statement.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Asiaticoside RS
  USP Powdered Centella asiatica Extract RS

Powdered *Centella asiatica* Extract

DEFINITION

Powdered *Centella asiatica* Extract is prepared from *Centella asiatica* by extraction with alcohol, methanol, acetone, or a mixture of these solvents. The ratio of plant material to extract is between 65:1 and 30:1. It contains NLT 90.0% and NMT 110.0% of the labeled amount of triterpene derivatives; the labeled amount of triterpene derivatives is NMT 40%, calculated on the dried basis as the sum of madecassoside, asiaticoside B, asiaticoside, madecassic acid, terminolic acid, and asiatic acid. It may contain suitable added substances as carriers.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**
  Standard solution A: 0.5 mg/mL of USP Asiaticoside RS in methanol
  Standard solution B: 10 mg/mL of USP Powdered Centella asiatica Extract RS in methanol. Sonicate for about 10 min, centrifuge, and use the supernatant.
  Sample solution: Transfer an amount of Powdered *Centella asiatica* Extract equivalent to about 5 mg of triterpene derivatives to a centrifuge tube. Add 5 mL of methanol, sonicate for 10 min, centrifuge, and use the supernatant.
  Adsorbent: Chromatographic silica gel with an average particle size of 10–15 µm (TLC plates) or with an average particle size of 5 µm (HPTLC plates)
  Application volume: 10 µL (TLC plates) or 4 µL (HPTLC plates)
  Developing solvent system: A mixture of methylene chloride, methanol, and water (14:6:1)
  Spray reagent: A solution of 10% sulfuric acid in methanol. [NOTE—Prepare fresh.]
  Analysis
  Samples: *Standard solution A, Standard solution B,* and *Sample solution*
  Apply the samples as bands to a suitable thin-layer chromatographic plate (see *Chromatography* ⟨621⟩). Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about three-fourths of the plate. Remove the plate from the chamber, dry, spray with the *Spray reagent,* heat for 3 min at 120°, and examine under visible light.
  Acceptance criteria: The *Sample solution* chromatogram exhibits a violet band in the lower third of the plate due to asiaticoside, corresponding in color and R_F to that in *Standard solution A*; a violet band due to madecassoside at an R_F lower than that of asiaticoside; and two additional violet bands in the upper third of the plate due to asiatic acid and madecassic acid. Bands detected in the *Sample solution* correspond in position and color to bands in *Standard solution B.* Other minor bands may be observed in the *Sample solution* and *Standard solution B.*
- **B. HPLC IDENTIFICATION TEST:** The *Sample solution* chromatogram from the test for *Content of Triterpene Derivatives* shows a peak at the retention time corresponding to that of asiaticoside in *Standard solution A.* Identify other triterpene derivative peaks in the *Sample solution* by comparison with the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Centella asiatica Extract RS being used. The *Sample solution* shows additional peaks corresponding to madecassoside and asiaticoside B (these two peaks may co-elute), madecassic acid, terminolic acid, and asiatic acid.

COMPOSITION

- **CONTENT OF TRITERPENE DERIVATIVES**
  Solution A: Dilute 3 mL of phosphoric acid with water to 1000 mL, mix, filter, and degas.
  Solution B: Acetonitrile
  Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	78	22
65	45	55
66	5	95
75	5	95
76	78	22
85	78	22

Standard solution A: 0.1 mg/mL of USP Asiaticoside RS in methanol
Standard solution B: Sonicate a portion of USP Powdered Centella asiatica Extract RS in methanol to obtain a solution with a concentration of about 5.0 mg/mL. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.
Sample solution: Sonicate a portion of Powdered *Centella asiatica* Extract in methanol to obtain a solution with a concentration of about 5.0 mg/mL. Before injection, pass

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through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 200 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Flow rate: 1.0 mL/min
Injection size: 10 µL

System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Centella asiatica Extract RS being used.
Tailing factor: Between 0.8 and 2.0 for the asiaticoside peak, *Standard solution A*
Resolution: NLT 1.5 between the madecassic acid and terminolic acid peaks, *Standard solution B*
Relative standard deviation: NMT 2.0% determined from the asiaticoside peak in repeated injections, *Standard solution A*

Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*. [NOTE—*Standard solution A*, *Standard solution B*, and the *Sample solution* are stable for 48 h at room temperature.]
Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Centella asiatica Extract RS being used, identify the retention times of the peaks corresponding to different triterpene derivatives. The approximate relative retention times of the different triterpene derivatives are provided in the following table.

Analyte	Approximate Relative Retention Time
Madecassoside	0.71
Asiaticoside B	0.72
Asiaticoside	1.00
Madecassic acid	2.40
Terminolic acid	2.44
Asiatic acid	3.12

Separately calculate the percentages of the sum of madecassoside and asiaticoside B (these two peaks may co-elute), asiaticoside, the sum of madecassic acid and terminolic acid, and asiatic acid in the portion of Powdered *Centella asiatica* Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak response(s) of the triterpene derivative(s) from the *Sample solution*
r_S = peak response of asiaticoside from *Standard solution A*
C_S = concentration of USP Asiaticoside RS in *Standard solution A* (mg/mL)
C_U = concentration of Powdered *Centella asiatica* Extract in the *Sample solution* (mg/mL)
F = conversion factors for analytes: 1.00 for asiaticoside, 1.017 for the sum of madecassoside and asiaticoside B, 0.526 for the sum of madecassic acid and terminolic acid, and 0.509 for asiatic acid

Acceptance criteria: Add the percentages of different triterpene derivatives: NLT 90.0% and NMT 110.0% of the labeled amount of triterpene derivatives; the labeled amount of triterpene derivatives is NMT 40%, calculated on the dried basis.

CONTAMINANTS

Change to read:

- **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020) **:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^4 cfu per g. The total combined yeast and mold count does not exceed 10^3 cfu per g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS
- **LOSS ON DRYING** ⟨731⟩**:** Dry 1.0 g of Powdered *Centella asiatica* Extract at 105° for 2 h: it loses NMT 5% of its weight.
- **OTHER REQUIREMENTS:** Meets the requirements of the test for *Residual Solvents* under *Botanical Extracts* ⟨565⟩

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived. It meets other labeling requirements under *Botanical Extracts* ⟨565⟩.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Asiaticoside RS
  USP Powdered Centella asiatica Extract RS

Centella asiatica Triterpenes

DEFINITION
Centella asiatica Triterpenes is a fraction enriched in *Centella asiatica* triterpenes derivatives. It is prepared from *Centella asiatica* Extract using suitable solvents or other means. It contains NLT 90.0% of triterpene derivatives, calculated on the anhydrous basis, as the sum of two or more of the following: madecassoside, asiaticoside B, asiaticoside, madecassic acid, terminolic acid, and asiatic acid.

IDENTIFICATION
- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution A: 0.5 mg/mL of USP Asiaticoside RS in methanol
Standard solution B: 10 mg/mL of USP Powdered Centella asiatica Extract RS in methanol. Sonicate for about 10 min, centrifuge, and use the supernatant.
Sample solution: Transfer an amount of *Centella asiatica* Triterpenes, equivalent to about 5 mg of triterpene derivatives, to a centrifuge tube. Add 5 mL of methanol, sonicate for 10 min, centrifuge, and use the supernatant.
Adsorbent: Chromatographic silica gel with an average particle size of 10–15 µm (TLC plates) or with an average particle size of 5 µm (HPTLC plates)
Application volume: 10 µL (TLC plates) or 4 µL (HPTLC plates)

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Developing solvent system: A mixture of methylene chloride, methanol, and water (14:6:1)
Spray reagent: A solution of 10% sulfuric acid in methanol. [NOTE—Prepare fresh.]
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Apply the samples as bands to a suitable thin-layer chromatographic plate (see *Chromatography* ⟨621⟩). Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about three-fourths of the plate. Remove the plate from the chamber, dry, spray with *Spray reagent*, heat for 3 min at 120°, and examine under visible light.
Acceptance criteria: The *Sample solution* chromatogram exhibits a violet band in the lower third of the plate due to asiaticoside, corresponding in color and R_F to that in *Standard solution A*. The *Sample solution* shows additional bands corresponding to some or all of the following triterpene derivatives: a violet band due to madecassoside at an R_F lower than that of asiaticoside, a violet band in the upper third of the plate due to asiatic acid, and a violet band due to madecassic acid at an R_F lower than that of asiatic acid. Bands detected in the *Sample solution* correspond in position and color to bands in *Standard solution B*. Other minor bands may be observed in the *Sample solution* and *Standard solution B*.

• **B. HPLC IDENTIFICATION TEST:** The *Sample solution* chromatogram from the test for *Content of Triterpene Derivatives* shows a peak at the retention time corresponding to that of asiaticoside in *Standard solution A*. Identify other triterpene derivative peaks in the *Sample solution* by comparison with the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Centella asiatica Extract RS being used. The *Sample solution* shows additional peaks corresponding to some or all of the following: madecassoside and asiaticoside B (these two peaks may co-elute), madecassic acid, terminolic acid, and asiatic acid.

COMPOSITION

• **CONTENT OF TRITERPENE DERIVATIVES**
Solution A: Dilute 3 mL of phosphoric acid with water to 1000 mL, mix, filter, and degas.
Solution B: Acetonitrile
Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	78	22
65	45	55
66	5	95
75	5	95
76	78	22
85	78	22

Standard solution A: 0.2 mg/mL of USP Asiaticoside RS in methanol
Standard solution B: Sonicate a portion of USP Powdered Centella asiatica Extract RS in methanol to obtain a solution with a concentration of about 5.0 mg/mL. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.
Sample solution: About 1.0 mg/mL of *Centella asiatica* Triterpenes in methanol; sonicate if necessary. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 200 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Flow rate: 1.0 mL/min
Injection size: 10 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Centella asiatica Extract RS being used.
Tailing factor: Between 0.8 and 2.0 for the asiaticoside peak, *Standard solution A*
Resolution: NLT 1.5 between the madecassic acid and terminolic acid peaks, *Standard solution B*
Relative standard deviation: NMT 2.0% determined from the asiaticoside peak in repeated injections, *Standard solution A*
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*. [NOTE—*Standard solution A*, *Standard solution B*, and the *Sample solution* are stable for 48 h at room temperature.]
Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Centella asiatica Extract RS being used, identify the retention times of the peaks corresponding to different triterpene derivatives. The approximate relative retention times of the different triterpene derivatives are provided in the following table.

Analyte	Approximate Relative Retention Time
Madecassoside	0.71
Asiaticoside B	0.72
Asiaticoside	1.00
Madecassic acid	2.40
Terminolic acid	2.44
Asiatic acid	3.12

Separately calculate the percentages of the triterpene derivatives in the portion of *Centella asiatica* Triterpenes taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak response(s) of the triterpene derivative(s) from the *Sample solution*
r_S = peak response of asiaticoside from *Standard solution A*
C_S = concentration of USP Asiaticoside RS in *Standard solution A* (mg/mL)
C_U = concentration of *Centella asiatica* Triterpenes in the *Sample solution* (mg/mL)
F = conversion factors for analytes: 1.00 for asiaticoside, 1.017 for madecassoside, 1.017 for asiaticoside B, 0.526 for madecassic acid, 0.526 for terminolic acid, and 0.509 for asiatic acid

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Acceptance criteria: Add the percentages of different triterpene derivatives: NLT 90.0% on the anhydrous basis.

CONTAMINANTS

Change to read:

- ▲**BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020) **:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^3 cfu/g. The total combined yeast and mold count does not exceed 10^2 cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Escherichia coli*

SPECIFIC TESTS

- **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 5%
- **OTHER REQUIREMENTS:** Meets the requirements of the test for *Residual Solvents* under *Botanical Extracts* ⟨565⟩

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Asiaticoside RS
  USP Powdered Centella asiatica Extract RS

Chamomile

DEFINITION

Chamomile consists of the dried flower heads of *Matricaria recutita* L. (*Matricaria chamomilla* L., *Matricaria chamomilla* L. var. *courrantiana*, *Chamomilla recutita* L.) Rauschert (Fam. Asteraceae alt. Compositae). It contains NLT 0.4% of blue volatile oil, NLT 0.3% of apigenin-7-glucoside, and NLT 0.15% of bisabolol derivatives, calculated as levomenol.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution: 1.0 mg/mL of borneol, 2.0 mg/mL of bornyl acetate, and 0.4 mg/mL of guaiazulene in toluene

Sample solution: Reduce 1.0 g of Chamomile to a coarse powder, using a porcelain pestle and mortar. Transfer to a 1.5-cm × 15-cm chromatographic column, and tamp lightly with a short length of rubber hose. Rinse the pestle and mortar twice, each time with 10 mL of methylene chloride. Pour the rinsings into the column. Collect the percolate in a flask with a long, narrow neck, and remove the solvent by evaporation on a water bath. Dissolve the residue in 0.5 mL of toluene.

Adsorbent: 0.25-mm layer of chromatographic silica gel

Developing solvent: Chloroform

Spray reagent: Mix anisaldehyde, glacial acetic acid, and methanol (0.5: 10: 85). Then carefully add 5 mL of sulfuric acid to this solution.

Application volume: 10 µL, as 3-mm × 20-mm bands

Analysis

Samples: *Standard solution* and *Sample solution*

Examine the plate under short-wavelength UV light: the *Sample solution* exhibits a number of quenching areas, the largest of which is due to en-yne-dicycloether and has the same R_F value as the band due to bornyl acetate in the *Standard solution*. There is also a band due to matricin near the line of application. Spray the plate evenly with the *Spray reagent*. Examine the plate in daylight while heating at 100°–105° for 5–10 min. The chromatogram obtained from the *Standard solution* shows in the lower third a brownish yellow zone that becomes violet-gray after a few hours and is due to borneol; in the middle a yellowish brown to gray zone due to bornyl acetate; and in the upper third a deep red zone with a blue edge due to guaiazulene.

Acceptance criteria: The *Sample solution* exhibits a blue zone due to matricin near the starting point; several violet-red zones, one of which is due to bisabolol, at R_F values between those of borneol and bornyl acetate; a brownish zone, due to en-yne-dicycloether, at an R_F value corresponding to that of bornyl acetate; red zones, due to terpenes, at R_F values similar to those of guaiazulene; and other zones that appear in the middle and lower parts of the chromatogram.

- **B.**

Analysis: Dissolve 0.25 g of dimethylaminobenzaldehyde in a mixture of 5 mL of phosphoric acid, 45 mL of acetic acid, and 45 mL of water. Transfer 2.5 mL of this solution and 0.1 mL of the *Sample solution*, prepared as directed for *Identification* test *A*, to a test tube. Heat on a water bath for 2 min, and allow to cool. Add 5 mL of solvent hexane, and shake.

Acceptance criteria: The aqueous layer has a distinct greenish blue or blue color.

COMPOSITION

- **CONTENT OF APIGENIN-7-GLUCOSIDE**

Dilute phosphoric acid: Mix 5.0 mL of phosphoric acid in 50 mL of water. Dilute with water to 100 mL.

Solution A: 0.005 M solution of monobasic potassium phosphate. Adjust with *Dilute phosphoric acid* to a pH of 2.55 ± 0.05.

Solution B: Acetonitrile and methanol (13:7)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	74	26
3	74	26
22	15	85
27	74	26
30	74	26

Standard solution: 25.0 µg/mL of USP Apigenin-7-glucoside RS and 10.0 µg/mL of 7-methoxycoumarin in methanol and water (1:1)

Sample solution: Transfer 1.0 g of Chamomile to a suitable flask fitted with a reflux condenser and a stirrer. Add 80.0 mL of methanol, and reflux the mixture with stirring for 1 h. Cool the flask to room temperature, pass the extract through a folded filter paper, and collect the filtrate in a 100-mL volumetric flask. Rinse the flask with 3 mL of methanol, pour the methanolic rinsings through the filter paper, and add the filtrate to the volumetric flask. Dilute with methanol to volume, and mix. Transfer 25.0 mL of the filtered solution to a round-bottom flask fitted with a reflux condenser and a stirrer; add 5.0 mL of sodium hydroxide solution, prepared by dissolving 0.4 g of sodium hydroxide in 5.0 mL of water; and reflux the mixture for 25 min. Cool the flask, and adjust the solution with hydrochloric acid to a

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pH of 5.0–6.2. Quantitatively transfer the solution to a 50-mL volumetric flask, dilute with methanol to volume, and filter, discarding the first 10 mL of the filtrate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 335 nm
Column: 4-mm × 12.5-cm; packing L1
Flow rate: 1 mL/min
[NOTE—Make adjustments, if necessary, to obtain relative retention times of 0.63 for apigenin-7-glucoside and 1.0 for 7-methoxycoumarin.]
Injection size: 15 µL

System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for apigenin-7-glucoside, 7-methoxycoumarin, apigenin, *trans*-spiroether, and *cis*-spiroether are about 0.63, 1.0, 1.2, 1.6, and 1.8, respectively.]
Suitability requirements
Resolution: NLT 3.5 between apigenin-7-glucoside and 7-methoxycoumarin
Relative standard deviation: NMT 2.0% for apigenin-7-glucoside

Analysis
Samples: *Standard solution* and *Sample solution*
[NOTE—Allow the *Sample solution* to elute for NLT 6 times the retention time of apigenin-7-glucoside.]
Calculate the percentage of apigenin-7-glucoside in the portion of Chamomile taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak response of apigenin-7-glucoside from the *Sample solution*
r_S = peak response of apigenin-7-glucoside from the *Standard solution*
C_S = concentration of USP Apigenin-7-glucoside RS in the *Standard solution* (mg/mL)
V = volume of *Sample solution* (mL)
W = weight of Chamomile taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 0.3%

• **CONTENT OF BISABOLANE DERIVATIVES**
Standard solution: 1 mg/mL of USP Levomenol RS in cyclohexane
Sample solution: Transfer the volatile oils obtained in the test for *Articles of Botanical Origin* ⟨561⟩, *Volatile Oil Determination* to a 25-mL volumetric flask, rinse the graduated tube of the apparatus with a small portion of cyclohexane, transfer the rinsing to the 25-mL volumetric flask, add cyclohexane to volume, and mix.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.32-mm × 30-m fused-silica capillary; coated with a 0.25-µm film of phase G16
Temperature
Column: See *Table 2*.

Table 2

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	4	230	10

Detector: 250°
Injection port: 220°
Carrier gas: Helium
Flow rate: 1.0 mL/min
Injection size: 1 µL

System suitability
Sample: *Standard solution*
Suitability requirements
Tailing factor: NMT 1.8 for levomenol
Relative standard deviation: NMT 2.0%

Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas. Identify the peaks due to levomenol, bisabolol oxide B, bisabolol oxide, and bisabolol oxide A in the *Sample solution*, using the retention time of levomenol in the *Standard solution* and the approximate relative retention times of 0.89, 0.97, and 1.1 for bisabol oxide B, bisabol oxide, and bisabol oxide A, respectively, with reference to the levomenol peak.
Calculate the percentage of bisabolane derivatives in the portion of Chamomile taken:

$$\text{Result} = (r_T/r_S) \times C_S \times (V/W) \times 100$$

r_T = sum of the peak areas for bisabolol oxide B, bisabolol oxide, levomenol, and bisabolol oxide A from the *Sample solution*
r_S = levomenol peak area from the *Standard solution*
C_S = concentration of USP Levomenol RS in the *Standard solution* (mg/mL)
V = volume of *Sample solution* (mL)
W = weight of Chamomile taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 0.15%

• **ARTICLES OF BOTANICAL ORIGIN,** *Volatile Oil Determination* ⟨561⟩
Analysis: Proceed as directed, except use 60 g of coarsely powdered Chamomile as the test specimen, a 2-L round-bottom flask, 300 mL of water as distillation liquid, and 0.5 mL of xylene in the graduated tube. Distill for 4 h at a rate of 3–4 mL/min.
Acceptance criteria: NLT 0.4% of blue volatile oil is found.
[NOTE—Retain the volatile oils for use in the test for *Content of Bisabolane Derivatives*.]

CONTAMINANTS

• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacterial count does not exceed 10^3 cfu/g.
• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.
• **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residues* ⟨561⟩**:** Meets the requirements

SPECIFIC TESTS

• **BOTANIC CHARACTERISTICS**
Macroscopic: Flower head is hemispherical, about 6 mm in diameter, composed of a few ray florets and numerous disk florets (distinction from *Matricaria discoidea*, which has disk florets only), carried on a receptacle surrounded by an involucre. Involucre is green, formed of two to three rows of lanceolate, glabrous, and imbricated bracts with blunt apices and scarious whitish edges. Ray florets, which usually have fallen off, have 10–20 pistils; corolla is ligulate, white, but darkens at a length of 6 mm and a width of about

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2 mm, 3-toothed, and traversed by four main veins. Disk florets are yellow, perfect, about 2 mm in length; corolla is tubular with five teeth; five stamens are epipetalous and syngenesious. Receptacle is hollow (distinction from *Chrysanthemum* and *Anthemis* species), hemispherical in the young and conical in the old flower head, 3–10 mm in width, and lacking paleae. Achene is ovoid, and has three to five longitudinal ribs.

Microscopic: Separate the capitulum into its parts, and examine under a microscope. The outer, abaxial epidermis of the involucral bracts shows a scarious margin with a single layer of radially elongated cells and a central part made up of chlorophyll tissue covered by elongated epidermal cells with sinuous lateral walls, stomata, and secretory trichomes. The vascular bundles are surrounded by numerous elongated, pitted sclereids with fairly large lumens. In surface view, ligulate and tubular corollas show isodiametric or elongated cells with more or less wavy walls and a few glandular trichomes. The outer part of the epidermis of the ligulate florets consists of papillary cells with cuticular striations radiating from their tips. In the mesophyll, very small clusters of calcium oxalate are sometimes seen. Four main veins run lengthwise through the entire mesophyll, sometimes accompanied by one or two other veins, which are shorter and run parallel to the main veins. Each of the two main median veins split into two near the tip and, with the lateral veins, anastomose two by two to form three arcs at the three terminal teeth of the ligule. The ovaries, oval to spherical, of both kinds of florets have at their base a sclerous ring consisting of a single row of cells. The epidermis of the ovary is made up of elongated cells with sinuous walls between which secretory trichomes are situated. The ovaries contain numerous, very small clusters of calcium oxalate. In the tubular florets, the low part of each stamen filament is surrounded by thick-walled cells. The ends of the two stigmata have papillose epidermal cells. The pollen grains have a diameter of about 30 µm and are rounded and triangular, with three germinal pores and a spiny exine.

- **Broken Flowers:** NMT 25% passes through a No. 25 standard-mesh sieve (see *Particle Size Distribution Estimation by Analytical Sieving* ⟨786⟩).
- **Articles of Botanical Origin,** *Foreign Organic Matter* ⟨561⟩**:** NMT 2.0%
- **Articles of Botanical Origin,** *Total Ash* ⟨561⟩**:** NMT 13.0%, determined on 1.0 g of powdered Chamomile

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in well-closed containers, protected from light.
- **Labeling:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article. This article is exempted from the requirements of the *Labeling,* ⟨7⟩*, Labels and Labeling for Products and Other Categories, Botanicals,* with respect to the pregnancy and lactation statement.
- **USP Reference Standards** ⟨11⟩
  USP Apigenin-7-glucoside RS
  USP Levomenol RS

Chaste Tree

DEFINITION

Chaste Tree consists of the dried ripe fruits of *Vitex agnus-castus* L. (*Verbenaceae*). It contains NLT 0.05% of agnuside and NLT 0.08% of casticin, calculated on the dried basis.

IDENTIFICATION

- **A. Thin-Layer Chromatography**

Standard solution: 100 mg of USP Powdered Chaste Tree Extract RS in 1 mL of methanol. Heat in a water bath at 60° for 10 min. Centrifuge, and use the clear supernatant.

Sample solution: Transfer about 1 g of powdered plant material to a screw-capped centrifuge tube. Add 10 mL of methanol, heat in a water bath at 60° for 10–15 min, cool, and filter.

Adsorbent: Chromatographic silica gel with an average particle size of 10–15 µm (TLC plates)

Application volume: 90 µL, *Standard solution*; 60 µL, *Sample solution*; in bands that are 2 cm in length

Developing solvent system: Ethyl acetate, methanol, and water (77:15:8)

Derivatization reagent: 10 mg/mL of *p*-dimethylaminobenzaldehyde in 1 N hydrochloric acid

Analysis

Samples: *Standard solution* and *Sample solution*

Develop to a length of NLT 12 cm, and dry the plate in a stream of air. Treat the plate with *Derivatization reagent*, and heat for 10 min at 120°.

Acceptance criteria: The *Sample solution* shows the following: a blue zone (at an R_F value of about 0.21) due to the presence of aucubin and that corresponds in color and R_F value to a similar zone for the *Standard solution*; a blue zone (at an R_F value of about 0.44) as a result of the presence of agnuside that corresponds in color and R_F value to a similar zone for the *Standard solution*; and one broad zone, violet in the middle, near the solvent front and that corresponds in color and R_F value to a similar zone for the *Standard solution*. Other colored zones of varying intensities may be observed for the *Sample solution*.

- **B.** In the test for *Content of Casticin*, the chromatogram of the *Sample solution* shows a peak at the retention time corresponding to the casticin peak in the chromatogram of the *Standard solution*.

COMPOSITION

- **Content of Casticin**

Standard solution: About 0.05 mg/mL of USP Casticin RS in methanol, with sonication. Pass through a cellulose membrane filter of 0.45-µm or finer pore size.

Sample solution: Place about 1000 mg of ground plant material in a container with a stopper. Extract twice with 40 mL of methanol, using a hand homogenizer at 19,000 rpm for 2 min. Filter each supernatant, and transfer to a 250-mL round-bottom flask. Rinse the residue with methanol, and filter the resulting solution into the flask. Evaporate the combined extract to dryness. Dissolve the residue in methanol, quantitatively transfer to a 20-mL volumetric flask, and dilute with methanol to volume. Pass through a cellulose membrane filter of 0.45-µm or finer pore size.

Solution A: Methanol

Solution B: 5.88 g/L of phosphoric acid in water

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	50	50
0	50	50
13	65	35
18	100	0
23	50	50

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Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 348 nm
Column: 3.1-mm × 12.5-cm; 5-µm packing L1
Column temperature: 25°
Flow rate: 1 mL/min
Injection volume: 10 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Tailing factor: NMT 2.0 for the casticin peak
Relative standard deviation: NMT 2.0% for the casticin peak, in repeated injections
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of casticin in the portion of Chaste Tree taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of casticin from the *Sample solution*
r_S = peak response of casticin from the *Standard solution*
C_S = concentration of USP Casticin RS in the *Standard solution* (mg/mL)
C_U = concentration of Chaste Tree in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 0.08% of casticin on the dried basis

• **CONTENT OF AGNUSIDE**
Solvent: Methanol and water (1:19)
Standard solution: Dissolve a quantity of USP Agnuside RS in *Solvent*, with sonication. Dilute with methanol to obtain a concentration of about 0.125 mg/mL. Pass through a cellulose membrane filter of 0.45-µm or finer pore size.
Sample solution: Place about 1000 mg of ground plant material in a container with a stopper. Extract twice with 40 mL of methanol, using a hand homogenizer at 19,000 rpm for 2 min. Centrifuge, and transfer each supernatant to a 250-mL round-bottom flask. Rinse the residue with methanol, and filter the resulting solution into the flask. Evaporate the combined extract to dryness, and dissolve the residue in 2 mL of *Solvent*. Quantitatively transfer the solution to a solid-phase extraction cartridge packed with neutral aluminum oxide previously conditioned with 5 mL of *Solvent*. Connect the cartridge to a vacuum pressure not exceeding 300 mbar, and collect the eluate. Rinse the round-bottom flask with 2 mL of *Solvent*, pass this solution through the cartridge, apply the vacuum, and collect the eluate. Rinse the cartridge with 4 mL of *Solvent*, and collect the eluate. Combine the eluates from the cartridge, transfer to a 10-mL volumetric flask, and dilute with *Solvent* to volume.
Solution A: Acetonitrile
Solution B: 5.88 g/L of phosphoric acid in water
Mobile phase: See *Table 2*.

Table 2

Time (min)	Solution A (%)	Solution B (%)
0	7	93
0.6	10	90
5	10	90
7	14	86
13	15	85

Table 2 *(continued)*

Time (min)	Solution A (%)	Solution B (%)
13.1	100	0
18	100	0
18.1	7	93
23	7	93

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 258 nm
Column: 3.1-mm × 12.5-cm; 5-µm packing L1
Column temperature: 25°
Flow rate: 1.3 mL/min
Injection volume: 10 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Tailing factor: NMT 2.0 for the agnuside peak
Relative standard deviation: NMT 2.0% for the agnuside peak, in repeated injections
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of agnuside in the portion of Chaste Tree taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of agnuside from the *Sample solution*
r_S = peak response of agnuside from the *Standard solution*
C_S = concentration of USP Agnuside RS in the *Standard solution* (mg/mL)
C_U = concentration of Chaste Tree in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 0.05% of agnuside on the dried basis

CONTAMINANTS

• **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
• **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements
• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^5 cfu/g, the total combined molds and yeast count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria count does not exceed 10^3 cfu/g.
• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

• **BOTANICAL CHARACTERISTICS**
Macroscopic: Mature chaste tree fruits are spherical to ovoid, 2–4 mm in diameter, very hard, usually with a short pedicel. The fruit is reddish brown to black, slightly rough, and covered with glandular hairs. There are four grooves perpendicular to one another, and a slight depression on the apex, more evident on large fruits. The internal appearance of the fruit is yellowish. The internal structure of the fruit includes four compartments, each containing an oblong seed. A group of up to six spongy, light tan, immature fruits may also accompany mature fruits. The fruit

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is often covered by a tubular, greenish-gray, fine tomentose calyx, which is persistent and has five teeth.

Microscopic: The exocarp is brown and narrow, consisting of parenchymatous cells with thin walls and partially lignified cells with many pitted thickenings on the inside. In surface view, the exocarp shows an epidermis of polygonal cells with irregular thickenings and glandular hairs, each with a short single-celled stalk and a four-celled head containing essential oil. The outer mesocarp consists of several layers of brown, isodiametric parenchyma cells. The inner mesocarp consists of finely pitted sclerenchymatous cells, some with moderately thickened walls, others consisting of isodiametric stone cells with small lumen. The endocarp consists of a layer of small brown sclereid cells. The seeds are small, having large cotyledons surrounded by thin-walled, large parenchymatous cells that have ribbed thickenings. The nutritive tissue and the cells of the germ contain aleuron grains and oil globules. Starch is absent. The outer epidermis of calyx is composed of polygonal cells, covered by abundant unicellular or multicellular curved trichomes. The inner epidermis of calyx is glabrous and composed of rectangular, elongated cells with slightly wavy walls.

- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩: NMT 3.0%
- **LOSS ON DRYING** ⟨731⟩
  Sample: 1.0 g of Chaste Tree, finely powdered
  Analysis: Dry the *Sample* at 105° for 2 h.
  Acceptance criteria: NMT 10.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩: NMT 8.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩: NMT 2.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Agnuside RS
  USP Casticin RS
  USP Powdered Chaste Tree Extract RS

Powdered Chaste Tree

DEFINITION

Powdered Chaste Tree is Chaste Tree reduced to a powder or a very fine powder. It contains NLT 0.05% of agnuside and NLT 0.08% of casticin, calculated on the dried basis.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHY**
  Standard solution: 100 mg of USP Powdered Chaste Tree Extract RS in 1 mL of methanol. Heat in a water bath at 60° for 10 min. Centrifuge, and use the clear supernatant.
  Sample solution: Transfer about 1 g of Powdered Chaste Tree to a screw-capped centrifuge tube. Add 10 mL of methanol, heat in a water bath at 60° for 10–15 min, cool, and filter.
  Adsorbent: Chromatographic silica gel with an average particle size of 10–15 µm (TLC plates)
  Application volume: 90 µL, *Standard solution*; 60 µL, *Sample solution*; in bands that are 2 cm in length
  Developing solvent system: Ethyl acetate, methanol, and water (77:15:8)
  Derivatization reagent: 10 mg/mL of *p*-dimethylaminobenzaldehyde in 1 N hydrochloric acid
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Develop to a length of NLT 12 cm, and dry the plate in a current of air. Treat the plate with *Derivatization reagent*, and heat for 10 min at 120°.
  Acceptance criteria: The *Sample solution* shows the following: a blue zone (at an R_F value of about 0.21) due to the presence of aucubin and that corresponds in color and R_F value to a similar zone for the *Standard solution*; a blue zone (at an R_F value of about 0.44) as a result of the presence of agnuside that corresponds in color and R_F value to a similar zone for the *Standard solution*; and one broad zone, violet in the middle, near the solvent front and that corresponds in color and R_F value to a similar zone for the *Standard solution*. Other colored zones of varying intensities may be observed for the *Sample solution*.
- **B.** In the test for *Content of Casticin*, the chromatogram of the *Sample solution* shows a peak at the retention time corresponding to the casticin peak in the chromatogram of the *Standard solution*.

COMPOSITION

- **CONTENT OF CASTICIN**
  Standard solution: About 0.05 mg/mL of USP Casticin RS in methanol, with sonication. Pass through a cellulose membrane filter of 0.45-µm or finer pore size.
  Sample solution: Place about 1000 mg of Powdered Chaste Tree in a container with a stopper. Extract twice with 40 mL of methanol, using a hand homogenizer at 19,000 rpm for 2 min. Filter each supernatant, and transfer to a 250-mL round-bottom flask. Rinse the residue with methanol, and filter the resulting solution into the flask. Evaporate the combined extract to dryness. Dissolve the residue in methanol, quantitatively transfer to a 20-mL volumetric flask, and dilute with methanol to volume. Pass through a cellulose membrane filter of 0.45-µm or finer pore size.
  Solution A: Methanol
  Solution B: 5.88 g/L of phosphoric acid in water
  Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	50	50
0	50	50
13	65	35
18	100	0
23	50	50

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 348 nm
Column: 3.1-mm × 12.5-cm; 5-µm packing L1
Column temperature: 25°
Flow rate: 1 mL/min
Injection volume: 10 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Tailing factor: NMT 2.0 for the casticin peak
Relative standard deviation: NMT 2.0% for the casticin peak, in repeated injections

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Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of casticin in the portion of Powdered Chaste Tree taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of casticin from the *Sample solution*
r_S = peak response of casticin from the *Standard solution*
C_S = concentration of USP Casticin RS in the *Standard solution* (mg/mL)
C_U = concentration of Powdered Chaste Tree in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 0.08% of casticin on the dried basis

• **CONTENT OF AGNUSIDE**
Solvent: Methanol and water (1:19)
Standard solution: Dissolve a quantity of USP Agnuside RS in *Solvent*, with sonication. Dilute with methanol to obtain a concentration of about 0.125 mg/mL. Pass through a cellulose membrane filter of 0.45-µm or finer pore size.
Sample solution: Place about 1000 mg of Powdered Chaste Tree in a container with a stopper. Extract twice with 40 mL of methanol, using a hand homogenizer at 19,000 rpm for 2 min. Centrifuge, and transfer each supernatant to a 250-mL round-bottom flask. Rinse the residue with methanol, and filter the resulting solution into the flask. Evaporate the combined extract to dryness, and dissolve the residue in 2 mL of *Solvent*. Quantitatively transfer the solution to a solid-phase extraction cartridge packed with neutral aluminum oxide previously conditioned with 5 mL of *Solvent*. Connect the cartridge to a vacuum pressure not exceeding 300 mbar, and collect the eluate. Rinse the round-bottom flask with 2 mL of *Solvent*, pass this solution through the cartridge, apply the vacuum, and collect the eluate. Rinse the cartridge with 4 mL of *Solvent*, and collect the eluate. Combine the eluates from the cartridge, transfer to a 10-mL volumetric flask, and dilute with *Solvent* to volume.
Solution A: Acetonitrile
Solution B: 5.88 g/L of phosphoric acid in water
Mobile phase: See *Table 2*.

Table 2

Time (min)	Solution A (%)	Solution B (%)
0	7	93
0.6	10	90
5	10	90
7	14	86
13	15	85
13.1	100	0
18	100	0
18.1	7	93
23	7	93

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 258 nm
Column: 3.1-mm × 12.5-cm; 5-µm packing L1
Column temperature: 25°
Flow rate: 1.3 mL/min
Injection volume: 10 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Tailing factor: NMT 2.0 for the agnuside peak
Relative standard deviation: NMT 2.0% for the agnuside peak, in repeated injections
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of agnuside in the portion of Powdered Chaste Tree taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of agnuside from the *Sample solution*
r_S = peak response of agnuside from the *Standard solution*
C_S = concentration of USP Agnuside RS in the *Standard solution* (mg/mL)
C_U = concentration of Powdered Chaste Tree in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 0.05% of agnuside on the dried basis

CONTAMINANTS

• **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
• **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements
• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^5 cfu/g, the total combined molds and yeast count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria count does not exceed 10^3 cfu/g.
• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

• **BOTANICAL CHARACTERISTICS:** Powdered Chaste Tree is dark brown, with a musty, slightly aromatic odor, and a taste resembling that of sage. The following characteristics are present: fragments of the calyx with covering and glandular trichomes on the outer side and rectangular, elongated cells with slightly wavy walls on the inner side; fragments of exocarp with trichomes and cells with large pits in the outer wall; thin-walled parenchymatous cells and globules of fixed oil; stone-pitted cells from the mesocarp; ovoid, lignified cells with bands of reticulate thickening from the testa; and endosperm and cotyledons with fixed oil.
• **LOSS ON DRYING** ⟨731⟩
Sample: 1.0 g of Powdered Chaste Tree
Analysis: Dry the *Sample* at 105° for 2 h.
Acceptance criteria: NMT 10.0%
• **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 8.0%
• **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 2.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at controlled room temperature.
• **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Agnuside RS

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USP Casticin RS
USP Powdered Chaste Tree Extract RS

Powdered Chaste Tree Extract

DEFINITION

Powdered Chaste Tree Extract is prepared from Chaste Tree by extraction with hydroalcoholic mixtures or other suitable solvents. It contains NLT 90.0% and NMT 110.0% of the labeled amount of casticin and agnuside, calculated on the dried basis. It may contain suitable added substances.

IDENTIFICATION

• **A. Thin-Layer Chromatographic Identification Test**

Standard solution: 100 mg of USP Powdered Chaste Tree Extract RS in 1 mL of methanol. Heat in a water bath at 60° for 10 min. Centrifuge, and use the clear supernatant.

Sample solution: Shake a quantity of Extract, equivalent to about 10 mg of the labeled amount of agnuside, in 10 mL of methanol. Heat in a water bath at 60°. Centrifuge or filter before use.

Adsorbent: Chromatographic silica gel with an average particle size of 10–15 µm (TLC plates)

Application volume: 90 µL, *Standard solution*; 60 µL, *Sample solution*; in bands that are 2 cm in length

Developing solvent system: Ethyl acetate, methanol, and water (77:15:8)

Spray reagent: 10 mg/mL of *p*-dimethylaminobenzaldehyde in 1 N hydrochloric acid

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the chromatograms to a length of NLT 12 cm, and dry the plate in a current of air. Spray the plate with *Spray reagent*, and heat for 10 min at 120°.

Acceptance criteria: The *Sample solution* shows the following: a blue zone (at an R_F value of about 0.21) due to the presence of aucubin and that corresponds in color and R_F value to a similar zone for the *Standard solution*; a blue zone (at an R_F value of about 0.44) as a result of the presence of agnuside that corresponds in color and R_F value to a similar zone for the *Standard solution*; and one broad zone, violet in the middle, near the solvent front and that corresponds in color and R_F value to a similar zone for the *Standard solution*. Other colored zones of varying intensities may be observed in the *Sample solution*.

• **B.** In the test for *Content of Casticin*, the chromatogram of the *Sample solution* exhibits a peak at the retention time corresponding to casticin.

• **C.** In the test for *Content of Agnuside*, the chromatogram of the *Sample solution* exhibits a peak at the retention time corresponding to agnuside.

COMPOSITION

• **Content of Casticin**

Standard solution: About 0.05 mg/mL of USP Casticin RS in methanol, with sonication. Pass through a cellulose membrane filter of 0.45-µm or finer pore size.

Sample solution: Transfer a quantity of Extract, equivalent to about 2.5 mg of the labeled content of casticin, into a 50-mL volumetric flask. Add 25 mL of methanol, and sonicate in a bath at 40° for 10 min, shaking to disperse the solid. Cool to room temperature, and dilute with methanol to volume. Centrifuge or pass through a filter of 0.45-µm or finer pore size.

Solution A: Methanol

Solution B: 5.88 g/L of phosphoric acid in water

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	50	50
0	50	50
13	65	35
18	100	0
23	50	50

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 348 nm

Column: 3.1-mm × 12.5-cm; 5-µm packing L1

Column temperature: 25°

Flow rate: 1 mL/min

Injection size: 10 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Tailing factor: NMT 2.0 for the casticin peak

Relative standard deviation: NMT 2.0% for the casticin peak, in repeated injections

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of casticin, *Pc*, in the portion of Extract taken:

$$Pc = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of casticin from the *Sample solution*
r_S = peak response of casticin from the *Standard solution*
C_S = concentration of USP Casticin RS in the *Standard solution* (mg/mL)
C_U = concentration of Extract in the *Sample solution* (mg/mL)

Calculate the percentage of the labeled amount of casticin in the portion of Extract taken:

$$\text{Result} = (Pc/L) \times 100$$

Pc = content of casticin as calculated above (%)
L = labeled amount of casticin (%)

Acceptance criteria: 90.0%–110.0% on the dried basis

• **Content of Agnuside**

Solvent: Methanol and water (1:19)

Standard solution: Dissolve a quantity of USP Agnuside RS in *Solvent*, with sonication. Dilute with methanol to obtain a concentration of about 0.125 mg/mL. Pass through a cellulose membrane filter of 0.45-µm or finer pore size.

Sample solution: Transfer an amount of Extract, equivalent to about 6.25 mg of the labeled content of agnuside, into a 50-mL volumetric flask. Add 25 mL of *Solvent*, and sonicate in a bath at 40° for 10 min, shaking to disperse the solid. Cool to room temperature, and dilute with *Solvent* to volume. Centrifuge or pass through a filter of 0.45-µm or finer pore size.

Solution A: Acetonitrile

Solution B: 5.88 g/L of phosphoric acid in water

Mobile phase: See *Table 2*.

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Table 2

Time (min)	Solution A (%)	Solution B (%)
0	7	93
0.6	10	90
5	10	90
7	14	86
13	15	85
13.1	100	0
18	100	0
18.1	7	93
23	7	93

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 258 nm
Column: 3.1-mm × 12.5-cm; 5-µm packing L1
Column temperature: 25°
Flow rate: 1.3 mL/min
Injection size: 10 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Tailing factor: NMT 2.0 for the agnuside peak
Relative standard deviation: NMT 2.0% for the agnuside peak, in repeated injections
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of agnuside, *Pa*, in the portion of Extract taken:

$$Pa = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of agnuside from the *Sample solution*
r_S = peak response of agnuside from the *Standard solution*
C_S = concentration of USP Agnuside RS in the *Standard solution* (mg/mL)
C_U = concentration of Extract in the *Sample solution* (mg/mL)

Calculate the percentage of the labeled amount of agnuside in the portion of Extract taken:

$$\text{Result} = (Pa/L) \times 100$$

Pa = content of agnuside calculated above (%)
L = labeled amount of agnuside (%)

Acceptance criteria: 90.0%–110.0% on the dried basis

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total bacterial count does not exceed 10^4 cfu/g. The total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*
- **OTHER REQUIREMENTS:** It meets the requirements for *Botanical Extracts* ⟨565⟩, *Residual Solvents and Pesticide Residues*.

SPECIFIC TESTS

- **LOSS ON DRYING** ⟨731⟩**:** NMT 6.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and store in a cool place, protected from light.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. The label also indicates the content of casticin and agnuside, the extracting solvent or solvent mixture used for preparation, the ratio of the starting crude plant material to Extract, the percentage of native extract, and the name and quantity of any added substances. It meets the requirements for *Botanical Extracts* ⟨565⟩, *Labeling*.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Agnuside RS
  USP Casticin RS
  USP Powdered Chaste Tree Extract RS

Horse Chestnut

DEFINITION

Horse Chestnut consists of the dried seeds of *Aesculus hippocastanum* L. (Fam. Hippocastanaceae) harvested in the fall. It contains NLT 3.0% of triterpene glycosides, calculated on the dried basis as escin ($C_{55}H_{86}O_{24}$).

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution: 5 mg/mL of USP Escin RS in methanol
Sample solution: Transfer 1 g of the powdered plant material to a screw-capped centrifuge tube, add 10 mL of a mixture of alcohol and water (7:3), and heat on a steam bath for 10 min. Centrifuge, and use the clear supernatant.
Chromatographic system
(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
Adsorbent: 0.25-mm layer of chromatographic silica gel (TLC plates)
Application volume: 10 µL
Developing solvent system: Use the upper phase of a mixture of 1-butanol, glacial acetic acid, and water (5:1:4).
Derivatization reagent: Methanol, glacial acetic acid, sulfuric acid, and *p*-anisaldehyde (85: 10: 5: 0.5)
Analysis
Samples: *Standard solution* and *Sample solution*
Develop the chromatograms to a length of NLT 15 cm, and dry the plate in a stream of air. Spray the plate with *Derivatization reagent*, heat at 100° for 5 min, and examine under white light.
Acceptance criteria: The chromatogram of the *Sample solution* shows a blue-violet zone corresponding to escin, comparable in position and color to the main zone in the chromatogram of the *Standard solution*. Above this zone, the chromatogram of the *Sample solution* shows several narrow, brown to brownish-red zones that are less intense than the zone corresponding to escin.

COMPOSITION

- **CONTENT OF TRITERPENE GLYCOSIDES**

Solvent A: Methanol and water (13:7)
Solvent B: Use the lower phase of a mixture of chloroform, 0.1 N hydrochloric acid, and 1-propanol (5:3:2).
Reagent: Dissolve 75 mg of ferric chloride in 50 mL of ice-cold glacial acetic acid. Add 50 mL of sulfuric acid, while swirling on an ice bath. Prepare immediately before use.
Standard solution A: 0.2 mg/mL of USP Escin RS in glacial acetic acid, shaken for 1 min

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Standard solution B: 0.4 mg/mL of USP Escin RS in glacial acetic acid, shaken for 1 min

Standard solution C: 0.6 mg/mL of USP Escin RS in glacial acetic acid, shaken for 1 min

Sample solution: Accurately weigh about 1 g of ground seeds, and transfer into a 250-mL round-bottom flask. Add exactly 100 mL of *Solvent A*, and weigh the filled flask with a precision of ±0.1 g. Attach a condenser, reflux for 30 min, and allow to cool. Adjust to the initial weight by adding *Solvent A*, and filter. Transfer 30.0 mL of the filtrate to a round-bottom flask, and evaporate under vacuum. Dissolve the residue in 20 mL of 0.1 N hydrochloric acid, and quantitatively transfer with the aid of two additional 5-mL portions of 0.1 N hydrochloric acid to a 250-mL separatory funnel. Add 20 mL of 1-propanol and 50 mL of chloroform, and shake vigorously for 2 min. Collect and retain the lower chloroform layer, and add 50 mL of *Solvent B* to the upper layer remaining in the separatory funnel. Shake vigorously for 2 min; collect and retain the lower chloroform layer. Combine the retained chloroform layers in a round-bottom flask, and evaporate to near-dryness under vacuum. Evaporate the remaining solvent under a stream of air. Wash the residue with two 10-mL aliquots of ether, filter, wash the filter with 10 mL of ether, and discard the ether filtrates. After evaporation of the residual ether, suspend the residue in 10 mL of glacial acetic acid, and pass through the previously used dried filter into a 50-mL volumetric flask. Repeat the addition of glacial acetic acid followed by filtration two additional times, combining the filtrates in the 50-mL volumetric flask. Wash the round-bottom flask with small quantities of glacial acetic acid, and filter into the volumetric flask. Dilute with glacial acetic acid to volume.

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Mode: Visible

Wavelength: 540 nm

Blank: Glacial acetic acid

Analysis: Accurately transfer 1.0 mL each of *Standard solutions A, B,* and *C, Sample solution,* and *Blank* into separate screw-cap test tubes. Add 4.0 mL of *Reagent* to each tube, cap the tubes, and keep them on a water bath at 60° for 25 min, shaking occasionally. Measure the absorbances of the reacted *Sample solution* and *Standard solutions A, B,* and *C,* corrected for the *Blank.* Plot the absorbances of *Standard solutions A, B,* and *C* against their respective concentrations, and establish the calibration line by linear regression. From the plot, determine the concentration, *C,* in mg/mL, of triterpene glycosides as escin in the *Sample solution.*

Calculate the percentage of triterpene glycosides as escin in the portion of Horse Chestnut taken:

$$\text{Result} = (C/W) \times (50/3)$$

C = concentration of triterpene glycosides in the *Sample solution* as obtained above (mg/mL)

W = weight of Horse Chestnut taken to prepare the *Sample solution* (g)

Acceptance criteria: NLT 3.0% of triterpene glycosides, calculated as escin ($C_{55}H_{86}O_{24}$), on the dried basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^6 cfu/g, the total combined molds and yeast count does not exceed 10^4 cfu/g, and the bile-tolerant Gram-negative bacteria count is NMT 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli.*

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: Horse chestnut seeds are dense and hard, subspherical to oval, slightly flattened, and from 2 to 4 cm in diameter. They have a dark brown seed coat from 1 to 1.5 mm thick, with a large, round, light brown spot (hilum). The seed coat is shiny, but only in fresh condition. The space under the coat is totally filled with the shiny, massive embryo and its large, pale yellow cotyledons lacking endosperm.

Microscopic: The epidermis of the testa in surface view has yellowish-brown cells of fairly uniform size, with the majority of cells rounded to polygonal, and a few that are square to obscurely triangular. The walls of these cells are considerably but rather unevenly thickened, and lack pits. In the sectional view, the cells are columnar, approximately 3–4 times as high as they are wide, with the outer periclinal wall markedly thickened, uneven, and becoming thinner toward the base; beneath the epidermis there are a few layers of small collenchymatously thickened cells with small intercellular spaces; the greater part of the testa consists of larger, loosely packed parenchymatous cells forming a spongy tissue; the walls are variably and unevenly thickened, with intercellular and large circular spaces well marked; the inner layer of the testa is a narrow zone, with ill-defined and thinner-walled cells. All the parenchymatous cells of the testa are darkly pigmented. The embryo has an outer layer of small colorless cells, almost square in sectional view, with outer and side walls thickened. In the surface view, only the irregular and more or less polygonal lumens are discernible, giving a reticulate, pitted appearance. Cotyledons are moderately thickened and indistinctly pitted, having round to ovoid parenchymatous cells densely filled with starch. Starch granules, mainly simple, are present in two size ranges: from 15 to 30 µm and from 3 to 10 µm. The largest granules vary from circular, ovoid, and bluntly polygonal to pyriform, most of them with a well-marked cleft or stellate hilum, and lacking striations. The smaller starch granules are less variable, spherical to ovoid, with the hilum more often a point. Compound starch granules are found very infrequently.

- **EXTRACTABLE MATTER**

Analysis: Proceed as directed for *Articles of Botanical Origin* ⟨561⟩, *Alcohol-Soluble Extractives, Method 2,* except use a mixture of methanol and water (8:2) instead of alcohol.

Acceptance criteria: NLT 18.0%

- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩**:** NMT 2.0%
- **LOSS ON DRYING** ⟨731⟩**:** Dry a sample at 105° for 2 h: it loses NMT 10.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 4.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in a well-closed, light-resistant container, protected from moisture.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Escin RS

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Powdered Horse Chestnut

DEFINITION

Powdered Horse Chestnut is Horse Chestnut reduced to a powder or very fine powder. It contains NLT 3.0% of triterpene glycosides, calculated on the dried basis as escin ($C_{55}H_{86}O_{24}$).

IDENTIFICATION

• **A. Thin-Layer Chromatographic Identification Test**

Standard solution: 5 mg/mL of USP Escin RS in methanol

Sample solution: Transfer 1 g of Powdered Horse Chestnut to a screw-capped centrifuge tube, add 10 mL of a mixture of alcohol and water (7:3), and heat on a steam bath for 10 min. Centrifuge, and use the clear supernatant.

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Adsorbent: 0.25-mm layer of chromatographic silica gel (TLC plates)

Application volume: 10 µL

Developing solvent system: Use the upper phase of a mixture of 1-butanol, glacial acetic acid, and water (5:1:4).

Derivatization reagent: Methanol, glacial acetic acid, sulfuric acid, and *p*-anisaldehyde (85: 10: 5: 0.5)

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the chromatograms to a length of NLT 15 cm, and dry the plate in a current of air. Spray the plate with *Derivatization reagent*, heat at 100° for 5 min, and examine under white light.

Acceptance criteria: The chromatogram of the *Sample solution* shows a blue-violet zone corresponding to escin, comparable in position and color to the main zone in the chromatogram of the *Standard solution*. Above this zone, the chromatogram of the *Sample solution* shows several narrow, brown to brownish-red zones that are less intense than the zone corresponding to escin.

COMPOSITION

• **Content of Triterpene Glycosides**

Solvent A: Methanol and water (13:7)

Solvent B: Use the lower phase of a mixture of chloroform, 0.1 N hydrochloric acid, and 1-propanol (5:3:2).

Reagent: Dissolve 75 mg of ferric chloride in 50 mL of ice-cold glacial acetic acid. Add 50 mL of sulfuric acid, while swirling on an ice bath. Prepare immediately before use.

Standard solution A: 0.2 mg/mL of USP Escin RS in glacial acetic acid, shaken for 1 min

Standard solution B: 0.4 mg/mL of USP Escin RS in glacial acetic acid, shaken for 1 min

Standard solution C: 0.6 mg/mL of USP Escin RS in glacial acetic acid, shaken for 1 min

Sample solution: Accurately weigh about 1 g of Powdered Horse Chestnut, and transfer into a 250-mL round-bottom flask. Add exactly 100 mL of *Solvent A*, and weigh the filled flask with a precision of ±0.1 g. Attach a condenser, reflux for 30 min, and allow to cool. Adjust to the initial weight by adding *Solvent A*, and filter. Transfer 30.0 mL of the filtrate to a round-bottom flask, and evaporate under vacuum. Dissolve the residue in 20 mL of 0.1 N hydrochloric acid, and quantitatively transfer with the aid of two additional 5-mL portions of 0.1 N hydrochloric acid to a 250-mL separatory funnel. Add 20 mL of 1-propanol and 50 mL of chloroform, and shake vigorously for 2 min. Collect and retain the lower chloroform layer, and add 50 mL of *Solvent B* to the upper layer remaining in the separatory funnel. Shake vigorously for 2 min; collect and retain the lower chloroform layer. Combine the retained chloroform layers in a round-bottom flask, and evaporate to near-dryness under vacuum. Evaporate the remaining solvent under a stream of air. Wash the residue with two 10-mL aliquots of ether, filter, wash the filter with 10 mL of ether, and discard the ether filtrates. After evaporation of the residual ether, suspend the residue in 10 mL of glacial acetic acid, and pass through the previously used dried filter into a 50-mL volumetric flask. Repeat the addition of glacial acetic acid followed by filtration two additional times, combining the filtrates in the 50-mL volumetric flask. Wash the round-bottom flask with small quantities of glacial acetic acid, and filter into the volumetric flask. Dilute with glacial acetic acid to volume.

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Wavelength: 540 nm

Mode: Visible

Blank: Glacial acetic acid

Analysis: Accurately transfer 1.0 mL each of *Standard solutions A*, *B*, and *C*, the *Sample solution*, and the *Blank* into separate screw-cap test tubes. Add 4.0 mL of *Reagent* to each tube, cap the tubes, and keep them on a water bath at 60° for 25 min, shaking occasionally. Measure the absorbances of the reacted *Sample solution* and *Standard solutions A*, *B*, and *C*, corrected for the *Blank*. Plot the absorbances of *Standard solutions A*, *B*, and *C* against their respective concentrations, and establish the calibration line by linear regression. From the plot, determine the concentration, *C*, in mg/mL, of triterpene glycosides as escin in the *Sample solution*.

Calculate the percentage of triterpene glycosides as escin in the portion of Powdered Horse Chestnut taken:

$$\text{Result} = (C/W) \times (50/3)$$

C = concentration of triterpene glycosides in the *Sample solution* as obtained above (mg/mL)

W = weight of Powdered Horse Chestnut taken to prepare the *Sample solution* (g)

Acceptance criteria: NLT 3.0% of triterpene glycosides, calculated as escin ($C_{55}H_{86}O_{24}$), on the dried basis

CONTAMINANTS

• **Articles of Botanical Origin,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements

• **Articles of Botanical Origin,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements

• **Microbial Enumeration Tests** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^6 cfu/g, the total combined molds and yeast count does not exceed 10^4 cfu/g, and the bile-tolerant Gram-negative bacteria count is NMT 10^3 cfu/g.

• **Absence of Specified Microorganisms** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

• **Botanical Characteristics:** Yellowish-brown powder, odorless, with a somewhat mealy, disagreeably bitter, and lingering taste. It shows numerous, different-sized fatty oil droplets that are free or within the thin-walled, colorless tissue of the cotyledons. Fragments of the testa consist of thick-walled pitted sclerenchymatous cells. The following are also present: pyriform, roundish or reniform larger individual starch granules from 15 to 30 µm in diameter, smaller individual granules from 3 to 10 µm, and only a few compounded granules consisting of 2–4 single grains that form rows up to 45 µm in length. Many of the starch granules have a bistellate or polystellate, but rarely simple, hilum.

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- **EXTRACTABLE MATTER**
  Analysis: Proceed as directed for *Articles of Botanical Origin* ⟨561⟩, *Alcohol-Soluble Extractives, Method 2*, except use a mixture of methanol and water (8:2) instead of alcohol.
  Acceptance criteria: NLT 18.0%
- **LOSS ON DRYING** ⟨731⟩**:** Dry a sample at 105° for 2 h: it loses NMT 10.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 4.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed, light-resistant containers, protected from moisture.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Escin RS

Powdered Horse Chestnut Extract

DEFINITION

Powdered Horse Chestnut Extract is prepared from Horse Chestnut by extraction with alcohol–water mixtures or methanol–water mixtures. The ratio of starting plant material to extract is between 5:1 and 8:1. It contains NLT 90.0% and NMT 110.0% of the labeled amount of triterpene glycosides, calculated on the dried basis as escin ($C_{55}H_{86}O_{24}$). It may contain suitable added substances.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**
  Standard solution: 5 mg/mL of USP Escin RS in methanol
  Sample solution: To 10 mL of methanol add a quantity of Powdered Extract equivalent to 25 mg of the labeled amount of triterpene glycosides, and shake. Allow to stand for 15 min before use.
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
  Adsorbent: 0.25-mm layer of chromatographic silica gel
  Application volume: 10 µL
  Developing solvent system: Use the upper phase of a mixture of 1-butanol, glacial acetic acid, and water (5:1:4).
  Spray reagent: Methanol, glacial acetic acid, sulfuric acid, and *p*-anisaldehyde (85: 10: 5: 0.5)
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Develop the chromatograms to a length of NLT 15 cm, and dry the plate in a current of air. Spray the plate with *Spray reagent,* heat the plate at 100° for 5 min, and examine the plate under daylight.
  Acceptance criteria: The chromatogram from the *Sample solution* shows a blue-violet zone corresponding to escin, comparable in position and color to the main zone in the chromatogram from the *Standard solution*. Above this zone, the chromatogram of the *Sample solution* shows several narrow, brown to brownish-red zones that are less intense than the zone corresponding to escin.

COMPOSITION

- **CONTENT OF TRITERPENE GLYCOSIDES**
  Solvent A: Methanol and water (13:7)
  Solvent B: Use the lower phase of a mixture of chloroform, 0.1 N hydrochloric acid, and 1-propanol (5:3:2).
  Reagent: Dissolve 75 mg of ferric chloride in 50 mL of glacial acetic acid. Add 50 mL of sulfuric acid, while shaking and cooling. Prepare immediately before use.
  Standard solution A: 0.2 mg/mL of USP Escin RS in glacial acetic acid, shaking for 1 min
  Standard solution B: 0.4 mg/mL of USP Escin RS in glacial acetic acid, shaking for 1 min
  Standard solution C: 0.6 mg/mL of USP Escin RS in glacial acetic acid, shaking for 1 min
  Sample solution: Transfer a quantity of Powdered Extract, equivalent to 50 mg of the labeled content of triterpene glycosides, into a 50-mL flask. Add 20 mL of 0.1 N hydrochloric acid, and shake for 5 min. Filter into a 250-mL separatory funnel with the aid of two additional 5-mL portions of 0.1 N hydrochloric acid. Add 20 mL of 1-propanol and 50 mL of chloroform, and shake vigorously for 2 min. Separate the chloroform layer, and add *Solvent B* to the upper phase remaining in the separation funnel. Shake vigorously for 2 min, and separate the chloroform layer. Combine the chloroform layers in a round-bottom flask, and evaporate to dryness under vacuum. Evaporate the remaining solvents with the aid of a current of air. Wash the residue with two 10-mL portions of ether, filter, wash the filter with 10 mL of ether, and discard the ether filtrates. After evaporation of the residual ether, add to the residue a 10-mL portion of glacial acetic acid, and pass through the previously used dried filter into a 100-mL volumetric flask. Repeat the addition of glacial acetic acid followed by filtration two additional times, combining the filtrates in the volumetric flask. Wash the round-bottom flask with small quantities of glacial acetic acid, and filter into the volumetric flask. Dilute with glacial acetic acid to volume.
  Instrumental conditions
  (See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
  Mode: Visible
  Wavelength: 540 nm
  Blank: Glacial acetic acid
  Analysis: Transfer 1 mL each of *Standard solutions A, B,* and *C,* the *Sample solution,* and the *Blank* to separate test tubes with stoppers. Add 4.0 mL of *Reagent* to each tube, cap the tubes, and place them in a water bath at 60° for 25 min, shaking occasionally. Measure the absorbances of the reacted *Sample solution* and the reacted *Standard solutions A, B,* and *C,* and correct for the *Blank*. Plot the absorbances of the reacted *Standard solutions A, B,* and *C* versus concentrations, in mg/mL of USP Escin RS in the corresponding *Standard solution*. From the graphs determine the concentration, *C,* in mg/mL, of triterpene glycosides as escin ($C_{55}H_{86}O_{24}$) in the *Sample solution*.
  Calculate the percentage of the labeled amount of triterpene glycosides in the portion of Powdered Extract taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of triterpene glycosides in the *Sample solution* as obtained above (mg/mL)
C_U = nominal concentration of triterpene glycosides in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–110.0% of the labeled amount of triterpene glycosides as escin ($C_{55}H_{86}O_{24}$) on the dried basis

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^4 cfu/g, the total combined molds and yeasts count does not exceed 10^2 cfu/g, and the count for enterobacteria does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

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SPECIFIC TESTS
- **LOSS ON DRYING** ⟨731⟩: Dry 1 g at 105° for 2 h: it loses NMT 5.0% of its weight.
- **OTHER REQUIREMENTS:** It meets the requirements in *Botanical Extracts* ⟨565⟩, *General Pharmacopeial Requirements*, for *Packaging and Storage*, *Residual Solvents*, and *Pesticide Residues* for powdered extracts.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers. Store in a cool place.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. The label also indicates the content of triterpene glycosides, the extracting solvent or solvent mixture used for preparation, the ratio of the starting crude plant material to Powdered Extract, and the name and content of any added substance. It meets the requirements for labeling in *Botanical Extracts* ⟨565⟩.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Escin RS

Chia Seed Oil

[93384-40-8].

DEFINITION
Chia Seed Oil is derived from the seeds of the Chia plant (*Salvia hispanica* L.). The oil is extracted from the seeds by cold pressing. No solvents or external heat are employed in the extraction process. Tocopherol may be added as an antioxidant.

IDENTIFICATION
- **A.** It meets the requirements in *Specific Tests* for *Fats and Fixed Oils* ⟨401⟩, *Procedures, Fatty Acid Composition*.
- **B. IDENTIFICATION OF FIXED OILS BY THIN-LAYER CHROMATOGRAPHY** ⟨202⟩: The R_F values of the principal spots of the *Sample solution* correspond to those of the *Standard solution*.
- **C.** It meets the requirements in *Specific Tests* for *Sterol Composition*.

SPECIFIC TESTS
- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Acid Value*: NMT 2.5
- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Peroxide Value*: NMT 10.0
- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Iodine Value*: 180–210
- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Saponification Value*: 180–230
- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Unsaponifiable Matter*: NMT 1.5
- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Fatty Acid Composition*: Chia Seed Oil exhibits the composition profile of fatty acids in *Table 1*.

Table 1

Fatty Acid	Shorthand Notation	Area Percentage (%)
Palmitic acid	16:0	5.0–9.0
Stearic acid	18:0	2.0–5.0
Oleic acid	18:1	4.0–9.0
Linoleic acid	18:2	17.0–22.0
Alpha-linolenic acid	18:3 (n–3)	57.0–70.0

Table 1 *(continued)*

Fatty Acid	Shorthand Notation	Area Percentage (%)
Gamma-linolenic acid	18:3 (n–6)	0.0–0.4

- **STEROL COMPOSITION**
  Internal standard solution: 0.3 mg/mL of USP Cholestanol RS in 2-propanol
  Standard solution: Transfer 50 mg of USP Chia Seed Oil RS to a 25-mL screw-cap test tube, add 2.0 mL of 2-propanol, and sonicate to dissolve. Add 3.0 mL of 1 M methanolic potassium hydroxide and 0.8 mL of *Internal standard solution* to the test tube. Cap the test tube and place in a hot water bath at 80° for 60 min. Remove the test tube from the water bath and cool to room temperature, then add 3 mL each of water and *n*-hexane. Mix the solution with a vortex mixer or shake vigorously for at least 30 s. Immediately add 5 mL of saturated sodium chloride, cap, and mix with a vortex mixer or shake thoroughly for at least 15 s. Let the solution stand for 5 min, or until the upper layer becomes clear, and transfer to a separate tube. Shake the lower layer once more with 3 mL of *n*-hexane, and combine the hexane extracts. Evaporate the hexane extracts with the aid of a nitrogen stream to dryness. Add 0.3 mL of pyridine silylated with 1.0 mL of BSA+TMCS +TMSI mixture (3:2:3)[1] and let it stand at room temperature for 15 min. Inject this solution into a gas chromatograph.
  Sample solution: Prepare as directed for the *Standard solution*, except replace USP Chia Seed Oil RS with Chia Seed Oil.
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: GC
  Detector: Flame ionization
  Column: 0.32-mm × 30-m fused silica capillary, bonded with a 0.25-µm film of phase G27
  Temperatures
  Injection port: 240°
  Detector: 325°
  Column: See *Table 2*.

Table 2

Initial Temperature (°)	Hold Time at 240° (min)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
240	3	2	300	7

  Carrier gas: Helium
  Flow rate: 1.5 mL/min
  Split ratio: 2:1
  Injection volume: 1 µL
  System suitability
  Sample: *Standard solution*
  Suitability requirements
  Resolution: NLT 1.5 between β-sitosterol and Δ5-avenasterol
  Relative standard deviation: NMT 2.0% for the ratios of β-sitosterol to internal standard peak responses from replicate injections
  Chromatogram similarity: The chromatogram from the *Standard solution* is similar to the reference

[1] BSA+TMCS+TMSI mixture (3:2:3) is available from Sigma-Aldrich, Product #33030, www.sigmaaldrich.com/catalog/product/supelco/33030.

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chromatogram supplied with USP Chia Seed Oil RS. Identify the retention times of six relevant sterol methyl esters by comparing the chromatogram of the *Standard solution* with the reference chromatogram supplied with USP Chia Seed Oil RS. The retention times of the sterols with reference to β-sitosterol are given in *Table 3*.

Table 3

Identification	Relative Retention Time
Cholestanol (internal standard)	0.73
Campesterol	0.91
Stigmasterol	0.94
β-Sitosterol	1.00
Δ5-Avenasterol	1.02
Cycloartenol	1.06
24-Methylenecycloartenol	1.11

Analysis

Sample: *Sample solution*

Calculate the area percentage of each individual sterol in the portion of Chia Seed Oil taken:

$$\text{Result} = (R_U/R_T) \times 100$$

R_U = peak response ratio of each sterol component to the internal standard (internal standard ratio) from the *Sample solution*

R_T = sum of six internal standard ratios from the *Sample solution*

Acceptance criteria: Chia Seed Oil exhibits six sterol components, each with the normalized area percentage shown in *Table 4*.

Table 4

Component	Normalized Area Percentage (%)
Campesterol	11.1–13.2
Stigmasterol	2.5–6.8
β-Sitosterol	66.2–68.9
Δ5-Avenasterol	6.8–10.4
Cycloartenol	3.0–5.6
24-Methylenecycloartenol	2.2–3.7

• **REFRACTIVE INDEX** ⟨831⟩: 1.460–1.490 at 20°

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed, tight, light-resistant containers.

• **LABELING:** Where Chia Seed Oil is intended for use in the manufacture of dosage forms, it is so labeled.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Chia Seed Oil RS

USP Cholestanol RS

3β-Hydroxy-5α-cholestane.

$C_{27}H_{48}O$ 388.67

Chinese Salvia

DEFINITION

Chinese Salvia consists of the dried roots and rhizomes of *Salvia miltiorrhiza* Bunge, also known as Danshen (Fam. Lamiaceae). It contains NLT 0.1% of tanshinone II_A; NLT 0.2% of total tanshinones, calculated as the sum of cryptotanshinone, tanshinone I, and tanshinone II_A; and NLT 3.0% of salvianolic acid B; all calculated on the dried basis. It is collected in spring or fall.

IDENTIFICATION

• **A.** Chinese Salvia meets the requirements for *Specific Tests, Botanic Characteristics*.

• **B. THIN-LAYER CHROMATOGRAPHY**

Standard solution A: A mixture of about 0.5 mg/mL of USP Tanshinone II_A RS and about 1.5 mg/mL of USP Salvianolic Acid B RS in alcohol

Standard solution B: About 0.25 g of USP Powdered Chinese Salvia Extract RS in 5.0 mL of alcohol. Sonicate for 15 min, centrifuge, and use the supernatant.

Sample solution: About 1.0 g of Chinese Salvia, finely powdered, in 5.0 mL of alcohol. Sonicate for 15 min, centrifuge, and use the supernatant.

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Adsorbent: Chromatographic silica gel mixture with an average particle size of 2–10 µm (HPTLC plates)

Application volume: 5 µL, as 8-mm bands

Developing solvent system A: A mixture of ethyl acetate, chloroform, toluene, formic acid, and methanol (8:6:4:4:1)

Developing solvent system B: A mixture of solvent hexane and ethyl acetate (4:1)

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the samples as bands to a suitable high performance thin-layer chromatographic plate. Use a saturated chamber, and condition the plate to a relative humidity of about 33% using a suitable device. Develop the chromatograms in *Developing solvent system A* until the solvent front has moved up about 40% of the plate. Remove the plate, and allow to dry. Develop the chromatograms in a saturated chamber containing *Developing solvent system B* until the solvent front has moved up about three-fourths of the plate. Remove the plate, dry, and examine under visible light and UV light at 254 nm and 365 nm.

Acceptance criteria

Under visible light, the chromatogram of the *Sample solution* exhibits three bands similar in positions and colors to bands in the chromatogram of *Standard solution B*. These include a pink band in the upper third of the chromatogram, similar in R_F and color to the tanshinone II_A band in the chromatogram of *Standard solution A*, a yellowish-orange band in the upper third of the chromatogram, and an orange band at about the middle of the chromatogram due to tanshinone I and cryptotanshinone, respectively.

Under UV light at 365 nm, the chromatogram of the *Sample solution* exhibits three blue bands similar in positions and colors to bands in the chromatogram of *Standard solution B*. These include an intense blue band in the lower third of the chromatogram corresponding in R_F and color to the salvianolic acid B band in the chromatogram of *Standard solution A*, and two minor blue bands in the lower third of the chromatogram and above the salvianolic acid B band, due to lithospermic acid and rosmarinic acid.

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Under UV light at 254 nm, the chromatogram of the *Sample solution* exhibits intense quenching bands at R_F corresponding to those for tanshinone II_A and salvianolic acid B in the chromatogram of *Standard solution A*. The chromatogram of the *Sample solution* also exhibits other quenching bands corresponding in R_F to the bands in the chromatogram of *Standard solution B*.

• **C. HPLC**

Analysis: Proceed as directed in the test for *Content of Tanshinones*.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits the most intense peak at a retention time corresponding to that of tanshinone II_A in the chromatogram of *Standard solution A*. The *Sample solution* chromatogram exhibits two additional peaks corresponding to tanshinone I and cryptotanshinone, of less intensity and accounting for about half of the total tanshinones content.

• **D. HPLC**

Analysis: Proceed as directed in the test for *Content of Salvianolic Acid B*.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits a peak at a retention time corresponding to that of salvianolic acid B in the chromatogram of *Standard solution A*.

COMPOSITION

• **CONTENT OF TANSHINONES**

Solution A: 0.02% phosphoric acid in water (v/v)

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	39	61
6	39	61
20	10	90
20.5	39	61
25	39	61

[NOTE—Proceed under subdued light or use low-actinic glassware. The *Standard solution* and *Sample solution* are stable for 24 h at room temperature.]

Standard solution A: 0.02 mg/mL of USP Tanshinone II_A RS in methanol

Standard solution B: 2 mg/mL of USP Powdered Chinese Salvia Extract RS in methanol. Sonicate for 15 min, and pass through a membrane filter having a 0.45-µm pore size. Discard the first few mL of the filtrate.

Sample solution: About 300 mg of Chinese Salvia, finely powdered and accurately weighed, in 40 mL of methanol. Sonicate for 30 min, filter into a 50-mL volumetric flask, and wash the residue and the filter paper with a few mL of methanol. Adjust with methanol to volume, mix, and pass through a membrane filter having a 0.45-µm pore size. Discard the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 270 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 20°

Flow rate: 1.0 mL/min

Injection volume: 10 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Chinese Salvia Extract RS being used.

Tailing factor: NMT 2.0 for the tanshinone II_A peak, *Standard solution A*

Relative standard deviation: NMT 2.0%, determined from the tanshinone II_A peak in repeated injections, *Standard solution A*

Resolution: NLT 1.5 between the cryptotanshinone and tanshinone I peaks, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Chinese Salvia Extract RS being used, identify the retention times of the peaks corresponding to different tanshinones in the *Sample solution* chromatogram. The approximate relative retention times of the different peaks for cryptotanshinone, tanshinone I, and tanshinone II_A are 0.75, 0.79, and 1.00, respectively.

Calculate the percentages of cryptotanshinone, tanshinone I, and tanshinone II_A in the portion of Chinese Salvia taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*

r_S = peak area of tanshinone II_A from *Standard solution A*

C_S = concentration of USP Tanshinone II_A RS in *Standard solution A* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Chinese Salvia taken to prepare the *Sample solution* (mg)

F = conversion factor for analytes (1.18 for cryptotanshinone, 1.31 for tanshinone I, and 1.00 for tanshinone II_A)

Add the percentages of cryptotanshinone, tanshinone I, and tanshinone II_A.

Acceptance criteria: NLT 0.1% tanshinone II_A and NLT 0.2% of total tanshinones, calculated on the dried basis

• **CONTENT OF SALVIANOLIC ACID B**

Solution A: 0.1% phosphoric acid in water (v/v)

Mobile phase: *Solution A* and acetonitrile (78:22)

[NOTE—The *Standard solution* and *Sample solution* are stable for 12 h at room temperature.]

Solvent: Methanol and water (8:2)

Standard solution: 0.1 mg/mL of USP Salvianolic Acid B RS in *Solvent*

Sample stock solution: About 150 mg of Chinese Salvia, finely powdered and accurately weighed, in 40 mL of *Solvent*. Sonicate for 30 min, filter into a 50-mL volumetric flask, and wash the residue and the filter paper with a few mL of *Solvent*. Adjust with *Solvent* to volume, mix, and centrifuge a portion.

Sample solution: Dilute a portion of the supernatant from the *Sample stock solution* (1:2) with *Solvent*, mix, and pass through a membrane filter having a 0.45-µm pore size. Discard the first 2 mL of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

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Mode: LC
Detector: UV 286 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 20 ± 1°
Flow rate: 1.2 mL/min
Injection volume: 10 µL

System suitability
Sample: *Standard solution*
Suitability requirements
Tailing factor: NMT 2.0 for the salvianolic acid B peak
Relative standard deviation: NMT 2.0%, determined from the salvianolic acid B peak in repeated injections

Analysis
Samples: *Standard solution* and *Sample solution*
Using the chromatogram of the *Standard solution*, identify the retention time of the peak corresponding to salvianolic acid B in the *Sample solution*.
Calculate the percentage of salvianolic acid B in the portion of Chinese Salvia taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times 100$$

r_U = peak area of salvianolic acid B from the *Sample solution*
r_S = peak area of salvianolic acid B from the *Standard solution*
C_S = concentration of USP Salvianolic Acid B RS in the *Standard solution* (mg/mL)
V = volume of the *Sample stock solution* (mL)
W = weight of Chinese Salvia used to prepare the *Sample stock solution* (mg)
D = dilution factor to prepare the *Sample solution* from the *Sample stock solution*, 2

Acceptance criteria: NLT 3.0%, calculated on the dried basis

CONTAMINANTS

- **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩

For deionized water: Use deionized water of at least 18 megaohm.
Sample solution: Use Chinese Salvia previously dried for 2 h at 60°, ground to coarse powder. Accurately weigh 0.5 g of the powder, transfer to a closed microwave vessel, and add 5–10 mL of concentrated nitric acid. [NOTE—In case of a severe reaction, set the vessel aside until the reaction ceases.] Digest under pressure following the instrument manufacturer's recommendations, cool to below 60°, and remove the vessel. Cool to room temperature, transfer the contents with the aid of three 10-mL portions of deionized water to a 250-mL volumetric flask, dilute with deionized water to volume, and mix. [NOTE—In case of deposits, centrifuge, and use the supernatant.]
Acceptance criteria
Arsenic: NMT 2 µg/g
Cadmium: NMT 0.3 µg/g
Lead: NMT 5 µg/g
Mercury: NMT 0.2 µg/g

Change to read:

- **▲ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩▲ (CN 1-May-2019)**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*
- **ARTICLES OF BOTANICAL ORIGIN,** *Test for Aflatoxins* ⟨561⟩**:** Meets the requirements

SPECIFIC TESTS

- **BOTANIC CHARACTERISTICS**

Macroscopic: Rhizomes short and thick, sometimes with remains of stems at the apex. Roots, long, cylindrical, slightly curved, some branched, with rootlets, 10–20 cm long, 0.3–1.5 cm in diameter. Externally brownish-red or dark brownish-red, rough, longitudinally wrinkled. The bark of old roots is loose, mostly purplish-brown, usually scaling off; the bark of young roots is closely adhering to wood and uneasy to be scaled off. Texture hard and fragile, fracture loose, with brownish-red bark and greyish-yellow or purplish-brown wood, showing bundles of vessels, yellowish-white, arranged radially.
Microscopic
Transverse section: Cork, 4–8 rows of cells with brown contents; rhytidome tissues may be present; cortex broad, parenchyma cells showing reddish-brown granules; phloem narrow, crescent shape; cambium in a ring; xylem vessels, lignified, mainly scalariform and reticulate, numerous near the cambium ring and fewer near the pith; xylem fibers in bundle, scattered radially; pith in the center.

- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩**:** NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Alcohol-Soluble Extractives,Method 1* ⟨561⟩**:** NLT 15.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Water-Soluble Extractives,Method 2* ⟨561⟩**:** NLT 35.0%
- **LOSS ON DRYING** ⟨731⟩

Sample: 1.0 g of finely powdered Chinese Salvia
Analysis: Dry at 105° for 2 h.
Acceptance criteria: NMT 13%

- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩

Sample: 4.0 g of finely powdered Chinese Salvia
Acceptance criteria: NMT 10%

- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 3.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Powdered Chinese Salvia Extract RS
USP Salvianolic Acid B RS
USP Tanshinone II_A RS

Powdered Chinese Salvia

DEFINITION

Powdered Chinese Salvia is Chinese Salvia reduced to a powder or very fine powder. It contains NLT 0.1% tanshinone II_A; NLT 0.2% of total tanshinones, calculated as the sum of cryptotanshinone, tanshinone I, and tanshinone II_A; and NLT 3.0% of salvianolic acid B; all calculated on the dried basis.

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IDENTIFICATION

• **A.** Powdered Chinese Salvia meets the requirements for *Specific Tests, Botanic Characteristics.*

• **B. THIN-LAYER CHROMATOGRAPHY**

Standard solution A: A mixture of about 0.5 mg/mL of USP Tanshinone II_A RS and about 1.5 mg/mL of USP Salvianolic Acid B RS in alcohol

Standard solution B: About 0.25 g of USP Powdered Chinese Salvia Extract RS in 5.0 mL of alcohol. Sonicate for 15 min, centrifuge, and use the supernatant.

Sample solution: About 1.0 g of Powdered Chinese Salvia in 5.0 mL of alcohol. Sonicate for 15 min, centrifuge, and use the supernatant.

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography.*)

Adsorbent: Chromatographic silica gel mixture with an average particle size of 2–10 µm (HPTLC plates)

Application volume: 5 µL, as 8-mm bands

Developing solvent system A: A mixture of ethyl acetate, chloroform, toluene, formic acid, and methanol (8:6:4:4:1)

Developing solvent system B: A mixture of solvent hexane and ethyl acetate (4:1)

Analysis

Samples: *Standard solution A, Standard solution B,* and *Sample solution*

Apply the samples as bands to a suitable high performance thin-layer chromatographic plate. Use a saturated chamber, and condition the plate to a relative humidity of about 33% using a suitable device. Develop the chromatograms in *Developing solvent system A* until the solvent front has moved up about 40% of the plate. Remove the plate, and allow to dry. Develop the chromatograms in a saturated chamber containing *Developing solvent system B* until the solvent front has moved up about three-fourths of the plate. Remove the plate, dry, and examine under visible light and UV light at 254 nm and 365 nm.

Acceptance criteria

Under visible light, the chromatogram of the *Sample solution* exhibits three bands similar in positions and colors to bands in the chromatogram of *Standard solution B.* These include a pink band in the upper third of the chromatogram, similar in R_F and color to the tanshinone II_A band in the chromatogram of *Standard solution A,* a yellowish-orange band in the upper third of the chromatogram, and an orange band at about the middle of the chromatogram due to tanshinone I and cryptotanshinone, respectively.

Under UV light at 365 nm, the chromatogram of the *Sample solution* exhibits three blue bands similar in positions and colors to bands in the chromatogram of *Standard solution B.* These include an intense blue band in the lower third of the chromatogram corresponding in R_F and color to the salvianolic acid B band in the chromatogram of *Standard solution A,* and two minor blue bands in the lower third of the chromatogram and above the salvianolic acid B band, due to lithospermic acid and rosmarinic acid.

Under UV light at 254 nm, the chromatogram of the *Sample solution* exhibits intense quenching bands at R_F corresponding to those for tanshinone II_A and salvianolic acid B in the chromatogram of *Standard solution A.* The chromatogram of the *Sample solution* also exhibits other quenching bands corresponding in R_F to the bands in the chromatogram of the *Standard solution B.*

• **C. HPLC**

Analysis: Proceed as directed in the test for *Content of Tanshinones.*

Acceptance criteria: The chromatogram of the *Sample solution* exhibits the most intense peak at a retention time corresponding to that of tanshinone II_A in the chromatogram of *Standard solution A.* The *Sample solution* chromatogram exhibits two additional peaks corresponding to tanshinone I and cryptotanshinone, of less intensity and accounting for about half of the total tanshinones content.

• **D. HPLC**

Analysis: Proceed as directed in the test for *Content of Salvianolic Acid B.*

Acceptance criteria: The chromatogram of the *Sample solution* exhibits a peak at a retention time corresponding to that of salvianolic acid B in the chromatogram of *Standard solution A.*

COMPOSITION

• **CONTENT OF TANSHINONES**

Solution A: 0.02% phosphoric acid in water (v/v)

Solution B: Acetonitrile

Mobile phase: See *Table 1.*

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	39	61
6	39	61
20	10	90
20.5	39	61
25	39	61

[NOTE—Proceed under subdued light or use low-actinic glassware. The *Standard solution* and *Sample solution* are stable for 24 h at room temperature.]

Standard solution A: 0.02 mg/mL of USP Tanshinone II_A RS in methanol

Standard solution B: 2 mg/mL of USP Powdered Chinese Salvia Extract RS in methanol. Sonicate for 15 min, and pass through a membrane filter having a 0.45-µm pore size. Discard the first few mL of the filtrate.

Sample solution: About 300 mg of Powdered Chinese Salvia, accurately weighed, in 40 mL of methanol. Sonicate for 30 min, and filter into a 50-mL volumetric flask. Wash the residue and the filter paper with a few mL of methanol, adjust to volume with methanol, mix, and pass through a membrane filter having a 0.45-µm pore size. Discard the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability.*)

Mode: LC

Detector: UV 270 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 20°

Flow rate: 1.0 mL/min

Injection volume: 10 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Chinese Salvia Extract RS being used.

Tailing factor: NMT 2.0 for the tanshinone II_A peak, *Standard solution A*

Relative standard deviation: NMT 2.0%, determined from the tanshinone II_A peak in repeated injections, *Standard solution A*

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Resolution: NLT 1.5 between the cryptotanshinone and tanshinone I peaks, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Chinese Salvia Extract RS being used, identify the retention times of the peaks corresponding to different tanshinones in the *Sample solution* chromatogram. The approximate relative retention times of the different peaks for cryptotanshinone, tanshinone I, and tanshinone II_A are 0.75, 0.79, and 1.00, respectively.

Calculate the percentages of cryptotanshinone, tanshinone I, and tanshinone II_A in the portion of Powdered Chinese Salvia taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*

r_S = peak area of tanshinone II_A from the *Standard solution A*

C_S = concentration of USP Tanshinone II_A RS in the *Standard solution A* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Powdered Chinese Salvia taken to prepare the *Sample solution* (mg)

F = conversion factor for analytes (1.18 for cryptotanshinone, 1.31 for tanshinone I, and 1.00 for tanshinone II_A)

Add the percentages of cryptotanshinone, tanshinone I, and tanshinone II_A.

Acceptance criteria: NLT 0.1% tanshinone II_A and NLT 0.2% of total tanshinones, calculated on the dried basis

- **CONTENT OF SALVIANOLIC ACID B**

Solution A: 0.1% phosphoric acid in water (v/v)

Mobile phase: *Solution A* and acetonitrile (78:22)

[NOTE—The *Standard solution* and *Sample solution* are stable for 12 h at room temperature.]

Solvent: Methanol and water (8:2)

Standard solution: 0.1 mg/mL of USP Salvianolic Acid B RS in *Solvent*

Sample stock solution: About 150 mg of Powdered Chinese Salvia, accurately weighed, in 40 mL of *Solvent*. Sonicate for 30 min, and filter into a 50-mL volumetric flask. Wash the residue and the filter paper with a few mL of *Solvent*, adjust to volume with *Solvent*, mix, and centrifuge a portion.

Sample solution: Dilute a portion of the supernatant from the *Sample stock solution* (1:2) with *Solvent*, mix, and pass through a membrane filter having a 0.45-µm pore size. Discard the first 2 mL of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 286 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 20 ± 1°

Flow rate: 1.2 mL/min

Injection volume: 10 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Tailing factor: NMT 2.0 for the salvianolic acid B peak

Relative standard deviation: NMT 2.0%, determined from the salvianolic acid B peak in repeated injections

Analysis

Samples: *Standard solution* and *Sample solution*

Using the chromatogram of *Standard solution*, identify the retention time of the peak corresponding to salvianolic acid B in the *Sample solution*.

Calculate the percentage of salvianolic acid B in the portion of Powdered Chinese Salvia taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times 100$$

r_U = peak area of salvianolic acid B from the *Sample solution*

r_S = peak area of salvianolic acid B from the *Standard solution*

C_S = concentration of USP Salvianolic Acid B RS in the *Standard solution* (mg/mL)

V = volume of *Sample stock solution* (mL)

W = weight of Powdered Chinese Salvia used to prepare the *Sample solution* (mg)

D = dilution factor to prepare the *Sample solution* from *Sample stock solution*, 2

Acceptance criteria: NLT 3.0%, calculated on the dried basis

CONTAMINANTS

- **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩

For deionized water: Use deionized water of at least 18 megaohm.

Sample solution: Use Powdered Chinese Salvia previously dried for 2 h at 60°, weigh accurately 0.5 g, transfer to a closed microwave vessel, and add 5–10 mL of concentrated nitric acid. [NOTE—In case of a severe reaction, set the vessel aside until the reaction ceases.] Digest under pressure following the instrument manufacturer's recommendations, cool to below 60°, remove the vessel, cool to room temperature, transfer the content with the aid of three 10-mL portions of deionized water to a 250-mL volumetric flask, complete to volume with deionized water, and mix. [NOTE—In case of deposits, centrifuge, and use the supernatant.]

Acceptance criteria

Arsenic: NMT 2 µg/g

Cadmium: NMT 0.3 µg/g

Lead: NMT 5 µg/g

Mercury: NMT 0.2 µg/g

Change to read:

- **▲ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩▲ (CN 1-May-2019)**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*
- **ARTICLES OF BOTANICAL ORIGIN,** *Test for Aflatoxins* ⟨561⟩**:** Meets the requirements

SPECIFIC TESTS

- **BOTANIC CHARACTERISTICS**

Macroscopic: Yellowish-brown to reddish-brown in color

Microscopic: It shows fragments of cork cells, subrectangular or polygonal, containing yellowish-brown pigments, 10–150 µm in diameter; parenchymatous cells of cortex, subsquare or polygonal, containing reddish-brown pigments; stone cells, subrounded,

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subtriangular, subrectangular or irregular shape, some elongated, mostly 14–70 µm in diameter, up to 270 µm in length; fibers mostly in bundles, long fusiform in shape, ends oblique-sharp or blunt-round, with oblique or criss-cross striations, 10–60 µm in diameter; and reticulate and pitted vessels, up to 120 µm in diameter.

- **ARTICLES OF BOTANICAL ORIGIN,** *Alcohol-Soluble Extractives,Method 1* ⟨561⟩**:** NLT 15.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Water-Soluble Extractives,Method 2* ⟨561⟩**:** NLT 35.0%
- **LOSS ON DRYING** ⟨731⟩
  Sample: 1.0 g of Powdered Chinese Salvia
  Analysis: Dry at 105° for 2 h.
  Acceptance criteria: NMT 13%
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩
  Sample: 4.0 g of Powdered Chinese Salvia
  Acceptance criteria: NMT 10%
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 3.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant from which the article was obtained.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Powdered Chinese Salvia Extract RS
  USP Salvianolic Acid B RS
  USP Tanshinone II_A RS

Cholecalciferol—*see Cholecalciferol General Monographs*

Cholecalciferol Capsules—*see Cholecalciferol Capsules General Monographs*

Add the following:

▲Chinese Skullcap Root

DEFINITION

Chinese Skullcap Root consists of the dried roots of *Scutellaria baicalensis* Georgi (Family Lamiaceae) collected in spring or autumn by removing fibrous roots and rough skin. It contains NLT 11% of total flavone glucuronides calculated as the sum of baicalein 7-*O*-glucuronide ($C_{21}H_{18}O_{11}$) and wogonin 7-*O*-glucuronide ($C_{22}H_{20}O_{11}$) on the dried basis; and NMT 3.5% of total flavone aglycones calculated as the sum of baicalein ($C_{15}H_{10}O_5$) and wogonin ($C_{16}H_{12}O_5$) on the dried basis.

IDENTIFICATION

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩
  Standard solution A: 1.0 mg/mL of USP Baicalein 7-*O*-Glucuronide RS and 0.5 mg/mL of USP Baicalein RS in methanol. Sonicate to dissolve.
  Standard solution B: 50 mg/mL of USP *Scutellaria baicalensis* Root Dry Extract RS in methanol. Sonicate for 15 min, centrifuge, and use the supernatant.
  Sample solution: 100 mg/mL of finely powdered Chinese Skullcap Root in methanol. Sonicate for 15 min, centrifuge, and use the supernatant.
  Chromatographic system
  Adsorbent: Chromatographic silica gel F_{254} mixture
  Application volume: 3 µL, as 8-mm bands
  Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
  Temperature: About 25°
  Developing solvent system: Toluene, ethyl acetate, methanol, and formic acid (6:4:1:2)
  Developing distance: 6 cm
  Derivatization reagent A: 10 mg/mL of 2-aminoethyl diphenylborinate in methanol
  Derivatization reagent B: 50 mg/mL of polyethylene glycol 4000 in alcohol
  Analysis
  Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
  Apply the *Samples* as bands and dry in air. Develop in a saturated chamber, remove the plate from the chamber, and dry the plate at 100° for 3 min. Treat the plate with *Derivatization reagent A*, and dry for 5 min with a current of cool air. Immediately treat the plate with *Derivatization reagent B*, dry for 5 min with a current of cool air, and examine under UV light at 365 nm.
  System suitability
  Samples: *Standard solution A* and *Standard solution B*
  Suitability requirements: *Standard solution A* exhibits two dark bands, one due to baicalein 7-*O*-glucuronide in the lower-third section and one due to baicalein in the middle-third section. *Standard solution B* exhibits four dark bands: two corresponding in R_F and color to the bands due to baicalein 7-*O*-glucuronide and baicalein in *Standard solution A*; one above baicalein 7-*O*-glucuronide due to wogonin 7-*O*-glucuronide; and one above baicalein due to wogonin. *Standard solution B* exhibits two blue bands below baicalein, a light-yellow band coeluted with wogonin 7-*O*-glucuronide, and a yellow band below baicalein 7-*O*-glucuronide.
  Acceptance criteria: The *Sample solution* exhibits four dark bands: two corresponding in R_F and color to the bands due to baicalein 7-*O*-glucuronide and baicalein in *Standard solution A* and *Standard solution B*; and one above baicalein 7-*O*-glucuronide due to wogonin 7-*O*-glucuronide and one above baicalein due to wogonin, both corresponding in R_F and color to similar bands in *Standard solution B*. The *Sample solution* exhibits additional colorful bands including two blue bands below baicalein, a light-yellow band coeluted with wogonin 7-*O*-glucuronide, a yellow band below baicalein 7-*O*-glucuronide, and some other faint bands corresponding in R_F and color to similar bands in *Standard solution B*. There are no significant red bands observed in the upper half of the chromatogram (the leaves of *S. lateriflora*, *S. scordiifolia*, *S. barbata*, and *S. indica* display multiple red bands in the upper-half section above baicalein).
- **B. HPLC**
  Analysis: Proceed as directed in the test for *Content of Flavone Glucuronides and Flavone Aglycones*.
  Acceptance criteria: The *Sample solution* exhibits the most intense peak corresponding to baicalein 7-*O*-glucuronide and a smaller peak corresponding to baicalein in *Standard solution A*. The *Sample solution* also exhibits peaks due to wogonin 7-*O*-glucuronide, wogonin, and two unidentified peaks with similar intensities as wogonin, between baicalein 7-*O*-glucuronide and wogonin 7-*O*-glucuronide, at

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retention times corresponding to the same constituents in *Standard solution B*. No other peak in the chromatogram between the relative retention time of 0.5 for baicalein 7-*O*-glucuronide and 1.2 for wogonin is more intense than the peak corresponding to wogonin. The content ratio of wogonin 7-*O*-glucuronide relative to baicalein 7-*O*-glucuronide is NLT 0.1 and NMT 0.3, and the content ratio of total flavone glucuronides to total flavone aglycones is NLT 3.0.

COMPOSITION

• **CONTENT OF FLAVONE GLUCURONIDES AND FLAVONE AGLYCONES**

[NOTE—Protect solutions from light and proceed under low actinic light. The standard solutions and the *Sample solution* are stable for 24 h at room temperature.]

Solution A: 0.1% phosphoric acid in water
Solution B: Acetonitrile
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	78	22
10	75	25
15	75	25
25	68	32
30	60	40
35	60	40
40	50	50
45	5	95
50	5	95
50.1	78	22
60	78	22

Solvent: Methanol and water (7:3)
Standard stock solution: 0.50 mg/mL of USP Baicalein 7-*O*-Glucuronide RS and 0.10 mg/mL of USP Baicalein RS in methanol
Standard solution A: 0.10 mg/mL of USP Baicalein 7-*O*-Glucuronide RS and 0.02 mg/mL of USP Baicalein RS from *Standard stock solution* diluted with *Solvent*
Standard solution B: 1 mg/mL of USP *Scutellaria baicalensis* Root Dry Extract RS in *Solvent*. Sonicate for 15 min, centrifuge, and pass through a suitable membrane filter of 0.45-µm or finer pore size.
Sample solution: Transfer about 100 mg of finely powdered Chinese Skullcap Root accurately weighed into a suitable flask, add 50.0 mL of *Solvent*, and close tightly. Weigh the filled flask accurately, and sonicate for 30 min. Cool to room temperature and adjust to the initial weight by adding *Solvent*, if needed. Before injection, pass through a suitable membrane filter of 0.45-µm or finer pore size, and discard the first portion of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 276 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 30°
Flow rate: 1.0 mL/min
Injection volume: 3 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Resolution: NLT 1.5 between the peak of baicalein 7-*O*-glucuronide and the small peak before it, *Standard solution B*
Tailing factor: NMT 2.0 for the baicalein 7-*O*-glucuronide and baicalein peaks, *Standard solution A*
Relative standard deviation: NMT 2.0% for the baicalein 7-*O*-glucuronide and baicalein peaks in repeated injections, *Standard solution A*
Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP *Scutellaria baicalensis* Root Dry Extract RS being used.
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP *Scutellaria baicalensis* Root Dry Extract RS being used, identify the peaks corresponding to baicalein 7-*O*-glucuronide, wogonin 7-*O*-glucuronide, baicalein, and wogonin in the *Sample solution*.
Separately calculate the percentage of baicalein 7-*O*-glucuronide and wogonin 7-*O*-glucuronide against USP Baicalein 7-*O*-Glucuronide RS and the percentage of baicalein and wogonin against USP Baicalein RS in the portion of Chinese Skullcap Root taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of baicalein 7-*O*-glucuronide or baicalein from *Standard solution A*
C_S = concentration of USP Baicalein 7-*O*-Glucuronide RS or USP Baicalein RS in *Standard solution A* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Chinese Skullcap Root taken to prepare the *Sample solution* (mg)
F = conversion factor for the relevant analyte (see *Table 2*)

Calculate the content of the total flavone glucuronides as the sum of the percentages of baicalein 7-*O*-glucuronide and wogonin 7-*O*-glucuronide.
Calculate the content of the total flavone aglycones as the sum of the percentages of baicalein and wogonin.

Table 2

Analyte	Conversion Factor for Flavone Glucuronides	Conversion Factor for Flavone Aglycones
Baicalein 7-*O*-glucuronide	1.00	—
Wogonin 7-*O*-glucuronide	0.86	—
Baicalein	—	1.00
Wogonin	—	0.82

Acceptance criteria
Total flavone glucuronides: NLT 11% on the dried basis
Total flavone aglycones: NMT 3.5% on the dried basis

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CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Limits of Elemental Impurities*: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Pesticide Residue Analysis*: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Test for Aflatoxins*: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacterial count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures*, *Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: The roots are conical and twisted, 8–25 cm long, 1–3 cm in diameter; the upper part rough with twisted longitudinal wrinkles or irregular reticular stripes; the lower part with longitudinal striations and fine wrinkles. The outer surface is brownish yellow or dark yellow with sparse and warty rootlet scars. Texture is hard and fragile, easily broken. The fracture is yellow but reddish brown in the center. The old roots have a dark-brown fracture with a withered or hollow center.

Microscopic

Transverse section: The cork consists of multiple layers of cells, up to 20 layers, mostly broken. The phelloderm is narrow with scattered sclereids. The phloem is broad with scattered sclereids in the outer part. The cambium is distinct. In the xylem, vessels are singly scattered or aggregated into groups and surrounded by lignified fiber bundles; xylem rays are broad. The parenchymatous cells contain starch granules and clusters of calcium oxalate. The central part of an old root usually appears with more than 1 cork tissue whorl.

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Foreign Organic Matter*: NMT 1.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Alcohol-Soluble Extractives, Method 1*: NLT 18.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Water-Soluble Extractives, Method 2*: NLT 28.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Total Ash*: NMT 6.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Acid-Insoluble Ash*: NMT 1.0%
- **LOSS ON DRYING** ⟨731⟩

Sample: The roots, finely powdered
Analysis: Dry the *Sample* at 105° for 5 h.
Acceptance criteria: NMT 12.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light and moisture, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial following the official name of the plant contained in the article. Dosage forms prepared with this article should bear the following statement: "Discontinue use and consult a healthcare practitioner if you develop symptoms of liver trouble, such as abdominal pain, dark urine, or jaundice (yellowing of the eyes or skin)."
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Baicalein RS
USP Baicalein 7-*O*-Glucuronide RS
USP *Scutellaria baicalensis* Root Dry Extract RS ▲(USP 1-Dec-2019)

Add the following:

▲Chinese Skullcap Root Powder

DEFINITION

Chinese Skullcap Root Powder consists of the dried roots of Chinese Skullcap (*Scutellaria baicalensis* Georgi) reduced to a fine or very fine powder. It contains NLT 11% of total flavone glucuronides calculated as the sum of baicalein 7-*O*-glucuronide ($C_{21}H_{18}O_{11}$) and wogonin 7-*O*-glucuronide ($C_{22}H_{20}O_{11}$) on the dried basis, and NMT 3.5% of total flavone aglycones calculated as the sum of baicalein ($C_{15}H_{10}O_5$) and wogonin ($C_{16}H_{12}O_5$) on the dried basis.

IDENTIFICATION

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 1.0 mg/mL of USP Baicalein 7-*O*-Glucuronide RS and 0.5 mg/mL of USP Baicalein RS in methanol. Sonicate to dissolve.
Standard solution B: 50 mg/mL of USP *Scutellaria baicalensis* Root Dry Extract RS in methanol. Sonicate for 15 min, centrifuge, and use the supernatant.
Sample solution: 100 mg/mL of Chinese Skullcap Root Powder in methanol. Sonicate for 15 min, centrifuge, and use the supernatant.
Chromatographic system
Adsorbent: Chromatographic silica gel F_{254} mixture
Application volume: 3 µL, as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
Temperature: About 25°
Developing solvent system: Toluene, ethyl acetate, methanol, and formic acid (6:4:1:2)
Developing distance: 6 cm
Derivatization reagent A: 10 mg/mL of 2-aminoethyl diphenylborinate in methanol
Derivatization reagent B: 50 mg/mL of polyethylene glycol 4000 in alcohol
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands and dry in air. Develop in a saturated chamber, remove the plate from the chamber, and dry the plate at 100° for 3 min. Treat the plate with *Derivatization reagent A*, and dry for 5 min with a current of cool air. Immediately treat the plate with *Derivatization reagent B*, dry for 5 min with a current of cool air, and examine under UV light at 365 nm.

System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements: *Standard solution A* exhibits two dark bands, one due to baicalein 7-*O*-glucuronide in the lower-third section and one due to baicalein in the middle-third section. *Standard solution B* exhibits four dark bands: two corresponding in R_F and color to the bands due to baicalein 7-*O*-glucuronide and baicalein in *Standard solution A*; one above baicalein 7-*O*-glucuronide due to wogonin 7-*O*-glucuronide; and one above baicalein due to wogonin. *Standard solution B* exhibits two blue bands below baicalein, a light-yellow band coeluted with wogonin 7-*O*-glucuronide, and a yellow band below baicalein 7-*O*-glucuronide.
Acceptance criteria: The *Sample solution* exhibits four dark bands: two corresponding in R_F and color to the bands due to baicalein 7-*O*-glucuronide and baicalein in *Standard solution A* and *Standard solution B*; and one above baicalein

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7-*O*-glucuronide due to wogonin 7-*O*-glucuronide and one above baicalein due to wogonin, both corresponding in R_F and color to similar bands in *Standard solution B*. The *Sample solution* exhibits additional colorful bands including two blue bands below baiclein, a light-yellow band coeluted with wogonin 7-*O*-glucuronide, a yellow band below baicalein 7-*O*-glucuronide, and some other faint bands corresponding in R_F and color to similar bands in *Standard solution B*. There are no significant red bands observed in the upper half of the chromatogram (the leaves of *S. lateriflora*, *S. scordiifolia*, *S. barbata*, and *S. indica* display multiple red bands in the upper-half section above baicalein).

• **B. HPLC**

Analysis: Proceed as directed in the test for *Content of Flavone Glucuronides and Flavone Aglycones*.

Acceptance criteria: The *Sample solution* exhibits the most intense peak corresponding to baicalein 7-*O*-glucuronide and a smaller peak corresponding to baicalein in *Standard solution A*. The *Sample solution* also exhibits peaks due to wogonin 7-*O*-glucuronide, wogonin, and two unidentified peaks with similar intensities as wogonin, between baicalein 7-*O*-glucuronide and wogonin 7-*O*-glucuronide, at retention times corresponding to the same constituents in *Standard solution B*. No other peak in the chromatogram between the relative retention time of 0.5 for baicalein 7-*O*-glucuronide and 1.2 for wogonin is more intense than the peak corresponding to wogonin. The content ratio of wogonin 7-*O*-glucuronide relative to baicalein 7-*O*-glucuronide is NLT 0.1 and NMT 0.3, and the content ratio of total flavone glucuronides to total flavone aglycones is NLT 3.0.

COMPOSITION

• **CONTENT OF FLAVONE GLUCURONIDES AND FLAVONE AGLYCONES**

[NOTE—Protect solutions from light and proceed under low actinic light. The standard solutions and the *Sample solution* are stable for 24 h at room temperature.]

Solution A: 0.1% phosphoric acid in water

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	78	22
10	75	25
15	75	25
25	68	32
30	60	40
35	60	40
40	50	50
45	5	95
50	5	95
50.1	78	22
60	78	22

Solvent: Methanol and water (7:3)

Standard stock solution: 0.50 mg/mL of USP Baicalein 7-*O*-Glucuronide RS and 0.10 mg/mL of USP Baicalein RS in methanol

Standard solution A: 0.10 mg/mL of USP Baicalein 7-*O*-Glucuronide RS and 0.02 mg/mL of USP Baicalein RS from *Standard stock solution* diluted with *Solvent*

Standard solution B: 1 mg/mL of USP *Scutellaria baicalensis* Root Dry Extract RS in *Solvent*. Sonicate for 15 min, centrifuge, and pass through a suitable membrane filter of 0.45-µm or finer pore size.

Sample solution: Transfer about 100 mg of Chinese Skullcap Root Powder accurately weighed into a suitable flask, add 50.0 mL of *Solvent*, and close tightly. Weigh the filled flask accurately, and sonicate for 30 min. Cool to room temperature and adjust to the initial weight by adding *Solvent*, if needed. Before injection, pass through a suitable membrane filter of 0.45-µm or finer pore size, and discard the first portion of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 276 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 30°

Flow rate: 1.0 mL/min

Injection volume: 3 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Resolution: NLT 1.5 between the peak of baicalein 7-*O*-glucuronide and the small peak before it, *Standard solution B*

Tailing factor: NMT 2.0 for the baicalein 7-*O*-glucuronide and baicalein peaks, *Standard solution A*

Relative standard deviation: NMT 2.0% for the baicalein 7-*O*-glucuronide and baicalein peaks in repeated injections, *Standard solution A*

Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP *Scutellaria baicalensis* Root Dry Extract RS being used.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP *Scutellaria baicalensis* Root Dry Extract RS being used, identify the peaks corresponding to baicalein 7-*O*-glucuronide, wogonin 7-*O*-glucuronide, baicalein, and wogonin in the *Sample solution*.

Separately calculate the percentage of baicalein 7-*O*-glucuronide and wogonin 7-*O*-glucuronide against USP Baicalein 7-*O*-Glucuronide RS and the percentage of baicalein and wogonin against USP Baicalein RS in the portion of Chinese Skullcap Root Powder taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*

r_S = peak area of baicalein 7-*O*-glucuronide or baicalein from *Standard solution A*

C_S = concentration of USP Baicalein 7-*O*-Glucuronide RS or USP Baicalein RS in *Standard solution A* (mg/mL)

V = volume of the *Sample solution* (mL)

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W = weight of Chinese Skullcap Root Powder taken to prepare the *Sample solution* (mg)
F = conversion factor for the relevant analyte (see *Table 2*)

Calculate the content of the total flavone glucuronides as the sum of the percentages of baicalein 7-*O*-glucuronide and wogonin 7-*O*-glucuronide.
Calculate the content of the total flavone aglycones as the sum of the percentages of baicalein and wogonin.

Table 2

Analyte	Conversion Factor for Flavone Glucuronides	Conversion Factor for Flavone Aglycones
Baicalein 7-*O*-glucuronide	1.00	—
Wogonin 7-*O*-glucuronide	0.86	—
Baicalein	—	1.00
Wogonin	—	0.82

Acceptance criteria
Total flavone glucuronides: NLT 11% on the dried basis
Total flavone aglycones: NMT 3.5% on the dried basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Limits of Elemental Impurities*: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Pesticide Residue Analysis*: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Test for Aflatoxins*: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacterial count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures*, *Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: Yellowish-white or yellowish-green powder
Microscopic: Phloem fibers are fusiform, 60–250 µm long, 9–33 µm in diameter, with thick walls and fine pit canals, singly scattered or aggregated in bundles. Sclereids are subround, subsquare, or rectangular, with thick or very thick walls. Cork cells are brownish yellow and polygonal in surface view. Reticulate vessels are dominating, 24–72 µm in diameter. Lignified fibers are mostly broken with sparse oblique pits, about 12 µm in diameter. Starch granules are abundant, simple granules spheroidal, 2–10 µm in diameter, hilum distinct, compound granules composed of 2–3 simple granules.

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Alcohol-Soluble Extractives, Method 1*: NLT 18.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Water-Soluble Extractives, Method 2*: NLT 28.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Total Ash*: NMT 6.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Acid-Insoluble Ash*: NMT 1.0%
- **LOSS ON DRYING** ⟨731⟩

Analysis: Dry at 105° for 5 h.
Acceptance criteria: NMT 12.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light and moisture, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial following the official name of the plant contained in the article. Dosage forms prepared with this article should bear the following statement: "Discontinue use and consult a healthcare practitioner if you develop symptoms of liver trouble, such as abdominal pain, dark urine, or jaundice (yellowing of the eyes or skin)."
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Baicalein RS
USP Baicalein 7-*O*-Glucuronide RS
USP *Scutellaria baicalensis* Root Dry Extract RS▲ (USP 1-Dec-2019)

Add the following:

▲Chinese Skullcap Root Dry Extract

DEFINITION

Chinese Skullcap Root Dry Extract is prepared from Chinese Skullcap Root (*Scutellaria baicalensis* Georgi) by extraction with water or hydroalcoholic mixtures. It contains NLT 90% and NMT 110% of the labeled amount of total flavone glucuronides calculated as the sum of baicalein 7-*O*-glucuronide ($C_{21}H_{18}O_{11}$) and wogonin 7-*O*-glucuronide ($C_{22}H_{20}O_{11}$) on the dried basis, and NLT 90% and NMT 110% of the labeled amount of total flavone aglycones calculated as the sum of baicalein ($C_{15}H_{10}O_5$) and wogonin ($C_{16}H_{12}O_5$) on the dried basis. It may contain suitable added substances as carriers.

IDENTIFICATION

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 1.0 mg/mL of USP Baicalein 7-*O*-Glucuronide RS and 0.5 mg/mL of USP Baicalein RS in methanol. Sonicate to dissolve.
Standard solution B: 50 mg/mL of USP *Scutellaria baicalensis* Root Dry Extract RS in methanol. Sonicate for 15 min, centrifuge, and use the supernatant.
Sample solution: 50 mg/mL of Chinese Skullcap Root Dry Extract in methanol. Sonicate for 15 min, centrifuge, and use the supernatant.
Chromatographic system
Adsorbent: Chromatographic silica gel F_{254} mixture
Application volume: 3 µL, as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
Temperature: About 25°
Developing solvent system: Toluene, ethyl acetate, methanol, and formic acid (6:4:1:2)
Developing distance: 6 cm
Derivatization reagent A: 10 mg/mL of 2-aminoethyl diphenylborinate in methanol
Derivatization reagent B: 50 mg/mL of polyethylene glycol 4000 in alcohol
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Apply the *Samples* as bands and dry in air. Develop in a saturated chamber, remove the plate from the chamber, and dry the plate at 100° for 3 min. Treat the plate with *Derivatization reagent A*, and dry for 5 min with a current of cool air. Immediately treat the plate with

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Derivatization reagent B, dry for 5 min with a current of cool air, and examine under UV light at 365 nm.

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements: *Standard solution A* exhibits two dark bands, one due to baicalein 7-*O*-glucuronide in the lower-third section and one due to baicalein in the middle-third section. *Standard solution B* exhibits four dark bands: two corresponding in R_F and color to the bands due to baicalein 7-*O*-glucuronide and baicalein in *Standard solution A*; one above baicalein 7-*O*-glucuronide due to wogonin 7-*O*-glucuronide; and one above baicalein due to wogonin. *Standard solution B* exhibits two blue bands below baicalein, a light-yellow band coeluted with wogonin 7-*O*-glucuronide, and a yellow band below baicalein 7-*O*-glucuronide.

Acceptance criteria: The *Sample solution* exhibits four dark bands: two corresponding in R_F and color to the bands due to baicalein 7-*O*-glucuronide and baicalein in *Standard solution A* and *Standard solution B*; and one above baicalein 7-*O*-glucuronide due to wogonin 7-*O*-glucuronide and one above baicalein due to wogonin, both corresponding in R_F and color to similar bands in *Standard solution B*. The *Sample solution* exhibits additional colorful bands including two blue bands below baicalein, a light-yellow band coeluted with wogonin 7-*O*-glucuronide, a yellow band below baicalein 7-*O*-glucuronide, and some other faint bands corresponding in R_F and color to similar bands in *Standard solution B*. There are no significant red bands observed in the upper half of the chromatogram (the leaves of *S. lateriflora*, *S. scordiifolia*, *S. barbata*, and *S. indica* display multiple red bands in the upper-half section above baicalein).

• **B. HPLC**

Analysis: Proceed as directed in the test for *Content of Flavone Glucuronides and Flavone Aglycones*.

Acceptance criteria: The *Sample solution* exhibits the most intense peak corresponding to baicalein 7-*O*-glucuronide and a smaller peak corresponding to baicalein in *Standard solution A*. The *Sample solution* also exhibits peaks due to wogonin 7-*O*-glucuronide, wogonin, and two unidentified peaks with similar intensities as wogonin, between baicalein 7-*O*-glucuronide and wogonin 7-*O*-glucuronide, at retention times corresponding to the same constituents in *Standard solution B*. No other peak in the chromatogram between the relative retention time of 0.5 for baicalein 7-*O*-glucuronide and 1.2 for wogonin is more intense than the peak corresponding to wogonin. The content ratio of wogonin 7-*O*-glucuronide relative to baicalein 7-*O*-glucuronide is NLT 0.1 and NMT 0.3, and the content ratio of total flavone glucuronides to total flavone aglycones is NLT 4.0.

COMPOSITION

• **CONTENT OF FLAVONE GLUCURONIDES AND FLAVONE AGLYCONES**

[NOTE—Protect solutions from light and proceed under low actinic light. The standard solutions and the *Sample solution* are stable for 24 h at room temperature.]

Solution A: 0.1% phosphoric acid in water

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	78	22
10	75	25
15	75	25
25	68	32
30	60	40
35	60	40
40	50	50
45	5	95
50	5	95
50.1	78	22
60	78	22

Solvent: Methanol and water (7:3)

Standard stock solution: 0.50 mg/mL of USP Baicalein 7-*O*-Glucuronide RS and 0.10 mg/mL of USP Baicalein RS in methanol

Standard solution A: 0.10 mg/mL of USP Baicalein 7-*O*-Glucuronide RS and 0.02 mg/mL of USP Baicalein RS from *Standard stock solution* diluted with *Solvent*

Standard solution B: 1 mg/mL of USP *Scutellaria baicalensis* Root Dry Extract RS in *Solvent*. Sonicate for 15 min, centrifuge, and pass through a suitable membrane filter of 0.45-µm or finer pore size.

Sample solution: Transfer about 50 mg of Chinese Skullcap Root Dry Extract accurately weighed into a suitable flask, add 50.0 mL of *Solvent*, and close tightly. Weigh the filled flask accurately, and sonicate for 30 min. Cool to room temperature and adjust to the initial weight by adding *Solvent*, if needed. Before injection, pass through a suitable membrane filter of 0.45-µm or finer pore size, and discard the first portion of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 276 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 30°

Flow rate: 1.0 mL/min

Injection volume: 3 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Resolution: NLT 1.5 between the peak of baicalein 7-*O*-glucuronide and the small peak before it, *Standard solution B*

Tailing factor: NMT 2.0 for the baicalein 7-*O*-glucuronide and baicalein peaks, *Standard solution A*

Relative standard deviation: NMT 2.0% for the baicalein 7-*O*-glucuronide and baicalein peaks in repeated injections, *Standard solution A*

Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP *Scutellaria baicalensis* Root Dry Extract RS being used.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

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Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP *Scutellaria baicalensis* Root Dry Extract RS being used, identify the peaks corresponding to baicalein 7-*O*-glucuronide, wogonin 7-*O*-glucuronide, baicalein, and wogonin in the *Sample solution*.

Separately calculate the percentage of baicalein 7-*O*-glucuronide and wogonin 7-*O*-glucuronide against USP Baicalein 7-*O*-Glucuronide RS and the percentage of baicalein and wogonin against USP Baicalein RS in the portion of Chinese Skullcap Root Dry Extract taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*

r_S = peak area of baicalein 7-*O*-glucuronide or baicalein from *Standard solution A*

C_S = concentration of USP Baicalein 7-*O*-Glucuronide RS or USP Baicalein RS in *Standard solution A* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Chinese Skullcap Root Dry Extract taken to prepare the *Sample solution* (mg)

F = conversion factor for the relevant analyte (see *Table 2*)

Calculate the content of the total flavone glucuronides as the sum of the percentages of baicalein 7-*O*-glucuronide and wogonin 7-*O*-glucuronide.

Calculate the percentage of the labeled amount of total flavone glucuronides in the portion of Chinese Skullcap Root Dry Extract taken:

$$\text{Result} = (P/L) \times 100$$

P = content of total flavone glucuronides as determined previously (%)

L = labeled amount of total flavone glucuronides (%)

Calculate the content of the total flavone aglycones as the sum of the percentages of baicalein and wogonin.

Calculate the percentage of the labeled amount of total flavone aglycones in the portion of Chinese Skullcap Root Dry Extract taken:

$$\text{Result} = (P/L) \times 100$$

P = content of total flavone aglycones as determined previously (%)

L = labeled amount of total flavone aglycones (%)

Table 2

Analyte	Conversion Factor for Flavone Glucuronides	Conversion Factor for Flavone Aglycones
Baicalein 7-*O*-glucuronide	1.00	—
Wogonin 7-*O*-glucuronide	0.86	—
Baicalein	—	1.00
Wogonin	—	0.82

Acceptance criteria

Total flavone glucuronides: 90%–110% of the labeled amount on the dried basis

Total flavone aglycones: 90%–110% of the labeled amount on the dried basis

CONTAMINANTS

- **BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Test for Aflatoxins*: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^4 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Total Ash*: NMT 6.0%
- **LOSS ON DRYING** ⟨731⟩

  Analysis: Dry at 105° for 5 h.

  Acceptance criteria: NMT 8.0%
- **BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Residual Solvents*: Meets the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light and moisture, and store in a cool place.
- **LABELING:** The label states the Latin binomial following the official name of the plant from which the article was prepared. The labeling indicates the content of total flavone glucuronides and the content of total flavone aglycones in percentage on the dried basis. Dosage forms prepared with this article should bear the following statement: "Discontinue use and consult a healthcare practitioner if you develop symptoms of liver trouble, such as abdominal pain, dark urine, or jaundice (yellowing of the eyes or skin)." The label also meets other labeling requirements in *Botanical Extracts* ⟨565⟩.
- **USP REFERENCE STANDARDS** ⟨11⟩

  USP Baicalein RS

  USP Baicalein 7-*O*-Glucuronide RS

  USP *Scutellaria baicalensis* Root Dry Extract RS▲ (USP 1-Dec-2019)

Cholecalciferol Chewable Gels

DEFINITION

Cholecalciferol Chewable Gels contain NLT 90.0% and NMT 140.0% of the labeled amount of cholecalciferol ($C_{27}H_{44}O$).

IDENTIFICATION

- **A.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Strength*.

STRENGTH

Change to read:

- **PROCEDURE**

  [NOTE—Use amber, low-actinic glassware. Use cryogenic gloves when handling liquid nitrogen.]

  Mobile phase: 1.2% isopropyl alcohol in hexane

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[NOTE—Wash the column periodically with 5% isopropyl alcohol in hexane to eliminate retained substances and avoid carryover interferences.]
Standard stock solution: 0.3 mg/mL of USP Cholecalciferol RS in 2,2,4-trimethylpentane. Shake on a mechanical shaker for 15 min.
Standard solution: 1 µg/mL of USP Cholecalciferol RS in 2,2,4-trimethylpentane from the *Standard stock solution*, prepared accurately
Sample solution: Immerse 25–30 Chewable Gels in liquid nitrogen in a cryogenic vessel for 10 min. Cool a blender jar by swirling liquid nitrogen for about 1 min and discard the contents. Add frozen Chewable Gels to the cooled blender jar and grind to a fine powder. Transfer a portion of the powder, nominally equivalent to 10 µg of cholecalciferol, into a 50-mL centrifuge tube. [NOTE—Proceed to this step immediately or keep the powdered Chewable Gels frozen until use.] Add 300 mg of pancreatin and 5 mL of water and shake on a shaker water bath at 55° until complete dissolution (about 20 min). Mix the solution on a vortex mixer to suspend any undissolved solids, add 20 mL of dimethyl sulfoxide, and mix again on a vortex mixer until well mixed. Pipet 10 mL of 2,2,4-trimethylpentane, mix on a vortex mixer until the phases are well mixed, then shake the tube on a horizontal shaker for 20 min, and centrifuge the sample at 2500 rpm for 10 min. Pass a portion of the 2,2,4-trimethylpentane layer through a 0.45-µm glass microfiber filter, discarding the first 2 mL of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 265 nm
Column: 4.6-mm × 25-cm; 5-µm packing L3
Flow rate: 1.3 mL/min
Injection volume: 80 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 3.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) in the portion of Chewable Gels taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of cholecalciferol from the *Sample solution*
r_S = peak area of cholecalciferol from the *Standard solution*
C_S = concentration of ▲USP Cholecalciferol RS▲ (USP 1-Dec-2019) in the *Standard solution* (µg/mL)
C_U = nominal concentration of cholecalciferol in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–140.0%

PERFORMANCE TESTS

Change to read:

• **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩, *Dissolution*
[NOTE—Perform this test under light conditions that minimize photodegradation.]
Medium: 0.1% (w/v) octoxynol-9 in 0.05 M phosphate buffer pH 6.8; 500 mL
Apparatus 2: 75 rpm
Time: 30 min
Mobile phase: Acetonitrile and water (70:30)
Standard stock solution: 0.5 mg/mL of USP Cholecalciferol RS in isopropyl alcohol
Standard solution: Dilute the *Standard stock solution* with *Medium* to obtain the concentration equivalent to that expected in the *Sample solution*.
Sample solution: Withdraw a portion of the solution under test, pass through a 0.45-µm polyvinylidene fluoride (PVDF) filter, and use the pooled sample as the test specimen.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 265 nm
Column: 4.6-mm × 15-cm; 2.7-µm packing L96
Column temperature: 40°
Flow rate: 1 mL/min
Injection volume: 100 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 2.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of cholecalciferol dissolved:

$$\text{Result} = (r_U/r_S) \times (C_S \times V/L) \times 100$$

r_U = peak area of cholecalciferol from the *Sample solution*
r_S = peak area of cholecalciferol from the *Standard solution*
C_S = concentration of ▲USP Cholecalciferol RS▲ (USP 1-Dec-2019) in the *Standard solution* (µg/mL)
V = volume of *Medium*, 500 mL
L = label claim of cholecalciferol (µg/Chewable Gel)

Tolerances: NLT 75% of the labeled amount of cholecalciferol is dissolved.

Change to read:

• **WEIGHT VARIATION** ⟨2091⟩▲▲ (USP 1-Dec-2019)**:** Meet the requirements

SPECIFIC TESTS

• **PH** ⟨791⟩
Sample solution: Cut the Chewable Gels into small pieces. Transfer about 15 g of the cut Chewable Gels into a 50-mL centrifuge tube, add 15 g of hot water (80°–90°), close the cap, place the centrifuge tube into a shaker water bath at 55°, and shake for 50–60 min or until all of the Chewable Gels are dissolved. Cool down to 30° and measure the pH.
Acceptance criteria: NMT 4.5
• **WATER ACTIVITY**
Sample: Slice the Chewable Gels into pieces about 2 mm thick.
Procedure: Measure the water activity using the AOAC's *Official Methods of Analysis*, Official Method #978.18.
Acceptance criteria: NMT 0.75

CONTAMINANTS

• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count is NMT 10^3 cfu/g, and the total combined yeasts and molds count is NMT 10^2 cfu/g.
• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli***:** Meet the requirements

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ADDITIONAL REQUIREMENTS

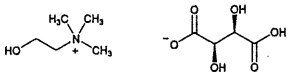
- **PACKAGING AND STORAGE:** Preserve in well-closed containers; protect from heat.

Change to read:

- **LABELING:** Label the Chewable Gels to indicate the content of cholecalciferol, in µg. ▲Expression of the amount of cholecalciferol in terms of units may be added in parentheses after the mass units.[1] ▲ (USP 1-Dec-2019)
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Cholecalciferol RS

Cholecalciferol Solution—*see Cholecalciferol Solution General Monographs*

Choline Bitartrate



$C_9H_{19}NO_7$ 253.25

2-Hydroxyethanaminium, -*N*,*N*,*N*-trimethyl-, [*R*-(*R**,*R**)]-2,3-dihydroxybutanedioate (1:1);
(2-Hydroxyethyl)trimethylammonium-L-(+)-tartrate salt (1:1) [87-67-2].

DEFINITION

Choline Bitartrate contains NLT 99.0% and NMT 100.5% of choline bitartrate ($C_9H_{19}NO_7$), calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
- **B.**

Sample: 1 g

Analysis: Dissolve the *Sample* in 20 mL of water, and add 2 mL of potassium chloride solution (1 in 4).

Acceptance criteria: A white precipitate of potassium bitartrate is formed.

ASSAY

- **PROCEDURE**

Sample: 200 mg

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Direct titration

Titrant: 0.1 N perchloric acid VS

Endpoint detection: Potentiometric

Blank: 50 mL of glacial acetic acid

Analysis: Dissolve the *Sample* in 50 mL of glacial acetic acid and titrate with *Titrant*.

Calculate the percentage of choline bitartrate ($C_9H_{19}NO_7$) in the *Sample* taken:

$$\text{Result} = [(V - B) \times N \times F \times 100]/W$$

V = *Sample* titrant volume (mL)
B = *Blank* titrant volume (mL)
N = titrant normality (mEq/mL)
F = equivalency factor, 253.2 mg/mEq
W = weight of the *Sample* (mg)

Acceptance criteria: 99.0%–100.5% on the anhydrous basis

IMPURITIES

- **RESIDUAL SOLVENTS** ⟨467⟩**:** Meet the requirements, except that the limit for 1,4-dioxane is 10 µg/g
- **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 0.1%
- **ARSENIC,** *Method I* ⟨211⟩

Analysis: Add 30 mL of water and 5 mL of hydrochloric acid to dissolve the sample.

Acceptance criteria: NMT 2 ppm

- **LEAD** ⟨251⟩

[NOTE—Use methylene chloride in place of chloroform to prepare the *Dithizone Extraction Solution* and *Standard Dithizone Solution*.]

Solution A: Transfer 8.4 g of sodium hydroxide solution (1 in 2) to a plastic bottle, add 100 mL of ammonium hydroxide, and mix.

Standard solution: Transfer 1.0 mL of the *Diluted Standard Lead Solution* to a separatory funnel containing 25.0 mL of water.

Sample solution: Dissolve 3.00 g of Choline Bitartrate in a separatory funnel containing 25.0 mL of water.

Analysis

Samples: *Standard solution* and *Sample solution*

Separately add 6.0 mL of *Ammonium Citrate Solution* and 3.0 mL of *Potassium Cyanide Solution* to the *Standard solution* and the *Sample solution*. Extract each of the resulting solutions three times with 5.0-mL portions of *Dithizone Extraction Solution*, shaking for 60 s and draining off each extract into another separator. Shake the combined dithizone solutions for 30 s with 20.0 mL of nitric acid (1 in 100), and discard the methylene chloride layer. Add 6.0 mL of *Ammonia-Cyanide Solution*, 2 mL of *Solution A*, and 10 mL of *Standard Dithizone Solution*, and shake for 45 s. Allow the phases to separate, and measure the absorbance of the lower layer at 510 nm with a suitable spectrophotometer.

Acceptance criteria: The absorbance of the *Sample solution* is NMT the absorbance of the *Standard solution* (NMT 0.3 ppm).

Change to read:

- **LIMIT OF TOTAL AMINES**

Standard solution: 500 µg/mL of trimethylamine hydrochloride

Sample solution: Transfer 10.0 g of Choline Bitartrate to a beaker containing a plastic-coated stirring bar, add 70 mL of sodium hydroxide TS and 130 mL of water, and stir until dissolved.

System suitability stock solution: 10 µg/mL of trimethylamine hydrochloride

System suitability solution: Transfer 10.0 mL of *System suitability stock solution* containing a plastic-coated stirring bar, add 160 mL water and 30.0 mL sodium hydroxide TS, and stir until dissolved.

Electrode system: Use a gas-sensing, ammonia-specific indicating electrode with internal reference connected to a pH meter capable of measuring potentials with a minimum reproducibility of ±0.1 mV (see *pH* ⟨791⟩).

Standard response line: Mix 30.0 mL of sodium hydroxide TS and 170 mL of water. Add a plastic-coated stirring bar, insert the electrode into the solution, and record the

[1] Where articles are labeled in terms of units in addition to the required labeling, the relationship of the USP Units or International Units (IU) to mass units is as follows: 1 µg of cholecalciferol = 40 USP Units or IU.

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potential, in mV. Continue stirring, and at 5-min intervals, add 0.200, 0.600, 1.00, and 2.00 mL of *Standard solution*, and record the potential after each addition. Plot the logarithms of the cumulative trimethylamine hydrochloride concentrations (0.50, 1.50, 2.50, and 5.00 µg/mL) versus potential, in mV, and determine the slope (S) of the *Standard response line* for the electrode.

System suitability

Sample: *System suitability solution*

Proceed as directed in *Analysis*, except to replace the *Sample solution* with the *System suitability solution* and in the formula below to replace W with V, which equals 10 mL.

Suitability requirements: The total change is NLT 10 mV for a 0.4-mL cumulative addition of the *Standard solution*; the amount of trimethylamine hydrochloride found is 8.5–11.5 ▲µg/mL.▲ (ERR 1-Jul-2019)

Analysis

Samples: *Standard solution* and *Sample solution*

Rinse the electrode, insert it into the *Sample solution*, stir, and record the potential, in mV. Add 0.100 mL of the *Standard solution*, and record the potential. Add another 0.100 mL of the *Standard solution*, and record the potential. [NOTE—If the total change after the second addition of the *Standard solution* is less than 10 mV, add a third aliquot of 0.200 mL.]

Calculate the content, in µg/g, of total amines as trimethylamine hydrochloride in the portion of sample taken:

$$\text{Result} = (C_S \times V_A)/[(F - 1) \times W]$$

C_S = concentration of the *Standard solution* (µg/mL)
V_A = total volume of the *Standard solution* added to the *Sample solution* (mL)
W = weight of Choline Bitartrate taken to prepare the *Sample solution* (g)
F = correction factor, calculated by the formula:

$$F = \text{antilog}\,[(mV_F - mV_0)/S]$$

mV_F = final reading after the additions of the *Standard solution* (mV)
mV_0 = initial reading of the *Sample solution* (mV)
S = slope of the *Standard response line* for the electrode

Acceptance criteria: NMT 10 µg/g

• **CHROMATOGRAPHIC PURITY**

Buffer solution: 7.1 g/L of anhydrous dibasic sodium phosphate. Adjust with phosphoric acid to a pH of 2.5.

Mobile phase: *Buffer solution* and acetonitrile (7:3)

Standard solution: Transfer an amount, NMT 100 mg, of USP Choline Chloride RS to a 24-mL screw-capped vial, and add 400 mg of 3,5-dinitrobenzoyl chloride and 10 mL of acetonitrile. Cap the vial, heat to 55°, and continue heating for 2 h. Cool to room temperature, and add 5 mL of water. Allow to stand for 5 min. Quantitatively transfer the solution to a 25-mL volumetric flask and dilute with acetonitrile to volume. Dilute a volume of this solution with *Mobile phase* to obtain a concentration of 2.0 µg/mL of USP Choline Chloride RS.

Sample solution: Transfer 500 mg of Choline Bitartrate to a centrifuge tube, add 2.0 mL of water, and swirl to dissolve. Add 0.5 mL of potassium chloride solution (7.5 in 25), centrifuge, and transfer 1.0 mL of the supernatant to a 24-mL screw-capped vial. Dry at 120° for 2 h. Add 400 mg of 3,5-dinitrobenzoyl chloride and 10 mL of acetonitrile. Cap the vial, and heat at 55° for 2 h. Cool to room temperature, add 5 mL of water, and allow to stand for 5 min. Quantitatively transfer this solution to a 50-mL volumetric flask, and dilute with *Mobile phase* to volume. Pipet 2.0 mL of the solution to a 25-mL volumetric flask, and dilute with *Mobile phase* to volume.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC
Detector: UV 208 nm
Column: 4.6-mm × 25-cm; packing L7
Column temperature: 30°
Flow rate: 1 mL/min
Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Capacity factor (k′): NLT 2
Relative standard deviation: NMT 5%, determined from the choline derivative peak

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of each impurity in the portion of Choline Bitartrate taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak response for each impurity, excluding that for the choline derivative and 3,5-dinitrobenzoic acid from the *Sample solution*
r_S = peak response for the choline derivative from the *Standard solution*
C_S = concentration of USP Choline Chloride RS in the *Standard solution* (mg/mL)
C_U = concentration of Choline Bitartrate in the *Sample solution* (mg/mL)
M_{r1} = molecular weight of choline bitartrate, 253.25
M_{r2} = molecular weight of choline chloride, 139.62

Acceptance criteria

Individual impurities: NMT 0.3%
Total impurity: NMT 2.0%

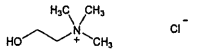
SPECIFIC TESTS

• **OPTICAL ROTATION,** *Specific Rotation* ⟨781S⟩
Sample solution: 400 mg/mL in water
Acceptance criteria: +17.5° to +18.5°
• **PH** ⟨791⟩**:** 3.0–4.0, in a solution (1 in 10)
• **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 0.5%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Choline Bitartrate RS
USP Choline Chloride RS

Choline Chloride



$C_5H_{14}ClNO$ 139.62

(2-Hydroxyethyl)trimethylammonium chloride; 2-Hydroxy-*N*,*N*,*N*-trimethylethanaminium chloride [67-48-1].

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DEFINITION

Choline Chloride contains NLT 99.0% and NMT 100.5% of choline chloride ($C_5H_{14}ClNO$), calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS ⟨197⟩, *Infrared Spectroscopy*: 197K▲** (CN 1-May-2020)
- **B. IDENTIFICATION TESTS—GENERAL, *Chloride* ⟨191⟩:** A solution (1 in 20) meets the requirements.

ASSAY

- **PROCEDURE**

Sample: 120 mg

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Direct titration

Titrant: 0.1 N silver nitrate VS

Endpoint detection: Potentiometric

Blank: 35 mL of water. Add 3 drops of acetic acid.

Analysis: Dissolve the *Sample* in 35 mL of water and add 3 drops of acetic acid. Titrate with *Titrant*.

Calculate the percentage of choline chloride ($C_5H_{14}ClNO$) in the *Sample* taken:

$$\text{Result} = [(V - B) \times N \times F \times 100]/W$$

V = *Sample* titrant volume (mL)
B = *Blank* titrant volume (mL)
N = titrant normality (mEq/mL)
F = equivalency factor, 139.6 mg/mEq
W = weight of the *Sample* (mg)

Acceptance criteria: 99.0%–100.5% on the anhydrous basis

IMPURITIES

- **RESIDUAL SOLVENTS ⟨467⟩:** Meets the requirements, except that the limit for 1,4-dioxane is 10 µg/g
- **RESIDUE ON IGNITION ⟨281⟩:** NMT 0.05%
- **ARSENIC, *Method I* ⟨211⟩**

Analysis: Add 30 mL of water and 5 mL of hydrochloric acid to dissolve the sample.

Acceptance criteria: NMT 2 ppm

- **LEAD ⟨251⟩**

[NOTE—Use methylene chloride in place of chloroform to prepare the *Dithizone Extraction Solution* and *Standard Dithizone Solution*.]

Solution A: Transfer 8.4 g of sodium hydroxide solution (1 in 2) to a plastic bottle, add 100 mL of ammonium hydroxide, and mix.

Standard solution: Transfer 1.0 mL of the *Diluted Standard Lead Solution* to a separatory funnel containing 25.0 mL of water.

Sample solution: Dissolve 3.00 g of Choline Chloride in a separatory funnel containing 25.0 mL of water.

Analysis

Samples: *Standard solution* and *Sample solution*

Separately add 6.0 mL of *Ammonium Citrate Solution* and 3.0 mL of *Potassium Cyanide Solution* to the *Standard solution* and the *Sample solution*. Extract each of the resulting solutions three times with 5.0-mL portions of *Dithizone Extraction Solution*, shaking for 60 s and draining off each extract into another separator. Shake the combined dithizone solutions for 30 s with 20.0 mL of nitric acid (1 in 100), and discard the methylene chloride layer. Add 6.0 mL of *Ammonia–Cyanide Solution*, 2 mL of *Solution A*, and 10 mL of *Standard Dithizone Solution*, and shake for 45 s. Allow the phases to separate, and measure the absorbance of the lower layer at 510 nm with a suitable spectrophotometer.

Acceptance criteria: The absorbance of the *Sample solution* is NMT the absorbance of the *Standard solution* (NMT 0.3 ppm).

Change to read:

- **LIMIT OF TOTAL AMINES**

Standard solution: 500 µg/mL of trimethylamine hydrochloride in water

Sample solution: Transfer 10.0 g of Choline Chloride to a beaker containing a plastic-coated stirring bar, add 170 mL of water and 30.0 mL of sodium hydroxide TS, and stir until dissolved.

System suitability stock solution: 10 µg/mL of trimethylamine hydrochloride in water

System suitability solution: Transfer 10.0 mL of *System suitability stock solution* to a beaker containing a plastic-coated stirring bar, add 160 mL of water and 30.0 mL of sodium hydroxide TS, and stir until dissolved.

Electrode system: Use a gas-sensing, ammonia-specific indicating electrode with internal reference connected to a pH meter capable of measuring potentials with a minimum reproducibility of ±0.1 mV (see *pH* ⟨791⟩).

Standard response line: Mix 30.0 mL of sodium hydroxide TS, and 170 mL of water. Add a plastic-coated stirring bar, insert the electrode into the solution, and record the potential, in mV. Continue stirring, and at 5-min intervals add 0.200, 0.600, 1.00, and 2.00 mL of *Standard solution*, and record the potential after each addition. Plot the logarithms of the cumulative trimethylamine hydrochloride concentrations (0.50, 1.50, 2.50, and 5.00 µg/mL) versus potential, in mV, and determine the slope (S) of the *Standard response line* for the electrode.

System suitability

Sample: *System suitability solution*

Proceed as directed in *Analysis*, except to replace the *Sample solution* with the *System suitability solution* and in the formula below to replace W with V, which equals 10 mL.

Suitability requirements: The total change is NLT 10 mV for a 0.4-mL cumulative addition of the *Standard solution*; the amount of trimethylamine hydrochloride found is 8.5–11.5 ▲µg/mL.▲ (ERR 1-Jul-2019)

Analysis

Samples: *Standard solution* and *Sample solution*

Rinse the electrode, insert it into the *Sample solution*, stir, and record the potential, in mV. Add 0.100 mL of the *Standard solution*, and record the potential. Add another 0.100 mL of the *Standard solution*, and record the potential. [NOTE—If the total change after the second addition of the *Standard solution* is less than 10 mV, add a third aliquot of 0.200 mL.]

Calculate the content, in µg/g, of total amines as trimethylamine hydrochloride in the portion of sample taken:

$$\text{Result} = (C_S \times V_A)/[(F - 1) \times W]$$

C_S = concentration of *Standard solution* (µg/mL)
V_A = total volume of the *Standard solution* added to the *Sample solution* (mL)
W = weight of Choline Chloride taken to prepare the *Sample solution* (g)
F = correction factor, calculated by the formula:

$$F = \text{antilog}\,[(mV_F - mV_0)/S]$$

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mV_F = final reading after the additions of the *Standard solution* (mV)
mV_0 = initial reading of the *Sample solution* (mV)
S = slope of the *Standard response line* for the electrode

Acceptance criteria: NMT 10 µg/g

• **CHROMATOGRAPHIC PURITY**

Buffer solution: 7.1 g/L of anhydrous dibasic sodium phosphate. Adjust with phosphoric acid to a pH of 2.5.
Mobile phase: *Buffer solution* and acetonitrile (7:3)
Standard solution: Transfer an amount, NMT 100 mg, of USP Choline Chloride RS to a 24-mL screw-capped vial, and add 400 mg of 3,5-dinitrobenzoyl chloride and 10 mL of acetonitrile. Cap the vial, heat to 55°, and continue heating for 2 h. Cool to room temperature, and add 5 mL of water. Allow to stand for 5 min. Quantitatively transfer the solution to a 25-mL volumetric flask, and dilute with acetonitrile to volume. Dilute a volume of this solution with *Mobile phase* to obtain a concentration of 2.0 µg/mL of USP Choline Chloride RS.
Sample solution: Transfer 110 mg of Choline Chloride to a 24-mL screw-capped vial. Dry at 120° for 2 h. Add 400 mg of 3,5-dinitrobenzoyl chloride and 10 mL of acetonitrile. Cap the vial, heat to 55°, and continue heating for 2 h. Cool to room temperature, and add 5 mL of water. Allow to stand for 5 min. Quantitatively transfer the solution to a 50-mL volumetric flask, and dilute with *Mobile phase* to volume. Pipet 2.0 mL of the solution to a 25-mL volumetric flask, and dilute with *Mobile phase* to volume.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 208 nm
Column: 4.6-mm × 25-cm; packing L7
Column temperature: 30°
Flow rate: 1.0 mL/min
Injection size: 20 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Capacity factor (k′): NLT 2
Relative standard deviation: NMT 5%, determined from the choline derivative peak
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of each impurity in the portion of Choline Chloride taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response for each impurity, excluding that for the choline derivative and 3,5-dinitrobenzoic acid from the *Sample solution*
r_S = peak response for the choline derivative from the *Standard solution*
C_S = concentration of USP Choline Chloride RS in the *Standard solution* (mg/mL)
C_U = concentration of Choline Chloride in the *Sample solution* (mg/mL)

Acceptance criteria
Individual impurities: NMT 0.3%
Total impurities: NMT 2.0%

SPECIFIC TESTS

• **pH** ⟨791⟩**:** 4.0–7.0, in a solution (1 in 10)
• **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 0.5%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Choline Chloride RS

Chondroitin Sulfate Sodium

Chondroitin, hydrogen sulfate, sodium salt [9082-07-9].

DEFINITION

Chondroitin Sulfate Sodium is the sodium salt of the sulfated linear glycosaminoglycan obtained from bovine, porcine, or avian cartilages of healthy and domestic animals used for food by humans. Chondroitin Sulfate Sodium consists mostly of the sodium salt of the sulfate ester of *N*-acetylchondrosamine (2-acetamido-2-deoxy-β-D-galactopyranose) and D-glucuronic acid copolymer. These hexoses are alternately linked β-1,4 and β-1,3 in the polymer. Chondrosamine moieties in the prevalent glycosaminoglycan are monosulfated primarily on position 4 and less so on position 6. It contains NLT 90.0% and NMT 105.0% of chondroitin sulfate sodium, calculated on the dried basis.
[NOTE—Chondroitin Sulfate Sodium is extremely hygroscopic once dried. Avoid exposure to the atmosphere, and weigh promptly.]

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
• **B. IDENTIFICATION TESTS—GENERAL** ⟨191⟩, *Sodium*
Sample solution: 0.5 g in 10 mL of water
Acceptance criteria: Meets the requirements
• **C. DISACCHARIDE COMPOSITION:** The chromatogram of the enzymatically digested *Sample solution* as obtained in the test for *Limit of Nonspecific Disaccharides* shows three main peaks corresponding to dehydrated glucuronic acid-[1→3]-chondrosamine-4-sulfated (ΔDi-4S), dehydrated glucuronic acid-[1→3]-chondrosamine-6-sulfated (ΔDi-6S), and nonsulfated dehydrated glucuronic acid-[1→3]-chondrosamine (ΔDi-0S) in the enzymatically digested *Standard solution*. By peak-area response, ΔDi-4S is the most abundant, followed by ΔDi-6S, with ΔDi-0S being the least abundant of the three. The ratio of the peak response of the ΔDi-4S to the ΔDi-6S is NLT 1.0.
• **D. SPECIFIC ROTATION:** Meets the requirements for *Optical Rotation* ⟨781S⟩, *Specific Rotation* in *Specific Tests*

COMPOSITION

• **CONTENT OF CHONDROITIN SULFATE SODIUM**
Standard solutions: 1.5, 1.0, and 0.5 mg/mL of USP Chondroitin Sulfate Sodium RS in water
Sample solution: Transfer 100 mg of dried Chondroitin Sulfate Sodium to a 100-mL volumetric flask, dissolve in 30 mL of water, and dilute with water to volume.
Diluent: Weigh about 297 mg of monobasic potassium phosphate, 492 mg of dibasic potassium phosphate, and 250 mg of polysorbate 80, and transfer to a 1-L beaker. Dissolve in 900 mL of water, and adjust with potassium hydroxide or phosphoric acid to a pH of 7.0 ± 0.2. Dilute with water to 1 L, and mix thoroughly.
Titrimetric system
(See *Titrimetry* ⟨541⟩.)

Supplement S3

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Mode: Photometric titration
Titrant: 1 mg/mL of cetylpyridinium chloride in water. Degas before use.
Endpoint detection: Turbidimetric with a photoelectric probe

Analysis
Samples: *Standard solutions*, *Sample solution*, and *Diluent*
Transfer 5.0 mL each of the *Standard solutions* and the *Sample solution* to separate titration vessels, and add 25 mL of *Diluent* to each. Stir until a steady reading is obtained with a phototrode either at 420, 550, or 660 nm. Set the instrument to zero in absorbance mode. Titrate with *Titrant* using the phototrode to determine the endpoint turbidimetrically. From a linear regression equation, calculated using the volumes of *Titrant* consumed versus concentrations of the *Standard solutions*, determine the concentration of chondroitin sulfate sodium in the *Sample solution*.
Calculate the percentage of chondroitin sulfate sodium in the portion of Chondroitin Sulfate Sodium taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of chondroitin sulfate sodium in the aliquot of the *Sample solution*, obtained from the regression equation (mg/mL)
C_U = concentration of Chondroitin Sulfate Sodium in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–105.0% on the dried basis

IMPURITIES

• **Residue on Ignition** ⟨281⟩**:** 20.0%–30.0% on the dried basis

• **Chloride and Sulfate** ⟨221⟩, *Chloride*: NMT 0.50%; a 0.10-g portion shows no more chloride than corresponds to 0.7 mL of 0.020 N hydrochloric acid.

• **Chloride and Sulfate** ⟨221⟩, *Sulfate*
Sample solution: Dissolve 200 mg in 40 mL of water. Add 10 mL of a 30-mg/mL solution of cetylpyridinium chloride, pass through a filter, and use a 25-mL portion of the filtrate.
Acceptance criteria: NMT 0.24%; the *Sample solution* shows no more sulfate than corresponds to 0.25 mL of 0.020 N sulfuric acid.

• **Electrophoretic Purity**
[**Caution**—Voltages used in electrophoresis can readily deliver a lethal shock. The hazard is increased by the use of aqueous buffer solutions and the possibility of working in damp environments. The equipment, with the possible exception of the power supply, should be enclosed in either a grounded metal case or a case made of insulating material. The case should have an interlock that deenergizes the power supply when the case is opened, after which reactivation should be prevented until activation of a reset switch is carried out. High-voltage cables from the power supply to the apparatus should preferably be a type in which a braided metal shield completely encloses the insulated central conductor, and the shield should be grounded. The base of the apparatus should be grounded metal or contain a grounded metal rim that is constructed in such a way that any leakage of electrolyte will produce a short that will deenergize the power supply before the electrolyte can flow beyond the protective enclosure. If the power supply contains capacitors as part of a filter circuit, it should also contain a bleeder resistor to ensure discharge of the capacitors before the protective case is opened. A shorting bar that is activated by opening the case may be considered as an added precaution. Because of the potential hazard associated with electrophoresis, laboratory personnel should be completely familiar with electrophoresis equipment before using it.]
Barium acetate buffer: Dissolve 25.24 g of barium acetate in 900 mL of water. Adjust with acetic acid to a pH of 5.0, and dilute with water to 1000 mL.
Staining reagent: Dissolve 1 g of toluidine blue in 1000 mL of 0.1 M acetic acid.
Standard solution A: 30 mg/mL of USP Chondroitin Sulfate Sodium RS in water
Standard solution B: Dilute 1 mL of *Standard solution A* with water to 50 mL.
Sample solution: 30 mg/mL of Chondroitin Sulfate Sodium in water
Analysis: Fill the chambers of an electrophoresis apparatus suitable for separations on cellulose acetate membranes[1] (a small submarine gel chamber or one dedicated to membrane media) with *Barium acetate buffer*. Soak a cellulose acetate membrane, 5–6 cm × 12–14 cm, in *Barium acetate buffer* for 10 min, or until evenly wetted, then blot dry between two sheets of absorbent paper. Using an applicator[2] suitable for electrophoresis, apply equal volumes (0.5 µL) of *Standard solution A*, *Standard solution B*, and *Sample solution* to the brighter side of the membrane held in position in an appropriate applicator stand or on a separating bridge in the chamber. Ensure that both ends of the membrane are dipped at least 0.5–1.0 cm deep into the buffer chambers. Apply a constant 60 V (6 mA at the start) for 2 h. [Note—Perform the application of solutions and voltage within 5 min because further drying of the blotted paper reduces sensitivity.]
Place the membrane in a plastic staining tray, and with the application side down, float or gently immerse in *Staining reagent* for 5 min. Then stir the solution gently for 1 min. Remove the membrane, and destain in 5% acetic acid until the background clears. Compare the bands. [Note—Document the results by taking a picture within 15 min of completion of destaining.]
Acceptance criteria: The electropherogram from the *Sample solution* exhibits a major band that is identical in position to the band from *Standard solution A*. The band from *Standard solution B* is clearly visible at a mobility similar to the band from *Standard solution A*. Any secondary band in the electropherogram of the *Sample solution* is not more intense than the band from *Standard solution B*. NMT 2% of any individual impurity is found. [Note—Document the results by taking a picture within 15 min of completion of destaining.]

• **Limit of Protein**
Solution A: 20 mg/mL of sodium tartrate dihydrate
Solution B: 10 mg/mL of cupric sulfate
Solution C: 20 mg/mL of anhydrous sodium carbonate in 0.1 M sodium hydroxide
Dilute Folin-Ciocalteu reagent: Dilute Folin-Ciocalteu phenol TS with water (1:5). Prepare immediately before use.
Alkaline cupric tartaric reagent: Mix 1 mL each of *Solution A* and *Solution B*, and to the mixture slowly add 100 mL of *Solution C* with stirring. Use within 24 h, and discard afterward.
Standard solution: 36 µg/mL of bovine serum albumin certified standard in water

[1] Suitable cellulose acetate membranes for electrophoresis are available from Malta Chemetron SRL, Milano, Italy; Fluka Chemical Corp., Milwaukee, WI; and DiaSys Corp., Waterbury, CT (www.diasys.com).
[2] Suitable applicators are available from DiaSys Corp., Waterbury, CT (www.diasys.com) and Helena Laboratories, Beaumont, TX (www.helena.com).

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Sample solution: Transfer a portion of Chondroitin Sulfate Sodium, equivalent to 60 mg of the dried substance, to a 100-mL volumetric flask, and dissolve in and dilute with water to volume.

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Analytical wavelength: 750 nm

Blank: Water

Analysis

Samples: *Standard solution*, *Sample solution*, and *Blank*

Add 2.0 mL of freshly prepared *Alkaline cupric tartaric reagent* to test tubes containing 2.0 mL of the *Standard solution*, 2.0 mL of the *Sample solution*, or 2.0 mL of the *Blank*. After 10 min, add 1.0 mL of *Dilute Folin-Ciocalteu reagent* to each test tube, and mix immediately and vigorously. After 30 min, measure the absorbance of the *Standard solution* and *Sample solution* against the *Blank*.

Acceptance criteria: NMT 6.0% on the dried basis; the absorbance of the *Sample solution* is NMT the absorbance of the *Standard solution*.

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total bacterial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

- **LIMIT OF NONSPECIFIC DISSACCHARIDES**

Solution A: Water adjusted with 0.1 N hydrochloric acid to a pH of 3.5

Solution B: 1 M sodium chloride adjusted with 0.1 N hydrochloric acid to a pH of 3.5

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0.0	100	0
4.5	100	0
21.0	61	39
21.1	100	0

Buffer solution: 50 mM tris(hydroxymethyl)aminomethane and 60 mM sodium acetate, adjusted with diluted hydrochloric acid to a pH of 8.0

Blank: Water

Chondroitinase AC solution: Use appropriate chondroitinase AC that is capable of cleaving the *N*-acetylhexosaminide linkage in chondroitin 4-sulfate and chondroitin 6-sulfate, yielding Δ^4-unsaturated disaccharides (ΔDi-0S, ΔDi-4S, and ΔDi-6S). The working concentration of the chondroitinase AC in *Buffer solution* must be sufficient for a complete digestion and meet the enzyme suitability requirement that follows.

[NOTE—If Chondroitinase AC from *Arthrobacter auresens*[3] is used, 0.2 Units/mL in *Buffer solution* is a typical working concentration; if Chondroitinase AC from *Flavobacterium heparium*[4] is used, 3 Units/mL in *Buffer solution* is a typical working concentration. The working enzyme concentration may be increased if a complete digestion could not be achieved. The working enzyme aliquots should be stored at −20° when not in use for a period of time to avoid a decrease in the enzyme activity. A working enzyme solution is typically stable for 4 days when stored at 4°.]

Enzyme suitability: Dilute the digested *Standard solution* and digested *Blank* (see *Analysis* section) (1 in 10), and measure the absorbance at 230 nm in 1-cm path cells. Make correction with the diluted *Blank*.

Calculate the absorptivity of USP Chondroitin Sulfate Sodium RS:

$$\text{Result} = A/(C \times D \times d)$$

A = absorbance of the diluted and digested *Standard solution*

C = concentration of USP Chondroitin Sulfate Sodium RS in the *Standard solution* (mg/mL)

D = dilution factor of digested *Standard solution* (1/5)

d = dilution factor for the UV measurement (1/10)

Enzyme suitability requirement: The absorptivity of the digested USP Chondroitin Sulfate Sodium RS is NLT 8 AU · mL · mg^{-1} · cm^{-1}.

Standard solution: 2.4 mg/mL of dried USP Chondroitin Sulfate Sodium RS in water

Sample solution: Transfer about 250 mg of dried (105° for 4 h) Chondroitin Sulfate Sodium to a 100-mL volumetric flask, and dissolve in and dilute with water to volume.

System suitability solution: Add 1 volume of *Standard solution* to 1 volume of *Sample solution*.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 230 nm

Column: 4.6-mm × 25-cm; 5-µm packing L14

Flow rate: 1 mL/min

Injection volume: 25 µL

[NOTE—The *Injection volume* may be decreased to improve the peak shape of the analytes.]

System suitability

Samples: *Standard solution*, *Sample solution*, and *System suitability solution* (prepared as directed for *Samples* in the *Analysis*)

[NOTE—The relative retention times for the ΔDi-0S, ΔDi-6S, and ΔDi-4S peaks are 0.80, 0.97, and 1.0, respectively.]

Suitability requirements

Chromatogram similarity: The chromatogram of the *Standard solution* is similar to that of the reference chromatogram provided with USP Chondroitin Sulfate Sodium RS.

Resolution: NLT 1.0 between the ΔDi-4S and ΔDi-6S peaks, *Standard solution*

Recovery factor: NLT 95% of the USP Chondroitin Sulfate Sodium RS added to the *Sample solution*

[NOTE—This test is intended to demonstrate the absence of enzyme inhibition by impurities in the articles being tested. Performance of this test is required only for the articles being tested not meeting the *Acceptance criteria*. The *Recovery factor* can be calculated as follows:

$$\text{Result} = \{[(2 \times \Sigma r_{SY}) - \Sigma r_U]/\Sigma r_S\} \times 100$$

Σr_{SY} = sum of the peak areas of ΔDi-0S, ΔDi-4S, and ΔDi-6S from the *System suitability solution*

Σr_U = sum of the peak areas of ΔDi-0S, ΔDi-4S, and ΔDi-6S from the *Sample solution*

[3] Chondroitinase AC from *Arthrobacter auresens*, Chromadex, part number ASB-00003613-10.

[4] Chondroitinase AC from *Flavobacterium heparium*, ≥200 units/mg protein, Sigma-Aldrich, catalog number E2039.

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Σr_S = sum of the peak areas of ΔDi-0S, ΔDi-4S, and ΔDi-6S from the *Standard solution*]

Analysis

Samples: *Blank*, *Standard solution*, *Sample solution*, and *System suitability solution*

In four separate vials, combine 4 volumes (e.g., 800 µL) of *Chondroitinase AC solution* with 1 volume (e.g., 200 µL) each of *Standard solution*, *Sample solution*, *System suitability solution*, and *Blank*. Mix thoroughly. Incubate at 37° for 3 h. [NOTE—the incubation period may be increased, if necessary, to complete the digestion.] Allow the solutions to cool before injection.

Calculate the percentage of specific disaccharides in the portion of Chondroitin Sulfate Sodium taken:

$$\text{Result} = (\Sigma r_U/\Sigma r_S) \times (C_S/C_U) \times 100$$

Σr_U = sum of the peak areas of ΔDi-0S, ΔDi-4S, and ΔDi-6S from the *Sample solution*

Σr_S = sum of the peak areas of ΔDi-0S, ΔDi-4S, and ΔDi-6S from the *Standard solution*

C_S = concentration of chondroitin sulfate sodium in the *Standard solution* (mg/mL)

C_U = concentration of Chondroitin Sulfate Sodium in the *Sample solution* (mg/mL)

Calculate the content of nonspecific disaccharides in the sample taken:

$$\text{Result} = CSC - SDC$$

CSC = chondroitin sulfate sodium content from the test for *Content of Chondroitin Sulfate Sodium* (%)

SDC = specific disaccharides content (%)

Acceptance criteria: NMT 10.0%

• **CLARITY AND COLOR OF SOLUTION**

Sample solution: Transfer 2.5 g of Chondroitin Sulfate Sodium to a 50-mL volumetric flask. Dissolve in and dilute with carbon dioxide-free water to volume, and examine immediately.

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Analytical wavelength: 420 nm

Cell: 1 cm

Blank: Carbon dioxide-free water

Analysis: Measure the absorbance of the *Sample solution*.

Acceptance criteria: NMT 0.35

• **OPTICAL ROTATION** ⟨781S⟩, *Specific Rotation*

Sample solution: 30 mg/mL

Acceptance criteria: –20.0° to –30.0°

• **pH** ⟨791⟩**:** 5.5–7.5, in a solution (1 in 100)

• **LOSS ON DRYING** ⟨731⟩

[NOTE—Chondroitin Sulfate Sodium is extremely hygroscopic once dried. Avoid exposure to the atmosphere, and weigh promptly.]

Analysis: Dry at 105° for 4 h.

Acceptance criteria: NMT 12.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers.

• **LABELING:** Label it to state the source(s) from which the article was derived, whether bovine, porcine, avian, or a mixture of any of them.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Chondroitin Sulfate Sodium RS

Chondroitin Sulfate Sodium Tablets

DEFINITION

Chondroitin Sulfate Sodium Tablets contain NLT 90.0% and NMT 120.0% of the labeled amount of chondroitin sulfate sodium.

[NOTE—Chondroitin Sulfate Sodium is extremely hygroscopic once dried. Avoid exposure to the atmosphere, and weigh promptly.]

IDENTIFICATION

• **A. ELECTROPHORESIS**

Barium acetate buffer: Dissolve 25.24 g of barium acetate in 900 mL of water. Adjust with acetic acid to a pH of 5.0, and dilute with water to 1000 mL.

Staining reagent: 0.1% (w/v) toluidine blue in 0.1 M acetic acid

Standard solution: Use the *Standard solution* of middle concentration from the *Content of Chondroitin Sulfate Sodium*.

Sample solution: Prepare as directed in the *Content of Chondroitin Sulfate Sodium*.

Analysis: Fill the chambers of an electrophoresis apparatus suitable for separations on cellulose acetate membranes[1] (a small submarine gel chamber or one dedicated to membrane media) with *Barium acetate buffer*. Soak a cellulose acetate membrane 5–6 cm × 12–14 cm in *Barium acetate buffer* for 10 min, or until evenly wetted, then blot dry between two sheets of absorbent paper. Using an applicator[2] suitable for electrophoresis, apply equal volumes (0.5 µL) of the *Sample solution* and *Standard solution* to the brighter side of the membrane held in position in an appropriate applicator stand or on a separating bridge in the chamber. Ensure that both ends of the membrane are dipped at least 0.5–1.0-cm deep into the buffer chambers. Apply a constant 60 volts (6 mA at the start) for 2 h. [NOTE—Perform the application of solutions and voltage within 5 min because further drying of the blotted paper reduces sensitivity.]

Place the membrane in a plastic staining tray, and with the application side down, float or gently immerse in *Staining reagent* for 5 min. Then stir the solution gently for 1 min. Remove the membrane, and destain in 5% acetic acid until the background clears.

Acceptance criteria: The principal spot from the *Sample solution* has the same migration as the principal spot from the *Standard solution*. [NOTE—Document the results by taking a picture within 15 min of completion of destaining.]

STRENGTH

• **CONTENT OF CHONDROITIN SULFATE SODIUM**

Standard solutions: 1.5, 1.0, and 0.5 mg/mL of USP Chondroitin Sulfate Sodium RS in water

Sample solution: Transfer an equivalent to 100 mg of chondroitin sulfate sodium from NLT 20 Tablets, finely powdered, to 60 mL of water, and shake to suspend the powder in solution. Sonicate in a 65° water bath for 20 min. Remove from the bath, stir or shake for 5 min, dilute with water to 100 mL, and centrifuge or pass through a suitable filter.

Diluent: Weigh about 297 mg of monobasic potassium phosphate, 492 mg of dibasic potassium phosphate, and 250 mg of polysorbate 80, and transfer into a 1-L beaker.

[1] Suitable cellulose acetate membranes for electrophoresis are available from Fluka Chemical Corp., Milwaukee, WI; and DiaSys Corp., Waterbury, CT (www.diasys.com).

[2] Suitable applicators are available from DiaSys Corp., Waterbury, CT (www.diasys.com) and Helena Laboratories, Beaumont, TX (www.helena.com).

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Dissolve in approximately 900 mL of water, and adjust with potassium hydroxide or phosphoric acid to a pH of 7.0 ± 0.2. Dilute with water to 1 L, and mix thoroughly.

Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Photometric titration
Titrant: 1 mg/mL of cetylpyridinium chloride in water
Endpoint detection: Turbidimetric with photoelectric probe

Analysis: Transfer 5.0 mL of each *Standard solution* and the *Sample solution* to separate titration vessels, and add 25 mL of *Diluent* to each. Stir until a steady reading is obtained with a photoelectric probe either at 420, 550, or 660 nm. Set the instrument to zero in absorbance mode. Titrate with *Titrant* using the photoelectric probe to determine the endpoint turbidimetrically. From a linear regression equation, calculated using the volumes of *Titrant* consumed versus concentrations of the *Standard solutions*, determine the concentration of chondroitin sulfate sodium in the *Sample solution*.

Calculate the percentage of the labeled amount of chondroitin sulfate sodium in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = determined concentration of chondroitin sulfate sodium in the *Sample solution* (mg/mL)
C_U = nominal concentration of chondroitin sulfate sodium in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–120.0% of the label claim

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** ⟨2040⟩**:** Meet the requirements for *Dissolution*

Medium: Water; 900 mL
Apparatus 2: 75 rpm
Time: 60 min
Titrant and **Diluent:** Prepare as directed as in *Content of Chondroitin Sulfate Sodium*.
Standard solutions: 1.5, 1.0, and 0.5 mg/mL of USP Chondroitin Sulfate Sodium RS in water
Sample solution: Combine equal portions of the solutions withdrawn from 6 dissolution vessels and pass through a suitable filter; use the pooled sample as the test specimen.
Analysis: Transfer 5.0 mL of each *Standard solution*, and an aliquot of the *Sample solution* equivalent to about 5 mg of chondroitin sulfate sodium, to separate titration vessels. Add 25 mL of *Diluent* to each titration vessel. Stir until a steady reading is obtained with a photoelectric probe. Set the instrument to zero in absorbance mode. Titrate with *Titrant* using the photoelectric probe to determine the endpoint turbidimetrically, either at 420, 550, or 660 nm. From a linear regression equation, calculated using the volumes of *Titrant* consumed versus amount, in mg, of chondroitin sulfate sodium from each *Standard solution*, determine the amount, in mg, of chondroitin sulfate sodium in the aliquot of *Sample solution* taken.

Calculate the percentage of the labeled amount of chondroitin sulfate sodium dissolved:

$$\text{Result} = (Ws/a) \times (V/L) \times 100$$

Ws = amount of chondroitin sulfate sodium in the aliquot of the *Sample solution* taken (mg)
a = volume of the aliquot of *Sample solution* taken
V = volume of *Medium*, 900 mL
L = label claim of chondroitin sulfate sodium (mg/Tablet)

Tolerances: NLT 75% of the labeled amount of chondroitin sulfate sodium is dissolved.

- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩**:** Meet the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at room temperature.
- **LABELING:** Label it to indicate the species of the source from which the chondroitin used to prepare the Tablets was derived. Label it to state the source(s) of chondroitin sulfate sodium, whether bovine, porcine, avian, or a mixture of any of them. The label states on the front panel the content of chondroitin sulfate sodium on the dried basis.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Chondroitin Sulfate Sodium RS

Chondroitin Sulfate Sodium, Shark

Chondroitin, hydrogen sulfate, sodium salt [9082-07-9].

DEFINITION

Chondroitin Sulfate Sodium, Shark is the sodium salt of the sulfated linear glycosaminoglycan obtained from shark cartilages used for human foods. Chondroitin Sulfate Sodium, Shark consists mostly of the sodium salt of the sulfate ester of *N*-acetylchondrosamine (2-acetamido-2-deoxy-β-D-galactopyranose) and D-glucuronic acid copolymer. These hexoses are alternately linked β-1,4 and β-1,3 in the polymer. Chondrosamine moieties in the prevalent glycosaminoglycan are monosulfated primarily on position 6 and less so on position 4 with minor disulfation on both positions 4 and 6. NLT 8% of the D-glucuronic acid moieties are monosulfated on position 2. It contains NLT 90.0% and NMT 105.0% of chondroitin sulfate sodium, calculated on the dried basis.

[NOTE—Chondroitin Sulfate Sodium, Shark is extremely hygroscopic once dried. Avoid exposure to the atmosphere, and weigh promptly.]

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
- **B. IDENTIFICATION TESTS—GENERAL** ⟨191⟩, *Sodium*
  Sample solution: 0.5 g in 10 mL of water
  Acceptance criteria: Meets the requirements
- **C. SPECIFIC DISACCHARIDES:** The chromatogram of the enzymatically digested *Sample solution* as obtained in the test for *Disaccharide Composition* shows three main peaks due to 6-sulfated (ΔDi-6S), 4-sulfated (ΔDi-4S), and 2,6-disulfated (ΔDi-2,6diS) disaccharides, corresponding to those of the enzymatically digested *Standard solution*, with ΔDi-6S being the most abundant, followed by ΔDi-4S, with NLT 8% corresponding to ΔDi-2,6diS. Additional minor peaks corresponding to nonsulfated (ΔDi-0S) and 4,6 disulfation may be detected.
- **D. SPECIFIC ROTATION:** Meets the requirements in the *Specific Tests*

DS Monographs

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COMPOSITION

• **CONTENT OF CHONDROITIN SULFATE SODIUM**

Standard solutions: 1.5, 1.0, and 0.5 mg/mL of dried USP Chondroitin Sulfate Sodium, Shark RS in water

Sample solution: Transfer 100 mg of dried Chondroitin Sulfate Sodium, Shark into a 100-mL volumetric flask, dissolve in 30 mL of water, and dilute with water to volume.

Diluent: Weigh about 297 mg of monobasic potassium phosphate, 492 mg of dibasic potassium phosphate, and 250 mg of polysorbate 80, and transfer to a 1-L beaker. Dissolve in 900 mL of water, and adjust with potassium hydroxide or phosphoric acid to a pH of 7.0 ± 0.2. Dilute with water to 1 L, and mix thoroughly.

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Photometric titration

Titrant: 1 mg/mL of cetylpyridinium chloride in water. Degas before use.

Endpoint detection: Turbidimetric with a photoelectric probe

Analysis: Transfer 5.0 mL each of the *Standard solution* and the *Sample solution* to separate titration vessels, and add 25 mL of *Diluent* to each. Stir until a steady reading is obtained with the photoelectric probe set either at 420, 550, or 660 nm. Set the instrument to zero in absorbance mode. Titrate with *Titrant* using the photoelectric probe to determine the endpoint turbidimetrically. From a linear regression equation, calculated using the volumes of *Titrant* consumed versus concentrations of the *Standard solutions*, determine the concentration of chondroitin sulfate sodium in the *Sample solution*.

Calculate the percentage of chondroitin sulfate sodium in the portion of Chondroitin Sulfate Sodium, Shark taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of chondroitin sulfate sodium in the aliquot of the *Sample solution*, obtained from the regression equation (mg/mL)

C_U = concentration of Chondroitin Sulfate Sodium, Shark in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–105.0% on the dried basis

• **DISACCHARIDE COMPOSITION**

Solution A: Water adjusted with 0.1 N hydrochloric acid to a pH of 3.5

Solution B: 1 M sodium chloride adjusted with 0.1 N hydrochloric acid to a pH of 3.5

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0.0	100	0
4.0	100	0
45.0	50	50
45.1	100	0

Buffer solution: 50 mM tris(hydroxymethyl)aminomethane and 60 mM sodium acetate, adjusted with diluted hydrochloric acid to a pH of 8.0

Standard solution: 2.4 mg/mL of dried USP Chondroitin Sulfate Sodium, Shark RS in water

Sample solution: Transfer about 250 mg of dried Chondroitin Sulfate Sodium, Shark to a 100-mL volumetric flask, and dissolve and dilute with water to volume. Filter to obtain a clear solution.

Blank: Water

Chondroitinase ABC solution: Dissolve 1 unit (U/mg of protein) of chondroitinase ABC[1] in 1.0 mL of *Buffer solution*. Mix thoroughly.

Chondroitinase ABC solution suitability: Dilute the incubated *Standard solution* (1 in 10), and measure its absorbance against the incubated *Blank* at 232 nm. The absorptivity is NLT 8 AU · mL · mg^{-1} · cm^{-1}.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 232 nm

Column: 4.6-mm × 25-cm; 5-µm packing L14

Flow rate: 1 mL/min

Injection volume: 20 µL

System suitability

Sample: *Standard solution* (prepared per *Analysis* below)

[NOTE—The relative retention times for the ΔDi-0S, ΔDi-6S, ΔDi-4S, and ΔDi-2,6diS peaks are 0.50, 0.75, 0.80, and 1.0, respectively.]

Suitability requirements

Chromatogram similarity: The chromatogram of the *Standard solution* is similar to the reference chromatogram provided with USP Chondroitin Sulfate Sodium, Shark RS.

Resolution: NLT 2.0 between the ΔDi-6S and ΔDi-4S peaks

Relative standard deviation: NMT 5.0% for the ΔDi-6S, ΔDi-4S, or ΔDi-2,6diS peaks

Analysis

Samples: *Standard solution*, *Sample solution*, and *Blank*

In three separate vials, combine 0.8 mL of *Buffer solution*, 0.1 mL of *Chondroitinase ABC solution*, and 0.1 mL each of the *Standard solution*, *Sample solution*, and *Blank*. Mix thoroughly. Incubate at 37° for 3 h. Allow the solution to cool to room temperature, and centrifuge prior to injection.

Calculate the percentage of each disaccharide in the sample taken:

$$\text{Result} = (r_U/\Sigma r_U) \times 100$$

r_U = peak area of ΔDi-0S, ΔDi-6S, ΔDi-4S, or ΔDi-2,6diS from the *Sample solution*

Σr_U = sum of the peak areas of ΔDi-0S, ΔDi-6S, ΔDi-4S, and ΔDi-2,6diS from the *Sample solution*

Acceptance criteria: The area percentage of the ΔDi-6S peak is greater than that of the ΔDi-4S peak, and the area percentage of the ΔDi-2,6diS peak is the lowest of the three. The area percentage of the ΔDi-2,6diS peak is NLT 8%.

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩: 20.0%–30.0% on the dried basis

• **CHLORIDE AND SULFATE** ⟨221⟩, *Chloride*

Standard solution: 0.7 mL of 0.020 N hydrochloric acid

Sample: 0.1 g

Acceptance criteria: NMT 0.50%

• **CHLORIDE AND SULFATE** ⟨221⟩, *Sulfate*

Standard solution: 0.25 mL of 0.020 N sulfuric acid

Sample solution: Dissolve 200 mg in 40 mL of water. Add 10 mL of a solution of cetylpyridinium chloride having a concentration of 30 mg/mL, and pass through a filter. Use a 25-mL portion of the filtrate.

Acceptance criteria: NMT 0.24%; the *Sample solution* shows no more sulfate than that of the *Standard solution*.

[1] Chondroitinase ABC from *Proteus vulgaris* is available from Sigma (www.sigmaaldrich.com), Catalog Number C3667.

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• **ELECTROPHORETIC PURITY**

[**CAUTION**—Voltages used in electrophoresis can readily deliver a lethal shock. The hazard is increased by the use of aqueous buffer solutions and the possibility of working in damp environments. The equipment, with the possible exception of the power supply, should be enclosed in either a grounded metal case or a case made of insulating material. The case should have an interlock that deenergizes the power supply when the case is opened, after which reactivation should be prevented until activation of a reset switch is carried out. High-voltage cables from the power supply to the apparatus should preferably be a type in which a braided metal shield completely encloses the insulated central conductor, and the shield should be grounded. The base of the apparatus should be grounded metal or contain a grounded metal rim which is constructed in such a way that any leakage of electrolyte will produce a short which will deenergize the power supply before the electrolyte can flow beyond the protective enclosure. If the power supply contains capacitors as part of a filter circuit, it should also contain a bleeder resistor to ensure discharge of the capacitors before the protective case is opened. A shorting bar that is activated by opening the case may be considered as an added precaution. Because of the potential hazard associated with electrophoresis, laboratory personnel should be completely familiar with electrophoresis equipment before using it.]

Barium acetate buffer: Dissolve 25.24 g of barium acetate in 900 mL of water. Adjust with acetic acid to a pH of 5.0, and dilute with water to 1000 mL.

Staining reagent: Dissolve 1 g of toluidine blue in 1000 mL of 0.1 M acetic acid.

Standard solution A: 30 mg/mL of USP Chondroitin Sulfate Sodium, Shark RS in water

Standard solution B: Dilute 1 mL of *Standard solution A* with water to 50 mL.

Sample solution: 30 mg/mL of Chondroitin Sulfate Sodium, Shark in water

Analysis: Fill the chambers of an electrophoresis apparatus suitable for separations on cellulose acetate membranes[2] (a small submarine gel chamber or one dedicated to membrane media) with *Barium acetate buffer*. Soak a cellulose acetate membrane, 5–6 cm × 12–14 cm, in *Barium acetate buffer* for 10 min, or until evenly wetted, then blot dry between two sheets of absorbent paper. Using an applicator[3] suitable for electrophoresis, apply equal volumes (0.5 µL) of the *Sample solution, Standard solution A,* and *Standard solution B* to the brighter side of the membrane held in position in an appropriate applicator stand or on a separating bridge in the chamber. Ensure that both ends of the membrane are dipped at least 0.5–1.0 cm deep into the buffer chambers. Apply a constant 60 volts (6 mA at the start) for 2 h. [NOTE—Perform the application of solutions, and voltage within 5 min because further drying of the blotted paper reduces sensitivity.]

Place the membrane in a plastic staining tray, and with the application side down, float or gently immerse in the *Staining reagent* for 5 min. Then stir the solution gently for 1 min. Remove the membrane, and destain in 5% acetic acid until the background clears. Compare the bands.

[NOTE—Document the results by taking a picture within 15 min of the completion of destaining.]

Acceptance criteria: The electropherogram from the *Sample solution* exhibits a major band that is identical in position to the band from *Standard solution A*. The band from *Standard solution B* is clearly visible at a mobility similar to the band from *Standard solution A*. Any secondary band in the electropherogram of the *Sample solution* is not more intense than the band from *Standard solution B*. NMT 2% of any individual impurity in Chondroitin Sulfate Sodium, Shark is found.

• **LIMIT OF PROTEIN**

Solution A: 20 mg/mL of sodium tartrate dihydrate

Solution B: 10 mg/mL of cupric sulfate

Solution C: 20 mg/mL of anhydrous sodium carbonate in 0.1 M sodium hydroxide

Dilute Folin-Ciocalteu reagent: Dilute Folin-Ciocalteu phenol TS with water (1:5). Prepare immediately before use.

Alkaline cupric tartaric reagent: Mix 1 mL each of *Solution A* and *Solution B*, and to the mixture slowly add 100 mL of *Solution C* with stirring. Use within 24 h, and discard afterward.

Standard solution: 36 µg/mL of bovine serum albumin certified standard in water

Sample solution: Transfer a portion of Chondroitin Sulfate Sodium, Shark, equivalent to 60 mg of the dried substance, to a 100-mL volumetric flask, and dissolve in and dilute with water to volume.

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Analytical wavelength: 750 nm

Blank: Water

Analysis

Samples: *Standard solution, Sample solution,* and *Blank*

Add 2.0 mL of freshly prepared *Alkaline cupric tartaric reagent* to three test tubes, each containing 2.0 mL of the *Standard solution,* 2.0 mL of the *Sample solution,* or 2.0 mL of the *Blank*. After 10 min, add 1.0 mL of *Dilute Folin-Ciocalteu reagent* to each test tube, and mix immediately and vigorously. After 30 min, measure the absorbance of the *Standard solution* and *Sample solution* against the *Blank*.

Acceptance criteria: NMT 6.0% on the dried basis; the absorbance of the *Sample solution* is NMT the absorbance of the *Standard solution.*

CONTAMINANTS

• **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩

Acceptance criteria

Arsenic: NMT 2.0 µg/g

Cadmium: NMT 1.0 µg/g

Lead: NMT 1.0 µg/g

Mercury: NMT 1.0 µg/g

• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total bacterial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli.*

SPECIFIC TESTS

• **CLARITY AND COLOR OF SOLUTION**

Sample solution: Transfer 2.5 g of Chondroitin Sulfate Sodium, Shark to a 50-mL volumetric flask. Dissolve in and dilute with carbon dioxide-free water to volume, and examine immediately.

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Analytical wavelength: 420 nm

[2] Suitable cellulose acetate membranes for electrophoresis are available from Malta Chemetron SRL, Milano, Italy; Fluka Chemical Corp., Milwaukee, WI; and Apacor Ltd., Berkshire, England (www.apacor.com/products/electrophoresis/cellulose-acetate-membranes).

[3] Suitable applicators are available from Apacor Ltd., Berkshire, England (www.apacor.com/PDF/APA092-ElectrophoresisEquipmentSupplies.pdf) and Helena Laboratories, Beaumont, TX (www.helena.com).

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Cell: 1 cm
Blank: Carbon dioxide-free water
Analysis: Measure the absorbance of the *Sample solution*.
Acceptance criteria: NMT 0.35

• **OPTICAL ROTATION** ⟨781S⟩, *Specific Rotation*
Sample solution: 30 mg/mL in water
Acceptance criteria: −12.0° to −23.0°

• **pH** ⟨791⟩
Sample solution: 10 mg/mL
Acceptance criteria: 5.5–7.5

• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry a sample at 105° for 4 h. [NOTE—Chondroitin Sulfate Sodium, Shark is extremely hygroscopic once dried. Avoid exposure to the atmosphere, and weigh promptly.]
Acceptance criteria: NMT 12.0%

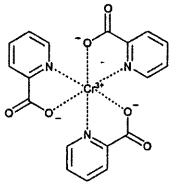
ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers.

• **LABELING:** Label it to state the source(s) from which the article was derived.

• **USP REFERENCE STANDARDS** ⟨11⟩
USP Chondroitin Sulfate Sodium, Shark RS

Chromium Picolinate



$C_{18}H_{12}N_3O_6Cr$ 418.30
Chromium Tripicolinate [14639-25-9].

DEFINITION
Chromium Picolinate contains NLT 98.0% and NMT 102.0% of chromium picolinate ($C_{18}H_{12}N_3O_6Cr$), calculated on the dried basis.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197M**▲ (CN 1-May-2020)

• **B.**
Sample solution: 4 mg/mL
Analysis: To 5 mL of the *Sample solution* add 1 mL of 5 N sodium hydroxide and 10 drops of 30% hydrogen peroxide, and heat gently for 2 min.
Acceptance criteria: A yellow color develops.

ASSAY

• **PROCEDURE**
Standard stock solution: 100 µg/mL of chromium. Transfer 0.283 g of potassium dichromate, previously dried at 120° for 4 h, to a 1000-mL volumetric flask, and dilute with water to volume. Store in a polyethylene bottle.
Standard solutions: 1.0, 2.0, 3.0, and 4.0 µg/mL of chromium. Separately transfer 1.0 and 2.0 mL of the *Standard stock solution* to 100-mL volumetric flasks, and transfer 1.5 and 2.0 mL of the *Standard stock solution* to separate 50-mL volumetric flasks. Add 1.0 mL of nitric acid to each flask, and dilute the contents of each flask with water to volume.
Sample solution: Transfer 200 mg of Chromium Picolinate to a 100-mL beaker, and add 25 mL of water. Slowly add 10 mL of nitric acid, and boil for 10 min with constant swirling. Cool the solution, quantitatively transfer to a 500-mL volumetric flask, and dilute with water to volume. Filter a portion of the solution, and transfer 5.0 mL of the filtrate to a 100-mL volumetric flask. Add 1 mL of nitric acid, and dilute with water to volume.
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Analytical wavelength: 357.9 nm
Lamp: Chromium hollow-cathode
Flame: Air–acetylene
Blank: Diluted nitric acid
Analysis
Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the *Standard solutions* and the *Sample solution*. Plot the absorbances of the *Standard solutions* versus the chromium concentration, in µg/mL, and draw the straight line best fitting the four plotted points. From the graph so obtained, determine the chromium concentration, in µg/mL, in the *Sample solution*.
Calculate the percentage of chromium picolinate ($C_{18}H_{12}N_3O_6Cr$) in the portion of Chromium Picolinate taken:

$$\text{Result} = (C_{Cr}/C_U) \times (M_r/A_r) \times 100$$

C_{Cr} = concentration of chromium in the *Sample solution*, obtained from the graph (µg/mL)
C_U = concentration of Chromium Picolinate in the *Sample solution* (µg/mL)
M_r = molecular weight of chromium picolinate, 418.31
A_r = atomic weight of chromium, 51.996

Acceptance criteria: 98.0%–102.0% on the dried basis

IMPURITIES

• **CHLORIDE AND SULFATE,** *Chloride* ⟨221⟩
Sample solution: Dissolve 30 mg of Chromium Picolinate in 30–40 mL of water, and heat to 70°. Cool overnight, and filter to remove the precipitate.
Analysis: Add 1 mL each of nitric acid and silver nitrate TS, and add sufficient water to make 50 mL. Mix, and allow to stand for 5 min, protected from direct sunlight.
Acceptance criteria: Any turbidity formed is NMT that produced in a similarly treated control solution containing 0.25 mL of 0.002 N hydrochloric acid (NMT 0.06%).

• **CHLORIDE AND SULFATE,** *Sulfate* ⟨221⟩
Sample solution: Dissolve 100 mg of Chromium Picolinate in 30–40 mL of water, and heat to 90°. Cool overnight, and filter to remove the precipitate.
Analysis: Add 1 mL of 3 N hydrochloric acid, 3 mL of barium chloride TS, and sufficient water to make 50 mL. Mix, and allow to stand for 10 min.
Acceptance criteria: Any turbidity formed is NMT that produced in a similarly treated control solution containing 0.2 mL of 0.02 N sulfuric acid (NMT 0.2%).

SPECIFIC TESTS

• **LOSS ON DRYING** ⟨731⟩: Dry a sample at 105° for 4 h: it loses NMT 4.0% of its weight.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers.

• **USP REFERENCE STANDARDS** ⟨11⟩
USP Chromium Picolinate RS

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Chromium Picolinate Tablets

DEFINITION

Chromium Picolinate Tablets contain NLT 95.0% and NMT 125.0% of the labeled amount of chromium (Cr).

IDENTIFICATION

- **A.** The *Sample solution* prepared as directed in the test for *Strength* gives a positive test for chromium, determined at 357.9 nm using the *Instrumental conditions* in the test for *Content of Chromium*.

STRENGTH

- **CONTENT OF CHROMIUM**

Standard stock solution A: 1000 µg/mL of chromium from potassium dichromate, previously dried at 120° for 4 h, in water. Store in a polyethylene bottle.

Standard stock solution B: Transfer 1.0 mL of *Standard stock solution A* to a 100-mL volumetric flask, add 5.0 mL of 6 N hydrochloric acid, and dilute with water to volume to obtain a solution having a concentration of 10 µg/mL of chromium.

Standard solutions: Dilute *Standard stock solution B* with 0.125 N hydrochloric acid to obtain concentrations of 1.0, 2.0, 3.0, and 4.0 µg/mL of chromium.

Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 5 Tablets, to a porcelain crucible, heat the crucible in a muffle furnace maintained at about 550° for 6–12 h, and cool. Add 60 mL of hydrochloric acid, and boil gently on a hot plate or steam bath for 30 min, intermittently rinsing the inner surface of the crucible with 6 N hydrochloric acid. Cool, and transfer the contents of the crucible to a 100-mL volumetric flask. Rinse the crucible with small portions of 6 N hydrochloric acid, and add the rinsings to the flask. Dilute with water to volume, mix, and filter, discarding the first 5 mL of the filtrate. Dilute this solution with 0.125 N hydrochloric acid to obtain a solution having a concentration of 2.5 µg/mL of chromium.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Lamp: Chromium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: 357.9 nm (chromium emission line)

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the *Samples*, using the *Blank*. From a linear regression equation, calculated using the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of chromium, determine the concentration, *C*, in µg/mL, of chromium in the *Sample solution*.

Calculate the percentage of the labeled amount of chromium (Cr) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = determined concentration of chromium in the *Sample solution* (µg/mL)

C_U = nominal concentration of chromium in the *Sample solution* (µg/mL)

Acceptance criteria: 95.0%–125.0%

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** ⟨2040⟩**:** Meet the requirements for *Disintegration*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩**:** Meet the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at controlled room temperature.

Cinnamomum cassia Twig

DEFINITION

Cinnamomum cassia Twig consists of the dried twigs of *Cinnamomum cassia* (L.) J.Presl [syn. *Cinnamomum aromaticum* Nees] (Fam. Lauraceae) collected in spring or summer. It contains NLT 0.8% of total phenylpropanoids, calculated as the sum of cinnamyl alcohol, cinnamic acid, 2-methoxycinnamic acid, cinnamaldehyde, and 2-methoxycinnamaldehyde on the anhydrous basis.

IDENTIFICATION

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 0.5 mg/mL each of USP Cinnamaldehyde RS and coumarin in methanol

Standard solution B: USP *Cinnamomum cassia* Twig Powder RS in methanol (1 in 5). Sonicate for 10 min, centrifuge or filter, and evaporate the solvent. Suspend the residue in a volume of toluene equivalent to one-fifth the volume of methanol used for the extraction. Sonicate for about 2 min, centrifuge or filter, and use the supernatant or the filtrate.

Sample solution: 2 g of *Cinnamomum cassia* Twig, finely powdered, in 10 mL of methanol. Sonicate for 10 min, centrifuge or filter, and transfer the extract to a round-bottom flask. Evaporate the solution under reduced pressure to dryness. Dissolve the residue in 2 mL of toluene, sonicate for about 2 min, centrifuge or filter, and use the supernatant or the filtrate.

Chromatographic system

Adsorbent: Chromatographic silica gel F_{254} mixture with an average particle size of about 5 µm

Application volume: 6 µL, as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

Temperature: About 25°

Developing solvent system: Toluene and ethyl acetate (19:1)

Developing distance: 6 cm

Derivatization reagent: Methanol, acetic acid, sulfuric acid, and *p*-anisaldehyde (170:20:10:1). [NOTE—Prepare fresh. Slowly add sulfuric acid to ice-cold methanol, followed by acetic acid and *p*-anisaldehyde.]

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands and dry in air. Develop in a saturated chamber (20 min with filter paper), remove the plate from the chamber, and dry in air. Examine under UV light at 254 nm. Then treat the plate with the *Derivatization reagent*, heat at 100° for 4 min, and examine under UV light at 366 nm.

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements: Prior to derivatization, under UV light at 254 nm, *Standard solution A* exhibits a quenching

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band due to cinnamaldehyde in the middle third of the chromatogram, and a quenching band due to coumarin in the upper part of the lower third.

Under UV light at 254 nm, *Standard solution B* exhibits, in the middle-third section, an intense quenching band corresponding in R_F to the cinnamaldehyde band in *Standard solution A*. In the upper part of the lower-third section, *Standard solution B* exhibits a weak quenching band due to coumarin, a quenching band close to the starting position due to the co-elution of cinnamic acid and 2-methoxycinnamic acid, and a quenching band between coumarin and cinnamic acid due to cinnamyl alcohol. After derivatization, under UV light at 366 nm, *Standard solution B* exhibits a band corresponding in R_F and color to the cinnamaldehyde band in *Standard solution A*, and a yellow band immediately below the cinnamaldehyde band.

Acceptance criteria: Under UV light at 254 nm, the *Sample solution* exhibits the most intense quenching band in the middle-third section, corresponding in R_F to the cinnamaldehyde band in *Standard solution A*. The *Sample solution* exhibits additional bands corresponding to similar bands in *Standard solution B*; these include a weak quenching band in the upper part of the lower-third section due to coumarin, a quenching band close to the starting position due to the co-elution of cinnamic acid and 2-methoxycinnamic acid, and a quenching band between the coumarin and cinnamic acid bands. After derivatization, under UV light at 366 nm, the *Sample solution* exhibits a band corresponding in R_F and color to the cinnamaldehyde band in *Standard solution A* and *Standard solution B*, and a yellow band immediately below the cinnamaldehyde band. There is no red band immediately above the cinnamaldehyde band (a distinction from *Cinnamomum verum*).

• **B. HPLC**

Analysis: Proceed as directed in the test for *Content of Total Phenylpropanoids and Coumarin*.

Acceptance criteria: The *Sample solution* exhibits the most intense peak at a retention time corresponding to cinnamaldehyde in *Standard solution B*; a peak with a retention time corresponding to cinnamic acid in *Standard solution A*; and peaks due to coumarin, cinnamyl alcohol, 2-methoxycinnamic acid, and 2-methoxycinnamaldehyde at retention times corresponding to the same constituents in *Standard solution B*. The content ratios of these constituents relative to cinnamic acid are within the typical ratio ranges listed in *Table 2*. The content of cinnamaldehyde is NLT 65% of the content of total phenylpropanoids.

COMPOSITION

• **CONTENT OF TOTAL PHENYLPROPANOIDS AND COUMARIN**

[NOTE—Protect from light and proceed under low actinic light. *Standard solution A*, *Standard solution B*, and the *Sample solution* are stable for 24 h at room temperature.]

Solution A: 0.05% phosphoric acid in water

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	75	25
1	75	25
21	62	38
30	60	40
35	60	40

Solvent: Methanol and water (7:3)

Standard solution A: 0.05 mg/mL of USP Cinnamic Acid RS in methanol

Standard solution B: Transfer about 250 mg of USP *Cinnamomum cassia* Twig Powder RS into a 50-mL round-bottom centrifuge tube, and add 25 mL of *Solvent*. Sonicate for 30 min, cool, and centrifuge. Before injection, pass through a membrane filter of 0.45-µm or finer pore size.

Sample solution: Accurately transfer about 500 mg of finely powdered *Cinnamomum cassia* Twig into a 50-mL round-bottom centrifuge tube, and add 25 mL of *Solvent*. Sonicate for 30 min (250 W, 33 kHz), cool, centrifuge, and transfer the supernatant to a 50-mL volumetric flask. Repeat the extraction one more time by adding 15 mL of *Solvent*, and transfer the supernatant to the same 50-mL volumetric flask, dilute with *Solvent* to volume, and mix. Before injection, pass through a membrane filter of 0.45-µm or finer pore size and discard the first portion of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 25°

Flow rate: 1.0 mL/min

Injection volume: 10 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Resolution: NLT 1.5 between the cinnamyl alcohol and cinnamic acid peaks and between the coumarin and cinnamyl alcohol peaks, *Standard solution B*

Tailing factor: NMT 2.0 for the cinnamic acid peak, *Standard solution A*

Relative standard deviation: NMT 2.0% for the cinnamic acid peak in repeated injections, *Standard solution A*

Chromatographic similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP *Cinnamomum cassia* Twig Powder RS being used.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatograms of *Standard solution A* and *Standard solution B* and the reference chromatogram provided with the lot of USP *Cinnamomum cassia* Twig Powder RS being used, identify the retention times of the peaks corresponding to coumarin, cinnamyl alcohol, cinnamic acid, 2-methoxycinnamic acid, cinnamaldehyde, and 2-methoxycinnamaldehyde in the *Sample solution*. [NOTE—see *Table 2* for relative retention times.]

DS Monographs

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Table 2

Analyte	Approximate Relative Retention Time	Conversion Factor	Typical Content Range (%)	Content Ratio Relative to Cinnamic Acid
Coumarin	0.8	2.10	0.02–0.15	0.2–1.3
Cinnamyl alcohol	0.9	2.12	0.003–0.05	0.02–0.6
Cinnamic acid	1.0	1.00	0.04–0.16	1.0
2-Methoxycinnamic acid	1.1	1.82	0.002–0.02	0.04–0.16
Cinnamaldehyde	1.3	1.47	0.6–2.0	6–30
2-Methoxycinnamaldehyde	1.5	2.69	0.08–0.5	0.5–6.0

Separately calculate the percentages of coumarin, cinnamyl alcohol, cinnamic acid, 2-methoxycinnamic acid, cinnamaldehyde, and 2-methoxycinnamaldehyde in the portion of *Cinnamomum cassia* Twig taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of cinnamic acid from *Standard solution A*
C_S = concentration of USP Cinnamic Acid RS in *Standard solution A* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of *Cinnamomum cassia* Twig taken to prepare the *Sample solution* (mg)
F = conversion factor for the analyte (see *Table 2*)

Calculate content of total phenylpropanoids as the sum of cinnamyl alcohol, cinnamic acid, 2-methoxycinnamic acid, cinnamaldehyde, and 2-methoxycinnamaldehyde.

Acceptance criteria: NLT 0.8% of total phenylpropanoids on the anhydrous basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Limits of Elemental Impurities*: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Pesticide Residue Analysis*: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures*, *Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: Long cylindrical, branched, 30–75 cm long, 0.3–1 cm in diameter at the thicker end. Externally brown to reddish brown, with longitudinal ridges, fine wrinkles, dotted with scars from leaves, branches or buds, and dotted lenticels. Texture hard and fragile, easily broken. Slices 2–4 mm thick, cut surface showing reddish-brown in bark, yellowish white to pale yellow-brown in wood, pith in subsquare.

Microscopic: In the transverse section, epidermis consists of one layer of cells, unicellular nonglandular hairs are visible in young branches. Cork consists of 3–5 layers of cells, the inner-most cells with thickened outer walls. Oil cells and stone cells are scattered in the cortex. Groups of stone cells in the pericycle are arranged in an interrupted ring, accompanied by fiber bundles. Secretory cells and fibers are scattered in phloem. Cambium is distinct. Xylem with rays 1–2 cells wide, containing brown contents; vessels are scattered single or two to several aggregated; lignified fibers have relatively thin walls and are difficult to differentiate from lignified parenchymatous cells. In pith, cell walls are slightly thickened and lignified; ray cells contain fine needle crystals of calcium oxalate.

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Foreign Organic Matter*: NMT 1.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Alcohol-Soluble Extractives, Method 1*: NLT 6.0%
- **WATER DETERMINATION** ⟨921⟩, *Method II*: NMT 15.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Total Ash*: NMT 3.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Acid-Insoluble Ash*: NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial following the official name of the plant from which the article was derived.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Cinnamaldehyde RS
  USP Cinnamic Acid RS
  USP *Cinnamomum cassia* Twig Powder RS

Cinnamomum cassia Twig Powder

DEFINITION

Cinnamomum cassia Twig Powder consists of the dried twigs of *Cinnamomum cassia* (L.) J.Presl [syn. *Cinnamomum aromaticum* Nees] (Fam. Lauraceae) collected in spring or summer, reduced to a fine or very fine powder. It contains NLT 0.8% of total phenylpropanoids, calculated as the sum of cinnamyl alcohol, cinnamic acid, 2-methoxycinnamic acid, cinnamaldehyde, and 2-methoxycinnamaldehyde on the anhydrous basis.

IDENTIFICATION

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 0.5 mg/mL each of USP Cinnamaldehyde RS and coumarin in methanol

Standard solution B: USP *Cinnamomum cassia* Twig Powder RS in methanol (1 in 5). Sonicate for 10 min, centrifuge or filter, and evaporate the solvent. Suspend the residue in a volume of toluene equivalent to one-fifth the volume of methanol used for the extraction. Sonicate for about 2 min, centrifuge or filter, and use the supernatant or the filtrate.

Sample solution: 2 g of Powder in 10 mL of methanol. Sonicate for 10 min, centrifuge or filter, and transfer the extract to a round-bottom flask. Evaporate the solution under reduced pressure to dryness. Dissolve the residue in 2 mL of toluene, sonicate for about 2 min, centrifuge or filter, and use the supernatant or the filtrate.

Chromatographic system

Adsorbent: Chromatographic silica gel F_{254} mixture with an average particle size of about 5 µm

Application volume: 6 µL, as 8-mm bands

DS Monographs

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Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
Temperature: About 25°
Developing solvent system: Toluene and ethyl acetate (19:1)
Developing distance: 6 cm
Derivatization reagent: Methanol, acetic acid, sulfuric acid, and *p*-anisaldehyde (170:20:10:1). [NOTE—Prepare fresh. Slowly add sulfuric acid to ice-cold methanol, followed by acetic acid and *p*-anisaldehyde.]

Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Apply the *Samples* as bands and dry in air. Develop in a saturated chamber (20 min with filter paper), remove the plate from the chamber, and dry in air. Examine under UV light at 254 nm. Then treat the plate with the *Derivatization reagent*, heat at 100° for 4 min, and examine under UV light at 366 nm.

System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements: Prior to derivatization, under UV light at 254 nm, *Standard solution A* exhibits a quenching band due to cinnamaldehyde in the middle third of the chromatogram, and a quenching band due to coumarin in the upper part of the lower third.
Under UV light at 254 nm, *Standard solution B* exhibits, in the middle-third section, an intense quenching band corresponding in R_F to the cinnamaldehyde band in *Standard solution A*. In the upper part of the lower-third section, *Standard solution B* exhibits a weak quenching band due to coumarin, a quenching band close to the starting position due to the co-elution of cinnamic acid and 2-methoxycinnamic acid, and a quenching band between coumarin and cinnamic acid due to cinnamyl alcohol. After derivatization, under UV light at 366 nm, *Standard solution B* exhibits a band corresponding in R_F and color to the cinnamaldehyde band in *Standard solution A*, and a yellow band immediately below the cinnamaldehyde band.

Acceptance criteria: Under UV light at 254 nm, the *Sample solution* exhibits the most intense quenching band in the middle-third section, corresponding in R_F to the cinnamaldehyde band in *Standard solution A*. The *Sample solution* exhibits additional bands corresponding to similar bands in *Standard solution B*; these include a weak quenching band in the upper part of the lower-third section due to coumarin, a quenching band close to the starting position due to the co-elution of cinnamic acid and 2-methoxycinnamic acid, and a quenching band between the coumarin and cinnamic acid bands. After derivatization, under UV light at 366 nm, the *Sample solution* exhibits a band corresponding in R_F and color to the cinnamaldehyde band in *Standard solution A* and *Standard solution B*, and a yellow band immediately below the cinnamaldehyde band. There is no red band immediately above the cinnamaldehyde band (a distinction from *Cinnamomum verum*).

• **B. HPLC**

Analysis: Proceed as directed in the test for *Content of Total Phenylpropanoids and Coumarin*.

Acceptance criteria: The *Sample solution* exhibits the most intense peak at a retention time corresponding to cinnamaldehyde in *Standard solution B*; a peak with a retention time corresponding to cinnamic acid in *Standard solution A*; and peaks due to coumarin, cinnamyl alcohol, 2-methoxycinnamic acid, and 2-methoxycinnamaldehyde at retention times corresponding to the same constituents in *Standard solution B*. The content ratios of these constituents relative to cinnamic acid are within the typical ratio ranges listed in *Table 2*. The content of cinnamaldehyde is NLT 65% of the content of total phenylpropanoids.

COMPOSITION

• **CONTENT OF TOTAL PHENYLPROPANOIDS AND COUMARIN**
[NOTE—Protect from light and proceed under low actinic light. *Standard solution A*, *Standard solution B*, and the *Sample solution* are stable for 24 h at room temperature.]
Solution A: 0.05% phosphoric acid in water
Solution B: Acetonitrile
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	75	25
1	75	25
21	62	38
30	60	40
35	60	40

Solvent: Methanol and water (7:3)
Standard solution A: 0.05 mg/mL of USP Cinnamic Acid RS in methanol
Standard solution B: Transfer about 250 mg of USP *Cinnamomum cassia* Twig Powder RS into a 50-mL round-bottom centrifuge tube, and add 25 mL of *Solvent*. Sonicate for 30 min, cool, and centrifuge. Before injection, pass through a membrane filter of 0.45-µm or finer pore size.
Sample solution: Accurately transfer about 500 mg of Powder into a 50-mL round-bottom centrifuge tube, and add 25 mL of *Solvent*. Sonicate for 30 min (250 W, 33 kHz), cool, centrifuge, and transfer the supernatant to a 50-mL volumetric flask. Repeat the extraction one more time by adding 15 mL of *Solvent*, and transfer the supernatant to the same 50-mL volumetric flask, dilute with *Solvent* to volume, and mix. Before injection, pass through a membrane filter of 0.45-µm or finer pore size and discard the first portion of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 265 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 25°
Flow rate: 1.0 mL/min
Injection volume: 10 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Resolution: NLT 1.5 between the cinnamyl alcohol and cinnamic acid peaks and between the coumarin and cinnamyl alcohol peaks, *Standard solution B*
Tailing factor: NMT 2.0 for the cinnamic acid peak, *Standard solution A*
Relative standard deviation: NMT 2.0% for the cinnamic acid peak in repeated injections, *Standard solution A*
Chromatographic similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP *Cinnamomum cassia* Twig Powder RS being used.

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Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatograms of *Standard solution A* and *Standard solution B* and the reference chromatogram provided with the lot of USP *Cinnamomum cassia* Twig Powder RS being used, identify the retention times of the peaks corresponding to coumarin, cinnamyl alcohol, cinnamic acid, 2-methoxycinnamic acid, cinnamaldehyde, and 2-methoxycinnamaldehyde in the *Sample solution*. [NOTE—See *Table 2* for relative retention times.]

Table 2

Analyte	Approximate Relative Retention Time	Conversion Factor	Typical Content Range (%)	Content Ratio Relative to Cinnamic Acid
Coumarin	0.8	2.10	0.02–0.15	0.2–1.3
Cinnamyl alcohol	0.9	2.12	0.003–0.05	0.02–0.6
Cinnamic acid	1.0	1.00	0.04–0.16	1.0
2-Methoxycinnamic acid	1.1	1.82	0.002–0.02	0.04–0.16
Cinnamaldehyde	1.3	1.47	0.6–2.0	6–30
2-Methoxycinnamaldehyde	1.5	2.69	0.08–0.5	0.5–6.0

Separately calculate the percentages of coumarin, cinnamyl alcohol, cinnamic acid, 2-methoxycinnamic acid, cinnamaldehyde, and 2-methoxycinnamaldehyde in the portion of Powder taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*

r_S = peak area of cinnamic acid from *Standard solution A*

C_S = concentration of USP Cinnamic Acid RS in *Standard solution A* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Powder taken to prepare the *Sample solution* (mg)

F = conversion factor for the analyte (see *Table 2*)

Calculate the content of total phenylpropanoids as the sum of cinnamyl alcohol, cinnamic acid, 2-methoxycinnamic acid, cinnamaldehyde, and 2-methoxycinnamaldehyde.

Acceptance criteria: NLT 0.8% of total phenylpropanoids on the anhydrous basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Limits of Elemental Impurities*: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Pesticide Residue Analysis*: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: Reddish-brown powder

Microscopic: Stone cells subsquare or subrounded, 30–65 µm in diameter with thickened walls, some with a very thin wall at one side. Phloem fibers mostly in bundles or scattered singly, colorless or brown, fusiform, some margins serrate, 10–40 µm in diameter, with heavily thickened walls, lignified, pit canals indistinct. Oil cells subrounded or elliptical, 40–105 µm in diameter. Xylary fibers numerous, usually in bundles, pits oblique or crossed. Cork cells yellowish brown, polygonal in surface view, containing reddish-brown content. Vessels mainly with bordered pits, up to 80 µm in diameter.

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Alcohol-Soluble Extractives, Method 1*: NLT 6.0%
- **WATER DETERMINATION** ⟨921⟩, *Method II*: NMT 15.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Total Ash*: NMT 3.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Acid-Insoluble Ash*: NMT 1.0%

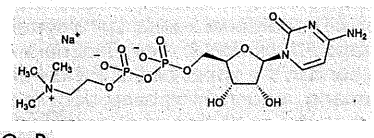
ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial following the official name of the plant from which the article was derived.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Cinnamaldehyde RS
USP Cinnamic Acid RS
USP *Cinnamomum cassia* Twig Powder RS

Add the following:

▲Citicoline Sodium



$C_{14}H_{25}N_4NaO_{11}P_2$ 510.31

Cytidine 5′-(trihydrogen diphosphate), P′-[2-(trimethylammonio)ethyl] ester, inner salt, monosodium salt;

CDP-choline (sodium) [33818-15-4].

DEFINITION

Citicoline Sodium contains NLT 98.0% and NMT 102.0% of citicoline sodium ($C_{14}H_{25}N_4NaO_{11}P_2$), calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

DS Monographs

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ASSAY

• **PROCEDURE**

Mobile phase: 10 mM tetrabutylammonium hydroxide and 15 mM dibasic sodium phosphate, anhydrous, adjusted with 20% phosphoric acid to a pH of 6.0

Standard solution: 24 µg/mL of USP Citicoline Sodium RS in water

Sample solution: 24 µg/mL of Citicoline Sodium in water

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 262 nm

Column: 4.0-mm × 25-cm; 5-µm packing L1

Column temperature: 40°

Flow rate: 1.0 mL/min

Injection volume: 15 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Column efficiency: NLT 5000 theoretical plates

Tailing factor: NMT 2.0

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of citicoline sodium ($C_{14}H_{25}N_4NaO_{11}P_2$) in the portion of Citicoline Sodium taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Citicoline Sodium RS in the *Standard solution* (mg/mL)

C_U = concentration of Citicoline Sodium in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–102.0% on the anhydrous basis

IMPURITIES

• **CHLORIDE AND SULFATE** ⟨221⟩, *Chloride*

Standard solution: 0.10 mL of 0.020 N hydrochloric acid

Sample: 0.47 g of Citicoline Sodium

Acceptance criteria: NMT 0.015%

• **CHLORIDE AND SULFATE** ⟨221⟩, *Sulfate*

Standard solution: 0.10 mL of 0.020 M sulfuric acid

Sample: 0.94 g of Citicoline Sodium

Acceptance criteria: NMT 0.020%

• **RELATED COMPOUNDS**

Solution A: 10 mM tetrabutylammonium hydroxide and 15 mM dibasic sodium phosphate, anhydrous, adjusted with 20% phosphoric acid to a pH of 6.0

Solution B: Methanol

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
5	100	0
15	85	15
20	85	15
21	100	0
35	100	0

System suitability solution: 24 µg/mL of USP Citicoline Sodium RS and 48 µg/mL of USP 5′-Cytidylic Acid RS in water

Standard solution: 2.4 µg/mL of USP Citicoline Sodium RS in water

5′-Cytidylic acid standard solution: 4.8 µg/mL of USP 5′-Cytidylic Acid RS in water

Sample solution: 2.4 mg/mL of Citicoline Sodium in water

Chromatographic system: Proceed as directed in the *Assay*.

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for citicoline and 5′-cytidylic acid are 1.00 and 2.2, respectively.]

Suitability requirements

Resolution: NLT 12 between citicoline and 5′-cytidylic acid

Column efficiency: NLT 5000 theoretical plates for the citicoline peak

Analysis

Samples: *Standard solution*, *5′-Cytidylic acid standard solution*, and *Sample solution*

Calculate the percentage of 5′-cytidylic acid in the portion of Citicoline Sodium taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of 5′-cytidylic acid from the *Sample solution*

r_S = peak response of 5′-cytidylic acid from the *5′-Cytidylic acid standard solution*

C_S = concentration of USP 5′-Cytidylic Acid RS in the *5′-Cytidylic acid standard solution* (mg/mL)

C_U = concentration of Citicoline Sodium in the *Sample solution* (mg/mL)

Calculate the percentage of cytidine-5′-monophosphate methyl ester and each unspecified impurity in the portion of Citicoline Sodium taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (1/F) \times 100$$

r_U = peak response of cytidine-5′-monophosphate methyl ester or each unspecified impurity from the *Sample solution*

r_S = peak response of citicoline from the *Standard solution*

C_S = concentration of USP Citicoline Sodium RS in the *Standard solution* (mg/mL)

C_U = concentration of Citicoline Sodium in the *Sample solution* (mg/mL)

F = relative response factor (see *Table 2*)

Acceptance criteria: See *Table 2*.

Table 2

Name	Relative Retention Time	Relative Response Factor	Acceptance Criteria, NMT (%)
Citicoline sodium	1.0	—	—
5′-Cytidylic acid	2.2	—	0.2
Cytidine-5′-monophosphate methyl ester	3.2	0.7	0.15
Any unspecified impurity	—	1.0	0.1

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Table 2 *(continued)*

Name	Relative Retention Time	Relative Response Factor	Acceptance Criteria, NMT (%)
Total impurities	—	—	0.7

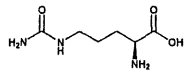
SPECIFIC TESTS

- **WATER DETERMINATION** ⟨921⟩, *Method I, Method Ia*: NMT 5.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Citicoline Sodium RS
  USP 5′-Cytidylic Acid RS ▲(USP 1-May-2019)

Citrulline



$C_6H_{13}N_3O_3$ 175.19

(*S*)-2-Amino-5-ureidopentanoic acid;
L-Citrulline [372-75-8].

DEFINITION

Citrulline contains NLT 98.0% and NMT 102.0% of L-citrulline ($C_6H_{13}N_3O_3$), calculated on the dried basis.

IDENTIFICATION

Change to read:

- **A.** ▲**SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
- **B.** It meets the requirements in *Specific Tests* for *Optical Rotation* ⟨781S⟩, *Procedures, Specific Rotation*.
- **C.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

ASSAY

- **PROCEDURE**

Solution A: 10 mM octanesulfonic acid sodium salt in water. Adjust with dilute phosphoric acid (1 in 10) to a pH of 2.5.
Solution B: Acetonitrile
Mobile phase: Gradient elution. See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	85	15
4	85	15
15	70	30
20	85	15
25	85	15

System suitability solution: 0.1 mg/mL of USP L-Citrulline RS and 0.05 mg/mL of USP *N*-Acetyl-L-leucine RS in water
Standard solution: 0.1 mg/mL of USP L-Citrulline RS in water
Sample solution: 0.1 mg/mL of Citrulline in water

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 200 nm
Column: 4.6-mm × 15-cm; 5-µm packing L1
Flow rate: 1 mL/min
Injection volume: 20 µL
System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The relative retention times for L-citrulline and *N*-acetyl-L-leucine are 1.0 and 2.4, respectively.]
Suitability requirements
Resolution: NLT 10.0 between L-citrulline and *N*-acetyl-L-leucine peaks, *System suitability solution*
Tailing factor: NMT 2.0, *Standard solution*
Relative standard deviation: NMT 2.0%, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of L-citrulline ($C_6H_{13}N_3O_3$) in the portion of Citrulline taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP L-Citrulline RS in the *Standard solution* (mg/mL)
C_U = concentration of Citrulline in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–102.0% on the dried basis

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%
- **CHLORIDE AND SULFATE** ⟨221⟩, *Chloride*
  Standard solution: 0.10 mL of 0.020 N hydrochloric acid
  Sample: 0.36 g of Citrulline
  Acceptance criteria: NMT 0.02%
- **CHLORIDE AND SULFATE** ⟨221⟩, *Sulfate*
  Standard solution: 0.10 mL of 0.020 N sulfuric acid
  Sample: 0.48 g of Citrulline
  Acceptance criteria: NMT 0.02%
- **RELATED COMPOUNDS**
  Mobile phase, System suitability solution, Chromatographic system, and System suitability: Proceed as directed in the *Assay*.
  Standard solution: 2.5 µg/mL of USP Citrulline RS in water
  Sample solution: 0.5 mg/mL of Citrulline in water
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Calculate the percentage of each impurity in the portion of Citrulline taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (1/F) \times 100$$

r_U = peak response of each impurity from the *Sample solution*
r_S = peak response of citrulline from the *Standard solution*
C_S = concentration of USP Citrulline RS in the *Standard solution* (mg/mL)
C_U = concentration of Citrulline in the *Sample solution* (mg/mL)
F = relative response factor (see *Table 2*)

Acceptance criteria: See *Table 2*. [NOTE—Depending on the manufacturing process, delta-Acetylornithine impurity may not be observed in the article being tested. Disregard any impurity less than 0.1%.]

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Table 2

Name	Relative Retention Time	Relative Response Factor	Acceptance Criteria, NMT (%)
Citrulline	1.0	—	—
delta-Acetylornithine[a]	1.1	2.8	0.5
Any unspecified impurity	—	1.0	0.1
Total impurities	—	—	2.0

[a] (2*S*)-5-Acetamido-2-aminopentanoic acid.

SPECIFIC TESTS

• **OPTICAL ROTATION** ⟨781S⟩, *Procedures, Specific Rotation*
Sample solution: 80 mg/mL in 6 N hydrochloric acid
Acceptance criteria: +24.5° to +26.5°

• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry at 105° for 3 h.
Acceptance criteria: NMT 0.2%

ADDITIONAL REQUIREMENTS

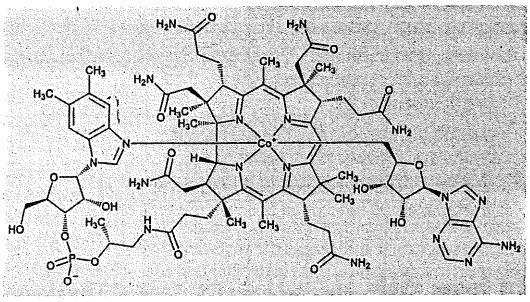
• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

• **USP REFERENCE STANDARDS** ⟨11⟩
USP *N*-Acetyl-L-leucine RS
Acetyl-L-leucine.
$C_8H_{15}NO_3$ 173.21
USP L-Citrulline RS

Clover, Red—*see Red Clover*

Add the following:

▲Cobamamide



$C_{72}H_{100}CoN_{18}O_{17}P$ 1579.58
5,6-Dimethylbenzimidazolyl-Co-5′-deoxyadenosylcobamide; Adenosylcobalamin [13870-90-1].

DEFINITION

Cobamamide contains NLT 96.0% and NMT 102.0% of cobamamide ($C_{72}H_{100}CoN_{18}O_{17}P$), calculated on the anhydrous basis.

IDENTIFICATION

• **A. SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: ⟨197K⟩

• **B. SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Ultraviolet-Visible Spectroscopy*: ⟨197U⟩
[NOTE—Use low-actinic glassware, and protect the *Sample solution* from exposure to light.]
Wavelength range: 200–700 nm
Buffer solution: Dissolve 6.8 g of monobasic potassium phosphate in 950 mL of water. Adjust with 50% sodium hydroxide solution to a pH of 7.0. Dilute with water to 1000 mL.
Sample solution: 50 µg/mL in *Buffer solution*
Acceptance criteria: The absorption spectrum exhibits maxima at 260, 288, 376, and 524 nm. The absorbance ratio A_{260}/A_{524} is 4.1–4.5; the absorbance ratio A_{376}/A_{260} is 0.29–0.33.

• **C.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

ASSAY

• **PROCEDURE**
Solution A: Methanol
Solution B: Dissolve 6.8 g of monobasic potassium phosphate in 950 mL of water. Adjust with 10% phosphoric acid to a pH of 3.2. Dilute with water to 1000 mL. Pass this solution through a filter of 0.2-µm pore size.
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	15	85
2	15	85
2.5	20	80
8	23	77
10	25	75
22	30	70
24	35	65
30	35	65
31	70	30
40	70	30
40.5	15	85
45	15	85

Diluent: Methanol and water (1:3)
[NOTE—Use low-actinic glassware, and protect the following solutions from exposure to light.]
System suitability solution: 0.05 mg/mL each of USP Cobamamide RS, USP Cyanocobalamin (Crystalline) RS, USP Hydroxocobalamin Chloride RS, and USP Methylcobalamin RS in *Diluent*
Standard solution: Allow an appropriate portion of the USP Cobamamide RS to equilibrate at room relative humidity and protected from light, until its weight shows no changes. The equilibration time may be up to 60 min or more depending on the room relative humidity. Use the equilibrated material to prepare a 1-mg/mL solution in *Diluent*. Concurrently determine the water content of the equilibrated sample and use it for the *Assay* correction.
Sample solution: Prepare as directed in the *Standard solution*, except replace USP Cobamamide RS with Cobamamide.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 260 nm
Column: 4.6-mm × 10-cm; 2.6-µm packing L1

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Column temperature: 40°
Flow rate: 1.2 mL/min
Injection volume: 5 µL
System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The relative retention times for cobamamide, hydroxocobalamin, cyanocobalamin, and methylcobalamin are 1.00, 0.15, 0.40, and 1.47, respectively, *System suitability solution*.]
Suitability requirements
Column efficiency: NLT 22,000 theoretical plates for the cobamamide peak, *Standard solution*
Tailing factor: NMT 2.0 for the cobamamide peak, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of cobamamide ($C_{72}H_{100}CoN_{18}O_{17}P$) in the portion of Cobamamide taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of cobamamide from the *Sample solution*
r_S = peak response of cobamamide from the *Standard solution*
C_S = concentration of USP Cobamamide RS (corrected for water content) in the *Standard solution* (mg/mL)
C_U = concentration of Cobamamide (corrected for water content) in the *Sample solution* (mg/mL)

Acceptance criteria: 96.0%–102.0% on the anhydrous basis

IMPURITIES

• **HYDROXOCOBALAMIN AND OTHER RELATED COMPOUNDS**
Mobile phase, Diluent, System suitability solution, Sample solution, Chromatographic system, and System suitability: Proceed as directed in the *Assay*.
[NOTE—Use low-actinic glassware, and protect the following solutions from exposure to light.]
Hydroxocobalamin chloride standard solution: 10 µg/mL of USP Hydroxocobalamin Chloride RS in *Diluent*
Cyanocobalamin standard solution: 20 µg/mL of USP Cyanocobalamin (Crystalline) RS in *Diluent*
Methylcobalamin standard solution: 20 µg/mL of USP Methylcobalamin RS in *Diluent*
Analysis
Samples: *Sample solution* and *Standard solutions*
Calculate the percentage of hydroxocobalamin in the portion of Cobamamide taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak response of hydroxocobalamin from the *Sample solution*
r_S = peak response of hydroxocobalamin from the *Hydroxocobalamin chloride standard solution*
C_S = concentration of USP Hydroxocobalamin Chloride RS in the *Hydroxocobalamin chloride standard solution* (mg/mL)
C_U = concentration of Cobamamide (corrected for water content) in the *Sample solution* (mg/mL)
M_{r1} = molecular weight of hydroxocobalamin, 1346.4
M_{r2} = molecular weight of hydroxocobalamin chloride, 1382.8

Calculate separately the percentages of cyanocobalamin and methylcobalamin in the portion of Cobamamide taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of cyanocobalamin or methylcobalamin from the *Sample solution*
r_S = peak response of cyanocobalamin or methylcobalamin from the *Cyanocobalamin standard solution* or *Methylcobalamin standard solution*
C_S = concentration of USP Cyanocobalamin (Crystalline) RS or USP Methylcobalamin RS in the *Cyanocobalamin standard solution* or *Methylcobalamin standard solution* (mg/mL)
C_U = concentration of Cobamamide (corrected for water content) in the *Sample solution* (mg/mL)

Calculate the percentage of any individual unspecified impurity in the portion of Cobamamide taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of any individual unspecified impurity from the *Sample solution*
r_T = sum of all the peak responses from the *Sample solution*

Acceptance criteria
Hydroxocobalamin: NMT 1.0% on the anhydrous basis
Cyanocobalamin: NMT 2.0% on the anhydrous basis
Methylcobalamin: NMT 2.0% on the anhydrous basis
Individual unspecified impurities: NMT 2.0% on the anhydrous basis
Total impurities: NMT 5.0% on the anhydrous basis

SPECIFIC TESTS

• **WATER DETERMINATION** ⟨921⟩, *Method I, Method Ia*: NMT 12.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at controlled room temperature.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Cobamamide RS
USP Cyanocobalamin (Crystalline) RS
USP Hydroxocobalamin Chloride RS
USP Methylcobalamin RS▲ (USP 1-Dec-2019)

Cod Liver Oil—*see Cod Liver Oil General Monographs*

Cod Liver Oil Capsules

Change to read:

DEFINITION

Cod Liver Oil Capsules contain NLT 95.0% and NMT 105.0% of the labeled amount of Cod Liver Oil, where Cod Liver Oil is the partially destearinated fixed oil obtained from fresh livers of *Gadus morrhua* L. and other species of Fam. Gadidae. Cod Liver Oil contains, in each gram, NLT 180 µg ▲▲ 2S (USP41)

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and NMT 750 µg ▲▲ 2S (USP41) of vitamin A and NLT 1.5 µg ▲▲ 2S (USP41) and NMT 6.25 µg ▲▲ 2S (USP41) of vitamin D. Cod Liver Oil may be flavored by the addition of NMT 1% of a suitable flavor or a mixture of flavors. A suitable antioxidant may be added.

IDENTIFICATION

• **A. Presence of Vitamin A**

Sample solution: 25 mg/mL of oil contained in the Capsules in chloroform

Analysis: To 1 mL of the *Sample solution* add 10 mL of antimony trichloride TS.

Acceptance criteria: A blue color results immediately.

Change to read:

• **B. Fatty Acid Profile**

Antioxidant solution: 0.05 mg/mL of butylated hydroxytoluene in hexanes

System suitability solution: Prepare a mixture containing equal amounts of methyl palmitate, methyl stearate, methyl arachidate, and methyl behenate in *Antioxidant solution*.

Standard stock solution: 45 mg/mL of USP Cod Liver Oil RS in *Antioxidant solution*

Standard solution: Transfer 2.0 mL of the *Standard stock solution* into a ▲glass[1] ▲ 2S (USP41) tube, and evaporate with a gentle stream of nitrogen. Add 1.5 mL of a 2% solution of sodium hydroxide in methanol. Cap tightly with a polytetrafluoroethylene-lined cap, mix, and heat in a water bath for 7 min. Cool, add 2 mL of a 120-mg/mL solution of boron trichloride in methanol. Cover with nitrogen, cap tightly, and mix. Heat in a water bath for 30 min. Cool to 40°–50°. Add 1 mL of isooctane, cap, and mix in a vortex mixer or shake vigorously for at least 30 s. Immediately add 5 mL of saturated sodium chloride solution. Cover with nitrogen, cap, and mix in a vortex mixer or shake thoroughly for at least 15 s. Allow the upper layer to become clear, and transfer to a separate tube. Shake the methanol layer once more with 1 mL of isooctane, and combine the isooctane extracts. Wash the combined extracts twice with 1 mL of water, and dry over anhydrous sodium sulfate.

Sample solution: Prepare as directed in the *Standard solution*, except use a weighed quantity of the oil contained in the Capsules.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.25-mm × 30-m fused silica capillary; coated with a 0.25-µm film of phase G16

Temperatures

Injector: 250°

Detector: 280°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
170	1	225	20

Carrier gas: Helium

Injection volume: 1 µL

Injection type: Split; split ratio, 200:1

[1] Suitable glass may be obtained from VWR cat. no. 391-0171.

System suitability

Samples: *System suitability solution* and *Standard solution*

Suitability requirements

Chromatogram similarity: The chromatogram from the *Standard solution* is similar to the reference chromatogram supplied with USP Cod Liver Oil RS. Identify the retention times of the relevant fatty acid methyl esters by comparing the chromatogram of the *Standard solution* with the reference chromatogram supplied with USP Cod Liver Oil RS.

Resolution: NLT 1.3 between methyl oleate and methyl *cis*-vaccinate, and that between methyl gadoleate and methyl gondoate is sufficient for purposes of identification and area measurement, *Standard solution*

Theoretical area percentages: 24.4 ± 1 for methyl palmitate, 24.8 ± 1 for methyl stearate, 25.2 ± 1 for methyl arachidate, and 25.6 ± 1 for methyl behenate, *System suitability solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Identify the retention times of the relevant fatty acid methyl esters in the *Sample solution* by comparing the chromatogram of the *Sample solution* with that of the *Standard solution*.

Determine the number of fatty acid methyl ester peaks in the *Sample solution*. The number of fatty acid methyl ester peaks exceeding 0.05% of the total area of fatty acid methyl esters is at least 24, and the 24 largest peaks of the methyl esters account for more than 90% of the total area. (These correspond to the following, in common elution order: 14:0, 15:0, 16:0, 16:1 *n*–7, 16:4 *n*–1, 18:0, 18:1 *n*–9, 18:1 *n*–7, 18:2 *n*–6, 18:3 *n*–3, 18:4 *n*–3, 20:1 *n*–11, 20:1 *n*–9, 20:1 *n*–7, 20:2 *n*–6, 20:4 *n*–6, 20:3 *n*–3, 20:4 *n*–3, 20:5 *n*–3, 22:1 *n*–11, 22:1 *n*–9, 21:5 *n*–3, 22:5 *n*–3, and 22:6 *n*–3.)

Calculate the area percentage for each fatty acid methyl ester in the portion of Capsules taken:

$$\text{Result} = (r_A/r_B) \times 100$$

r_A = peak area of each individual fatty acid methyl ester in the *Sample solution*

r_B = total area from all peaks, except the solvent peak and butylated hydroxytoluene, in the *Sample solution*

Acceptance criteria: The *Sample solution* meets the limits described in *Table 2*.

Table 2

Fatty Acid	Shorthand Notation	Lower Limit (Area %)	Upper Limit (Area %)
Saturated fatty acids			
Myristic acid	14:0	2.0	6.0
Palmitic acid	16:0	7.0	14.0
Stearic acid	18:0	1.0	4.0
Monounsaturated fatty acids			
Palmitoleic acid	16:1 *n*–7	4.5	11.5
cis-Vaccenic acid	18:1 *n*–7	2.0	7.0
Oleic acid	18:1 *n*–9	12.0	21.0
Gadoleic acid	20:1 *n*–11	1.0	5.5
Gondoic acid	20:1 *n*–9	5.0	17.0
Erucic acid	22:1 *n*–9	0	1.5

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Table 2 *(continued)*

Fatty Acid	Shorthand Notation	Lower Limit (Area %)	Upper Limit (Area %)
Cetoleic acid	22:1 *n*–11	5.0	12.0
Polyunsaturated fatty acids			
Linoleic acid	18:2 *n*–6	0.5	3.0
α-Linolenic acid	18:3 *n*–3	0	2.0
Moroctic acid	18:4 *n*–3	0.5	4.5
Eicosapentaenoic acid	20:5 *n*–3	7.0	16.0
Docosahexaenoic acid	22:6 *n*–3	6.0	18.0

STRENGTH

Change to read:

- **VITAMIN A**

Sample: 500 mg to 1 g of oil contained in the Capsules
Analysis: Proceed as directed in *Vitamin A Assay* ⟨571⟩, ▲*Assay, Chemical Methods, Procedure 1*▲ 2S (USP41).
Acceptance criteria: 180 ▲▲ 2S (USP41) to 750 µg ▲▲ 2S (USP41) of vitamin A per gram of oil contained in the Capsules

Change to read:

- **VITAMIN D**

▲Proceed as directed in *Vitamin D Assay* ⟨581⟩, *Assay, Chromatographic Methods, Procedure 8*.▲ 2S (USP41)
Acceptance criteria: 1.5 ▲▲ 2S (USP41) to 6.25 µg ▲▲ 2S (USP41) of vitamin D per gram of oil contained in the Capsules

- **CONTENT OF COD LIVER OIL**

Analysis: Weigh NLT 10 Capsules in a tared weighing bottle. With a sharp blade, or by other appropriate means, carefully open the Capsules, without loss of shell material, and transfer the combined Capsule contents to a 100-mL beaker. Remove any adhering substance from the emptied Capsules by washing with several small portions of isooctane. Discard the washings, and allow the empty Capsules to dry in a current of dry air until the isooctane is completely evaporated. Weigh the empty Capsules in the original tared weighing bottle, and calculate the average net weight per Capsule.
Acceptance criteria: 95.0%–105.0% of the labeled amount of cod liver oil

PERFORMANCE TESTS

- **WEIGHT VARIATION** ⟨2091⟩**:** Meet the requirements
- **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩, *Rupture Test for Soft Shell Capsules***:** Meet the requirements

SPECIFIC TESTS

- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Unsaponifiable Matter***:** NMT 1.30%
- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Acid Value*

Sample solution: Mix 15 mL of alcohol with 15 mL of ether, add 5 drops of phenolphthalein TS, and neutralize with 0.1 N sodium hydroxide. Dissolve 2.0 g of oil contained in the Capsules in the mixture, and boil the oil solution gently under a reflux condenser for 10 min.
Analysis: Cool, and titrate the mixture with 0.1 N sodium hydroxide VS to the production of a pink color that persists after shaking for 30 s.
Acceptance criteria: NMT 1.0 mL of 0.1 N sodium hydroxide is required.

- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Iodine Value***:** 145–180
- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Saponification Value***:** 180–192

[NOTE—If carbon dioxide has been used as a preservative, expose the oil contained in the Capsules in a shallow dish in a vacuum desiccator for 24 h before weighing the specimen for determination of the saponification value.]

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers at room temperature. Protect from light.

Change to read:

- **LABELING:** ▲The label states the amount of vitamin A and vitamin D in µg/Capsule.▲ 2S (USP41) The label emphasizes the need to avoid freezing or exposure to excessive humidity or to a temperature above 40°. Where the content of docosahexaenoic acid and eicosapentaenoic acid is claimed, state the amount in mg/Capsule on the label.

Change to read:

- **USP REFERENCE STANDARDS** ⟨11⟩

▲▲ (CN 1-May-2018)
USP Cod Liver Oil RS
▲▲ (CN 1-May-2018)
▲▲ 2S (USP41)

Coffee Fruit Dry Extract

DEFINITION

Coffee Fruit Dry Extract is prepared from the dried semi-ripened light red entire fruit of *Coffea arabica* L. (Fam. Rubiaceae) by extraction with hydroalcoholic mixtures. It contains NLT 40% of caffeoylquinic acids, calculated as the sum of neochlorogenic acid, chlorogenic acid, cryptochlorogenic acid, isochlorogenic acid B, isochlorogenic acid A, and isochlorogenic acid C on the dried basis; and NLT 15% of chlorogenic acid on the dried basis.

IDENTIFICATION

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 0.2 mg/mL of USP Chlorogenic Acid RS in methanol
Standard solution B: 4 mg/mL of USP Coffee Fruit Dry Extract RS in methanol. Sonicate for 10 min, centrifuge or filter, and use the supernatant or filtrate.
Sample solution: 4 mg/mL of Coffee Fruit Dry Extract in methanol. Sonicate for 10 min, centrifuge or filter, and use the supernatant or filtrate.
Chromatographic system
Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm
Application volume: 5 µL, as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
Temperature: About 25°
Developing solvent system: The upper layer solution of a mixture of *n*-butyl acetate, formic acid, and water (7:5:5)
Developing distance: 6 cm
Derivatization reagent A: 10 mg/mL of 2-aminoethyl diphenylborinate in methanol
Derivatization reagent B: 50 mg/mL of polyethylene glycol 4000 in alcohol

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Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Apply the *Samples* as bands and dry in air. Develop in a saturated chamber, remove the plate from the chamber, and dry in air. Treat the plate with *Derivatization reagent A*, and dry in air. Immediately treat the plate with *Derivatization reagent B*, dry in air, and examine under UV light at 366 nm.
Suitability requirements: Under UV light at 366 nm, *Standard solution A* exhibits a blue fluorescent band due to chlorogenic acid in the lower-third section of the chromatogram. *Standard solution B* exhibits an intense blue fluorescent band corresponding in R_F to chlorogenic acid in *Standard solution A*; a band above chlorogenic acid in deeper blue color; and a blue fluorescent band below chlorogenic acid. In the middle-third section, *Standard solution B* exhibits at least three blue fluorescent bands.
Acceptance criteria: Under UV light at 366 nm, the *Sample solution* exhibits an intense band corresponding in R_F and color to chlorogenic acid in *Standard solution A*. The *Sample solution* exhibits additional bands corresponding to similar bands in *Standard solution B*, including a band above chlorogenic acid in deeper blue color; a blue fluorescent band below chlorogenic acid; three blue fluorescent bands in the middle-third section with the top one due to isochlorogenic acid A, and below that, a pair of bands corresponding to other isochlorogenic acids; and several faint blue fluorescent bands above isochlorogenic acid A.

• **B. HPLC**
Analysis: Proceed as directed in the test for *Content of Caffeoylquinic Acids*.
Acceptance criteria: The chromatogram of the *Sample solution* exhibits the most intense peak corresponding to chlorogenic acid in *Standard solution A*, and peaks corresponding to neochlorogenic acid; cryptochlorogenic acid; 5-*O*-feruloylquinic acid, which typically coelutes with another constituent and sometimes can be resolved into two peaks; isochlorogenic acid B; isochlorogenic acid A; and isochlorogenic acid C in the chromatogram of *Standard solution B*. The peaks of neochlorogenic acid and cryptochlorogenic acid are of comparable medium intensity. The peaks of 5-*O*-feruloylquinic acid, isochlorogenic acid B, isochlorogenic acid A, and isochlorogenic acid C are of lower intensity.

COMPOSITION

• **CONTENT OF CAFFEOYLQUINIC ACIDS**
Solution A: 2% acetic acid in water
Solution B: Acetonitrile
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	95	5
9	95	5
11.5	91	9
21.5	91	9
60	75	25
75	75	25
75.1	95	5
80	95	5

[NOTE—Protect from light and proceed under low-actinic light. Each *Standard solution* and the *Sample solution* are stable for 24 h at room temperature.]
Solvent: Methanol and water (1:1)
Standard solution A: 0.2 mg/mL of USP Chlorogenic Acid RS in *Solvent*
Standard solution B: 1.0 mg/mL of USP Coffee Fruit Dry Extract RS in *Solvent*. Sonicate for 10 min, and pass through a hydrophilic polypropylene (GHP) membrane filter of 0.45-µm or finer pore size.
Sample solution: Accurately transfer a quantity of Coffee Fruit Dry Extract, equivalent to about 10 mg of total caffeoylquinic acids calculated based on the labeled content, into a 25-mL volumetric flask, and add 15 mL of *Solvent*. Sonicate the sample for 10 min, cool to room temperature, dilute with *Solvent* to volume, and mix. Before injection, pass through a GHP membrane filter of 0.45-µm or finer pore size and discard the first portion of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 325 nm
Column: 4.6-mm × 15-cm; 5-µm packing L1
Temperatures
Autosampler: 4°
Column: 25°
Flow rate: 1.0 mL/min
Injection volume: 20 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Resolution: NLT 1.5 between the peaks of chlorogenic acid and cryptochlorogenic acid; and peaks of isochlorogenic acid B and isochlorogenic acid A, *Standard solution B*
Tailing factor: NMT 2.0 for the chlorogenic acid peak, *Standard solution A*
Relative standard deviation: NMT 2.0% for the chlorogenic acid peak in repeated injections, *Standard solution A*
Chromatographic similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Coffee Fruit Dry Extract RS being used.
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Coffee Fruit Dry Extract RS being used, identify the peaks for neochlorogenic acid, chlorogenic acid, cryptochlorogenic acid, 5-*O*-feruloylquinic acid, isochlorogenic acid B, isochlorogenic acid A, and isochlorogenic acid C in the *Sample solution*. [NOTE—See *Table 2* for relative retention times. These values may vary due to differences in the chromatographic conditions allowed by the *Suitability requirements*.]

Table 2

Analyte	Approximate Relative Retention Time	Conversion Factor
Neochlorogenic acid[a]	0.57	1.00
Chlorogenic acid[b]	1.00	1.00
Cryptochlorogenic acid[c]	1.06	1.00
5-*O*-Feruloylquinic acid[d]	1.84	—
Isochlorogenic acid B[e]	2.90	0.84

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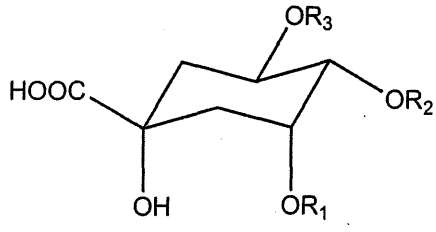
Table 2 *(continued)*

Analyte	Approximate Relative Retention Time	Conversion Factor
Isochlorogenic acid A[f]	2.94	0.80
Isochlorogenic acid C[g]	3.20	0.80

[a] (1*R*,3*R*,4*S*,5*R*)-3-{[(*E*)-3-(3,4-Dihydroxyphenyl)acryloyl]oxy}-1,4,5-trihydroxycyclohexane-1-carboxylic acid.
[b] (1*S*,3*R*,4*R*,5*R*)-3-{[(*E*)-3-(3,4-Dihydroxyphenyl)acryloyl]oxy}-1,4,5-trihydroxycyclohexane-1-carboxylic acid.
[c] (1*S*,3*R*,4*S*,5*R*)-4-{[(*E*)-3-(3,4-Dihydroxyphenyl)acryloyl]oxy}-1,3,5-trihydroxycyclohexane-1-carboxylic acid.
[d] (1*S*,3*R*,4*R*,5*R*)-1,3,4-Trihydroxy-5-{[(*E*)-3-(4-hydroxy-3-methoxyphenyl)acryloyl]oxy}cyclohexane-1-carboxylic acid.
[e] (1*S*,3*R*,4*R*,5*R*)-3,4-Bis{[(*E*)-3-(3,4-dihydroxyphenyl)acryloyl]oxy}-1,5-dihydroxycyclohexane-1-carboxylic acid.
[f] (1*S*,3*R*,4*S*,5*R*)-3,5-Bis{[(*E*)-3-(3,4-dihydroxyphenyl)acryloyl]oxy}-1,4-dihydroxycyclohexane-1-carboxylic acid.
[g] (1*R*,3*R*,4*S*,5*R*)-3,4-Bis{[(*E*)-3-(3,4-dihydroxyphenyl)acryloyl]oxy}-1,5-dihydroxycyclohexane-1-carboxylic acid.

[NOTE—These compounds are often referred in the literature as 3-/4-/5-/mono-/di-substituted quinic acids. Due to the confusion in historic and evolving IUPAC ring numbering, that nomenclature is not used in this Pharmacopeia. Instead, the compounds are described by their conformational structures in the *Table 3*.]

Table 3. Graphic Conformational Structures of Caffeoylquinic Acids



Caffeoylquinic Acids	R1	R2	R3
Neochlorogenic acid	Caffeoyl	H	H
Chlorogenic acid	H	H	Caffeoyl
Cryptochlorogenic acid	H	Caffeoyl	H
5-*O*-Feruloylquinic acid	H	H	Caffeoyl
Isochlorogenic acid A	Caffeoyl	H	Caffeoyl
Isochlorogenic acid B	H	Caffeoyl	Caffeoyl
Isochlorogenic acid C	Caffeoyl	Caffeoyl	H

Separately calculate the percentages of neochlorogenic acid, chlorogenic acid, cryptochlorogenic acid, isochlorogenic acid B, isochlorogenic acid A, and isochlorogenic acid C in the portion of Coffee Fruit Dry Extract taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of chlorogenic acid from *Standard solution A*
C_S = concentration of USP Chlorogenic Acid RS in *Standard solution A* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Coffee Fruit Dry Extract taken to prepare the *Sample solution* (mg)
F = conversion factor for the analyte (see *Table 2*)

Calculate the content of caffeoylquinic acids as the sum of the percentages of neochlorogenic acid, chlorogenic acid, cryptochlorogenic acid, isochlorogenic acid B, isochlorogenic acid A, and isochlorogenic acid C.

Acceptance criteria: NLT 40% of total caffeoylquinic acids on the dried basis; NLT 15% of chlorogenic acid on the dried basis

Change to read:

CONTAMINANTS

- **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩

Acceptance criteria
Arsenic: NMT 2.0 µg/g
Cadmium: NMT 0.5 µg/g
Lead: NMT 5.0 µg/g
Mercury: NMT 0.2 µg/g

- ▲**BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020)
: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS

- **LIMIT OF CAFFEINE**

Mobile phase: 0.1% perchloric acid in a mixture of water and acetonitrile (9:1)
Standard solution: 0.05 mg/mL of USP Caffeine RS in *Mobile phase*
Sample solution: Accurately transfer a quantity of Coffee Fruit Dry Extract, equivalent to 1.0–2.0 mg of caffeine calculated based on the labeled amount, into a 25-mL volumetric flask, and add 15 mL of *Mobile phase*. Sonicate for 10 min, cool to room temperature, dilute with *Mobile phase* to volume, and mix. Transfer 5.0 mL of the solution into a 15-mL centrifuge tube containing 1 g of PSA (primary and secondary amine) bonded silica. Mix on a vortex mixer for 3 min and allow to sit at room temperature for 10 min. Before injection, pass through a GHP membrane filter of 0.45-µm or finer pore size, and discard the first portion of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 275 nm
Column: 3.0-mm × 15-cm; 2.7-µm packing L11
Temperatures
Autosampler: 4°
Column: 30°
Flow rate: 0.6 mL/min
Injection volume: 10 µL
Run time: 15 min
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 2.0% for the caffeine peak in repeated injections
Analysis
Samples: *Standard solution* and *Sample solution*
Using the chromatogram of the *Standard solution*, identify the peak corresponding to caffeine in the *Sample solution*.

DS Monographs

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Calculate the percentage of caffeine in the portion of Coffee Fruit Dry Extract taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of caffeine from the *Sample solution*
r_S = peak area of caffeine from the *Standard solution*
C_S = concentration of USP Caffeine RS in the *Standard solution* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Coffee Fruit Dry Extract taken to prepare the *Sample solution* (mg)

Acceptance criteria: NMT 2.0% on the dried basis

• **Loss on Drying** ⟨731⟩
Sample: 1.0 g of Coffee Fruit Dry Extract
Analysis: Dry the *Sample* at 105° for 2 h.
Acceptance criteria: NMT 5.0%

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Preserve in well-closed containers, protected from light and moisture, and store in a cool place.
• **Labeling:** The label states the Latin binomial of the plant from which the article was prepared following the official name. It meets other labeling requirements in *Botanical Extracts* ⟨565⟩.
• **USP Reference Standards** ⟨11⟩
USP Caffeine RS
USP Chlorogenic Acid RS
USP Coffee Fruit Dry Extract RS

Cohosh, Black—*see Black Cohosh*

Coix Seed

DEFINITION

Coix Seed, also known as Job's tears, consists of the dried ripe caryopsis, freed from the shell, of *Coix lacryma-jobi* var. *ma-yuen* (Rom.Caill.) Stapf (Fam. Poaceae, alt. Gramineae) collected in the fall. It contains NLT 3.5% of triglycerides calculated as the sum of trilinolein ($C_{57}H_{98}O_6$), 1,2-dilinoleoyl-3-palmitin ($C_{55}H_{98}O_6$), 1,2-dilinoleoyl-3-olein ($C_{57}H_{100}O_6$), 1-palmitoyl-2-oleoyl-3-linolein ($C_{55}H_{100}O_6$), 1,2-dioleoyl-3-linolein ($C_{57}H_{102}O_6$), 1,2-dioleoyl-3-palmitin ($C_{55}H_{102}O_6$), and triolein ($C_{57}H_{104}O_6$) on the dried basis.

IDENTIFICATION

• **A. HPTLC for Articles of Botanical Origin** ⟨203⟩
Standard solution A: 2 mg/mL of USP Oleic Acid RS in methanol
Standard solution B: 40 mg/mL (about 50 µL/mL) of USP Coix Seed Oil Extract RS in methylene chloride
Sample solution: Sonicate 0.5 g of finely powdered Coix Seed in 5 mL of methylene chloride for 10 min. Centrifuge at 5000 rpm for 5 min and use the supernatant.
Chromatographic system and **System suitability**
Use *Identification of Fixed Oils by Thin-Layer Chromatography* ⟨202⟩, *Identification, Method II*.
Derivatization reagent: 25 mg/mL of phosphomolybdic acid in 96% alcohol
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Apply the *Samples* as bands and dry in air. Develop in a saturated chamber up to 7 cm, remove the plate from the chamber, and dry in air. Treat the plate with *Derivatization reagent*, heat at 120° for 3 min, and examine in white light.
Acceptance criteria: The *Sample solution* exhibits, in the upper half, a band corresponding in R_F and color to the oleic acid band in *Standard solution A*. The *Sample solution* exhibits additional bands that correspond to similar bands in *Standard solution B*, including one band right above oleic acid with the same color, a cluster of four blue bands in the lower half, and some faint bands in the middle third.

• **B. HPLC**
Analysis: Proceed as directed in the test for *Content of Triglycerides*.
Acceptance criteria: The *Sample solution* exhibits a peak with a retention time corresponding to triolein in *Standard stock solution* and peaks due to trilinolein, 1,2-dilinoleoyl-3-palmitin,1,2-dilinoleoyl-3-olein, 1-palmitoyl-2-oleoyl-3-linolein, 1,2-dioleoyl-3-linolein, and 1,2-dioleoyl-3-palmitin at retention times corresponding to the same triglycerides in *Standard solution 6*. The 1,2-dilinoleoyl-3-olein and 1,2-dioleoyl-3-linolein are the most intense peaks; 1-palmitoyl-2-oleoyl-3-linolein and triolein are medium-intensity peaks; trilinolein, 1,2-dilinoleoyl-3-palmitin, and 1,2-dioleoyl-3-palmitin are low-intensity peaks (distinction from *Sorghum bicolor* (L.) Moench, which has similar triglyceride peaks, all of low intensity, and *Hordeum vulgare* L., which has four triglyceride peaks, all of low intensity).

COMPOSITION

• **Content of Triglycerides**
Mobile phase: Methanol
Standard stock solution: 0.20 mg/mL of USP Triolein RS in methanol. Sonicate to dissolve.
Standard solutions 1–5: Dilute the *Standard stock solution* with methanol to obtain solutions of 0.0125, 0.025, 0.05, 0.10, and 0.20 mg/mL of USP Triolein RS. Pass each through a suitable membrane filter of 0.45-µm or finer pore size.
Standard solution 6: 0.5 mg/mL of USP Coix Seed Oil Extract RS in methanol
Sample solution: Accurately transfer about 500 mg of finely powdered Coix Seed into a suitable flask and add 35 mL of methanol. Sonicate for 30 min and filter under vacuum. Rinse the original flask and the residue left in the flask with 5 mL of methanol, and wash the residue and paper on the filter with the rinsing. Repeat the rinse and wash procedures one more time, and transfer the filtrate from the vacuum flask to a 50-mL volumetric flask. Wash the vacuum flask twice with about 2.5 mL of methanol each time, transfer the washings into the volumetric flask, dilute with methanol to volume, and mix. Before injection, pass through a suitable membrane filter of 0.45-µm or finer pore size, and discard the first portion of the filtrate. [Note—Dried filter and vacuum flask should be used.]
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Evaporative light-scattering. [Note—The parameters should be adjusted to achieve the best signal-to-noise ratio, according to the manufacturer's recommendations.]
Column: 3.0-mm × 10-cm; 2.7-µm packing L7
Column temperature: 20°
Flow rate: 0.3 mL/min

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Injection volume: 5 µL

System suitability

Samples: *Standard stock solution* and *Standard solutions 1–6*

Suitability requirements

Resolution: NLT 1.5 between the peak of 1,2-dilinoleoyl-3-olein and the small peak following it, *Standard solution 6*

Tailing factor: NMT 1.5 for the triolein peak, *Standard stock solution*

Relative standard deviation: NMT 5.0% for the triolein peak in repeated injections, *Standard stock solution*

Correlation coefficient: NLT 0.995 for the regression line as determined in *Analysis*, *Standard solutions 1–5*

Signal-to-noise ratio: NLT 15 for the triolein peak, *Standard solution 1* (0.0125 mg/mL)

Chromatogram similarity: The chromatogram of *Standard solution 6* is similar to the reference chromatogram provided with the lot of USP Coix Seed Oil Extract RS being used.

Analysis

Samples: *Standard solutions 1–6* and *Sample solution*

Using the chromatograms of *Standard solutions 5–6* and the reference chromatogram provided with the lot of USP Coix Seed Oil Extract RS being used, identify the retention times of the peaks of the relevant analytes in the *Sample solution*. [NOTE—See *Table 1* for the approximate relative retention times.]

Table 1

Analyte	Approximate Relative Retention Time
Trilinolein	0.61
1,2-Dilinoleoyl-3-palmitin	0.67
1,2-Dilinoleoyl-3-olein	0.71
1-Palmitoyl-2-oleoyl-3-linolein	0.79
1,2-Dioleoyl-3-linolein	0.84
1,2-Dioleoyl-3-palmitin	0.94
Triolein	1.00

Plot the logarithms of peak responses versus the logarithms of concentrations, in mg/mL, of triolein from *Standard solutions 1–5*. Using a least-squares analysis, establish the regression line or determine a linear regression equation.

Determine the concentration (C), in mg/mL, of the relevant analytes in the *Sample solution* by using the regression line or linear regression equation.

Separately calculate the percentages of trilinolein, 1,2-dilinoleoyl-3-palmitin, 1,2-dilinoleoyl-3-olein, 1-palmitoyl-2-oleoyl-3-linolein, 1,2-dioleoyl-3-linolein, 1,2-dioleoyl-3-palmitin, and triolein in the portion of Coix Seed taken:

$$\text{Result} = C \times (V/W) \times 100$$

C = concentration of the relevant analyte in the *Sample solution* as determined above (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Coix Seed taken to prepare the *Sample solution* (mg)

Calculate the content of triglycerides as the sum of the percentages of trilinolein, 1,2-dilinoleoyl-3-palmitin, 1,2-dilinoleoyl-3-olein, 1-palmitoyl-2-oleoyl-3-linolein, 1,2-dioleoyl-3-linolein, 1,2-dioleoyl-3-palmitin, and triolein.

Acceptance criteria: NLT 3.5% on the dried basis

CONTAMINANTS

• LIMIT OF ZEARALENONE

Mobile phase: Acetonitrile and water (1:1)

Standard stock solution: 250 ng/mL of zearalenone in methanol

Standard solution: Accurately transfer 1 mL of *Standard stock solution* to a 10-mL volumetric flask and add methanol to volume.

Sample solution: Accurately transfer about 20 g of finely powdered Coix Seed to a suitable centrifuge tube. Add 4.0 g of sodium chloride, and accurately add 100 mL of 90% acetonitrile. Mix for 2 min with a high speed disperser (NLT 11,000 rpm), and centrifuge for 5 min (4000 rpm). Immediately pipet 10.0 mL of the supernatant into a 50-mL volumetric flask, add water to volume, mix, and centrifuge. Pipet 10.0 mL of the supernatant onto an immunoaffinity column[1] capable of binding zearalenone, and elute at a flow rate of approximately 3 mL/min (60 drops/min). Wash the column with 10 mL of water at a flow rate of approximately 6 mL/min (120 drops/min), let the column run dry, and discard the water eluate. Wash the column with 1.5 mL of methanol at a flow rate of approximately 1 mL/min (20 drops/min), collect the methanol eluate into a 2-mL volumetric flask, and let the column run dry. Add methanol to volume, and mix.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Fluorometric

Excitation wavelength: 232 nm

Emission wavelength: 460 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Column temperature: 30°

Flow rate: 1.0 mL/min

Injection volume: 20 µL of *Sample solution*

System suitability

Sample: *Standard solution*

Suitability requirements

Column efficiency: NLT 10,000 theoretical plates

Relative standard deviation: NMT 10.0% for the zearalenone peak

Correlation coefficient: NLT 0.999 for the regression line as determined in *Analysis*

Analysis

Samples: *Standard solution* and *Sample solution*

Inject the *Standard solution* in volumes of 5, 10, 15, 20, and 25 µL, and measure zearalenone peak areas for each injection. Plot the peak areas versus the amounts, in ng, of zearalenone in the injections, and establish the regression line using a least-squares analysis.

Using the chromatogram of the *Standard solution*, identify the retention time of the peak corresponding to zearalenone in the *Sample solution*.

From the graph, determine the content (C), in ng, of zearalenone in the *Sample solution*.

Calculate content, in ng/g, of zearalenone in the portion of Coix Seed taken:

$$\text{Result} = 5000 \times (C/W)$$

C = content of zearalenone as determined above (ng)

W = weight of Coix Seed taken to prepare the *Sample solution* (g)

[1] Suitable grade of ZearalaTest™. Available from Vicam, Cat. No. G1012 (ZearalaTest) or Cat. No. G1026 (ZearalaTest WB).

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Acceptance criteria: NMT 60 ng/g on the dried basis

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Limits of Elemental Impurities*: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Pesticide Residue Analysis*: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Test for Aflatoxins*: Meets the requirements

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: Broad ovoid or elongated-elliptical, 4–8 mm long, 3–6 mm wide. Externally milky white, smooth, with yellowish-brown testa occasionally. One end obtusely rounded, the other end relatively broad and slightly dented with one pale, brown dotted hilum. Dorsal surface rounded and protruding; ventral surface with one relatively broad and deep longitudinal furrow. Texture hard, fracture white and starchy.

Microscopic: The powder contains numerous starch granules usually in clumps, simple granule spherical or polyhedral, 2–20 µm in diameter with a stellate, y-shaped hilum; compound granules rare, usually consisting of two to three simple granules.

- **LOSS ON DRYING** ⟨731⟩

Analysis: Finely powder and dry at 105° for 2 h.

Acceptance criteria: NMT 12.0%

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Foreign Organic Matter*: NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Alcohol-Soluble Extractives, Method 1*: NLT 5.5%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Total Ash*: NMT 3.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at 4°–10°.
- **LABELING:** The label states the Latin binomial following the official name of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Coix Seed Oil Extract RS
USP Oleic Acid RS
USP Triolein RS

Coix Seed Powder

DEFINITION

Coix Seed Powder, also known as Job's tears powder, consists of the dried ripe caryopsis, freed from the shell, of *Coix lacryma-jobi* var. *ma-yuen* (Rom.Caill.) Stapf (Fam. Poaceae, alt. Gramineae) collected in the fall and reduced to a fine or very fine powder. It contains NLT 3.5% of triglycerides calculated as the sum of trilinolein ($C_{57}H_{98}O_6$), 1,2-dilinoleoyl-3-palmitin ($C_{55}H_{98}O_6$), 1,2-dilinoleoyl-3-olein ($C_{57}H_{100}O_6$), 1-palmitoyl-2-oleoyl-3-linolein ($C_{55}H_{100}O_6$), 1,2-dioleoyl-3-linolein ($C_{57}H_{102}O_6$), 1,2-dioleoyl-3-palmitin ($C_{55}H_{102}O_6$), and triolein ($C_{57}H_{104}O_6$) on the dried basis.

IDENTIFICATION

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 2 mg/mL of USP Oleic Acid RS in methanol

Standard solution B: 40 mg/mL (about 50 µL/mL) of USP Coix Seed Oil Extract RS in methylene chloride

Sample solution: Sonicate 0.5 g of Coix Seed Powder in 5 mL of methylene chloride for 10 min. Centrifuge at 5000 rpm for 5 min and use the supernatant.

Chromatographic system and **System suitability**
Use *Identification of Fixed Oils by Thin-Layer Chromatography* ⟨202⟩, *Identification, Method II*.

Derivatization reagent: 25 mg/mL of phosphomolybdic acid in 96% alcohol

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands and dry in air. Develop in a saturated chamber up to 7 cm, remove the plate from the chamber, and dry in air. Treat the plate with *Derivatization reagent*, heat at 120° for 3 min, and examine in white light.

Acceptance criteria: The *Sample solution* exhibits, in the upper half, a band corresponding in R_F and color to the oleic acid band in *Standard solution A*. The *Sample solution* exhibits additional bands that correspond to similar bands in *Standard solution B*, including one band right above oleic acid with the same color, a cluster of four blue bands in the lower half, and some faint bands in the middle third.

- **B. HPLC**

Analysis: Proceed as directed in the test for *Content of Triglycerides*.

Acceptance criteria: The *Sample solution* exhibits a peak with a retention time corresponding to triolein in *Standard stock solution* and peaks due to trilinolein, 1,2-dilinoleoyl-3-palmitin,1,2-dilinoleoyl-3-olein, 1-palmitoyl-2-oleoyl-3-linolein, 1,2-dioleoyl-3-linolein, and 1,2-dioleoyl-3-palmitin at retention times corresponding to the same triglycerides in *Standard solution 6*. The 1,2-dilinoleoyl-3-olein and 1,2-dioleoyl-3-linolein are the most intense peaks; 1-palmitoyl-2-oleoyl-3-linolein and triolein are medium-intensity peaks; trilinolein, 1,2-dilinoleoyl-3-palmitin, and 1,2-dioleoyl-3-palmitin are low-intensity peaks (distinction from *Sorghum bicolor* (L.) Moench, which has similar triglyceride peaks, all of low intensity, and *Hordeum vulgare* L., which has four triglyceride peaks, all of low intensity).

COMPOSITION

- **CONTENT OF TRIGLYCERIDES**

Mobile phase: Methanol

Standard stock solution: 0.20 mg/mL of USP Triolein RS in methanol. Sonicate to dissolve.

Standard solutions 1–5: Dilute the *Standard stock solution* with methanol to obtain solutions of 0.0125, 0.025, 0.05, 0.10, and 0.20 mg/mL of USP Triolein RS. Pass each through a suitable membrane filter of 0.45-µm or finer pore size.

Standard solution 6: 0.5 mg/mL of USP Coix Seed Oil Extract RS in methanol

Sample solution: Accurately transfer about 500 mg of Coix Seed Powder into a suitable flask and add 35 mL of methanol. Sonicate for 30 min and filter under vacuum. Rinse the original flask and the residue left in the flask with 5 mL of methanol, and wash the residue and paper on the filter with the rinsing. Repeat the rinse and wash procedures one more time, and transfer the filtrate from the vacuum flask to a 50-mL volumetric flask. Wash the vacuum flask twice with about 2.5 mL of methanol each time, transfer the washings into the volumetric flask, dilute with methanol

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to volume, and mix. Before injection, pass through a suitable membrane filter of 0.45-µm or finer pore size, and discard the first portion of the filtrate. [NOTE—Dried filter and vacuum flask should be used.]

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Evaporative light-scattering. [NOTE—The parameters should be adjusted to achieve the best signal-to-noise ratio, according to manufacturer's recommendations.]
Column: 3.0-mm × 10-cm; 2.7-µm packing L7
Column temperature: 20°
Flow rate: 0.3 mL/min
Injection volume: 5 µL

System suitability
Samples: *Standard stock solution* and *Standard solutions 1–6*.
Resolution: NLT 1.5 between the peak of 1,2-dilinoleoyl-3-olein and the small peak following it, *Standard solution 6*
Tailing factor: NMT 1.5 for the triolein peak, *Standard stock solution*
Relative standard deviation: NMT 5.0% for the triolein peak in repeated injections, *Standard stock solution*
Correlation coefficient: NLT 0.995 for the regression line as determined in *Analysis*, *Standard solutions 1–5*
Signal-to-noise ratio: NLT 15 for the triolein peak, *Standard solution 1* (0.0125 mg/mL)
Chromatogram similarity: The chromatogram of *Standard solution 6* is similar to the reference chromatogram provided with the lot of USP Coix Seed Oil Extract RS being used.

Analysis
Samples: *Standard solutions 1–6* and *Sample solution*
Using the chromatograms of *Standard solutions 5–6* and the reference chromatogram provided with the lot of USP Coix Seed Oil Extract RS being used, identify the retention times of the peaks of the relevant analytes in the *Sample solution*. [NOTE—See *Table 1* for the approximate relative retention times.]

Table 1

Analyte	Approximate Relative Retention Time
Trilinolein	0.61
1,2-Dilinoleoyl-3-palmitin	0.67
1,2-Dilinoleoyl-3-olein	0.71
1-Palmitoyl-2-oleoyl-3-linolein	0.79
1,2-Dioleoyl-3-linolein	0.84
1,2-Dioleoyl-3-palmitin	0.94
Triolein	1.00

Plot the logarithms of peak responses versus the logarithms of concentrations, in mg/mL, of triolein from *Standard solutions 1–5*. Using a least-squares analysis, establish a regression line or determine a linear regression equation.
Determine the concentration (*C*), in mg/mL, of the relevant analytes in the *Sample solution* by using the regression line or linear regression equation.
Separately calculate the percentages of trilinolein, 1,2-dilinoleoyl-3-palmitin, 1,2-dilinoleoyl-3-olein, 1-palmitoyl-2-oleoyl-3-linolein, 1,2-dioleoyl-3-linolein, 1,2-dioleoyl-3-palmitin, and triolein in the portion of Coix Seed Powder taken:

$$\text{Result} = C \times (V/W) \times 100$$

C = concentration of relevant analyte in the *Sample solution* as determined above (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Coix Seed Powder taken to prepare the *Sample solution* (mg)

Calculate the content of triglycerides as the sum of the percentages of trilinolein, 1,2-dilinoleoyl-3-palmitin, 1,2-dilinoleoyl-3-olein, 1-palmitoyl-2-oleoyl-3-linolein, 1,2-dioleoyl-3-linolein, 1,2-dioleoyl-3-palmitin, and triolein.
Acceptance criteria: NLT 3.5% on the dried basis

CONTAMINANTS

• LIMIT OF ZEARALENONE

Mobile phase: Acetonitrile and water (1:1)
Standard stock solution: 250 ng/mL of zearalenone in methanol
Standard solution: Accurately transfer 1 mL of *Standard stock solution* to a 10-mL volumetric flask and add methanol to volume.
Sample solution: Accurately transfer about 20 g of Coix Seed Powder to a suitable centrifuge tube. Add 4.0 g of sodium chloride, and accurately add 100 mL of 90% acetonitrile. Mix for 2 min with a high-speed disperser (NLT 11,000 rpm), and centrifuge for 5 min (4000 rpm). Immediately pipet 10.0 mL of the supernatant into a 50-mL volumetric flask, add water to volume, mix, and centrifuge. Pipet 10.0 mL of the supernatant onto an immunoaffinity column[1] capable of binding zearalenone, and elute at a flow rate of approximately 3 mL/min (60 drops/min). Wash the column with 10 mL of water at a flow rate of approximately 6 mL/min (120 drops/min), let the column run dry, and discard the water eluate. Wash the column with 1.5 mL of methanol at a flow rate of approximately 1 mL/min (20 drops/min), collect the methanol eluate into a 2-mL volumetric flask, and let the column run dry. Add methanol to volume, and mix.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Fluorometric
Excitation wavelength: 232 nm
Emission wavelength: 460 nm
Column: 4.6-mm × 15-cm; 5-µm packing L1
Column temperature: 30°
Flow rate: 1.0 mL/min
Injection volume: 20 µL of *Sample solution*

System suitability
Sample: *Standard solution*
Suitability requirements
Column efficiency: NLT 10,000 theoretical plates
Relative standard deviation: NMT 10.0% for the zearalenone peak
Correlation coefficient: NLT 0.999 for the regression line as determined in *Analysis*

Analysis
Samples: *Standard solution* and *Sample solution*
Inject the *Standard solution* with volumes of 5, 10, 15, 20, and 25 µL, and measure zearalenone peak areas for each injection. Plot the peak areas versus the amounts, in ng, of zearalenone in the injections, and establish the regression line using a least-squares analysis.

[1] Suitable grade of ZearalaTest™. Available from Vicam, Cat. No. G1012 (ZearalaTest) or Cat. No. G1026 (ZearalaTest WB).

DS Monographs

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Using the chromatogram of the *Standard solution*, identify the retention time of the peak corresponding to zearalenone in the *Sample solution*.
From the graph, determine the content (*C*), in ng, of zearalenone in the *Sample solution*.
Calculate content, in ng/g, of zearalenone in the portion of Coix Seed Powder taken:

$$\text{Result} = 5000 \times (C/W)$$

C = content of zearalenone as determined above (ng)
W = weight of Coix Seed Powder taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 60 ng/g on the dried basis
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Limits of Elemental Impurities*: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Pesticide Residue Analysis*: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Test for Aflatoxins*: Meets the requirements

SPECIFIC TESTS
- **BOTANICAL CHARACTERISTICS**
  Macroscopic: Pale, whitish powder
  Microscopic: Starch granules numerous, usually in clumps, simple granules spherical or polyhedral, 2–20 µm in diameter with a stellate, y-shaped hilum; compound granules rare, usually consisting of two to three simple granules.
- **LOSS ON DRYING** ⟨731⟩
  Analysis: Dry at 105° for 2 h.
  Acceptance criteria: NMT 12.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Alcohol-Soluble Extractives, Method 1*: NLT 5.5%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Total Ash*: NMT 3.0%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at 4°–10°.
- **LABELING:** The label states the Latin binomial following the official name of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Coix Seed Oil Extract RS
  USP Oleic Acid RS
  USP Triolein RS

Copper Gluconate—*see Copper Gluconate General Monographs*

Co-Q10—*see Ubidecarenone in Dietary Supplements section*

Cranberry Liquid Preparation

DEFINITION
Cranberry Liquid Preparation is a bright red juice derived from the fruits of *Vaccinium macrocarpon* Ait. or *Vaccinium oxycoccos* L. (Fam. Ericaceae). It contains no added substances.

IDENTIFICATION
- **A. HPLC** The retention times of the quinic acid, malic acid, and citric acid peaks of the *Sample solution* correspond to those of the *Standard solution*, as obtained in the test for *Content of Organic Acids*.
- **B. ABSENCE OF ADULTERANTS**
  Standard solution: 1.0 mg/mL of tartaric acid and 0.1 mg/mL of fumaric acid
  Sample solution: Use the Liquid Preparation.
  Mobile phase and **Chromatographic system:** Proceed as directed in the test for *Content of Organic Acids*.
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Injection size: 20 µL
  Acceptance criteria: The retention times of the tartaric acid and fumaric acid peaks of the *Standard solution* do not correspond to any of the retention times for peaks observed from the *Sample solution*.

COMPOSITION
- **CONTENT OF DEXTROSE AND FRUCTOSE**
  Mobile phase: Water
  Standard solution: 6.0 mg/mL of USP Dextrose RS and 2.0 mg/mL of USP Fructose RS in water
  Sample solution: Transfer 1.0 g of sodium carboxylate cation-exchange resin to a 50-mL beaker, add 5 mL of water to make a slurry, and transfer the slurry to a polypropylene automatic pipet fitted with a small plug of silanized glass wool. Quantitatively transfer the slurry to a small chromatographic tube, rinsing the beaker with water and packing the column evenly. Keep the column wet until ready for use. Using a volumetric pipet, transfer 1.0 mL of Liquid Preparation to the column, collect the eluate, and discard it. Pipet 4.0 mL of water onto the top of the column, collect the eluate in a clean vial, and filter if necessary.
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: LC
  Detector: Refractive index
  Columns
  Guard: Packing L19
  Analytical: 7.8-mm × 30-cm; packing L19
  Column temperature: 85°
  Flow rate: 0.6 mL/min
  Injection size: 20 µL
  System suitability
  Sample: *Standard solution*
  [NOTE—The approximate relative retention times for dextrose and fructose are about 0.8 and 1.0, respectively.]
  Suitability requirements
  Resolution: NLT 1.8 between the dextrose and fructose peaks
  Relative standard deviation: NMT 2.0%
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Calculate the percentages of dextrose and fructose in the volume of Liquid Preparation taken:

$$\text{Result} = (r_U/r_S) \times (C_S/V) \times 0.5$$

DS Monographs

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r_U = peak response of each appropriate analyte from the *Sample solution*
r_S = peak response of each appropriate analyte from the *Standard solution*
C_S = concentration of the appropriate USP Reference Standard in the *Standard solution* (mg/mL)
V = volume of Liquid Preparation taken for the *Sample solution* (mL)

Acceptance criteria: NLT 2.4% dextrose and NLT 0.7% fructose

• **CONTENT OF ORGANIC ACIDS**

Mobile phase: Transfer 27.2 g of monobasic potassium phosphate to a 1000-mL volumetric flask, and dissolve in 950 mL of water. Adjust with phosphoric acid to a pH of 2.4, and dilute with water to volume.
Standard solution: 1.0 mg/mL each of USP Citric Acid RS, USP Malic Acid RS, and USP Quinic Acid RS
Sample solution: Use the filtered Liquid Preparation.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 214 nm
Columns
Guard: 5-µm; packing L1
[NOTE—Before use, condition the column with methanol, then with water, and finally with *Mobile phase*.]
Analytical: 4.6-mm × 25-cm analytical; packing L1
Flow rate: 0.6 mL/min
Injection size: 20 µL
System suitability
Sample: *Standard solution*
[NOTE—The approximate relative retention times for quinic acid, malic acid, and citric acid are 0.4, 0.5, and 1.0, respectively.]
Suitability requirements
Resolution: NLT 2.5 between quinic acid and malic acid
Relative standard deviation: NMT 2.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas. Calculate the percentages of quinic acid, malic acid, and citric acid in the volume of Liquid Preparation taken:

$$\text{Result} = (r_U/r_S) \times C_S \times 0.1$$

r_U = peak area of each appropriate analyte from the *Sample solution*
r_S = peak area of each appropriate analyte from the *Standard solution*
C_S = concentration of the appropriate USP Reference Standard in the *Standard solution* (mg/mL)

Acceptance criteria: NLT 0.9% each of quinic acid and citric acid; NLT 0.7% of malic acid. The ratio of quinic acid to malic acid is NLT 1.0.

ADULTERANTS

• **LIMIT OF SORBITOL AND SUCROSE**

Mobile phase and **Sample solution:** Prepare as directed in the test for *Content of Dextrose and Fructose*.
Standard solution: 0.5 mg/mL each of USP Sorbitol RS and USP Sucrose RS
Chromatographic system: Proceed as directed in the test for *Content of Dextrose and Fructose*.
System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for sucrose and sorbitol are about 0.4 and 1.0, respectively.]
Suitability requirements
Resolution: NLT 1.8 between the sucrose and sorbitol peaks
Relative standard deviation: NMT 2.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Injection size: 20 µL
Calculate the percentages of sucrose and sorbitol in the volume of Liquid Preparation taken:

$$\text{Result} = (r_U/r_S) \times (C_S/V) \times 0.5$$

r_U = peak response of each appropriate analyte from the *Sample solution*
r_S = peak response of each appropriate analyte from the *Standard solution*
C_S = concentration of the appropriate USP Reference Standard in the *Standard solution* (mg/mL)
V = volume of Liquid Preparation taken for the *Sample solution* (mL)

Acceptance criteria: NMT 0.05% each of sorbitol and sucrose

SPECIFIC TESTS

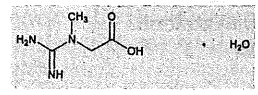
• **REFRACTIVE INDEX** ⟨831⟩: 1.3435–1.3445
• **pH** ⟨791⟩: 2.5 ± 0.1

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store in a refrigerator.
• **LABELING:** The label states the Latin binomial name and, following the official name, the parts of the plant source from which the article was derived. The label also states that it is for manufacturing purposes only. This article is exempt from the requirements of the *Labeling* ⟨7⟩, *Labels and Labeling for Products and Other Categories, Botanicals*, with respect to the pregnancy and lactation statement.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Citric Acid RS
USP Dextrose RS
USP Fructose RS
USP Malic Acid RS
USP Quinic Acid RS
USP Sorbitol RS
USP Sucrose RS

Add the following:

▲Creatine



$C_4H_9N_3O_2 \cdot H_2O$ 149.15
Glycine, *N*-(aminoiminomethyl)-*N*-methyl-, monohydrate; *N*-Carbamimidoyl-*N*-methylglycine monohydrate [6020-87-7].

$C_4H_9N_3O_2$ 131.14
Anhydrous [57-00-1].

DEFINITION

Creatine contains one molecule of water of hydration. It contains NLT 98.0% and NMT 102.0% of creatine ($C_4H_9N_3O_2$), calculated on the dried basis.

DS Monographs

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IDENTIFICATION

• **A. SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197K⟩

• **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

ASSAY

• **PROCEDURE**

Mobile phase: Dissolve 23 g of monobasic ammonium phosphate in 1 L of water and adjust with phosphoric acid to a pH of 4.0.

Diluent: Water

System suitability solution: 0.1 mg/mL of USP Creatine RS, 0.1 mg/mL of USP Creatinine RS, and 0.002 mg/mL of dicyandiamide[1] in *Diluent*

Standard solution: 2.5 mg/mL of USP Creatine RS in *Diluent*

Sample solution: 2.5 mg/mL of Creatine in *Diluent*

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 225 nm

Column: 4.0-mm × 25-cm; 5-µm packing L9

Column temperature: 25°

Flow rate: 1.0 mL/min

Injection volume: 20 µL

System suitability

Samples: *System suitability solution* and *Standard solution*

[NOTE—The relative retention times for dicyandiamide, creatine, and creatinine are about 0.8, 1.0, and 2.0, respectively, *System suitability solution*.]

Suitability requirements

Resolution: NLT 2.0 between dicyandiamide and creatine, *System suitability solution*

Column efficiency: NLT 1000 theoretical plates, *Standard solution*

Relative standard deviation: NMT 2.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of creatine ($C_4H_9N_3O_2$) in the portion of Creatine taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of creatine from the *Sample solution*

r_S = peak response of creatine from the *Standard solution*

C_S = concentration of USP Creatine RS in the *Standard solution* (mg/mL)

C_U = concentration of Creatine in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–102.0% on the dried basis

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%

• **CHLORIDE AND SULFATE** ⟨221⟩, *Sulfate*

Standard solution: 0.10 mL of 0.020 M sulfuric acid

Sample: 200 mg of Creatine

Acceptance criteria: NMT 0.1%

PROCESS-RELATED IMPURITIES

[NOTE—On the basis of information regarding the manufacturing process, perform either *Procedure 1* or *Procedure 2*.]

• **PROCEDURE 1**

Mobile phase, Diluent, System suitability solution, Chromatographic system, and **System suitability:** Proceed as directed in the *Assay*.

Standard solution: 2.5 µg/mL of USP Creatine RS and 2.5 µg/mL of USP Creatinine RS in *Diluent*

Sample solution: 2.5 mg/mL of Creatine in *Diluent*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of creatinine ($C_4H_7N_3O$) in the portion of Creatine taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of creatinine from the *Sample solution*

r_S = peak response of creatinine from the *Standard solution*

C_S = concentration of USP Creatinine RS in the *Standard solution* (mg/mL)

C_U = concentration of Creatine in the *Sample solution* (mg/mL)

Separately calculate the percentage of dicyandiamide and dihydrotriazine in the portion of Creatine taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (1/F) \times 100$$

r_U = peak response of dicyandiamide or dihydrotriazine from the *Sample solution*

r_S = peak response of creatine from the *Standard solution*

C_S = concentration of USP Creatine RS in the *Standard solution* (mg/mL)

C_U = concentration of Creatine in the *Sample solution* (mg/mL)

F = relative response factor (see *Table 1*)

Calculate the percentage of any unspecified impurity in the portion of Creatine taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of any unspecified impurity from the *Sample solution*

r_S = peak response of creatine from the *Standard solution*

C_S = concentration of USP Creatine RS in the *Standard solution* (mg/mL)

C_U = concentration of Creatine in the *Sample solution* (mg/mL)

Acceptance criteria: See *Table 1*.

Table 1

Name	Relative Retention Time	Relative Response Factor	Acceptance Criteria, NMT (%)
Dicyandiamide	0.8	68.5	0.1
Creatine	1.0	1.0	—
Dihydrotriazine	1.3	44.5	0.0005
Creatinine	2.0	—	0.1
Any unspecified impurity	—	1.0	0.1
Total unspecified impurities	—	—	1.5
Total impurities	—	—	2.0

[1] Use ACS reagent grade.

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[NOTE—On the basis of information regarding the manufacturing process, perform either *Procedure 1* or *Procedure 2*.]

• **PROCEDURE 2**

Buffer solution: 10 mM of monobasic sodium phosphate in water

Mobile phase: Acetonitrile and *Buffer solution* (83:17)

Diluent: Acetonitrile and water (50:50)

System suitability solution: 0.05 mg/mL of USP Creatine RS, 0.02 mg/mL of USP Creatinine RS, and 2 mg/mL of urea

Standard solution: 2 µg/mL of USP Creatine RS and 2 µg/mL of USP Creatinine RS in *Diluent*

Sample solution: 2 mg/mL of Creatine in *Diluent*

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 208 nm

Column: 4.6-mm × 15-cm; 5-µm packing L3

Column temperature: 30°

Flow rate: 1.5 mL/min

Injection volume: 10 µL

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for urea, creatinine, and creatine are 0.15, 0.30, and 1.0, respectively.]

Suitability requirements

Resolution: NLT 3.0 between urea and creatinine

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of creatinine ($C_4H_7N_3O$) in the portion of Creatine taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of creatinine from the *Sample solution*

r_S = peak response of creatinine from the *Standard solution*

C_S = concentration of USP Creatinine RS in the *Standard solution* (mg/mL)

C_U = concentration of Creatine in the *Sample solution* (mg/mL)

Separately calculate the percentage of dicyandiamide, urea, and sarcosine in the portion of Creatine taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (1/F) \times 100$$

r_U = peak response of dicyandiamide, urea, or sarcosine from the *Sample solution*

r_S = peak response of creatine from the *Standard solution*

C_S = concentration of USP Creatine RS in the *Standard solution* (mg/mL)

C_U = concentration of Creatine in the *Sample solution* (mg/mL)

F = relative response factor (see *Table 2*)

Calculate the percentage of any unspecified impurity in the portion of Creatine taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of any unspecified impurity from the *Sample solution*

r_S = peak response of creatine from the *Standard solution*

C_S = concentration of USP Creatine RS in the *Standard solution* (mg/mL)

C_U = concentration of Creatine in the *Sample solution* (mg/mL)

Acceptance criteria: See *Table 2*.

Table 2

Name	Relative Retention Time	Relative Response Factor	Acceptance Criteria, NMT (%)
Dicyandiamide	0.11	3.0	0.1
Urea	0.15	0.016	0.1
Creatinine	0.28	—	0.1
Sarcosine	0.73	0.016	0.1
Creatine	1.0	—	—
Any unspecified impurity	—	1.0	0.1
Total impurities	—	—	2.0

SPECIFIC TESTS

• **LOSS ON DRYING** ⟨731⟩

Analysis: Dry at 105° for 2 h.

Acceptance criteria: 10.5%–12.5%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

• **LABELING:** If *Procedure 2* for *Process-Related Impurities* is used, then the labeling states that the article complies with *Process-Related Impurities, Procedure 2*.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Creatine RS

USP Creatinine RS▲ (USP 1-May-2020)

Crypthecodinium cohnii Oil

DEFINITION

Crypthecodinium cohnii Oil is obtained from the fermentation and extraction of algae of the species *Crypthecodinium cohnii* and contains NLT 35.0% (w/w) of docosahexaenoic acid (DHA, $C_{22}H_{32}O_2$) (C22: 6 n–3), as the only significant polyunsaturated fatty acid present. Suitable antioxidants in appropriate concentration may be added.

IDENTIFICATION

• **A. LONG-CHAIN UNSATURATED FATTY ACID PROFILE:** Proceed as directed in *Fats and Fixed Oils* ⟨401⟩, *Omega-3 Fatty Acids Determination and Profile, Content of EPA and DHA*.

Analysis

Samples: *Standard Solution 2a*, *Standard Solution 2b*, and *Test Solution 1*

Calculate the area percentage for each fatty acid as methyl ester in *Test Solution 1*:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of each individual fatty acid as methyl ester

r_T = sum of all the peak responses, except the solvent and butylated hydroxytoluene peaks

DS Monographs

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Acceptance criteria: The retention times of the peaks for the docosahexaenoic acid methyl ester and the eicosapentaenoic acid methyl ester from *Test Solution 1* correspond to those from *Standard Solution 2a* and *Standard Solution 2b*, respectively. The area percentage for the methyl esters of the fatty acids from *Test Solution 1* meets the requirements for each fatty acid indicated in *Table 1*.

Table 1

Fatty Acid	Relative Retention Time	Shorthand Notation	Lower Limit (Area, %)	Upper Limit (Area, %)
Linoleic acid	0.52	18:2 n–6	0	1.0
Eicosapentaenoic acid	0.79	20:5 n–3	0	0.1
Docosapentaenoic acid	0.94	22:5 n–6	0	0.1
Docosahexaenoic acid	1.00	22:6 n–3	35.0	47.0

COMPOSITION

• **CONTENT OF DHA**

Analysis: Proceed as directed in *Fats and Fixed Oils* ⟨401⟩, *Omega-3 Fatty Acids Determination and Profile, Content of EPA and DHA*.

Acceptance criteria: NLT 35.0% (w/w) of docosahexaenoic acid (DHA)

IMPURITIES

• **LIMIT OF ARSENIC**

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytef, and plastic vessels before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin before use. Select all reagents to have as low a content of arsenic as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytef, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

Solution A: Transfer 1 g of ultrapure palladium metal to a Teflon beaker. Add 20 mL of water and 10 mL of nitric acid, and warm on a hot plate to dissolve. Allow the solution to cool to room temperature, transfer it into a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution B: Transfer 1 g of ultrapure magnesium nitrate to a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it to a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution C: *Solution A*, *Solution B*, and 2% nitric acid (3:2:5). A volume of 5 µL provides 0.015 mg of palladium and 0.01 mg of magnesium nitrate.

Standard stock solution: Transfer 10.0 mL of *Standard Arsenic Solution*, prepared as directed in *Arsenic* ⟨211⟩, to a 100-mL volumetric flask. Add 40 mL of water and 5 mL of nitric acid, and dilute with water to volume. This solution contains 0.10 µg/mL of arsenic.

Blank: Nitric acid and water (1:19)

Standard solutions: Dilute the *Standard stock solution* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 µg/mL of arsenic.

Sample solution: For preparation, use a microwave oven with a magnetron frequency of 2455 MHz and a selectable output power of 0–950 watts in 1% increments, equipped with advanced composite vessels with 100-mL polytef liners. Use rupture membranes to vent vessels should the pressure exceed 125 psi. The vessels fit into a turntable, and each vessel can be vented into an overflow container. Equip the microwave oven with an exhaust tube to ventilate fumes. [**CAUTION**—Wear proper eye protection and protective clothing and gloves.]

Transfer approximately 500 mg of *Crypthecodinium cohnii* Oil, weighed to the nearest 0.1 mg, into a Teflon digestion vessel liner. Prepare samples in duplicate. Add 15 mL of nitric acid, and swirl gently. Cover the vessels with lids, leaving the vent fitting off. Predigest overnight under a hood. Place the rupture membrane in the vent fitting, and tighten the lid. Place all vessels on the microwave oven turntable. Connect the vent tubes to the vent trap, and connect the pressure-sensing line to the appropriate vessel. Initiate a two-stage digestion procedure by heating the microwave at 15% power for 15 min, followed by 25% power for 45 min. Remove the turntable of vessels from the oven, and allow the vessels to cool to room temperature. [NOTE—A cool water bath may be used to speed the cooling process.] Vent the vessels when they reach room temperature. Remove the lids, and slowly add 2 mL of 30% hydrogen peroxide to each. Allow the reactions to subside, and seal the vessels. Return the vessels on the turntable to the microwave oven, and heat for an additional 15 min at 30% power. Remove the vessels from the oven, and allow them to cool to room temperature. Transfer the cooled digests into 25-mL volumetric flasks, and dilute with water to volume.

Analysis: Program the graphite furnace as follows. Dry at 115°, using a 1-s ramp, a 65-s hold, and an argon flow of 300 mL/min; char the sample at 1000°, using a 1-s ramp, a 20-s hold, and an airflow of 300 mL/min; cool down, and purge the air from the furnace for 10 s, using a 20° set temperature and an argon flow of 300 mL/min; atomize at 2400°, using a 0-s ramp and a 5-s hold with the argon flow stopped; and clean out at 2600° with a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 µL) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by an injection of 5 µL of *Solution C* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrophotometer equipped with a hollow-cathode lamp for arsenic. Determine the peak area at the arsenic emission line at 193.7 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of arsenic, in µg/mL, and calculate the regression line best fitting the points. Determine the concentration, C, in µg/mL, of arsenic in each mL of the *Sample solution* by interpolation from the regression line.

Calculate the content of arsenic in the portion of *Crypthecodinium cohnii* Oil taken:

$$\text{Result} = (C/W) \times 25$$

C = concentration, as obtained above
W = weight of *Crypthecodinium cohnii* Oil taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g

• **LIMIT OF LEAD**

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytef, and plastic vessels before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin before use. Select all reagents to have as low a content of lead as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytef, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

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Solution A: 10 g of ultrapure monobasic ammonium phosphate in 1 mL of nitric acid and 40 mL of water to dissolve the phosphate. Dilute with deionized water to 100 mL.

Solution B: Transfer 1 g of ultrapure magnesium nitrate to a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it to a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution C: *Solution A*, *Solution B*, and 2% nitric acid (2:1:2). A volume of 5 µL provides 0.2 mg of phosphate and 0.01 mg of magnesium nitrate.

Standard stock solution: Transfer 10.0 mL of lead nitrate stock solution TS to a 100-mL volumetric flask. Add 40 mL of water and 5 mL of nitric acid, and dilute with water to volume. Transfer 1.0 mL of this solution to a second 100-mL volumetric flask, add 50 mL of water and 1 mL of nitric acid, and dilute with water to volume. This solution contains 0.10 µg/mL of lead.

Blank: Nitric acid and water (1:19)

Standard solutions: Dilute the *Standard stock solution* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 µg/mL of lead.

Sample solution: Prepare as directed for the *Sample solution* in the test for *Limit of Arsenic*.

Analysis: Program the graphite furnace as follows. Dry at 120°, using a 1-s ramp, a 55-s hold, and an argon flow of 300 mL/min; char the sample at 850°, using a 1-s ramp, a 30-s hold, and an airflow of 300 mL/min; cool down, and purge the air from the furnace for 10 s, using a 20° set temperature and an argon flow of 300 mL/min; atomize at 2100°, using a 0-s ramp and a 5-s hold with the argon flow stopped; and clean out at 2600° with a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 µL) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by an injection of 5 µL of *Solution C* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrophotometer equipped with a hollow-cathode lamp for lead. Determine the peak area at the lead emission line at 283.3 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of lead, in µg/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in µg/mL, of lead in each mL of the *Sample solution* by interpolation from the regression line.

Calculate the content of lead in the portion of *Crypthecodinium cohnii* Oil taken:

$$\text{Result} = (C/W) \times 25$$

C = concentration, as obtained above
W = weight of *Crypthecodinium cohnii* Oil taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g

- **LIMIT OF CADMIUM**

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytef, and plastic vessels before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin before use. Select all reagents to have as low a content of cadmium as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytef, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

Solution A: 10 g of ultrapure monobasic ammonium phosphate in 40 mL of water and 1 mL of nitric acid to dissolve the phosphate. Dilute with deionized water to 100 mL.

Solution B: Transfer 1 g of ultrapure magnesium nitrate to a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it to a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution C: *Solution A*, *Solution B*, and 2% nitric acid to volume (2:1:2). A volume of 5 µL provides 0.2 mg of phosphate and 0.01 mg of magnesium nitrate.

Standard stock solution A: 0.1372 mg/mL of cadmium nitrate

Standard stock solution B: *Standard stock solution A*, nitric acid, and water (2:1:97). This solution contains 0.10 µg/mL of cadmium. [NOTE—Before make-up to final volume dissolve in a portion of water and nitric acid.]

Blank: Nitric acid and water (1:19)

Standard solutions: Dilute *Standard stock solution B* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 µg/mL of cadmium.

Sample solution: Prepare as directed for the *Sample solution* in the test for *Limit of Arsenic*.

Analysis: Program the graphite furnace as follows. Dry at 120°, using a 1-s ramp, a 55-s hold, and an argon flow of 300 mL/min; char the sample at 850°, using a 1-s ramp, a 30-s hold, and an airflow of 300 mL/min; cool down, and purge the air from the furnace for 10 s, using a 20° set temperature and an argon flow of 300 mL/min; atomize at 2400°, using a 0-s ramp and a 5-s hold with the argon flow stopped; and clean out at 2600° with a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 µL) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by an injection of 5 µL of *Solution C* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrophotometer equipped with a hollow-cathode lamp for cadmium. Determine the peak area at the cadmium emission line at 228.8 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of cadmium, in µg/mL, and calculate the regression line best fitting the points.

Determine the concentration, *C*, in µg/mL, of cadmium in each mL of the *Sample solution* by interpolation from the regression line.

Calculate the content of cadmium in the portion of *Crypthecodinium cohnii* Oil taken:

$$\text{Result} = (C/W) \times 25$$

C = concentration, as obtained above
W = weight of *Crypthecodinium cohnii* Oil taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g

- **LIMIT OF MERCURY:** Proceed as directed in *Mercury* ⟨261⟩, *Method IIa*, except use a *Standard Mercury Solution* having the equivalent of 0.1 µg/mL of mercury.

Sample solution: Prepare as directed for the *Sample solution* in the test for *Limit of Arsenic*, combining the two duplicate cooled digests into 1.0 mL of *Potassium Permanganate Solution*.

Acceptance criteria: NMT 0.1 µg/g

SPECIFIC TESTS

- **FATS AND FIXED OILS** ⟨401⟩, *Anisidine Value*: NMT 20.0
- **FATS AND FIXED OILS** ⟨401⟩, *Acid Value (Free Fatty Acids)*: The free fatty acids in 10 g require NMT 1.42 mL of 0.1 N sodium hydroxide for neutralization.
- **FATS AND FIXED OILS** ⟨401⟩, *Peroxide Value*: NMT 5.0

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- **Fats and Fixed Oils** ⟨401⟩, *Total Oxidation Value (TOTOX)* : NMT 26, calculated as:

$$\text{Result} = (2 \times PV) + AV$$

PV = peroxide value
AV = anisidine value

- **Fats and Fixed Oils** ⟨401⟩, *Unsaponifiable Matter*: NMT 3.5%
- **Specific Gravity** ⟨841⟩: 0.91–0.93

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in tight, light-resistant containers, and avoid exposure to excessive heat.
- **Labeling:** The label states the content of docosahexaenoic acid in mg/g. It also states the name and concentration of any added antioxidant.

Crypthecodinium cohnii Oil Capsules

DEFINITION

Crypthecodinium cohnii Oil Capsules are prepared from *Crypthecodinium cohnii* Oil and contain NLT 95.0% and NMT 105.0% of the labeled amount of docosahexaenoic acid (DHA, $C_{22}H_{32}O_2$) (C22:6 n–3).

IDENTIFICATION

- **Long-Chain Unsaturated Fatty Acid Profile:** Proceed as directed in *Content of DHA*.

Analysis

Samples: *Standard Solution 2a*, *Standard Solution 2b*, and *Test solution 1*

Calculate the area percentage for each fatty acid as methyl ester in *Test solution 1*:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of each individual fatty acid as methyl ester
r_T = sum of all the peak responses, except the solvent and butylated hydroxytoluene peaks

Acceptance criteria: The retention time of the peaks of the docosahexaenoic acid methyl ester and the eicosapentaenoic acid methyl ester from *Test solution 1* corresponds to that from the docosahexaenoic acid methyl ester and eicosapentanoic acid methyl ester peaks from *Standard Solution 2a* and *Standard Solution 2b*, respectively, as obtained in the test for *Content of EPA and DHA*. The area percent for the methyl esters of the fatty acids from *Test solution 1* in the test for *Content of EPA and DHA* meet the requirements for each fatty acid indicated in *Table 1*.

Table 1

Fatty Acid	Relative Retention Time	Shorthand Notation	Lower Limit (Area, %)	Upper Limit (Area, %)
Linoleic acid	0.52	18:2 n–6	0	1.0
Eicosapentanoic acid	0.79	20:5 n–3	0	0.1
Docosapentaenoic acid	0.94	22:5 n–6	0	0.1
Docosahexaenoic acid	1.00	22:6 n–3	35.0	47.0

STRENGTH

- **Content of DHA**

Test solution 1 and **Test solution 2:** Weigh NLT 10 Capsules in a tared weighing bottle. With a sharp blade or other appropriate means, carefully open the Capsules, without loss of the shell material, and transfer the combined Capsule contents to a 100-mL beaker. Remove any adhering substance from the emptied Capsules by washing with several small portions of isooctane. Discard the washings, and allow the empty Capsules to dry in a current of dry air until the isooctane is completely evaporated. Weigh the empty Capsules in the original tared weighing bottle, and calculate the average fill weight (AFW) of *Crypthecodinium cohnni* oil/Capsule. Proceed with the content of Capsules as directed in the *Analysis*.

Analysis: Proceed as directed in *Fats and Fixed Oils* ⟨401⟩, *Omega-3 Fatty Acids Determination and Profile, Content of EPA and DHA*.

Calculate the percentage of the labeled amount of docosahexaenoic acid (DHA) in the Capsules taken:

$$\text{Result} = R \times AFW/L$$

R = determined percentage of DHA in the portion of oil taken from the Capsules (%)
AFW = average fill weight of the Capsules taken (mg)
L = labeled amount of DHA (mg/Capsule)

Acceptance criteria: NLT 95.0% and NMT 105.0% of the labeled amount of DHA

PERFORMANCE TESTS

- **Disintegration and Dissolution** ⟨2040⟩: Meet the requirements for *Rupture Test for Soft Shell Capsules*
- **Weight Variation of Dietary Supplements** ⟨2091⟩ : Meet the requirements

IMPURITIES

- **Limit of Arsenic**

[Note—For the preparation of all aqueous solutions and for the rinsing of glass, polytef, and plastic vessels before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin before use. Select all reagents to have as low a content of arsenic as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytef, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

Solution A: Transfer 1 g of ultrapure palladium metal into a Teflon beaker. Add 20 mL of water and 10 mL of nitric acid, and warm on a hot plate to dissolve. Allow the solution to cool to room temperature, transfer it into a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution B: Transfer 1 g of ultrapure magnesium nitrate into a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it into a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution C: *Solution A*, *Solution B*, and 2% nitric acid (3:2:5). A volume of 5 µL provides 0.015 mg of palladium and 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (1:19)

Standard stock solution: Transfer 10.0 mL of *Standard Arsenic Solution*, prepared as directed in the test for *Arsenic* ⟨211⟩, to a 100-mL volumetric flask. Add 40 mL of water and 5 mL of nitric acid, and dilute with water to volume. This solution contains 0.10 µg/mL of arsenic.

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Standard solutions: Dilute the *Standard stock solution* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 µg/mL of arsenic.

Sample solution: For preparation of the *Sample solution*, use a microwave oven with a magnetron frequency of 2455 MHz and a selectable output power of 0–950 watts in 1% increments, equipped with advanced composite vessels with 100-mL polytef liners. Use rupture membranes to vent vessels should the pressure exceed 125 psi. The vessels fit into a turntable, and each vessel can be vented into an overflow container. Equip the microwave oven with an exhaust tube to ventilate fumes. [**CAUTION**—Wear proper eye protection and protective clothing and gloves.] Transfer approximately 500 mg of *Crypthecondinium cohnii* oil from Capsules, weighed to the nearest 0.1 mg, into a Teflon digestion vessel liner. Prepare samples in duplicate. Add 15 mL of nitric acid, and swirl gently. Cover the vessels with lids, leaving the vent fitting off. Predigest overnight under a hood. Place the rupture membrane in the vent fitting, and tighten the lid. Place all vessels on the microwave oven turntable. Connect the vent tubes to the vent trap, and connect the pressure-sensing line to the appropriate vessel. Initiate a two-stage digestion procedure by heating the microwave at 15% power for 15 min, followed by 25% power for 45 min. Remove the turntable of vessels from the oven, and allow the vessels to cool to room temperature. [NOTE—A cool water bath may be used to speed the cooling process.] Vent the vessels when they reach room temperature. Remove the lids, and slowly add 2 mL of 30% hydrogen peroxide to each. Allow the reactions to subside, and seal the vessels. Return the vessels on the turntable to the microwave oven, and heat for an additional 15 min at 30% power. Remove the vessels from the oven, and allow them to cool to room temperature. Transfer the cooled digests into 25-mL volumetric flasks, and dilute with water to volume.

Analysis: Program the graphite furnace as follows. Dry at 115°, using a 1-s ramp, a 65-s hold, and an argon flow of 300 mL/min; char the sample at 1000°, using a 1-s ramp, a 20-s hold, and an airflow of 300 mL/min; cool down, and purge the air from the furnace for 10 s, using a 20° set temperature and an argon flow of 300 mL/min; atomize at 2400°, using a 0-s ramp and a 5-s hold with the argon flow stopped; and clean out at 2600° with a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 µL) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by an injection of 5 µL of *Solution C* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for arsenic. Determine the peak area at the arsenic emission line at 193.7 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of arsenic, in µg/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in µg/mL, of arsenic in each mL of the *Sample solution* by interpolation from the regression line.

Calculate the content of arsenic in the portion of Capsules taken:

$$\text{Result} = (C/W) \times 25$$

C = concentration as obtained above
W = weight of Capsules content taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g

• **LIMIT OF LEAD**

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytef, and plastic vessels before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin before use. Select all reagents to have as low a content of lead as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytef, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

Solution A: 10 g of ultrapure monobasic ammonium phosphate in 1 mL of nitric acid and 40 mL of water to dissolve the phosphate. Dilute with deionized water to 100 mL.

Solution B: Transfer 1 g of ultrapure magnesium nitrate to a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it to a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution C: *Solution A*, *Solution B*, and 2% nitric acid (2:1:2). A volume of 5 µL provides 0.2 mg of phosphate and 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (1:19)

Standard stock solution: Transfer 10.0 mL of lead nitrate stock solution TS to a 100-mL volumetric flask. Add 40 mL of water and 5 mL of nitric acid, and dilute with water to volume. Transfer 1.0 mL of this solution to a second 100-mL volumetric flask, add 50 mL of water and 1 mL of nitric acid, and dilute with water to volume. This solution contains 0.10 µg/mL of lead.

Standard solutions: Dilute the *Standard stock solution* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 µg/mL of lead.

Sample solution: Prepare as directed for *Sample solution* in the test for *Limit of Arsenic*.

Analysis: Program the graphite furnace as follows. Dry at 120°, using a 1-s ramp, a 55-s hold, and an argon flow of 300 mL/min; char the sample at 850°, using a 1-s ramp, a 30-s hold, and an airflow of 300 mL/min; cool down, and purge the air from the furnace for 10 s, using a 20° set temperature and an argon flow of 300 mL/min; atomize at 2100°, using a 0-s ramp and a 5-s hold with the argon flow stopped; and clean out at 2600° with a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 µL) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by an injection of 5 µL of the *Solution C* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for lead. Determine the peak area at the lead emission line at 283.3 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of lead, in µg/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in µg/mL, of lead in each mL of the *Sample solution* by interpolation from the regression line.

Calculate the content of lead in the portion of Capsules taken:

$$\text{Result} = (C/W) \times 25$$

C = concentration as obtained above
W = weight of Capsules content taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g

• **LIMIT OF CADMIUM**

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytef, and plastic vessels before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange

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resin before use. Select all reagents to have as low a content of cadmium as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytef, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

Solution A: 10 g of ultrapure monobasic ammonium phosphate in 40 mL of water and 1 mL of nitric acid to dissolve the phosphate. Dilute with deionized water to 100 mL.

Solution B: Transfer 1 g of ultrapure magnesium nitrate to a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it to a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution C: *Solution A*, *Solution B*, and 2% nitric acid to volume (2:1:2). A volume of 5 µL provides 0.2 mg of phosphate and 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (1:19)

Standard stock solution A: 0.1372 mg/mL of cadmium nitrate

Standard stock solution B: *Standard stock solution A*, nitric acid, and water (2:1:97). This solution contains 0.10 µg/mL of cadmium. [NOTE—Before make up to final volume dissolve in a portion of water and nitric acid.]

Standard solutions: Dilute *Standard stock solution B* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 µg/mL of cadmium.

Sample solution: Prepare as directed for *Sample solution* in the test for *Limit of Arsenic*.

Analysis: Program the graphite furnace as follows. Dry at 120°, using a 1-s ramp, a 55-s hold, and an argon flow of 300 mL/min; char the sample at 850°, using a 1-s ramp, a 30-s hold, and an airflow of 300 mL/min; cool down, and purge the air from the furnace for 10 s, using a 20° set temperature and an argon flow of 300 mL/min; atomize at 2400°, using a 0-s ramp and a 5-s hold with the argon flow stopped; and clean out at 2600° with a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 µL) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by an injection of 5 µL of the *Solution C* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for cadmium. Determine the peak area at the cadmium emission line at 228.8 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of cadmium, in µg/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in µg/mL, of cadmium in each mL of the *Sample solution* by interpolation from the regression line.

Calculate the content of cadmium in the Capsules taken:

$$\text{Result} = (C/W) \times 25$$

C = concentration as obtained above
W = weight of Capsules content taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g

- **LIMIT OF MERCURY:** Proceed as directed in *Mercury* ⟨261⟩, *Method IIa*, except use a *Standard Mercury Solution* having the equivalent of 0.1 µg/mL of mercury.

Sample solution: Prepare as directed for the *Sample solution* in the test for *Limit of Arsenic*, combining the two duplicate cooled digests into 1.0 mL of *Potassium Permanganate Solution*.

Acceptance criteria: NMT 0.1 µg/g

SPECIFIC TESTS

- **FATS AND FIXED OILS,** *Anisidine Value* ⟨401⟩: NMT 20.0, determined on the contents of the Capsules
- **FATS AND FIXED OILS,** *Free Fatty Acids* ⟨401⟩: The free fatty acids in 10 g require for neutralization NMT 1.42 mL of 0.1 N sodium hydroxide.
- **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩: NMT 5.0, determined on the contents of the Capsules
- **FATS AND FIXED OILS,** *Total Oxidation Value (TOTOX)* ⟨401⟩: NMT 26 (determined on the contents of the Capsules), calculated as:

$$\text{Result} = (2 \times PV) + AV$$

PV = peroxide value
AV = anisidine value

- **FATS AND FIXED OILS,** *Unsaponifiable Matter* ⟨401⟩: NMT 3.5%, determined on the contents of the Capsules
- **SPECIFIC GRAVITY** ⟨841⟩: 0.91–0.93, determined on the contents of the Capsules

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and avoid exposure to excessive heat.
- **LABELING:** The label states the content of docosahexaenoic acid in mg/Capsule. It also states the name and concentration of any added antioxidant.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Docosahexaenoic Acid Ethyl Ester RS
  USP Eicosapentaenoic Acid Ethyl Ester RS
  USP Methyl Tricosanoate RS

Curcuminoids

DEFINITION

Curcuminoids is a partially purified natural complex of diaryl heptanoid derivatives isolated from Turmeric, *Curcuma longa* L. It contains NLT 95.0% of curcuminoids, calculated on the dried basis, as the sum of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin. It contains NLT 70.0% and NMT 80.0% of curcumin, NLT 15.0% and NMT 25.0% of desmethoxycurcumin, and NLT 2.5% and NMT 6.5% of bisdesmethoxycurcumin.

IDENTIFICATION

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution: 1 mg/mL of USP Curcuminoids RS in methanol

Sample solution: Suspend about 5 mg of Curcuminoids in 5 mL of methanol, and sonicate briefly.

Chromatographic system

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plate)[1]

Application volume: 2 µL each of the *Standard solution* and the *Sample solution* as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of 33%.

Temperature: Ambient, not to exceed 30°

Developing solvent system: Toluene and glacial acetic acid (4:1)

Developing distance: 6 cm

Derivatization reagent: 85 mL of ice-cold methanol combined with 10 mL of glacial acetic acid, 5 mL of sulfuric acid, and 0.5 mL of *p*-anisaldehyde

[1] Suitable commercially available plates are HPTLC Silica Gel 60 F_{254} from EMD Millipore (e.g., Part No. 1.05642.0001).

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Analysis
Samples: *Standard solution* and *Sample solution*
Apply the *Samples* as bands and dry in air. Develop in a saturated chamber and dry in air. Treat with *Derivatization reagent*, heat at 100° for 3 min, and examine under long-wave UV light (365 nm) and under white light.
System suitability: Under long-wave UV light (365 nm), the derivatized chromatogram of the *Standard solution* exhibits, in its lower half, three bands in the order of increasing R_F: an orange band due to bisdesmethoxycurcumin, an orange band due to desmethoxycurcumin, and the red band due to curcumin. Under white light, the two lower bands appear orange, while the topmost band is reddish-pink.
Acceptance criteria: Under long-wave UV light (365 nm), the derivatized chromatogram of the *Sample solution* displays two orange bands and one red band, similar in position and color to those observed in the *Standard solution*. At the bottom part of the upper half of the plate, two purple bands are seen. Under white light, two orange bands and a darker red band are seen coincident with the bands due to bisdesmethoxycurcumin, desmethoxycurcumin, and curcumin in the *Standard solution*, in the order of increasing R_F. In the upper half of the plate, the lower of the two bands appears purple, while the upper band is brown. No bands appear in the topmost quarter of the plate, which is characteristic of *Curcuma zanthorrhiza* Roxb. and *Curcuma aromatica* Salisb. These confounders, and occasional adulterants, also lack the lower orange band corresponding to bisdesmethoxycurcumin. Additional weak bands may be observed in the *Sample solution* under either illumination condition.

- **B. HPLC**

Analysis: Proceed as directed in the test for *Content of Curcuminoids*.
Acceptance criteria: The retention times of the peaks for curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin of the *Sample solution* correspond to those of *Standard solution A* and *Standard solution B*.

COMPOSITION

- **CONTENT OF CURCUMINOIDS**

Mobile phase: Tetrahydrofuran and 1 mg/mL of citric acid in water (4:6)
Standard solution A: 40 µg/mL of USP Curcuminoids RS in *Mobile phase*
Standard solution B: A composite solution containing 40 µg/mL of USP Curcumin RS, 10 µg/mL of USP Desmethoxycurcumin RS, and 2.0 µg/mL of USP Bisdesmethoxycurcumin RS in *Mobile phase*. Use sonication if necessary. Before injection, pass through a filter of 0.45-µm pore size, and discard the initial 10 mL of the filtrate.
Sample stock solution: Transfer about 20 mg of Curcuminoids, accurately weighed, to a 50-mL volumetric flask, add 30 mL of acetone, and sonicate for 30 min. Dilute with acetone to volume, mix, and centrifuge.
Sample solution: Transfer 5.0 mL of the *Sample stock solution* to a 50-mL volumetric flask. Dilute with *Mobile phase* to volume, and mix. Before injection, pass through a filter of 0.45-µm pore size, and discard the initial 10 mL of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Vis 420 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Flow rate: 1.0 mL/min
Injection volume: 20 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
[NOTE—The relative retention times for the curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin peaks are 1.0, 1.2, and 1.4, respectively.]
Suitability requirements
Chromatogram similarity: The chromatogram of *Standard solution A* is similar to the reference chromatogram provided with USP Curcuminoids RS.
Resolution: NLT 2.0 between curcumin and desmethoxycurcumin peaks and desmethoxycurcumin and bisdesmethoxycurcumin peaks, *Standard solution B*
Tailing factor: NMT 1.5 for bisdesmethoxycurcumin, desmethoxycurcumin, and curcumin peaks, *Standard solution B*
Relative standard deviation: NMT 2.0% for the desmethoxycurcumin peak, in replicate injections, *Standard solution B*
Analysis
Samples: *Standard solution B* and *Sample solution*
Calculate the percentages of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin in the portion of Curcuminoids taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of the relevant analyte from *Standard solution B*
C_S = concentration of the relevant analyte in *Standard solution B* (mg/mL)
V = volume of the *Sample stock solution* (mL)
W = weight of Curcuminoids used to prepare the *Sample stock solution* (mg)
D = dilution factor to obtain the *Sample solution* from the *Sample stock solution*, 10

Acceptance criteria: Curcuminoids contains NLT 95.0% of curcuminoids, calculated on the dried basis, as the sum of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin. It contains 70.0%–80.0% of curcumin, 15.0%–25.0% of desmethoxycurcumin, and 2.5%–6.5% of bisdesmethoxycurcumin.

Change to read:

CONTAMINANTS

- **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues:*▲ (CN 1-May-2020) Meets the requirements
- **BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Residual Solvents*: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Test for Aflatoxins*: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS

- **MELTING RANGE OR TEMPERATURE** ⟨741⟩, *Procedures, Procedure for Class I, Apparatus II*: 172°–178°
- **LOSS ON DRYING** ⟨731⟩

Sample: 1.0 g of Curcuminoids

DS Monographs

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Analysis: Dry the *Sample* at 105° for 2 h.
Acceptance criteria: NMT 2.0%
• **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Total Ash*: NMT 1.0%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers; protect from light and moisture, and store at room temperature.
• **LABELING:** The label states the content of curcuminoids and the content of the individual curcuminoids, on the dried basis; the Latin binomial; and the part of the plant used to prepare the article.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Bisdesmethoxycurcumin RS
USP Curcumin RS
USP Curcuminoids RS
USP Desmethoxycurcumin RS

Curcuminoids Capsules

DEFINITION
Curcuminoids Capsules are prepared from Curcuminoids and contain NLT 90.0% and NMT 110.0% of the labeled amount of curcuminoids, calculated as the sum of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin.

IDENTIFICATION
• **A. THIN-LAYER CHROMATOGRAPHY**
Standard solution: 0.2 mg/mL of USP Curcuminoids RS in acetone
Sample solution: Weigh and finely powder the contents of NLT 20 Capsules. Transfer a portion of the powder, equivalent to about 10 mg of curcuminoids, to a suitable container, add 5 mL of acetone, shake for 1 min, and sonicate for 10 min. Allow to stand for 15 min before use.
Adsorbent: Chromatographic silica gel mixture with an average particle size of 10–15 µm (TLC plates)
Application volume: 10 µL, as bands
Developing solvent system: Chloroform, methanol, and formic acid (96:4:1)
Analysis
Samples: *Standard solution* and *Sample solution*
Apply the samples as bands to a suitable thin-layer chromatographic plate (see *Chromatography* ⟨621⟩). Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about three-fourths of the length of the plate. Remove the plate from the chamber, dry, and examine under UV light at 365 nm.
Acceptance criteria: The *Sample solution* chromatogram shows yellowish-brown bands due to bisdesmethoxycurcumin, desmethoxycurcumin, and curcumin at R_F values of about 0.4, 0.6, and 0.7, respectively, corresponding in position and color to those obtained from the *Standard solution*.
• **B.** The retention times of the peaks for curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin of the *Sample solution* correspond to those of the *Standard solution* for the appropriate USP Reference Standard, as obtained in the test for *Content of Curcuminoids*.

STRENGTH
• **CONTENT OF CURCUMINOIDS**
Mobile phase: Tetrahydrofuran and 1 mg/mL of citric acid in water (4:6)
[NOTE—Sonication may be necessary to dissolve the RS in each *Standard solution*; all solutions should be passed through a filter with 0.45-µm pore size before injection. USP Curcumin RS, USP Desmethoxycurcumin RS, and USP Bisdesmethoxycurcumin RS can also be prepared in one standard solution containing the final concentration specified below for each.]
Standard solution A: 40 µg/mL of USP Curcuminoids RS in *Mobile phase*
Standard solution B: 40 µg/mL of USP Curcumin RS in *Mobile phase*
Standard solution C: 10 µg/mL of USP Desmethoxycurcumin RS in *Mobile phase*
Standard solution D: 2 µg/mL of USP Bisdesmethoxycurcumin RS in *Mobile phase*
Sample stock solution: Weigh and finely powder the contents of NLT 20 Capsules. Transfer an accurately weighed amount of the powder, equivalent to about 20 mg of curcuminoids, to a 50-mL volumetric flask. Add about 30 mL of acetone, sonicate for 30 min, dilute with acetone to volume, mix, and centrifuge.
Sample solution: Dilute a portion of the *Sample stock solution* 1 in 10 with *Mobile phase*, and mix.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV-Vis 420 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Flow rate: 1 mL/min
Injection size: 20 µL
System suitability
Sample: *Standard solution A*
[NOTE—The relative retention times for the curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin peaks are about 1.0, 1.2, and 1.4, respectively.]
Suitability requirements
Chromatogram similarity: The chromatogram from *Standard solution A* is similar to the Reference Chromatogram provided with the lot of USP Curcuminoids RS being used.
Resolution: NLT 2.0 between the curcumin and desmethoxycurcumin peaks and the desmethoxycurcumin and bisdesmethoxycurcumin peaks
Tailing factor: NMT 1.5 for the bisdesmethoxycurcumin, desmethoxycurcumin, and curcumin peaks
Relative standard deviation: NMT 2.0% for desmethoxycurcumin peak, in repeated injections
Analysis
Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, *Standard solution D*, and *Sample solution*
Calculate the quantity, in mg, of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin in each Capsule:

$$\text{Result} = (r_U/r_S) \times C_S \times D \times V \times (W_F/W_U)$$

r_U = peak area for curcumin, desmethoxycurcumin, or bisdesmethoxycurcumin from the *Sample solution*
r_S = peak area for curcumin, desmethoxycurcumin, or bisdesmethoxycurcumin from the appropriate *Standard solution*
C_S = concentration of the appropriate *Standard solution* (mg/mL)
D = dilution factor to prepare the *Sample solution* from *Sample stock solution*
V = volume of *Sample stock solution* (mL)
W_F = average fill weight of Capsules (mg)
W_U = weight of content of Capsules taken to prepare the *Sample stock solution* (mg)

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Calculate the percentage of the labeled amount of curcuminoids in the Capsule:

$$\text{Result} = (\Sigma Q/L) \times 100$$

ΣQ = sum of the quantities of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin in the Capsule (mg)
L = labeled amount of curcuminoids (mg/Capsule)

Acceptance criteria: 90.0%–110.0% of the label claim

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩
  Mode: Dissolution
  Medium: Water containing 1% sodium lauryl sulfate; 900 mL
  Apparatus 2: 100 rpm
  Time: 60 min
  Sample solution: Combine 25-mL portions of the solution under test from each of the six dissolution vessels, and mix. Transfer 5 mL to a 25-mL volumetric flask, and dilute with *Mobile phase* to volume.
  Analysis: Determine the amount of curcumin ($C_{21}H_{20}O_6$) dissolved by using the method used in *Strength*, making any necessary modifications.
  Tolerances: NLT 75% of the content of curcumin ($C_{21}H_{20}O_6$) is dissolved.
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩: Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩: Meet the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protect from light and moisture, and store at room temperature.
- **LABELING:** The label states the content of curcuminoids in mg/Capsule.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Bisdesmethoxycurcumin RS
  USP Curcumin RS
  USP Curcuminoids RS
  USP Desmethoxycurcumin RS

Curcuminoids Tablets

DEFINITION

Curcuminoids Tablets are prepared from Curcuminoids and contain NLT 90.0% and NMT 110.0% of the labeled amount of curcuminoids, calculated as the sum of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHY**
  Standard solution: 0.2 mg/mL of USP Curcuminoids RS in acetone
  Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to about 10 mg of curcuminoids, to a suitable container, add 5 mL of acetone, shake for 1 min, and sonicate for 10 min. Allow to stand for 15 min before use.
  Adsorbent: Chromatographic silica gel mixture with an average particle size of 10-15 µm (TLC plates)
  Application volume: 10 µL, as bands
  Developing solvent system: Chloroform, methanol, and formic acid (96:4:1)
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Apply the samples as bands to a suitable thin-layer chromatographic plate (see *Chromatography* ⟨621⟩). Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about three-fourths of the length of the plate. Remove the plate from the chamber, dry, and examine under UV light at 365 nm.
  Acceptance criteria: The *Sample solution* chromatogram shows yellowish-brown bands due to bisdesmethoxycurcumin, desmethoxycurcumin, and curcumin at R_F values of about 0.4, 0.6, and 0.7, respectively, corresponding in position and color to those obtained from the *Standard solution*.
- **B.** The retention times of the peaks for curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin of the *Sample solution* correspond to those of the *Standard solution* for the appropriate USP Reference Standard, as obtained in the test for *Content of Curcuminoids*.

STRENGTH

- **CONTENT OF CURCUMINOIDS**
  Mobile phase: Tetrahydrofuran and 1 mg/mL of citric acid in water (4:6)
  [NOTE—Sonication may be necessary to dissolve the RS in each *Standard solution*; all solutions should be passed through a filter with 0.45-µm pore size before injection. USP Curcumin RS, USP Desmethoxycurcumin RS and USP Bisdesmethoxycurcumin RS can also be prepared in one standard solution containing the final concentration specified below for each.]
  Standard solution A: 40 µg/mL of USP Curcuminoids RS in *Mobile phase*
  Standard solution B: 40 µg/mL of USP Curcumin RS in *Mobile phase*
  Standard solution C: 10 µg/mL of USP Desmethoxycurcumin RS in *Mobile phase*
  Standard solution D: 2 µg/mL of USP Bisdesmethoxycurcumin RS in *Mobile phase*
  Sample stock solution: Weigh and finely powder NLT 20 Tablets. Transfer an accurately weighed amount of the powder, equivalent to about 20 mg of curcuminoids, to a 50-mL volumetric flask. Add about 30 mL of acetone, sonicate for 30 min, dilute with acetone to volume, mix, and centrifuge.
  Sample solution: Dilute a portion of the *Sample stock solution* (1 in 10) with *Mobile phase*, and mix.
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: LC
  Detector: UV-Vis 420 nm
  Column: 4.6-mm × 25-cm; 5-µm packing L1
  Flow rate: 1 mL/min
  Injection size: 20 µL
  System suitability
  Sample: *Standard solution A*
  [NOTE—The relative retention times for the curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin peaks are about 1.0, 1.2, and 1.4, respectively.]
  Suitability requirements
  Chromatogram similarity: The chromatogram from *Standard solution A* is similar to the Reference

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Chromatogram provided with the lot of USP Curcuminoids RS being used.
Resolution: NLT 2.0 between the curcumin and desmethoxycurcumin peaks and the desmethoxycurcumin and bisdesmethoxycurcumin peaks
Tailing factor: NMT 1.5 for the bisdesmethoxycurcumin, desmethoxycurcumin, and curcumin peaks
Relative standard deviation: NMT 2.0% for desmethoxycurcumin peak, in repeated injections
Analysis
Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, *Standard solution D*, and *Sample solution*
Calculate the quantity, in mg, of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin in each Tablet:

$$\text{Result} = (r_U/r_S) \times C_S \times D \times V \times (W_F/W_U)$$

r_U = peak area for curcumin, desmethoxycurcumin, or bisdesmethoxycurcumin from the *Sample solution*
r_S = peak area for curcumin, desmethoxycurcumin, or bisdesmethoxycurcumin from the appropriate *Standard solution*
C_S = concentration of the appropriate *Standard solution* (mg/mL)
D = dilution factor to prepare the *Sample solution* from the *Sample stock solution*
V = volume of *Sample solution* (mL)
W_F = average weight of Tablets (mg)
W_U = weight of Tablets powder taken to prepare the *Sample stock solution* (mg)

Calculate the percentage of the labeled amount of curcuminoids in the Tablet:

$$\text{Result} = (\Sigma Q/L) \times 100$$

ΣQ = sum of the quantities of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin in the Tablet (mg)
L = labeled amount of curcuminoids (mg/Tablet)

Acceptance criteria: 90.0%–110.0% of the label claim

PERFORMANCE TESTS
• **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩
Mode: Dissolution
Medium: Water containing 1% sodium lauryl sulfate; 900 mL
Apparatus 2: 100 rpm
Time: 60 min
Sample solution: Combine 25-mL portions of the solution under test from each of the six dissolution vessels, and mix. Transfer 5 mL to a 25-mL volumetric flask, and dilute with *Mobile phase* to volume.
Analysis: Determine the amount of curcumin ($C_{21}H_{20}O_6$) dissolved by using the method used in the *Content of Curcuminoids*, making any necessary modifications.
Tolerances: NLT 75% of the content of curcumin ($C_{21}H_{20}O_6$) is dissolved.
• **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩**:** Meet the requirements

CONTAMINANTS
• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meet the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers, protect from light and moisture, and store at room temperature.
• **LABELING:** The label states the content of curcuminoids in mg/Tablet.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Bisdesmethoxycurcumin RS
USP Curcumin RS
USP Curcuminoids RS
USP Desmethoxycurcumin RS

Cyanocobalamin—*see Cyanocobalamin General Monographs*

Add the following:

▲Cyanocobalamin Chewable Gels

DEFINITION
Cyanocobalamin Chewable Gels contain NLT 90.0% and NMT 155.0% of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$).

IDENTIFICATION
• **A.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Strength*, *Method 1* or *Method 2*.

STRENGTH
[NOTE—The requirements may be met by following any one of the methods specified in this section. The method used is stated in the labeling only if *Method 1* is not used.]
• **METHOD 1**
[NOTE—Protect all samples from light. Use cryogenic gloves when handling liquid nitrogen.]
Solution A: 0.1% formic acid in water
Solution B: 0.1% formic acid in acetonitrile
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	98.0	2.0
7.0	80.0	20.0
8.0	65.0	35.0
9.0	65.0	35.0
10.0	98.0	2.0

System suitability solution: Dissolve 5 mg of USP Cyanocobalamin (Crystalline) RS in 10 mL of water, warming if necessary. Allow to cool. Add 1 mL of a 1.0-mg/mL solution of tosylchloramide sodium, 0.5 mL of 0.01 M hydrochloric acid, and then dilute with water to 25 mL. Shake and allow to stand for 5 min. Dilute with *Solution A* to 1 µg/mL of cyanocobalamin, and inject immediately.

DS Monographs

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[NOTE—This procedure generates 7β, 8β-lactocyanocobalamin.]

Standard solution: 1 µg/mL of cyanocobalamin from USP Cyanocobalamin (Crystalline) RS in water

Sample solution: Immerse 25–30 Chewable Gels in liquid nitrogen in a cryogenic vessel for 10 min. Cool a blender jar by swirling liquid nitrogen for about 1 min and discard. Add frozen Chewable Gels to the cooled blender jar, and grind to a fine powder. Transfer a portion of the powder, nominally equivalent to 25 µg of cyanocobalamin, into a 50-mL centrifuge tube. [NOTE—Proceed to the next step immediately or keep the powdered Chewable Gels frozen until use.]

Add 200 mg of pancreatin and 25.0 mL of water, accurately measured, and mix on a vortex mixer for 30 s. Shake on a water bath shaker at 40° until complete dissolution (about 10 min). Cool to room temperature, add 10 mL of methylene chloride, and mix on a vortex mixer until well mixed. Shake on a horizontal shaker for 15 min. Centrifuge the sample at 2000 rpm for 5 min. Pass a portion of the top aqueous layer through a 0.2-µm nylon filter, and discard the first few milliliters of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 361 nm

Column: 2.1-mm × 5-cm; 1.8-µm packing L96

Column temperature: 25°

Flow rate: 0.5 mL/min

Injection volume: 10 µL

System suitability

Samples: *System suitability solution* and *Standard solution*

Suitability requirements

Resolution: NLT 5.0 between the cyanocobalamin and 7β,8β-lactocyanocobalamin peaks, *System suitability solution*

Relative standard deviation: NMT 2.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$) in the portion of Chewable Gels taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of cyanocobalamin from the *Sample solution*

r_S = peak area of cyanocobalamin from the *Standard solution*

C_S = concentration of cyanocobalamin in the *Standard solution* (µg/mL)

C_U = nominal concentration of cyanocobalamin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–155.0%

• **METHOD 2**

[NOTE—Protect all samples from light. Use cryogenic gloves when handling liquid nitrogen.]

Solution A, Solution B, System suitability solution, Standard solution, Sample solution, and Analysis: Proceed as directed in *Strength, Method 1*.

Mobile phase: See *Table 2*.

Table 2

Time (min)	Solution A (%)	Solution B (%)
0	98.0	2.0
13	80.0	20.0
14.5	65.0	35.0
16.0	65.0	35.0
17.0	98.0	2.0
20.0	98.0	2.0

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 361 nm

Column: 2.1-mm × 10-cm; 1.8-µm packing L1

Column temperature: 30°

Flow rate: 0.5 mL/min

Injection volume: 10 µL

System suitability

Samples: *System suitability solution* and *Standard solution*

Suitability requirements

Resolution: NLT 4.0 between the cyanocobalamin and 7β,8β-lactocyanocobalamin peaks, *System suitability solution*

Relative standard deviation: NMT 2.0%, *Standard solution*

Acceptance criteria: 90.0%–155.0%

PERFORMANCE TESTS

• **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩, *Dissolution*

Buffer: Mix 95 mL of 0.1 M citric acid monohydrate and 405 mL of 0.1 M sodium citrate dihydrate, dilute with water to 1000 mL, and mix. Adjust with either 0.1 M hydrochloric acid or 0.1 M sodium hydroxide solution to a pH of 6.0.

Medium: *Buffer*, 500 mL

Apparatus 2: 50 rpm

Time: 30 min

Standard solution: Relevant concentration of cyanocobalamin from USP Cyanocobalamin (Crystalline) RS in *Medium*

Sample solution: Withdraw a portion of the solution under test, pass through a 0.2-µm nylon membrane filter, and use the pooled sample as the test specimen.

Analysis: Proceed as directed in *Strength, Method 1* or *Method 2*, making any necessary modifications.

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$) dissolved:

$$\text{Result} = (r_U/r_S) \times (C_S \times V/L) \times 100$$

r_U = peak area of cyanocobalamin from the *Sample solution*

r_S = peak area of cyanocobalamin from the *Standard solution*

C_S = concentration of cyanocobalamin in the *Standard solution* (µg/mL)

V = volume of *Medium*, 500 mL

L = label claim of cyanocobalamin (µg/Chewable Gel)

Tolerances: NLT 75% of the labeled amount of cyanocobalamin is dissolved.

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• **Weight Variation** ⟨2091⟩, *Tablets, Uncoated Tablets and Film-Coated Tablets*: Meet the requirements

SPECIFIC TESTS

• **pH** ⟨791⟩

Sample solution: Cut the Chewable Gels into small pieces. Transfer about 15 g of the cut Chewable Gels into a 50-mL centrifuge tube, add 15 g of hot (80°–90°) water, and close the cap. Put the centrifuge tube into a shaker water bath at 55°. Shake for 50–60 min or until all of the Chewable Gels are dissolved. Cool to 30° and measure the pH.

Acceptance criteria: NMT 4.5

• **Water Activity**

Sample: Slice the Chewable Gels into pieces about 2 mm thick.

Analysis: Measure the water activity by using the *Official Methods of Analysis of AOAC International*, No. 978.18.

Acceptance criteria: NMT 0.75

CONTAMINANTS

• **Microbial Enumeration Tests** ⟨2021⟩: The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined yeasts and molds count does not exceed 10^2 cfu/g.

• **Absence of Specified Microorganisms** ⟨2022⟩, *Test Procedures*, *Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meet the requirements

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Preserve in tight containers; protect from heat.

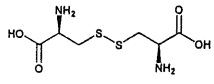
• **Labeling:** The label states the quantity of cyanocobalamin in µg/Chewable Gel. The *Strength* method used to determine compliance is stated in the labeling, if different from *Method 1*.

• **USP Reference Standards** ⟨11⟩

USP Cyanocobalamin (Crystalline) RS▲ (USP 1-Aug-2019)

Cysteine Hydrochloride—*see Cysteine Hydrochloride General Monographs*

Cystine



$C_6H_{12}N_2O_4S_2$ 240.30

L-Cystine;

3,3′-Disulfanediylbis [(2*R*)-2-aminopropanoic acid] [56-89-3].

DEFINITION

Cystine contains NLT 98.5% and NMT 101.5% of cystine ($C_6H_{12}N_2O_4S_2$), as L-cystine, calculated on the dried basis.

IDENTIFICATION

Change to read:

• **A.** ▲**Spectroscopic Identification Tests** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

• **B. Optical Rotation** ⟨781S⟩, *Procedures, Specific Rotation*

Sample solution: 20 mg/mL, in 1 N hydrochloric acid. Perform the measurements immediately after preparation.

Acceptance criteria: −215° to −225°, determined at 20°

• **C.** The R_F value of the principal spot of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Organic Impurities*.

ASSAY

Change to read:

• **Procedure**

Sample solution: Transfer about 0.1 g of Cystine to a glass-stoppered flask, and dissolve in a mixture of 2 mL of dilute sodium hydroxide ▲(50 g/L in water)▲ (USP 1-Dec-2019) and 10 mL of water. Add 10 mL of ▲freshly prepared▲ (USP 1-Dec-2019) potassium bromide solution (200 g/L in water), 50.0 mL of 0.1 N potassium bromate VS, and 15 mL of dilute hydrochloric acid ▲(170 mL/L in water).▲ (USP 1-Dec-2019) Immediately insert the stopper into the flask, ▲ mix the solution well, and cool in an ice water bath for 10 min in the dark.▲ (USP 1-Dec-2019)

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Residual titration

Titrant: 0.1 N potassium bromate VS

Back-titrant: 0.1 N sodium thiosulfate VS

Endpoint detection: Visual

Equivalency: Each milliliter of 0.1 N potassium bromate VS is equivalent to 2.403 mg of cystine ($C_6H_{12}N_2O_4S_2$) on the dried basis.

Analysis: ▲Immediately add 5 mL of potassium iodide solution (1.5 g in 5 mL of water) to the *Sample solution*, and mix well. Titrate slowly (dropwise addition)▲ (USP 1-Dec-2019) with *Back-titrant*, using starch TS as the indicator. Perform a blank determination, and make any necessary correction.

Acceptance criteria: 98.5%–101.5% on the dried basis

IMPURITIES

• **Residue on Ignition** ⟨281⟩: NMT 0.1%

• **Chloride and Sulfate** ⟨221⟩, *Chloride*: NMT 200 ppm. A 0.7-g portion shows no more chloride than corresponds to 0.40 mL of 0.01 N hydrochloric acid.

Change to read:

• **Chloride and Sulfate** ⟨221⟩, *Sulfate*

▲**Standard solution:** Dissolve 0.10 mL of 0.020 N sulfuric acid in 5 mL of dilute hydrochloric acid (0.2 g/mL in water), and dilute with water to 40 mL.

Sample solution: Dissolve 0.48 g of Cystine in 5 mL of dilute hydrochloric acid (0.2 g/mL in water) and dilute with water to 40 mL.

Analysis: Add 5 mL of barium chloride TS to the *Sample solution* and to the *Standard solution*. Add sufficient water to bring the final volume of each solution to 50 mL. Mix well, and allow the solutions to stand for 10 min.

Acceptance criteria: The observed turbidity of the *Sample solution* is NMT that of the *Standard solution* (NMT 200 ppm).▲ (USP 1-Dec-2019)

• **Iron** ⟨241⟩: NMT 10 ppm

• **Organic Impurities**

System suitability solution: Dissolve quantities of USP Cystine RS and USP Arginine Hydrochloride RS in 1 N hydrochloric acid, and dilute with water to obtain a solution having a known concentration of about 0.4 mg/mL each.

Standard solution: Dissolve a quantity of USP Cystine RS in 1 N hydrochloric acid, and dilute with water to obtain a solution having a known concentration of about 0.02 mg/mL.

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Sample solution: Dissolve a quantity of Cystine in 1 N hydrochloric acid, and dilute with water to obtain a solution having a known concentration of about 10 mg/mL.
Chromatographic system
(See *Chromatography* ⟨621⟩, *General Procedures, Thin-Layer Chromatography.*)
Mode: TLC
Adsorbent: 0.25-mm layer of chromatographic silica gel mixture
Application volume: 5 µL
Developing solvent system: Ammonia and 2-propanol (3:7)
Spray reagent: Dissolve 0.2 g of ninhydrin in 100 mL of a mixture of butanol and 2 N acetic acid (95:5).
Analysis
Samples: *System suitability solution, Standard solution,* and *Sample solution*
Proceed as directed in the chapter. After air-drying the plate, spray with *Spray reagent,* and heat between 100° and 105° for about 15 min. Examine the plate. The chromatogram from the *System suitability solution* exhibits 2 clearly separated spots.
Acceptance criteria: Any secondary spot from the *Sample solution* is not larger or more intense than the principal spot from the *Standard solution.*
Individual impurities: NMT 0.2%
Total impurities: NMT 2.0%

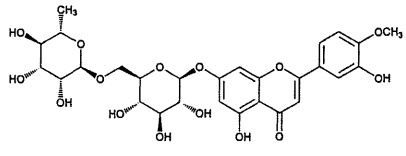
SPECIFIC TESTS
• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry at 105° for 3 h.
Acceptance criteria: NMT 0.2%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at controlled room temperature.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Arginine Hydrochloride RS
USP Cystine RS

Dexpanthenol—*see Dexpanthenol General Monographs*

Dexpanthenol Preparation—*see Dexpanthenol Preparation General Monographs*

Diosmin



$C_{28}H_{32}O_{15}$ 608.54
5-Hydroxy-2-(3-hydroxy-4-methoxyphenyl)-7-[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-[[(2*R*,3*R*,4*R*,5*R*,6*S*)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxymethyl]oxan-2-yl]oxychromen-4-one;
7-[[6-*O*-(6-Deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4*H*-1-benzopyran-4-one [520-27-4].

DEFINITION
Diosmin contains NLT 90.0% and NMT 102.0% of diosmin ($C_{28}H_{32}O_{15}$), calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy:* **197K**▲ (CN 1-May-2020)
• **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution,* as obtained in the *Assay.*

ASSAY
• **PROCEDURE**
Mobile phase: Methanol, acetonitrile, acetic acid, and water (27:2:6:65)
Standard solution: 1.0 mg/mL of USP Diosmin RS in dimethyl sulfoxide
System suitability solution: 1 mg/mL of USP Diosmin for System Suitability RS in dimethyl sulfoxide
Sample solution: 1.0 mg/mL of Diosmin in dimethyl sulfoxide
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability.*)
Mode: LC
Detector: UV 275 nm
Column: 4.6-mm × 10-cm; 3-µm packing L1
Column temperature: 40°
Flow rate: 1.2 mL/min
Injection size: 10 µL
System suitability
Samples: *System suitability solution* and *Standard solution.*
[NOTE—Allow the run time about 6 times the diosmin retention time. The relative retention times for diosmin, acetoisovanillone, hesperidin, isorhoifolin, linarin, and diosmetin are 1, 0.5, 0.6, 0.8, 2.6, and 4.5, respectively.]
Suitability requirements
Chromatogram similarity: The chromatogram from the *System suitability solution* is similar to the Reference Chromatogram provided with the USP Diosmin for System Suitability RS being used.
Resolution: NLT 2.5 between hesperidin and isorhoifolin, *System suitability solution*
Relative standard deviation: NMT 2.0%, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of diosmin ($C_{28}H_{32}O_{15}$), in the portion of Diosmin taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP Diosmin RS in the *Standard solution* (mg/mL)
C_U = concentration of Diosmin in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–102.0% on the anhydrous basis

IMPURITIES
• **RESIDUE ON IGNITION** ⟨281⟩
Sample: 1.0 g
Acceptance criteria: NMT 0.2%

USP Monographs

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• **Limit of Iodine**

Determine the total content of iodine by potentiometry, using an iodide-selective electrode, after oxygen flask combustion (see *Oxygen Flask Combustion* ⟨471⟩).

Sample solution: [**Caution**—Observe rigorously the precautions set forth for *Procedure* under *Oxygen Flask Combustion* ⟨471⟩.] Wrap 0.100 g of Diosmin in a piece of free-halide filter paper, and place it in the platinum gauze specimen holder. Introduce into the flask 50.0 mL of a 0.2 g/L solution of hydrazine. Flush the flask with oxygen for 10 min. Ignite the filter paper. Stir the contents of the flask immediately after the end of the combustion to dissolve completely the combustion products. Continue stirring for 1 h.

Standard solution: 33.2 µg/mL of potassium iodide in water, equivalent to 25.4 µg/mL of iodine

Potassium nitrate solution: 200 mg/mL of potassium nitrate in 0.1 M nitric acid

Analysis

Samples: *Sample solution* and *Standard solution*

Transfer 30 mL of *Potassium nitrate solution* to a beaker, immerse the electrodes, and stir for 10 min. The potential (nU_1) must remain stable. Measure the potential (nU_1). Add 1 mL of the *Sample solution*, and measure the potential (nU_2).

Transfer 30 mL of *Potassium nitrate solution* to a beaker, immerse the electrodes, and stir for 10 min. The potential (nS_1) must remain stable. Measure the potential (nS_1). Add 80 µL of the *Standard solution*, and measure the potential (nS_2).

Acceptance criteria: NMT 0.1%: The absolute value $|nU_2| - |nU_1|$ is not higher than the absolute value $|nS_2| - |nS_1|$.

• **Related Compounds**

Mobile phase, System suitability solution, Sample solution, and **Chromatographic system:** Proceed as directed in the *Assay*.

Standard solution: 0.05 mg/mL of USP Diosmin RS in dimethyl sulfoxide

System suitability

Sample: *System suitability solution*. [Note—Allow the run time about 6 times that of the diosmin retention time. The relative retention times for diosmin, acetoisovanillone, hesperidin, isorhoifolin, linarin, and diosmetin are 1, 0.5, 0.6, 0.8, 2.6, and 4.5, respectively.]

System suitability requirements

Chromatogram similarity: The chromatogram from the *System suitability solution* is similar to the Reference Chromatogram provided with the USP Diosmin for System Suitability RS being used.

Resolution: NLT 2.5 between hesperidin and isorhoifolin, *System suitability solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of each impurity in the portion of Diosmin taken: [Note—Disregard any impurity less than 0.1%.]

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak response for each impurity from the *Sample solution*

r_S = peak response for diosmin from the *Standard solution*

C_S = concentration of USP Diosmin RS in the *Standard solution* (mg/mL)

C_U = concentration of Diosmin in the *Sample solution* (mg/mL)

F = correction factor for each individual impurity (see *Table 1*)

Acceptance criteria

Total impurities: NMT 10%

Individual impurities: See *Table 1*.

Total other impurities and acetoisovanillone: NMT 1%

Table 1

Name	Relative Retention Time	Correction Factor (*F*)	Acceptance Criteria, NMT (%)
Acetoisovanillone[a]	0.5	0.3	1
Hesperidin[b]	0.6	1	5
Isorhoifolin[c]	0.8	1	3
Linarin[d]	2.6	1	3
Diosmetin[e]	4.5	0.5	3
Any other impurity	—	1	1
Total impurities	—	—	10

[a] 1-(3-Hydroxy-4-methoxyphenyl)ethanone.

[b] (2*S*)-7-[[6-*O*-(6-Deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-2,3-dihydro-4*H*-1-benzopyran-4-one.

[c] 7-[[6-*O*-(6-Deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-5-hydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one.

[d] 7-[[6-*O*-(6-Deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-5-hydroxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one.

[e] 5,7-Dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-4*H*-1-benzopyran-4-one.

SPECIFIC TESTS

• **Water Determination,** *Method Ia* ⟨921⟩

Sample: 0.3 g

Acceptance criteria: NMT 6.0%

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Preserve in well-closed, tight containers.

• **USP Reference Standards** ⟨11⟩

USP Diosmin RS

USP Diosmin for System Suitability RS

Echinacea angustifolia

DEFINITION

Echinacea angustifolia consists of the dried rhizome and roots of *Echinacea angustifolia* DC. (Fam. Asteraceae). It is harvested in the fall after one or more years of growth. It contains NLT 0.5% of total phenols, calculated on the dried basis as the sum of echinacoside ($C_{35}H_{46}O_{20}$), dicaffeoylquinic acid ($C_{25}H_{24}O_{12}$), and chlorogenic acid ($C_{16}H_{18}O_9$). It contains NLT 0.075% of dodecatetraenoic acid isobutylamides ($C_{16}H_{25}NO$) on the dried basis.

IDENTIFICATION

• **A. Thin-Layer Chromatography**

Presence of echinacoside and dicaffeoylquinic acid (cynarin(e))

Standard solution A: 0.2 mg/mL of USP Echinacoside RS and 0.2 mg/mL of dicaffeoylquinic acid (cynarin) in methanol

Standard solution B: 0.05 mg/mL of USP Caftaric Acid RS, 0.1 mg/mL of USP Chlorogenic Acid RS, and 0.05 mg/mL of USP Chicoric Acid RS in methanol

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Standard solution C: 20 mg/mL of USP Powdered *Echinacea angustifolia* Extract RS in methanol. Shake to disperse, sonicate for 5 min, and centrifuge. Use the supernatant.
Sample solution: Transfer 1 g of finely pulverized *Echinacea angustifolia* to a centrifuge tube, add 10 mL of methanol, mix well, and sonicate for 10 min. Centrifuge, and use the supernatant.
Chromatographic system
(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)
Application volume: 5 µL *Standard solution C* and *Sample solution*, and 2 µL *Standard solution A* and *Standard solution B* as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
Developing solvent system: A mixture of ethyl acetate, methylethyl ketone, water, and formic acid (5:3:1:1)
Developing distance: 6 cm
Derivatization reagent: 5 mg/mL of 2-aminoethyl diphenylborinate in ethyl acetate
Analysis
Samples: *Standard solution A, Standard solution B, Standard solution C,* and *Sample solution*
Apply the *Samples* as bands to a suitable thin-layer chromatographic plate, and dry in air. Develop the chromatograms in a saturated chamber. Remove the plate from the chamber, heat at 100° for 5 min, derivatize the plate while still warm with *Derivatization reagent*, dry in air, and examine under UV light at 366 nm.
System suitability: *Standard solution A* shows two major blue bands, one in the lower third of the chromatogram due to echinacoside, and the other band in the middle section of the chromatogram due to dicaffeoylquinic acid (cynarin). *Standard solution B* shows two major blue bands at about the middle of the chromatogram due to caftaric acid (higher R_F) and chlorogenic acid (lower R_F) that are clearly separated, and a blue band for chicoric acid in the upper third section of the chromatogram.
Acceptance criteria: The most prominent band in the *Sample solution* chromatogram is a blue band in the lower third section of the chromatogram at an R_F corresponding to the echinacoside band in the chromatogram of *Standard solution A* and *Standard solution C* (absent in *Echinacea purpurea*). The *Sample solution* chromatogram exhibits a prominent greenish blue band in the middle section of the chromatogram at an R_F corresponding to the dicaffeoylquinic acid (cynarin) band in the chromatogram of *Standard solution A* and *Standard solution C* (absent in *Echinacea pallida* and *Echinacea purpurea*). The *Sample solution* chromatogram does not exhibit, or exhibits very faint blue bands at an R_F corresponding to the caftaric acid and chicoric acid bands in *Standard solution B* (difference from *Echinacea pallida* and *Echinacea purpurea*). The *Sample solution* chromatogram exhibits minor bands between the positions of echinacoside and cynarin. One of these is due to chlorogenic acid at an R_F corresponding to that of chlorogenic acid in *Standard solution B*.

• **B. THIN-LAYER CHROMATOGRAPHY**
Presence of alkylamides
Standard solution A: 0.2 mg/mL of USP β-Sitosterol RS in methanol
Standard solution B: 100 mg/mL of USP Powdered *Echinacea angustifolia* Extract RS in dichloromethane. Shake to disperse, sonicate for 5 min, and centrifuge. Use the supernatant.
Sample solution: Transfer 1 g of finely pulverized *Echinacea angustifolia* to a centrifuge tube, add 10 mL of dichloromethane, mix well, and sonicate for 10 min. Centrifuge, and use the supernatant.
Chromatographic system
(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plates)
Application volume: 5 µL *Standard solution B* and *Sample solution*, and 2 µL *Standard solution A* as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
Developing solvent system: A mixture of toluene, ethyl acetate, cyclohexane, and formic acid (8: 2: 1: 0.3)
Developing distance: 6 cm
Derivatization reagent: Place 85 mL of methanol in a 100-mL glass bottle, and cool it down in a water–ice cubes–salt bath or in a freezer. To the ice-cold methanol, slowly and carefully add 10 mL of acetic acid and 5 mL of sulfuric acid, and mix well. Allow the mixture to cool to room temperature, then add 0.5 mL of *p*-anisaldehyde.
Analysis
Samples: *Standard solution A, Standard solution B,* and *Sample solution*
Apply the *Samples* as bands to a suitable thin-layer chromatographic plate, and dry in air. Develop the chromatograms in a saturated chamber. Remove the plate from the chamber, dry in air, derivatize with *Derivatization reagent*, heat at 100° for 3–5 min, set aside to cool, and examine under visible light.
System suitability: The β-sitosterol band of the *Standard solution B* chromatogram and the two bands below are clearly separated from one another. These two bands, in decreasing R_F, include a major blue violet band and a yellow band.
Acceptance criteria: The most prominent band of the *Sample solution* chromatogram is a blue violet band in the lower-third section of the chromatogram (much less prominent in *Echinacea purpurea* and absent in *Echinacea pallida*). This blue violet band is between two bands: a less prominent blue violet band at a higher R_F corresponding to the β-sitosterol band in the chromatograms of *Standard solution A* and *Standard solution B*, and a characteristic yellow band at a lower R_F (absent in *Echinacea purpurea* and *Echinacea pallida*). The yellow band turns reddish pink when the plate is heated at 100° for more than 10 min. The *Sample solution* chromatogram exhibits a minor pink-violet band at about the middle of the chromatogram (much more prominent in *Echinacea purpurea*), a minor pink-violet band at about two-thirds of the chromatogram (much more prominent in *Echinacea pallida*), and a broad pink-violet band close to the solvent front.

• **C.** The retention time of the major peak of the *Sample solution* corresponds to that of the echinacoside peak of *Standard solution A* and *Standard solution C;* and the retention time of the peak for 1,3-dicaffeoylquinic acid from the *Sample solution* corresponds to that of *Standard solution A,* all peaks as obtained in the test for *Content of Total Phenols*.

COMPOSITION

• **CONTENT OF TOTAL PHENOLS**
Solution A: Phosphoric acid (0.1 in 100) in water
Solution B: Acetonitrile

Supplement 2

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Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	90	10
3	90	10
16	78	22
17	60	40
20	60	40
20.5	90	10
25	90	10

Solvent: Alcohol and water (7:3)
Standard solution A: Dissolve USP Powdered *Echinacea angustifolia* Extract RS in *Solvent*, shaking and heating in a water bath. Dilute with *Solvent* to obtain a solution having a known concentration of 1 mg/mL. Pass through a membrane filter having a 0.45-µm or finer pore size.
Standard solution B: 40 µg/mL of USP Chlorogenic Acid RS in *Solvent*
Standard solution C: 80 µg/mL of USP Echinacoside RS in *Solvent*
Sample solution: Transfer about 125 mg of finely powdered *Echinacea angustifolia* (capable of passing through a 40-mesh sieve), accurately weighed, to a round-bottom flask equipped with a condenser. Add 25.0 mL of *Solvent*, and heat under reflux, while shaking by mechanical means for 15 min. Centrifuge, or pass through a membrane filter having a 0.45-µm or finer pore size.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 330 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 35°
Flow rate: 1.5 mL/min
Injection volume: 5 µL
System suitability
Samples: *Standard solution A* and *Standard solution C*
Suitability requirements
Chromatogram similarity: The chromatogram from *Standard solution A* is similar to the Reference Chromatogram for total phenols provided with the USP Powdered *Echinacea angustifolia* Extract RS.
Resolution: NLT 1.0 between the 1,3-dicaffeoylquinic acid isomer and echinacoside, *Standard solution A*. [NOTE—Echinacoside peak may be resolved in two components.]
Capacity factor (k′): NLT 3.0 from *Standard solution C*
Tailing factor: NMT 2.0 for the echinacoside peak, *Standard solution C*
Relative standard deviation: NMT 2.5% for the echinacoside peaks in repeated injections, *Standard solution C*
Analysis
Samples: *Standard solution A, Standard solution B, Standard solution C,* and *Sample solution*
Identify the relevant analytes in the chromatogram from the *Sample solution* by comparison with the chromatogram from *Standard solution A*. Measure the areas for the relevant peaks.
Separately calculate the percentage of chlorogenic acid ($C_{16}H_{18}O_9$), dicaffeoylquinic acids ($C_{25}H_{24}O_{12}$), and echinacoside ($C_{35}H_{46}O_{20}$) in the portion of *Echinacea angustifolia* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak response for the relevant analyte from the *Sample solution*
r_S = peak response for chlorogenic acid or both components of echinacoside from the corresponding *Standard solution*
C_S = concentration of chlorogenic acid or echinacoside in the corresponding *Standard solution* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of *Echinacea angustifolia* taken to prepare the *Sample solution* (mg)
F = response factor and is equal to 0.729 for dicaffeoylquinic acids, relative to chlorogenic acid, 1.00 for chlorogenic acid, and 1.00 for echinacoside components

Calculate the percentage of total phenols in the portion of *Echinacea angustifolia* taken by adding the individual percentages calculated.
Acceptance criteria: NLT 0.5% of total phenols on the dried basis

• **CONTENT OF DODECATETRAENOIC ACID ISOBUTYLAMIDES**
Mobile phase: Acetonitrile and water (55:45)
Standard solution A: Dissolve, with sonication, USP Powdered *Echinacea angustifolia* Extract RS in methanol, shaking for 10 min, and dilute with methanol to obtain a solution having a concentration of 5 mg/mL. Pass through a membrane filter having a 0.45-µm or finer pore size.
Standard solution B: 10 µg/mL of USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS in methanol
Sample solution: Transfer about 2.5 g, accurately weighed, of finely powdered *Echinacea angustifolia* (capable of passing through a 40-mesh sieve) to a round-bottom flask. Add 80 mL of methanol, and reflux for 30 min. Cool to room temperature, and filter into a 100-mL volumetric flask, using small portions of methanol to rinse the flask and the filter. Dilute with methanol to volume. Pass through a membrane filter having a 0.45-µm or finer pore size.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 30°
Flow rate: 1.5 mL/min
Injection volume: 25 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram from *Standard solution A* is similar to the reference chromatogram for alkamides provided with USP Powdered *Echinacea angustifolia* Extract RS.
Resolution: NLT 1.0 between dodecatetraenoic acid isobutylamide peaks, *Standard solution A*
Tailing factor: NMT 2.0 for the 2*E*,4*E*-hexadienoic acid isobutylamide peak, *Standard solution B*
Relative standard deviation: NMT 2.5% for the 2*E*,4*E*-hexadienoic acid isobutylamide peak in repeated injections, *Standard solution B*
Analysis
Samples: *Standard solution A, Standard solution B,* and *Sample solution*

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Identify the peaks due to 2*E*,4*E*,8*Z*,10*E*-dodecatetraenoic acid isobutylamide and 2*E*,4*E*,8*Z*,10*Z*-dodecatetraenoic acid isobutylamide in the chromatogram from the *Sample solution* by comparison with the chromatogram from *Standard solution A*. Measure the areas for the relevant peaks.

Calculate the percentage of dodecatetraenoic acid isobutylamides in the portion of *Echinacea angustifolia* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = sum of the peak responses of the relevant analytes from the *Sample solution*

r_S = peak response for 2*E*,4*E*-hexadienoic acid isobutylamide from *Standard solution B*

C_S = concentration of USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS in *Standard solution B* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of *Echinacea angustifolia* taken to prepare the *Sample solution* (mg)

F = response factor for 2*E*,4*E*-hexadienoic acid isobutylamide, 1.353

Acceptance criteria: NLT 0.075% of dodecatetraenoic acid isobutylamides ($C_{16}H_{25}NO$) on the dried basis

CONTAMINANTS

- **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩

Acceptance criteria

Arsenic: NMT 1.0 µg/g
Cadmium: NMT 0.5 µg/g
Lead: NMT 5.0 µg/g
Mercury: NMT 1.0 µg/g

Change to read:

- **▲ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩▲ (CN 1-May-2019)**:** Meets the requirements

SPECIFIC TESTS

- **BOTANIC CHARACTERISTICS**

Macroscopic: The outer surface of the rhizome is pale to yellowish brown, crowned with remains of the aerial stem, and sometimes showing surface annulations up to 15 mm in diameter. The roots are also pale to yellowish brown, cylindrical or slightly tapering, sometimes spirally twisted, longitudinally wrinkled and deeply furrowed, up to 4–10 mm in diameter, and passing imperceptibly into rhizome. The fracture is short when dry and becomes tough and pliable on exposure to air.

Microscopic: The rhizomes and roots in transverse section show a thin outer bark separated from a wide xylem by a distinct cambial line. The cork is composed of several rows of thin-walled cells containing yellowish-brown pigment. The rhizome has a small circular pith, occasional small groups of thick-walled, lignified fibers in the pericycle, and a parenchymatous cortex. The phloem and xylem are composed of narrow strands of vascular tissue separated by wide, nonlignified medullary rays. Xylem vessels are lignified, 25–75 µm in diameter, usually with reticulate thickening but occasionally with spiral or annular thickening. Sclereids occur singly or in small groups, varying considerably in size and shape from rounded to rectangular to elongated and fiber-like, up to 300 µm long and 20–40 µm wide, with intercellular spaces forming schizogenous oleoresin canals that are 80–150 µm in diameter and contain a dense black deposit. The canals are present outside of the central cylinder only (unlike *Echinacea pallida*, where they are present both inside and outside of the central cylinder). Spherocrystalline masses of inulin occur throughout the parenchymatous tissues. Lignified fibers, 300–800 µm long, are present in scattered groups, and are usually surrounded by phytomelanin (unlike fibers in *Echinacea pallida*, where they usually occur singly in the periphery of the cortex and are 100–300 µm long, with phytomelanin often absent).

- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩**:** NMT 3.0%
- **LOSS ON DRYING** ⟨731⟩

Analysis: Dry a sample at 105° for 2 h.
Acceptance criteria: NMT 10.0%

- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 7.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 4.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Store in well-closed, light-resistant containers.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Caftaric Acid RS
USP Chicoric Acid RS
USP Chlorogenic Acid RS
USP Powdered *Echinacea angustifolia* Extract RS
USP Echinacoside RS
USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS
USP β-Sitosterol RS

Powdered *Echinacea angustifolia*

DEFINITION

Powdered *Echinacea angustifolia* consists of the dried rhizome and roots of *Echinacea angustifolia* DC. (Fam. Asteraceae), harvested in the fall after one or more years of growth, and reduced to powder. It contains NLT 0.5% of total phenols, calculated on the dried basis as the sum of echinacoside ($C_{35}H_{46}O_{20}$), dicaffeoylquinic acid ($C_{25}H_{24}O_{12}$), and chlorogenic acid ($C_{16}H_{18}O_9$). It contains NLT 0.075% of dodecatetraenoic acid isobutylamides ($C_{16}H_{25}NO$) on the dried basis.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHY**

Presence of echinacoside and dicaffeoylquinic acid (cynarin(e))

Standard solution A: A mixture of 0.2 mg/mL of USP Echinacoside RS and 0.2 mg/mL of dicaffeoylquinic acid (cynarin) in methanol

Standard solution B: 0.05 mg/mL of USP Caftaric Acid RS, 0.1 mg/mL of USP Chlorogenic Acid RS, and 0.05 mg/mL of USP Chicoric Acid RS in methanol

Standard solution C: 20 mg/mL of USP Powdered *Echinacea angustifolia* Extract RS in methanol. Shake to disperse, sonicate for 5 min, and centrifuge. Use the supernatant.

Sample solution: Transfer 1 g of Powdered *Echinacea angustifolia* to a centrifuge tube, add 10 mL of methanol, mix well, and sonicate for 10 min. Centrifuge, and use the supernatant.

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

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Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)
Application volume: 5 µL *Standard solution C* and *Sample solution*, and 2 µL *Standard solution A* and *Standard solution B*, as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
Developing solvent system: A mixture of ethyl acetate, methylethyl ketone, water, and formic acid (5:3:1:1)
Developing distance: 6 cm
Derivatization reagent: 5 mg/mL of 2-aminoethyl diphenylborinate in ethyl acetate

Analysis
Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*
Apply the *Samples* as bands to a suitable thin-layer chromatographic plate, and dry in air. Develop the chromatograms in a saturated chamber. Remove the plate from the chamber, heat at 100° for 5 min, derivatize the plate while still warm with *Derivatization reagent*, dry in air, and examine under UV light at 366 nm.

System suitability: *Standard solution A* shows two major blue bands, one in the lower third of the chromatogram due to echinacoside, and the other band in the middle section of the chromatogram due to dicaffeoylquinic acid (cynarin). *Standard solution B* shows two major blue bands at about the middle of the chromatogram due to caftaric acid (higher R_F) and chlorogenic acid (lower R_F) that are clearly separated, and a blue band for chicoric acid in the upper third section of the chromatogram.

Acceptance criteria: The most prominent band in the *Sample solution* chromatogram is a blue band in the lower third section of the chromatogram at an R_F corresponding to the echinacoside band in the chromatogram of *Standard solution A* and *Standard solution C* (absent in *Echinacea purpurea*). The *Sample solution* chromatogram exhibits a prominent greenish blue band in the middle section of the chromatogram at an R_F corresponding to the dicaffeoylquinic acid (cynarin) band in the chromatogram of *Standard solution A* and *Standard solution C* (absent in *Echinacea pallida* and *Echinacea purpurea*). The *Sample solution* chromatogram does not exhibit, or exhibits very faint blue bands at an R_F corresponding to the caftaric acid and chicoric acid bands in *Standard solution B* (difference from *Echinacea pallida* and *Echinacea purpurea*). The *Sample solution* chromatogram exhibits minor bands between the positions of echinacoside and cynarin. One of these is due to chlorogenic acid at an R_F corresponding to that of chlorogenic acid in *Standard solution B*.

• **B. THIN-LAYER CHROMATOGRAPHY**

Presence of alkylamides
Standard solution A: 0.2 mg/mL of USP β-Sitosterol RS in methanol
Standard solution B: 100 mg/mL of USP Powdered *Echinacea angustifolia* Extract RS in dichloromethane. Shake to disperse, sonicate for 5 min, and centrifuge. Use the supernatant.
Sample solution: Transfer 1 g of Powdered *Echinacea angustifolia* to a centrifuge tube, add 10 mL of dichloromethane, mix well, and sonicate for 10 min. Centrifuge, and use the supernatant.
Chromatographic system
(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plates)
Application volume: 5 µL *Standard solution B* and *Sample solution*, and 2 µL *Standard solution A* as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
Developing solvent system: A mixture of toluene, ethyl acetate, cyclohexane, and formic acid (8: 2: 1: 0.3)
Developing distance: 6 cm
Derivatization reagent: Place 85 mL of methanol in a 100-mL glass bottle, and cool it down in a water–ice cubes–salt bath or in a freezer. To the ice-cold methanol, slowly and carefully add 10 mL of acetic acid and 5 mL of sulfuric acid, and mix well. Allow the mixture to cool to room temperature, then add 0.5 mL of *p*-anisaldehyde.

Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Apply the *Samples* as bands to a suitable thin-layer chromatographic plate, and dry in air. Develop the chromatograms in a saturated chamber. Remove the plate from the chamber, dry in air, derivatize with *Derivatization reagent*, heat at 100° for 3–5 min, dry in air, and examine under visible light.

System suitability: The β-sitosterol band of the *Standard solution B* chromatogram and the two bands underneath are clearly separated from one another. These two bands, in decreasing R_F, include a major blue violet band and a yellow band.

Acceptance criteria: The most prominent band of the *Sample solution* chromatogram is a blue violet band in the lower-third section of the chromatogram (much less prominent in *Echinacea purpurea* and absent in *Echinacea pallida*). This blue violet band is between two bands: a less prominent blue violet band at a higher R_F corresponding to the β-sitosterol band in the chromatograms of *Standard solution A* and *Standard solution B*, and a characteristic yellow band at a lower R_F (absent in *Echinacea purpurea* and *Echinacea pallida*). The yellow band turns reddish pink when the plate is heated at 100° for more than 10 min. The *Sample solution* chromatogram exhibits a minor pink-violet band at about the middle of the chromatogram (much more prominent in *Echinacea purpurea*), a minor pink-violet band at about two-thirds of the chromatogram (much more prominent in *Echinacea pallida*), and a broad pink-violet band close to the solvent front.

• **C.** The retention time of the major peak of the *Sample solution* corresponds to that of the echinacoside peak of *Standard solution A* and *Standard solution C*, and the retention time of the 1,3-dicaffeoylquinic acid peak from the *Sample solution* corresponds to that of *Standard solution A*, all peaks as obtained in the test for *Content of Total Phenols*.

COMPOSITION

• **CONTENT OF TOTAL PHENOLS**
Solution A: Phosphoric acid (0.1 in 100) in water
Solution B: Acetonitrile
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	90	10
3	90	10
16	78	22
17	60	40

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Table 1 *(continued)*

Time (min)	Solution A (%)	Solution B (%)
20	60	40
20.5	90	10
25	90	10

Solvent: Alcohol and water (7:3)
Standard solution A: Dissolve USP Powdered *Echinacea angustifolia* Extract RS in *Solvent*, shaking and heating in a water bath. Dilute with *Solvent* to obtain a solution having a known concentration of 1 mg/mL. Pass through a membrane filter having a 0.45-µm or finer pore size.
Standard solution B: 40 µg/mL of USP Chlorogenic Acid RS in *Solvent*
Standard solution C: 80 µg/mL of USP Echinacoside RS in *Solvent*
Sample solution: Transfer about 125 mg of Powdered *Echinacea angustifolia* (capable of passing through a 40-mesh sieve), accurately weighed, to a round-bottom flask equipped with a condenser. Add 25.0 mL of *Solvent*, and heat under reflux while shaking by mechanical means for 15 min. Centrifuge, or pass through a membrane filter having a 0.45-µm or finer pore size.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 330 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 35°
Flow rate: 1.5 mL/min
Injection volume: 5 µL
System suitability
Samples: *Standard solution A* and *Standard solution C*
Suitability requirements
Chromatogram similarity: The chromatogram from *Standard solution A* is similar to the Reference Chromatogram for total phenols provided with the USP Powdered *Echinacea angustifolia* Extract RS.
Resolution: NLT 1.0 between the 1,3-dicaffeoylquinic acid isomer and echinacoside peaks, *Standard solution A*. [NOTE—Echinacoside peak may be resolved in two components.]
Capacity factor (k′): NLT 3.0, *Standard solution C*
Tailing factor: NMT 2.0 for the echinacoside peak, *Standard solution C*
Relative standard deviation: NMT 2.5% for the echinacoside peaks in repeated injections, *Standard solution C*
Analysis
Samples: *Standard solution A*, *Standard solution B*, *Standard solution C* and *Sample solution*
Identify the relevant analytes in the chromatogram from the *Sample solution* by comparison with the chromatogram from *Standard solution A*. Measure the areas for the relevant peaks.
Separately calculate the percentage of chlorogenic acid ($C_{16}H_{18}O_9$), dicaffeoylquinic acids ($C_{25}H_{24}O_{12}$), and echinacoside ($C_{35}H_{46}O_{20}$) in the portion of Powdered *Echinacea angustifolia* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak response for the relevant analyte from the *Sample solution*
r_S = peak response for chlorogenic acid or both components of echinacoside from the corresponding *Standard solution*
C_S = concentration of chlorogenic acid or echinacoside in the corresponding *Standard solution* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Powdered *Echinacea angustifolia* used to prepare the *Sample solution* (mg)
F = response factor: 0.729 for dicaffeoylquinic acids, relative to chlorogenic acid, 1.00 for chlorogenic acid, and 1.00 for echinacoside components

Calculate the percentage of total phenols in the portion of Powdered *Echinacea angustifolia* taken by adding the individual percentages calculated.
Acceptance criteria: NLT 0.5% of total phenols on the dried basis

• **CONTENT OF DODECATETRAENOIC ACID ISOBUTYLAMIDES**
Standard solution A: Dissolve, with sonication, USP Powdered *Echinacea angustifolia* Extract RS in methanol, shaking for 10 min, and dilute with methanol to obtain a solution having a concentration of 5 mg/mL. Pass through a membrane filter having a 0.45-µm or finer pore size.
Standard solution B: 10 µg/mL of USP *2E,4E*-Hexadienoic Acid Isobutylamide RS in methanol
Sample solution: Transfer about 2.5 g of Powdered *Echinacea angustifolia* (capable of passing through a 40-mesh sieve), accurately weighed, into a round-bottom flask. Add 80 mL of methanol, and reflux for 30 min. Cool to room temperature, and filter into a 100-mL volumetric flask, using small portions of methanol to rinse the flask and the filter. Dilute with methanol to volume. Pass through a membrane filter having a 0.45-µm or finer pore size.
Mobile phase: Acetonitrile and water (55:45)
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 30°
Flow rate: 1.5 mL/min
Injection volume: 25 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram from *Standard solution A* is similar to the Reference Chromatogram for alkamides provided with USP Powdered *Echinacea angustifolia* Extract RS.
Resolution: NLT 1.0 between dodecatetraenoic acid isobutylamide peaks, *Standard solution A*
Tailing factor: NMT 2.0 for the *2E,4E*-hexadienoic acid isobutylamide peak, *Standard solution B*
Relative standard deviation: NMT 2.5% for the *2E,4E*-hexadienoic acid isobutylamide peak in repeated injections, *Standard solution B*
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Identify the peaks due to *2E,4E,8Z,10E*-dodecatetraenoic acid isobutylamide and *2E,4E,8Z,10Z*-dodecatetraenoic acid isobutylamide in the chromatogram from the *Sample solution* by comparison with the chromatogram from *Standard solution A*. Measure the areas for the relevant peaks.
Calculate the percentage of dodecatetraenoic acid isobutylamides in the portion of Powdered *Echinacea angustifolia* taken:

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$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = sum of the peak responses of the relevant analytes from the *Sample solution*
r_S = peak response of 2*E*,4*E*-hexadienoic acid isobutylamide from *Standard solution B*
C_S = concentration of USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS in *Standard solution B* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Powdered *Echinacea angustifolia* used to prepare the *Sample solution* (mg)
F = response factor for 2*E*,4*E*-hexadienoic acid isobutylamide, 1.353

Acceptance criteria: NLT 0.075% of dodecatetraenoic acid isobutylamides ($C_{16}H_{25}NO$) on the dried basis

CONTAMINANTS

- **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩

Acceptance criteria
Arsenic: NMT 1.0 µg/g
Cadmium: NMT 0.5 µg/g
Lead: NMT 5.0 µg/g
Mercury: NMT 1.0 µg/g

Change to read:

- **▲ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩▲ (CN 1-May-2019)**:** Meets the requirements

SPECIFIC TESTS

- **BOTANIC CHARACTERISTICS:** Powdered *Echinacea angustifolia* is a brown powder with a slight aromatic odor and a sweet taste that quickly becomes bitter, leaving a tingling sensation on the tongue. Under a microscope, the following characteristics are observed: thin-walled polygonal cork cells with red-brown contents; lignified reticulate vessels; abundant stone cells of various shapes; fragments of oleoresin canals with reddish-brown contents; and abundant thin-walled parenchyma with spherocrystalline masses of inulin.
- **LOSS ON DRYING** ⟨731⟩

Analysis: Dry a sample at 105° for 2 h.
Acceptance criteria: NMT 10.0%

- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 7.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 4.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed, light-resistant containers.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Caftaric Acid RS
USP Chicoric Acid RS
USP Chlorogenic Acid RS
USP Powdered *Echinacea angustifolia* Extract RS
USP Echinacoside RS
USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS
USP β-Sitosterol RS

Powdered *Echinacea angustifolia* Extract

DEFINITION

Powdered *Echinacea angustifolia* Extract is prepared from *Echinacea angustifolia* roots by extraction with hydroalcoholic mixtures or other suitable solvents. The ratio of the starting crude plant material to Powdered Extract is 2:1–8:1. It contains NLT 4.0% and NMT 5.0% of total phenols, calculated on the dried basis as the sum of chlorogenic acid ($C_{16}H_{18}O_9$), dicaffeoylquinic acids ($C_{25}H_{24}O_{12}$), and echinacoside ($C_{35}H_{46}O_{20}$). It contains NLT 0.1% of dodecatetraenoic acid isobutylamides ($C_{16}H_{25}NO$) on the dried basis.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHY**

Presence of echinacoside and dicaffeoylquinic acid (cynarin(e))
Standard solution A: A mixture of 0.2 mg/mL of USP Echinacoside RS and 0.2 mg/mL of dicaffeoylquinic acid (cynarin) in methanol
Standard solution B: 0.05 mg/mL of USP Caftaric Acid RS, 0.1 mg/mL of USP Chlorogenic Acid RS, and 0.05 mg/mL of USP Chicoric Acid RS in methanol
Standard solution C: 20 mg/mL of USP Powdered *Echinacea angustifolia* Extract RS in methanol. Shake to disperse, sonicate for 5 min, and centrifuge. Use the supernatant.
Sample solution: 20 mg/mL of Powdered *Echinacea angustifolia* Extract in methanol. Shake to disperse, sonicate for 5 min, and centrifuge. Use the supernatant.
Chromatographic system
(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)
Application volume: 5 µL *Standard solution C* and *Sample solution*, and 2 µL *Standard solution A* and *Standard solution B* as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
Developing solvent system: A mixture of ethyl acetate, methylethyl ketone, water, and formic acid (5:3:1:1)
Developing distance: 6 cm
Derivatization reagent: 5 mg/mL of 2-aminoethyl diphenylborinate in ethyl acetate
Analysis
Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*
Apply the samples as bands to a suitable thin-layer chromatographic plate, and dry in air. Develop the chromatograms in a saturated chamber. Remove the plate from the chamber, heat at 100° for 5 min, derivatize the plate while still warm with *Derivatization reagent*, dry in air, and examine under UV light at 366 nm.
System suitability: *Standard solution A* shows two major blue bands, one in the lower third of the chromatogram due to echinacoside, and the other band in the middle section of the chromatogram due to dicaffeoylquinic acid (cynarin). *Standard solution B* shows two major blue bands at about the middle of the chromatogram due to caftaric acid (higher R_F) and chlorogenic acid (lower R_F) that are clearly separated, and a blue band for chicoric acid in the upper third of the chromatogram.
Acceptance criteria: The most prominent band in the *Sample solution* chromatogram is a blue band in the lower third of the chromatogram at an R_F corresponding to the echinacoside band in the chromatograms of *Standard*

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solution A and *Standard solution C* (absent in *Echinacea purpurea*). The *Sample solution* chromatogram exhibits a prominent greenish blue band in the middle section of the chromatogram at an R_F corresponding to the dicaffeoylquinic acid (cynarin) band in the chromatograms of *Standard solution A* and *Standard solution C* (absent in *Echinacea pallida* and *Echinacea purpurea*). The *Sample solution* chromatogram does not exhibit, or exhibits very faint blue bands at an R_F corresponding to the caftaric acid and chicoric acid bands in *Standard solution B* (difference from *Echinacea pallida* and *Echinacea purpurea*). The *Sample solution* chromatogram exhibits minor bands between the positions of echinacoside and cynarin. One of these is due to chlorogenic acid at an R_F corresponding to that of chlorogenic acid in *Standard solution B*.

• **B. THIN-LAYER CHROMATOGRAPHY**

Presence of alkylamides

Standard solution A: 0.2 mg/mL of USP β-Sitosterol RS in methanol

Standard solution B: 100 mg/mL of USP Powdered *Echinacea angustifolia* Extract RS in dichloromethane. Shake to disperse, sonicate for 5 min, and centrifuge. Use the supernatant.

Sample solution: 100 mg/mL of Powdered *Echinacea angustifolia* Extract in dichloromethane. Shake to disperse, sonicate for 5 min, and centrifuge. Use the supernatant.

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plates)

Application volume: 5 µL *Standard solution B* and *Sample solution*, and 2 µL *Standard solution A* as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

Developing solvent system: A mixture of toluene, ethyl acetate, cyclohexane, and formic acid (8: 2: 1: 0.3)

Developing distance: 6 cm

Derivatization reagent: Place 85 mL of methanol in a 100-mL glass bottle, and cool it down in a water–ice cubes–salt bath or in a freezer. To the ice-cold methanol, slowly and carefully add 10 mL of acetic acid and 5 mL of sulfuric acid, and mix well. Allow the mixture to cool to room temperature, then add 0.5 mL of *p*-anisaldehyde.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the samples as bands to a suitable thin-layer chromatographic plate, and dry in air. Develop the chromatograms in a saturated chamber. Remove the plate from the chamber, dry in air, derivatize with *Derivatization reagent*, heat at 100° for 3–5 min, dry in air, and examine under visible light.

System suitability: The β-sitosterol band of the *Standard solution B* chromatogram and the two bands underneath are clearly separated from one another. These two bands, in decreasing R_F, include a major blue violet band and a yellow band.

Acceptance criteria: The most prominent band of the *Sample solution* chromatogram is a blue violet band in the lower-third section of the chromatogram (much less prominent in *Echinacea purpurea* and absent in *Echinacea pallida*). This blue violet band is between two bands: a less prominent blue violet band at a higher R_F corresponding to the β-sitosterol band in the chromatograms of *Standard solution A* and *Standard solution B*, and a characteristic yellow band at a lower R_F (absent in *Echinacea purpurea* and *Echinacea pallida*). The yellow band turns reddish pink when the plate is heated at 100° for more than 10 min. The *Sample solution* chromatogram exhibits a minor pink-violet band at about the middle of the chromatogram (much more prominent in *Echinacea purpurea*), a minor pink-violet band at about two-thirds of the chromatogram (much more prominent in *Echinacea pallida*), and a broad pink-violet band close to the solvent front.

• **C.** The retention time of the major peak of the *Sample solution* corresponds to that of the echinacoside peak of *Standard solution A* and *Standard solution C;* and the retention time of the peak for 1,3-dicaffeoylquinic acid from the *Sample solution* corresponds to that of *Standard solution A*, all peaks as obtained in the test for *Content of Total Phenols*.

COMPOSITION

• **CONTENT OF TOTAL PHENOLS**

Solution A: Phosphoric acid (0.1 in 100) in water

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	90	10
3	90	10
16	78	22
17	60	40
20	60	40
20.5	90	10
25	90	10

Solvent: Alcohol and water (7:3)

Standard solution A: Dissolve USP Powdered *Echinacea angustifolia* Extract RS in *Solvent*, shaking and heating in a water bath. Dilute with *Solvent* to obtain a solution having a known concentration of 1 mg/mL. Pass through a membrane filter having a 0.45-µm or finer pore size.

Standard solution B: 40 µg/mL of USP Chlorogenic Acid RS in *Solvent*

Standard solution C: 80 µg/mL of USP Echinacoside RS in *Solvent*

Sample solution: Transfer about 60 mg of Powdered Extract, accurately weighed, to an appropriate round-bottom flask equipped with a condenser. Add 25.0 mL of *Solvent*, and heat under reflux while shaking by mechanical means for 15 min. Centrifuge, or pass through a membrane filter having a 0.45-µm or finer pore size.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 330 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 35°

Flow rate: 1.5 mL/min

Injection volume: 5 µL

System suitability

Samples: *Standard solution A* and *Standard solution C*

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution A* is similar to the Reference Chromatogram for total phenols provided with the USP Powdered *Echinacea angustifolia* Extract RS.

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Resolution: NLT 1.0 between the 1,3-dicaffeoylquinic acid isomer and echinacoside peaks, *Standard solution A*. [NOTE—Echinacoside peak may be resolved in two components.]
Capacity factor (k′): NLT 3.0, *Standard solution C*
Tailing factor: NMT 2.0 for the echinacoside peak, *Standard solution C*
Relative standard deviation: NMT 2.5% for the echinacoside peaks, *Standard solution C*

Analysis
Samples: *Standard solution A*, *Standard solution B*, *Standard solution C* and *Sample solution*
Identify the relevant analytes in the chromatogram from the *Sample solution* by comparison with the chromatogram from *Standard solution A*. Measure the areas for the relevant peaks.
Separately calculate the percentage of chlorogenic acid ($C_{16}H_{18}O_9$), dicaffeoylquinic acids ($C_{25}H_{24}O_{12}$), and echinacoside ($C_{35}H_{46}O_{20}$) in the portion of Powdered Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak response for the relevant analyte from the *Sample solution*
r_S = peak response for chlorogenic acid or both components of echinacoside from the corresponding *Standard solution*
C_S = concentration of chlorogenic acid or echinacoside in the corresponding *Standard solution* (mg/mL)
C_U = concentration of Powdered *Echinacea angustifolia* Extract in the *Sample solution* (mg/mL)
F = 0.729 for dicaffeoylquinic acids, 1.00 for chlorogenic acid, and 1.00 for echinacoside components

Calculate the percentage of total phenols in the portion of Powdered Extract taken by adding the individual percentages.
Acceptance criteria: NLT 4.0% and NMT 5.0% of total phenols on the dried basis

• **CONTENT OF DODECATETRAENOIC ACID ISOBUTYLAMIDES**
Standard solution A: Dissolve USP Powdered *Echinacea angustifolia* Extract RS in methanol, shaking for 1 min, and dilute with methanol to volume to obtain a solution having a known concentration of 1 mg/mL. Pass through a membrane filter having a 0.45-µm or finer pore size.
Standard solution B: 10 µg/mL of USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS in methanol
Sample solution: Transfer about 500 mg of Powdered Extract, accurately weighed, to a 100-mL volumetric flask. Add 80 mL of methanol, and sonicate for 30 min. Dilute with methanol to volume, and pass through a membrane filter having a 0.45-µm or finer pore size.
Mobile phase: Acetonitrile and water (55:45)
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 30°
Flow rate: 1.5 mL/min
Injection volume: 25 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram from *Standard solution A* is similar to the Reference Chromatogram for alkamides provided with USP Powdered *Echinacea angustifolia* Extract RS.
Resolution: NLT 1.0 between dodecatetraenoic acid isobutylamide peaks, *Standard solution A*
Tailing factor: NMT 2.0 for the 2*E*,4*E*-hexadienoic acid isobutylamide peak, *Standard solution B*
Relative standard deviation: NMT 2.5% for the 2*E*,4*E*-hexadienoic acid isobutylamide peak in repeated injections, *Standard solution B*

Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Identify the peaks due to 2*E*,4*E*,8*Z*,10*E*-dodecatetraenoic acid isobutylamide and 2*E*,4*E*,8*Z*,10*Z*-dodecatetraenoic acid isobutylamide in the chromatogram from the *Sample solution* by comparison with the chromatogram from *Standard solution A*. Measure the areas for the relevant peaks.
Calculate the percentage of dodecatetraenoic acid isobutylamides in the portion of Powdered Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = sum of the peak responses of the relevant analytes from the *Sample solution*
r_S = peak response for 2*E*,4*E*-hexadienoic acid isobutylamide from *Standard solution B*
C_S = concentration of USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS in *Standard solution B* (mg/mL)
C_U = concentration of Powdered *Echinacea angustifolia* Extract in the *Sample solution* (mg/mL)
F = response factor for 2*E*,4*E*-hexadienoic acid isobutylamide, 1.353

Acceptance criteria: NLT 0.1% of dodecatetraenoic acid isobutylamides ($C_{16}H_{25}NO$) on the dried basis

CONTAMINANTS

• **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩
Acceptance criteria
Arsenic: NMT 1.0 µg/g
Cadmium: NMT 0.5 µg/g
Lead: NMT 5.0 µg/g
Mercury: NMT 1.0 µg/g
• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total bacterial count does not exceed 10^4 cfu/g and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.
• **OTHER REQUIREMENTS:** It meets the requirements for *Botanical Extracts* ⟨565⟩, *Residual Solvents* and *Pesticide Residues*.

SPECIFIC TESTS

• **LOSS ON DRYING** ⟨731⟩
Sample: 1 g
Analysis: Dry the *Sample* at 105° for 2 h.
Acceptance criteria: NMT 5.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, in a cool place.
• **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. If standardized by the content of alkamides, label it to indicate the targeted content of dodecatraenoic acid isobutylamides. The label bears a statement indicating that *Echinacea angustifolia* may cause

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rare allergic reactions, rashes, or aggravate asthma. It meets the requirements for *Botanical Extracts* ⟨565⟩, *Labeling.*

• **USP REFERENCE STANDARDS** ⟨11⟩
USP Caftaric Acid RS
USP Chicoric Acid RS
USP Chlorogenic Acid RS
USP Powdered *Echinacea angustifolia* Extract RS
USP Echinacoside RS
USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS
USP β-Sitosterol RS

Echinacea pallida

DEFINITION

Echinacea pallida consists of the dried rhizome and roots of *Echinacea pallida* (Nutt.) Nutt. (Fam. Asteraceae). It is harvested in the fall after three or more years of growth. It contains NLT 0.5% of total phenols, calculated on the dried basis as the sum of caftaric acid ($C_{13}H_{12}O_9$), chicoric acid ($C_{22}H_{18}O_{12}$), chlorogenic acid ($C_{16}H_{18}O_9$), and echinacoside ($C_{35}H_{46}O_{20}$).

IDENTIFICATION

• **A. THIN-LAYER CHROMATOGRAPHY**

Presence of echinacoside and absence of dicaffeoylquinic acid (cynarin(e))

Standard solution A: A mixture of 0.2 mg/mL of USP Echinacoside RS and 0.2 mg/mL of dicaffeoylquinic acid (cynarin) in methanol

Standard solution B: 0.05 mg/mL of USP Caftaric Acid RS, 0.1 mg/mL of USP Chlorogenic Acid RS, and 0.05 mg/mL of USP Chicoric Acid RS in methanol

Standard solution C: 20 mg/mL of USP Powdered *Echinacea pallida* Extract RS in methanol. Shake to disperse, sonicate for 5 min, and centrifuge. Use the supernatant.

Sample solution: Transfer 1 g of finely pulverized *Echinacea pallida* to a centrifuge tube, add 10 mL of methanol, mix well, and sonicate for 10 min. Centrifuge, and use the supernatant.

Chromatographic system
(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography.*)

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)

Application volume: 5 µL *Standard solution C* and *Sample solution*, and 2 µL *Standard solution A* and *Standard solution B* as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

Developing solvent system: A mixture of ethyl acetate, methylethyl ketone, water, and formic acid (5:3:1:1)

Developing distance: 6 cm

Derivatization reagent: 5 mg/mL of 2-aminoethyl diphenylborinate in ethyl acetate

Analysis

Samples: *Standard solution A, Standard solution B, Standard solution C,* and *Sample solution*

Apply the *Samples* as bands to a suitable thin-layer chromatographic plate, and dry in air. Develop the chromatograms in a saturated chamber. Remove the plate from the chamber, heat at 100° for 5 min, derivatize the plate while still warm with *Derivatization reagent,* dry in air, and examine under UV light at 366 nm.

System suitability: *Standard solution A* shows two major blue bands, one in the lower third section of the chromatogram due to echinacoside, and the other band in the middle section of the chromatogram due to dicaffeoylquinic acid (cynarin). *Standard solution B* shows two major blue bands at about the middle of the chromatogram due to caftaric acid (higher R_F) and chlorogenic acid (lower R_F) that are clearly separated, and a blue band for chicoric acid in the upper third section of the chromatogram.

Acceptance criteria: The most prominent band in the *Sample solution* chromatogram is a blue band in the lower third section of the chromatogram at an R_F corresponding to the echinacoside band in the chromatograms of *Standard solution A* and *Standard solution C* (absent in *Echinacea purpurea*). The *Sample solution* chromatogram does not exhibit a blue band at an R_F corresponding to the dicaffeoylquinic acid (cynarin) band in the chromatogram of *Standard solution A* (present in *Echinacea angustifolia*). The *Sample solution* chromatogram may exhibit bands of lesser intensity at the R_F of caftaric acid and chicoric acid bands in chromatograms of *Standard solution B* and *Standard solution C* (absent in *Echinacea angustifolia* and much more prominent in *Echinacea purpurea*). The *Sample solution* chromatogram exhibits minor bands between the positions of echinacoside and caftaric acid. One of these is due to chlorogenic acid at an R_F corresponding to that of chlorogenic acid in *Standard solution B.*

• **B. THIN-LAYER CHROMATOGRAPHY**

Presence of alkylamides

Standard solution A: 0.2 mg/mL of USP β-Sitosterol RS in methanol

Standard solution B: 100 mg/mL of USP Powdered *Echinacea pallida* Extract RS in dichloromethane. Shake to disperse, sonicate for 5 min, and centrifuge. Use the supernatant.

Sample solution: Transfer 1 g of finely pulverized *Echinacea pallida* to a centrifuge tube, add 10 mL of dichloromethane, mix well, and sonicate for 10 min. Centrifuge, and use the supernatant.

Chromatographic system
(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography.*)

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plates)

Application volume: 5 µL *Standard solution B* and *Sample solution*, and 2 µL *Standard solution A* as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

Developing solvent system: A mixture of toluene, ethyl acetate, cyclohexane, and formic acid (8: 2: 1: 0.3)

Developing distance: 6 cm

Derivatization reagent: Place 85 mL of methanol in a 100-mL glass bottle, and cool it down in a water–ice cubes–salt bath or in a freezer. To the ice-cold methanol, slowly and carefully add 10 mL of acetic acid and 5 mL of sulfuric acid, and mix well. Allow the mixture to cool to room temperature, then add 0.5 mL of *p*-anisaldehyde.

Analysis

Samples: *Standard solution A, Standard solution B,* and *Sample solution*

Apply the *Samples* as bands to a suitable thin-layer chromatographic plate, and dry in air. Develop the chromatograms in a saturated chamber. Remove the plate from the chamber, dry in air, derivatize with *Derivatization reagent,* heat at 100° for 3–5 min, set aside to cool, and examine under visible light.

System suitability: The *Standard solution A* chromatogram exhibits a violet band corresponding to β-sitosterol. The

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Standard solution B shows the most prominent band as a violet band in the upper third section of the chromatogram. The *Standard solution B* chromatogram exhibits a less prominent violet band in the lower third section of the chromatogram and a broad pink violet band close to the solvent front.

Acceptance criteria: The most prominent band of the *Sample solution* chromatogram is a violet band in the upper third section of the chromatogram, corresponding in R_F to a similar band observed in *Standard solution B* (much less prominent in *Echinacea angustifolia* and absent in *Echinacea purpurea*). The *Sample solution* chromatogram exhibits a less prominent violet band in the lower third section of the chromatogram corresponding in R_F to a similar band observed in *Standard solution B* (much less prominent in *Echinacea purpurea* and absent in *Echinacea angustifolia*). The *Sample solution* chromatogram exhibits a minor violet band at an R_F corresponding to the β-sitosterol band in the chromatograms of *Standard solution A* and *Standard solution B* and a broad pink violet band close to the solvent front.

• **C.** The retention time of the major peak in the *Sample solution* corresponds to that of the echinacoside peak in *Standard solution A* and *Standard solution C*, as obtained in the test for *Content of Total Phenols*. The peak area of any peak detected in the *Sample solution* chromatogram at the locus of 1,3-dicaffeoylquinic acid (*Standard solution D*) is NMT 1% of the peak area for the echinacoside peak.

COMPOSITION

• **CONTENT OF TOTAL PHENOLS**

Solution A: Phosphoric acid (0.1 in 100) in water
Solution B: Acetonitrile
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	90	10
3	90	10
16	78	22
17	60	40
20	60	40
20.5	90	10
25	90	10

Solvent: Alcohol and water (7:3)
Standard solution A: Dissolve USP Powdered *Echinacea pallida* Extract RS in *Solvent* by shaking and heating in a water bath. Dilute with *Solvent* to obtain a solution having a known concentration of 1 mg/mL. Pass through a membrane filter having a 0.45-µm or finer pore size.
Standard solution B: 40 µg/mL of USP Chlorogenic Acid RS in *Solvent*
Standard solution C: 80 µg/mL of USP Echinacoside RS in *Solvent*
Standard solution D: 40 µg/mL of dicaffeoylquinic acid (cynarin) in *Solvent*
Sample solution: Transfer 125 mg of finely powdered *Echinacea pallida* (capable of passing through a 40-mesh sieve) to a round-bottom flask equipped with a condenser. Add 25.0 mL of *Solvent*, and heat under reflux while shaking by mechanical means for 15 min. Centrifuge, or pass through a membrane filter having a 0.45-µm or finer pore size.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 330 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 35°
Flow rate: 1.5 mL/min
Injection volume: 5 µL

System suitability
Samples: *Standard solution A* and *Standard solution C*
Suitability requirements
Chromatogram similarity: The chromatogram of *Standard solution A* is similar to the Reference Chromatogram for total phenols provided with USP Powdered *Echinacea pallida* Extract RS.
Capacity factor (k′): NLT 3.0 for the echinacoside peak, *Standard solution C*. [NOTE—Echinacoside peak may be resolved in two components.]
Tailing factor: NMT 2.0 for the echinacoside peak, *Standard solution C*
Relative standard deviation: NMT 2.5% for the echinacoside peaks in repeated injections, *Standard solution C*

Analysis
Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, *Standard solution D*, and *Sample solution*
Identify the relevant analytes in the chromatogram from the *Sample solution* by comparison with the chromatogram from *Standard solution A*. Measure the areas for the relevant peaks.
Separately calculate the percentage of caftaric acid ($C_{13}H_{12}O_9$), chicoric acid ($C_{22}H_{18}O_{12}$), chlorogenic acid ($C_{16}H_{18}O_9$), and echinacoside ($C_{35}H_{46}O_{20}$) in the portion of *Echinacea pallida* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak response for the relevant analyte from the *Sample solution*
r_S = peak response for chlorogenic acid or both components of echinacoside from the corresponding *Standard solution*
C_S = concentration of chlorogenic acid or echinacoside in the corresponding *Standard solution* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of powdered *Echinacea pallida* used to prepare the *Sample solution* (mg)
F = response factor: chicoric acid, 0.695; caftaric acid, 0.881; chlorogenic acid, 1.000; relative to chlorogenic acid; and echinacoside components, 1.000

Calculate the percentage of total phenols in the portion of *Echinacea pallida* taken by adding the individual percentages calculated.
Acceptance criteria: NLT 0.5% of total phenols on the dried basis

CONTAMINANTS

• **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩

Acceptance criteria
Arsenic: NMT 1.0 µg/g
Cadmium: NMT 0.5 µg/g
Lead: NMT 5.0 µg/g
Mercury: NMT 1.0 µg/g

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Change to read:

- **▲ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩▲ (CN 1-May-2019)**:** Meets the requirements

SPECIFIC TESTS

- **BOTANIC CHARACTERISTICS**

Macroscopic: The outer surface of the rhizome is pale to yellowish-brown, crowned with the remains of the aerial stem, and sometimes shows surface annulations up to 15 mm in diameter. The roots are pale to yellowish-brown, cylindrical or slightly tapering, sometimes spirally twisted, longitudinally wrinkled and deeply furrowed, up to 4–10 mm in diameter, and pass imperceptibly into rhizome. The short fracture, when dry, becomes tough and pliable on exposure to air.

Microscopic: The rhizomes and roots in transverse section show a thin outer bark separated from a wide xylem by a distinct cambial line. The cork is composed of several rows of thin-walled cells containing yellowish-brown pigment. The rhizome has a small circular pith, occasional small groups of thick-walled, lignified fibers in the pericycle, and a parenchymatous cortex. The phloem and xylem are composed of narrow strands of vascular tissue separated by wide, nonlignified medullary rays. Xylem vessels are lignified, 25–75 µm in diameter, usually with reticulate thickening but occasionally with spiral or annular thickening. Sclereids occur singly or in small groups, varying considerably in size and shape from rounded to rectangular to elongated and fiber-like, are up to 300 µm long and 20–40 µm wide, with intercellular spaces forming schizogenous oleoresin canals that are 80–150 µm in diameter and contain a dense black deposit present both inside and outside of the central cylinder (unlike *Echinacea angustifolia*, where the canals are present only outside of the central cylinder). Spherocrystalline masses of inulin occur throughout the parenchymatous tissues. Lignified fibers, present in the periphery of the cortex, are 100–300 µm long and occur singly with phytomelanin often absent (unlike *Echinacea angustifolia*, where the fibers occur scattered in groups, are 300–800 µm long, and are usually surrounded by phytomelanin).

- **ARTICLES OF BOTANICAL ORIGIN,** *Volatile Oil Determination* ⟨561⟩**:** 1.0–2.0 mL/100 g
- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩**:** NMT 3.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 7.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 4.0%
- **LOSS ON DRYING** ⟨731⟩

Analysis: Dry a sample at 105° for 2 h.
Acceptance criteria: NMT 10.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed, light-resistant containers.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Caftaric Acid RS
USP Chicoric Acid RS
USP Chlorogenic Acid RS
USP Powdered *Echinacea pallida* Extract RS
USP Echinacoside RS
USP β-Sitosterol RS

Powdered *Echinacea pallida*

DEFINITION

Powdered *Echinacea pallida* consists of the dried rhizome and roots of *Echinacea pallida* (Nutt.) Nutt. (Fam. Asteraceae) harvested in the fall after three or more years of growth, reduced to powder. It contains NLT 0.5% of total phenols, calculated on the dried basis as the sum of caftaric acid ($C_{13}H_{12}O_9$), chicoric acid ($C_{22}H_{18}O_{12}$), chlorogenic acid ($C_{16}H_{18}O_9$), and echinacoside ($C_{35}H_{46}O_{20}$).

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHY**

Presence of echinacoside and absence of dicaffeoylquinic acid (cynarin(e))

Standard solution A: A mixture of 0.2 mg/mL of USP Echinacoside RS and 0.2 mg/mL of dicaffeoylquinic acid (cynarin) in methanol

Standard solution B: 0.05 mg/mL of USP Caftaric Acid RS, 0.1 mg/mL of USP Chlorogenic Acid RS, and 0.05 mg/mL of USP Chicoric Acid RS in methanol

Standard solution C: 20 mg/mL of USP Powdered *Echinacea pallida* Extract RS in methanol. Shake to disperse, sonicate for 5 min, and centrifuge. Use the supernatant.

Sample solution: Transfer 1 g of Powdered *Echinacea pallida* to a centrifuge tube, add 10 mL of methanol, mix well, and sonicate for 10 min. Centrifuge, and use the supernatant.

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)

Application volume: 5 µL *Standard solution C* and *Sample solution*, and 2 µL *Standard solution A* and *Standard solution B* as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

Developing solvent system: A mixture of ethyl acetate, methylethyl ketone, water, and formic acid (5:3:1:1)

Developing distance: 6 cm

Derivatization reagent: 5 mg/mL of 2-aminoethyl diphenylborinate in ethyl acetate

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Apply the *Samples* as bands to a suitable thin-layer chromatographic plate, and dry in air. Develop the chromatograms in a saturated chamber. Remove the plate from the chamber, heat at 100° for 5 min, derivatize the plate while still warm with *Derivatization reagent*, dry in air, and examine under UV light at 366 nm.

System suitability: *Standard solution A* shows two major blue bands, one in the lower third of the chromatogram, due to echinacoside, and the other band in the middle section of the chromatogram due to dicaffeoylquinic acid (cynarin). *Standard solution B* shows two major blue bands at about the middle of the chromatogram due to caftaric acid (higher R_F) and chlorogenic acid (lower R_F) that are clearly separated, and a blue band for chicoric acid in the upper third of the chromatogram.

Acceptance criteria: The most prominent band in the *Sample solution* chromatogram is a blue band in the lower third section of the chromatogram at an R_F corresponding to the echinacoside band in the chromatograms of *Standard solution A* and *Standard solution C* (absent in *Echinacea purpurea*). The *Sample solution* chromatogram

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does not exhibit a blue band at an R_F corresponding to the dicaffeoylquinic acid (cynarin) band in the chromatogram of *Standard solution A* (present in *Echinacea angustifolia*). The *Sample solution* chromatogram may exhibit bands of lesser intensity at the R_F of caftaric acid and chicoric acid bands in chromatograms of *Standard solution B* and *Standard solution C* (absent in *Echinacea angustifolia* and much more prominent in *Echinacea purpurea*). The *Sample solution* chromatogram exhibits minor bands between the positions of echinacoside and caftaric acid. One of these is due to chlorogenic acid at an R_F corresponding to that of chlorogenic acid in *Standard solution B*.

• **B. THIN-LAYER CHROMATOGRAPHY**

Presence of alkylamides

Standard solution A: 0.2 mg/mL of USP β-Sitosterol RS in methanol

Standard solution B: 100 mg/mL of USP Powdered *Echinacea pallida* Extract RS in dichloromethane. Shake to disperse, sonicate for 5 min, and centrifuge. Use the supernatant.

Sample solution: Transfer 1 g of Powdered *Echinacea pallida* to a centrifuge tube, add 10 mL of dichloromethane, mix well, and sonicate for 10 min. Centrifuge, and use the supernatant.

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plates)

Application volume: 5 µL *Standard solution B* and *Sample solution*, and 2 µL *Standard solution A* as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

Developing solvent system: A mixture of toluene, ethyl acetate, cyclohexane, and formic acid (8: 2: 1: 0.3)

Developing distance: 6 cm

Derivatization reagent: Place 85 mL of methanol in a 100-mL glass bottle, and cool it down in a water–ice cubes–salt bath or in a freezer. To the ice-cold methanol, slowly and carefully add 10 mL of acetic acid and 5 mL of sulfuric acid, and mix well. Allow the mixture to cool to room temperature, then add 0.5 mL of *p*-anisaldehyde.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands to a suitable thin-layer chromatographic plate, and dry in air. Develop the chromatograms in a saturated chamber. Remove the plate from the chamber, dry in air, derivatize with *Derivatization reagent*, heat at 100° for 3–5 min, set aside to cool, and examine under visible light.

System suitability: The *Standard solution A* chromatogram exhibits a violet band corresponding to β-sitosterol. *Standard solution B* shows the most prominent band as a violet band in the upper third section of the chromatogram. The *Standard solution B* chromatogram exhibits a less prominent violet band in the lower third section of the chromatogram, and a broad pink violet band close to the solvent front.

Acceptance criteria: The most prominent band of the *Sample solution* chromatogram is a violet band in the upper third section of the chromatogram, corresponding in R_F to a similar band observed with *Standard solution B* (much less prominent in *Echinacea angustifolia* and absent in *Echinacea purpurea*). The *Sample solution* chromatogram exhibits a less prominent violet band in the lower third section of the chromatogram corresponding in R_F to a similar band observed in *Standard solution B* (much less prominent in *Echinacea purpurea* and absent in *Echinacea angustifolia*). The *Sample solution* chromatogram exhibits a minor violet band at an R_F corresponding to the β-sitosterol band in the chromatograms of *Standard solution A* and *Standard solution B*, and a broad pink violet band close to the solvent front.

• **C.** The retention time of the major peak in the *Sample solution* corresponds to that of the echinacoside peak in *Standard solution A* and *Standard solution C*, as obtained in the test for *Content of Total Phenols*. Peak area of any peak detected in the *Sample solution* chromatogram at the locus of 1,3-dicaffeoylquinic acid (*Standard solution D*) is NMT 1% of the peak area for the echinacoside peak.

COMPOSITION

• **CONTENT OF TOTAL PHENOLS**

Solution A: Phosphoric acid (0.1 in 100) in water

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	90	10
3	90	10
16	78	22
17	60	40
20	60	40
20.5	90	10
25	90	10

Solvent: Alcohol and water (7:3)

Standard solution A: Dissolve USP Powdered *Echinacea pallida* Extract RS in *Solvent* by shaking and heating in a water bath. Dilute with *Solvent* to obtain a solution having a known concentration of about 1 mg/mL. Pass through a membrane filter having a 0.45-µm or finer pore size.

Standard solution B: 40 µg/mL of USP Chlorogenic Acid RS in *Solvent*

Standard solution C: 80 µg/mL of USP Echinacoside RS in *Solvent*

Standard solution D: 40 µg/mL of dicaffeoylquinic acid (cynarin) in *Solvent*

Sample solution: Transfer 125 mg of Powdered *Echinacea pallida* (capable of passing through a 40-mesh sieve) to a round-bottom flask equipped with a condenser. Add 25.0 mL of *Solvent*, and heat under reflux while shaking by mechanical means for 15 min. Centrifuge, or pass through a membrane filter having a 0.45-µm or finer pore size.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 330 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 35°

Flow rate: 1.5 mL/min

Injection volume: 5 µL

System suitability

Samples: *Standard solution A* and *Standard solution C*

Suitability requirements

Chromatogram similarity: The chromatogram of *Standard solution A* is similar to the Reference

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Chromatogram for total phenols provided with USP Powdered *Echinacea pallida* Extract RS.

Capacity factor (k′): NLT 3.0 for the echinacoside peak, *Standard solution C*. [NOTE—Echinacoside peak may be resolved in two components.]

Tailing factor: NMT 2.0 for the echinacoside peak, *Standard solution C*

Relative standard deviation: NMT 2.5% for the echinacoside peaks in repeated injections, *Standard solution C*

Analysis

Samples: *Standard solution A, Standard solution B, Standard solution C, Standard solution D,* and *Sample solution*

Identify the relevant analytes in the chromatogram from the *Sample solution* by comparison with the chromatogram from *Standard solution A*. Measure the areas for the relevant peaks.

Separately calculate the percentage of caftaric acid ($C_{13}H_{12}O_9$), chicoric acid ($C_{22}H_{18}O_{12}$), chlorogenic acid ($C_{16}H_{18}O_9$), and echinacoside ($C_{35}H_{46}O_{20}$) in the portion of Powdered *Echinacea pallida* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak response for the relevant analyte from the *Sample solution*

r_S = peak response for chlorogenic acid or both echinacoside components from the corresponding *Standard solution*

C_S = concentration of chlorogenic acid or echinacoside in the corresponding *Standard solution* (mg/mL)

V = final volume of the *Sample solution* (mL)

W = weight of Powdered *Echinacea pallida* taken to prepare the *Sample solution* (mg)

F = response factor: chicoric acid, 0.695; caftaric acid, 0.881; chlorogenic acid, 1.000; relative to chlorogenic acid; and echinacoside components, 1.000

Calculate the percentage of total phenols in the portion of *Echinacea pallida* taken by adding the individual percentages calculated.

Acceptance criteria: NLT 0.5% of total phenols on the dried basis

CONTAMINANTS

- **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩

Acceptance criteria

Arsenic: NMT 1.0 µg/g
Cadmium: NMT 0.5 µg/g
Lead: NMT 5.0 µg/g
Mercury: NMT 1.0 µg/g

Change to read:

- **▲ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩▲ (CN 1-May-2019)**:** Meets the requirements

SPECIFIC TESTS

- **BOTANIC CHARACTERISTICS:** Powdered *Echinacea pallida* is a brown powder with a faint aromatic odor and a slightly acrid, persistent taste. It turns yellow when mounted in sodium hydroxide solution. Under a microscope, the following characteristics are observed: groups of secretory canals with brown contents, surrounded by parenchymatous cells containing cluster crystals of calcium oxalate; and parenchymatous cells with small starch granules; thick-walled lignified fibers and fragments of reticulate and pitted vessels.
- **ARTICLES OF BOTANICAL ORIGIN,** *Volatile Oil Determination* ⟨561⟩**:** 1.0–2.0 mL/100 g
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 7.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 4.0%
- **LOSS ON DRYING** ⟨731⟩

Analysis: Dry a sample at 105° for 2 h.
Acceptance criteria: NMT 10.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Caftaric Acid RS
USP Chicoric Acid RS
USP Chlorogenic Acid RS
USP Powdered *Echinacea pallida* Extract RS
USP Echinacoside RS
USP β-Sitosterol RS

Powdered *Echinacea pallida* Extract

DEFINITION

Powdered *Echinacea pallida* Extract is prepared from *Echinacea pallida* roots by extraction with hydroalcoholic mixtures or other suitable solvents. The ratio of the starting crude plant material to Powdered Extract is between 2:1 and 8:1. It contains NLT 4.0% and NMT 5.0% of total phenols, calculated as the sum of caftaric acid ($C_{13}H_{12}O_9$), chicoric acid ($C_{22}H_{18}O_{12}$), chlorogenic acid ($C_{16}H_{18}O_9$), and echinacoside ($C_{35}H_{46}O_{20}$), on the dried basis.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHY**

Presence of echinacoside and absence of dicaffeoylquinic acid (cynarin(e))

Standard solution A: A mixture of 0.2 mg/mL of USP Echinacoside RS and 0.2 mg/mL of dicaffeoylquinic acid (cynarin) in methanol

Standard solution B: 0.05 mg/mL of USP Caftaric Acid RS, 0.1 mg/mL of USP Chlorogenic Acid RS, and 0.05 mg/mL of USP Chicoric Acid RS in methanol

Standard solution C: 20 mg/mL of USP Powdered *Echinacea pallida* Extract RS in methanol. Shake to disperse, sonicate for 5 min, and centrifuge. Use the supernatant.

Sample solution: 20 mg/mL of Powdered Extract in methanol. Shake to disperse, sonicate for 5 min, and centrifuge. Use the supernatant.

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)

Application volume: 5 µL *Standard solution C* and *Sample solution*, and 2 µL *Standard solution A* and *Standard solution B* as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

Developing solvent system: A mixture of ethyl acetate, methylethyl ketone, water, and formic acid (5:3:1:1)

Developing distance: 6 cm

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Derivatization reagent: 5 mg/mL of 2-aminoethyl diphenylborinate in ethyl acetate

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Apply the *Samples* as bands to a suitable thin-layer chromatographic plate, and dry in air. Develop the chromatograms in a saturated chamber. Remove the plate from the chamber, heat at 100° for 5 min, derivatize the plate while still warm with *Derivatization reagent*, dry in air, and examine under UV light at 366 nm.

System suitability: *Standard solution A* shows two major blue bands, one in the lower third section of the chromatogram due to echinacoside, and the other band in the middle section of the chromatogram due to dicaffeoylquinic acid (cynarin). *Standard solution B* shows two major blue bands at about the middle of the chromatogram due to caftaric acid (higher R_F) and chlorogenic acid (lower R_F) that are clearly separated, and a blue band for chicoric acid in the upper third section of the chromatogram.

Acceptance criteria: The most prominent band in the *Sample solution* chromatogram is a blue band in the lower third section of the chromatogram at an R_F corresponding to the echinacoside band in the chromatograms of *Standard solution A* and *Standard solution C* (absent in *Echinacea purpurea*). The *Sample solution* chromatogram does not exhibit a blue band at an R_F corresponding to the dicaffeoylquinic acid (cynarin) band in the chromatogram of *Standard solution A* (present in *Echinacea angustifolia*). The *Sample solution* chromatogram may exhibit bands of lesser intensity at the R_F of caftaric acid and chicoric acid bands in the chromatograms of *Standard solution B* and *Standard solution C* (absent in *Echinacea angustifolia* and much more prominent in *Echinacea purpurea*). The *Sample solution* chromatogram exhibits minor bands between the positions of echinacoside and caftaric acid. One of these is due to chlorogenic acid at an R_F corresponding to that of chlorogenic acid in *Standard solution B*.

• **B. THIN-LAYER CHROMATOGRAPHY**

Presence of alkylamides

Standard solution A: 0.2 mg/mL of USP β-Sitosterol RS in methanol.

Standard solution B: 100 mg/mL of USP Powdered *Echinacea pallida* Extract RS in dichloromethane. Shake to disperse, sonicate for 5 min, and centrifuge. Use the supernatant.

Sample solution: 100 mg/mL of Powdered Extract in dichloromethane. Shake to disperse, sonicate for 5 min, and centrifuge. Use the supernatant.

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plates)

Application volume: 5 µL *Standard solution B* and *Sample solution*, and 2 µL *Standard solution A* as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

Developing solvent system: A mixture of toluene, ethyl acetate, cyclohexane, and formic acid (8: 2: 1: 0.3)

Developing distance: 6 cm

Derivatization reagent: Place 85 mL of methanol in a 100-mL glass bottle, and cool it down in a water–ice cubes–salt bath or in a freezer. To the ice-cold methanol, slowly and carefully add 10 mL of acetic acid and 5 mL of sulfuric acid, and mix well. Allow the mixture to cool to room temperature, then add 0.5 mL of *p*-anisaldehyde.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands to a suitable thin-layer chromatographic plate, and dry in air. Develop the chromatograms in a saturated chamber. Remove the plate from the chamber, dry in air, derivatize with *Derivatization reagent*, heat at 100° for 3–5 min, set aside to cool, and examine under visible light.

System suitability: The *Standard solution A* chromatogram exhibits a violet band corresponding to β-sitosterol. *Standard solution B* shows the most prominent band as a violet band in the upper third section of the chromatogram. The *Standard solution B* chromatogram exhibits a less prominent violet band in the lower third section of the chromatogram, and a broad pink violet band close to the solvent front.

Acceptance criteria: The most prominent band of the *Sample solution* chromatogram is a violet band in the upper third section of the chromatogram, corresponding in R_F to a similar band observed in *Standard solution B* (much less prominent in *Echinacea angustifolia* and absent in *Echinacea purpurea*). The *Sample solution* chromatogram exhibits a less prominent violet band in the lower third section of the chromatogram corresponding in R_F to a similar band observed in *Standard solution B* (much less prominent in *Echinacea purpurea* and absent in *Echinacea angustifolia*). The *Sample solution* chromatogram exhibits a minor violet band at an R_F corresponding to the β-sitosterol band in the chromatograms of *Standard solution A* and *Standard solution B*, and a broad pink violet band close to the solvent front.

• **C.** The retention time of the major peak in the *Sample solution* corresponds to that of the echinacoside peak in *Standard solution A* and *Standard solution C*, as obtained in the test for *Content of Total Phenols*. The peak area of any peak detected in the *Sample solution* chromatogram at the locus of 1,3-dicaffeoylquinic acid (*Standard solution D*) is NMT 1% of the peak area for the echinacoside peak.

COMPOSITION

• **CONTENT OF TOTAL PHENOLS**

Solution A: Phosphoric acid (0.1 in 100)

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	90	10
3	90	10
16	78	22
17	60	40
20	60	40
20.5	90	10
25	90	10

Solvent: Alcohol and water (7:3)

Standard solution A: Dissolve USP Powdered *Echinacea pallida* Extract RS in *Solvent* by shaking and heating in a water bath. Dilute with *Solvent* to obtain a solution having a known concentration of 1 mg/mL. Pass through a membrane filter having a 0.45-µm or finer pore size.

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Standard solution B: 40 µg/mL of USP Chlorogenic Acid RS in *Solvent*
Standard solution C: 80 µg/mL of USP Echinacoside RS in *Solvent*
Standard solution D: 40 µg/mL of dicaffeoylquinic acid (cynarin) in *Solvent*
Sample solution: Transfer about 60 mg of Powdered Extract, accurately weighed, to an appropriate round-bottom flask equipped with a condenser. Add 25.0 mL of *Solvent*, and heat under reflux while shaking by mechanical means for 15 min. Centrifuge, or pass through a membrane filter having a 0.45-µm or finer pore size.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 330 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 35°
Flow rate: 1.5 mL/min
Injection volume: 5 µL
System suitability
Samples: *Standard solution A* and *Standard solution C*.
[NOTE—Echinacoside peak may be resolved in two components.]
Suitability requirements
Chromatogram similarity: The chromatogram of *Standard solution A* is similar to the Reference Chromatogram for total phenols provided with USP Powdered *Echinacea pallida* Extract RS.
Capacity factor (k′): NLT 3.0 for the echinacoside peak, *Standard solution C*
Tailing factor: NMT 2.0 for the echinacoside peak, *Standard solution C*
Relative standard deviation: NMT 2.5% for the echinacoside peaks in repeated injections, *Standard solution C*
Analysis
Samples: *Sample solution*, *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Standard solution D*
Identify the relevant analytes in the chromatogram from the *Sample solution* by comparison with the chromatogram from *Standard solution A*. Measure the areas for the relevant peaks.
Separately calculate the percentage of caftaric acid ($C_{13}H_{12}O_9$), chicoric acid ($C_{22}H_{18}O_{12}$), chlorogenic acid ($C_{16}H_{18}O_9$), and echinacoside ($C_{35}H_{46}O_{20}$) in the portion of Powdered Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak response for the relevant analyte from the *Sample solution*
r_S = peak response for chlorogenic acid or both echinacoside components from the corresponding *Standard solution*
C_S = concentration of chlorogenic acid or echinacoside in the corresponding *Standard solution* (mg/mL)
C_U = concentration of Powdered Extract in the *Sample solution* (mg/mL)
F = response factor: chicoric acid, 0.695; caftaric acid, 0.881; chlorogenic acid, 1.000; relative to chlorogenic acid; and echinacoside components, 1.000

Calculate the percentage of total phenols in the portion of Powdered Extract taken by adding the individual percentages calculated.
Acceptance criteria: NLT 4.0% and NMT 5.0% of total phenols on the dried basis

CONTAMINANTS
• **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩
Acceptance criteria
Arsenic: NMT 1.0 µg/g
Cadmium: NMT 0.5 µg/g
Lead: NMT 5.0 µg/g
Mercury: NMT 1.0 µg/g
• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*
• **OTHER REQUIREMENTS:** It meets the requirements for *Botanical Extracts* ⟨565⟩, *Residual Solvents* and *Pesticide Residues*.

SPECIFIC TESTS
• **LOSS ON DRYING** ⟨731⟩
Sample: 1 g
Analysis: Dry the *Sample* at 105° for 2 h.
Acceptance criteria: NMT 5.0%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store in a cool place.
• **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant from which the article was prepared. The label bears a statement indicating that *Echinacea pallida* may cause rare allergic reactions, rashes, or aggravate asthma. It meets the requirements for *Botanical Extracts* ⟨565⟩, *Labeling*.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Caftaric Acid RS
USP Chicoric Acid RS
USP Chlorogenic Acid RS
USP Powdered *Echinacea pallida* Extract RS
USP Echinacoside RS
USP β-Sitosterol RS

Echinacea purpurea Aerial Parts

DEFINITION
Echinacea purpurea Aerial Parts consists of the aerial parts of *Echinacea purpurea* (L.) Moench (Fam. Asteraceae). It is harvested during the flowering stage. It contains NLT 1.0% of the sum of caftaric acid ($C_{13}H_{12}O_9$) and chicoric acid ($C_{22}H_{18}O_{12}$), and NLT 0.01% of dodecatetraenoic acid isobutylamides ($C_{16}H_{25}NO$), calculated on the dried basis.

IDENTIFICATION
• **A. THIN-LAYER CHROMATOGRAPHY**
Presence of chicoric acid and absence of echinacoside
Standard solution A: 0.2 mg/mL of USP Echinacoside RS in methanol
Standard solution B: 0.2 mg/mL of USP Caftaric Acid RS, 0.1 mg of USP Chlorogenic Acid RS, and 0.2 mg/mL of USP Chicoric Acid RS in methanol
Standard solution C: 20 mg/mL of USP Powdered *Echinacea purpurea* Extract RS in methanol. Shake to disperse, sonicate for 5 min, and centrifuge. Use the supernatant.
Sample solution: Transfer 1 g of finely pulverized *Echinacea purpurea* Aerial Parts to a centrifuge tube, add 10 mL of

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methanol, mix well, and sonicate for 10 min. Centrifuge, and use the supernatant.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)
Application volume: 5 µL *Standard solution C* and *Sample solution*, and 2 µL *Standard solution A* and *Standard solution B* as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
Developing solvent system: A mixture of ethyl acetate, methylethyl ketone, water, and formic acid (5:3:1:1)
Developing distance: 6 cm
Derivatization reagent: 5 mg/mL of 2-aminoethyl diphenylborinate in ethyl acetate

Analysis
Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*
Apply the *Samples* as bands to a suitable thin-layer chromatographic plate, and dry in air. Develop the chromatograms in a satutated chamber. Remove the plate from the chamber, heat at 100° for 5 min, derivatize the plate while still warm with *Derivatization reagent*, dry in air, and examine under UV light at 366 nm.

System suitability: *Standard solution A* shows one major blue band in the lower third section of the chromatogram due to echinacoside. *Standard solution B* shows two major blue bands at about the middle of the chromatogram due to caftaric acid (higher R_F) and chlorogenic acid (lower R_F) that are clearly separated, and a blue band for chicoric acid in the upper third section of the chromatogram.

Acceptance criteria: The most prominent band in the *Sample solution* chromatogram is a blue band in the upper third section of the chromatogram at an R_F corresponding to the chicoric acid band in the chromatogram of *Standard solution B* and *Standard solution C*. The second most prominent band in the *Sample solution* chromatogram is a blue band at about the middle of the chromatogram due to caftaric acid, corresponding to a band in the chromatogram of *Standard solution C*. The *Sample solution* chromatogram does not exhibit a band at the R_F of echinacoside in *Standard solution A* (difference from *Echinacea pallida* and *Echinacea angustifolia*). The *Sample solution* chromatogram exhibits minor blue bands corresponding to similar bands in the chromatogram of *Standard solution C*. One of these bands is due to chlorogenic acid at an R_F corresponding to chlorogenic acid in *Standard solution B*. The *Sample solution* chromatogram exhibits a red band due to chlorophyll close to the solvent front.

- **B.** The retention time of the major peak in the *Sample solution* corresponds to that of the chicoric acid peak in *Standard solution A*, and the second most prominent peak corresponds to that of the caftaric acid peak in *Standard solution B*. The *Sample solution* chromatogram shows no peak or a very minor peak at the retention time corresponding to the echinacoside peak in the *Standard solution C* chromatogram, all peaks as obtained in the test for *Content of Chicoric Acid and Caftaric Acid*.
- **C.** The retention times for the relevant peaks of the *Sample solution*, mainly due to dodecatetraenoic isobutyl amides, correspond to those of *Standard solution A*, as obtained in the test for *Content of Dodecatetraenoic Isobutylamides*.

COMPOSITION

- **Content of Chicoric Acid and Caftaric Acid**

Solution A: Phosphoric acid (0.1 in 100) in water
Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	90	10
13	78	22
14	60	40
17.5	60	40
18	90	10
30	90	10

Solvent: Alcohol and water (7:3)
Standard solution A: 30 µg/mL of USP Chicoric Acid RS in *Solvent*
Standard solution B: 20 µg/mL of USP Caftaric Acid RS in *Solvent*
Standard solution C: 20 µg/mL of USP Echinacoside RS in *Solvent*
Sample solution: Transfer about 125 mg, accurately weighed, of finely powdered *Echinacea purpurea* Aerial Parts (capable of passing through a 40-mesh sieve) to a round-bottom flask equipped with a condenser. Add 25.0 mL of *Solvent*, and heat under reflux while shaking by mechanical means for 15 min. Centrifuge, or pass through a membrane filter of 0.45-µm or finer pore size.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 330 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 35°
Flow rate: 1.5 mL/min
Injection size: 5 µL

System suitability
Samples: *Standard solution A*
Suitability requirements
Relative standard deviation: NMT 2.0% for the chicoric acid peak in *Standard solution A*

Analysis
Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*
Separately calculate the percentages of caftaric acid ($C_{13}H_{12}O_9$) and chicoric acid ($C_{22}H_{18}O_{12}$) in the portion of *Echinacea purpurea* Aerial Parts taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of the relevant analyte from the corresponding *Standard solution*
C_S = concentration of the relevant analyte in the corresponding *Standard solution* (mg/mL)
V = final volume of the *Sample solution* (mL)
W = weight of *Echinacea purpurea* Aerial Parts taken to prepare the *Sample solution* (mg)

Calculate the percentage of the sum of chicoric acid and caftaric acid in the portion of *Echinacea purpurea* Aerial Parts taken by adding the individual percentages calculated.

Acceptance criteria: NLT 1.0% on the dried basis

- **Content of Dodecatetraenoic Acid Isobutylamides**

Mobile phase: Acetonitrile and water (55:45)

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Standard solution A: 5 mg/mL of USP Powdered *Echinacea purpurea* Extract RS in methanol. Dissolve using sonication and shaking for 10 min. After dilution, pass through a membrane filter of 0.45-µm or finer pore size.
Standard solution B: 10 µg/mL of USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS in methanol
Sample solution: Transfer about 2.5 g of finely powdered *Echinacea purpurea* Aerial Parts (capable of passing through a 40-mesh sieve), accurately weighed, into a round-bottom flask. Add 80 mL of methanol, and reflux for 30 min. Cool to room temperature, and filter into a 100-mL volumetric flask, using small portions of methanol to rinse the flask and the filter. Dilute with methanol to volume. Pass through a membrane filter of 0.45-µm or finer pore size.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 30°
Flow rate: 1.5 mL/min
Injection size: 25 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram from *Standard solution A* is similar to the Reference Chromatogram for alkamides provided with USP Powdered *Echinacea purpurea* Extract RS.
Resolution: NLT 1.0 between dodecatetraenoic acid isobutylamide peaks, *Standard solution A*
Tailing factor: NMT 2.0 for 2*E*,4*E*-hexadienoic acid isobutylamide, *Standard solution B*
Relative standard deviation: NMT 2.5% for the 2*E*,4*E*-hexadienoic acid isobutylamide peak in repeated injections, *Standard solution B*
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Identify the peaks of the two isomers of dodecatetraenoic acid isobutylamides in the chromatogram from the *Sample solution* by comparison with the chromatogram from *Standard solution A*. Measure the areas for the relevant peaks.
Calculate the percentage of dodecatetraenoic acid isobutylamides in the portion of *Echinacea purpurea* Aerial Parts taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = sum of the peak areas of the relevant analytes from the *Sample solution*
r_S = peak area of 2*E*,4*E*-hexadienoic acid isobutylamide from *Standard solution B*
C_S = concentration of USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS in *Standard solution B* (mg/mL)
V = final volume of the *Sample solution* (mL)
W = weight of *Echinacea purpurea* Aerial Parts taken to prepare the *Sample solution* (mg)
F = response factor to convert 2*E*,4*E*-hexadienoic acid isobutylamide into dodecatetraenoic acid isobutylamides, 1.353

Acceptance criteria: NLT 0.01% of dodecatetraenoic acid isobutylamides on the dried basis

CONTAMINANTS
• **Elemental Impurities—Procedures** ⟨233⟩
Acceptance criteria
Arsenic: NMT 1.0 µg/g
Cadmium: NMT 0.5 µg/g
Lead: NMT 5.0 µg/g
Mercury: NMT 1.0 µg/g

Change to read:

• **▲Articles of Botanical Origin,** *Pesticide Residue Analysis* ⟨561⟩▲ (CN 1-May-2019)**:** Meet the requirements
• **Microbial Enumeration Tests** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the enterobacterial count does not exceed 10^3 cfu/g.
• **Absence of Specified Microorganisms** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS
• **Botanic Characteristics**
Macroscopic: The herb is an erect, coarse, rough-hairy perennial, usually up to 90 cm tall, rarely up to 180 cm. The leaves are alternate and simple; the lowermost leaves are slender, long, and petioled, ovate to broadly lanceolate, mostly penta-nerved, acute or acuminated at the apex, abruptly narrowed or rarely cordate at the base, usually sharply dentate, and 7–20 cm long and 2.5–7.5 cm wide; the petioles are mostly winged at the summit. The upper leaves are narrower, often almost entirely sessile, lanceolate or ovate lanceolate, and usually with 3 veins.
The flower heads are radiate, up to 15 cm across, solitary or few, and long-peduncled, with 12–20 rays, purple, crimson, or rarely pale; the bristle disks are often orange, 3.5–7.5 cm long; the involucre is depressed-hemispheric; the bracts are lanceolate, spreading or appressed, imbricated in 2–4 series, and hairy on the outer surface with ciliate margins; the receptacle is conical, the scales of the receptacle stiff, spinescent, and conspicuously longer than the disc flowers; the chaff is carinate and cuspidate; the achenes are 3–4 mm in length, tetrasided, obypyramidal, and thick; the pappus has a short, dentate crown.
Microscopic
Leaf: The leaf has a thickness of 200–350 µm, with an epidermis 9–13 µm thick, largely without chloroplasts; the stomata are 28–35 µm, abundant on the ventral surface and fewer on the dorsal surface; the mesophyll is clearly divided into palisade parenchyma and sponge parenchyma. The palisade parenchyma is one layer thick, with elongated cells 50–65 µm in length, oriented at right angles to the leaf surface, containing numerous chloroplasts. The sponge parenchyma is 150–250 µm thick, with cells of irregular shape, and has multiple cell layers, few chloroplasts, and large intercellular spaces. The phloem bundles of the lateral veins within the sponge parenchyma are bound by a one-layer sheath of small parenchymous cells, with vascular elements of the midrib surrounded by large-celled parenchyma. The uniseriate trichomes are few in the ventral surface, numerous on the dorsal surface, typically tricelled, occasionally tetra- or pentacelled, 250–500 µm in length, each arising from an epidermal cell; the epidermal cell walls appear with moderate thickening; the vessels are various, scalariform, with variable reticulated width.
Petiole: The parenchyma appear without chloroplasts, in several layers adjacent to a layer of collenchyma; 5–7 phloem bundles of small- to medium-sized vessels are

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weakly lignified and embedded in the parenchyma in the form of an arc; the wing ribs of the upper surface of the slightly hollowed petiole are marginal.

Inflorescence: The epidermal cells of the ray florets are square, 50 µm, with a transparent, beaded cell wall; various elements of the *Asteraceous* exhibit inflorescence; numerous multicellular jointed trichomes of the involucral bracts are 500–800 µm in length; tangential sections of the paleae with numerous fiber bundles are 10–15 µm in diameter and 100–150 µm in length; cell walls are thin. The epidermis of ray florets is reddish to violet; the epidermal cells from the end of the corolla form rounded papillae; a stigma of papillary cells is present; *Asteraceous* pollen grains are 20–30 µm and spherical with a warty exine.

Calcium oxalate is negative; crystals of inulin and starch granules are rare.

- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩: NMT 3.0%
- **LOSS ON DRYING** ⟨731⟩

Sample: 1 g of the powdered plant material
Analysis: Dry the *Sample*.
Acceptance criteria: NMT 12%

- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩: NMT 10.0%, determined on 3 g
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩: NMT 2.5%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Store in tight, light-resistant containers at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Chlorogenic Acid RS
USP Caftaric Acid RS
USP Chicoric Acid RS
USP Echinacoside RS
USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS
USP Powdered *Echinacea purpurea* Extract RS

Echinacea purpurea Root

DEFINITION

Echinacea purpurea Root consists of the dried rhizome and roots of *Echinacea purpurea* (L.) Moench (Fam. Asteraceae). It is harvested in the fall after three or more years of growth. It contains NLT 0.5% of total phenols, calculated on the dried basis as the sum of caftaric acid ($C_{13}H_{12}O_9$), chicoric acid ($C_{22}H_{18}O_{12}$), and chlorogenic acid ($C_{16}H_{18}O_9$). It contains NLT 0.025% of alkamides calculated as dodecatetraenoic acid isobutylamides ($C_{16}H_{25}NO$).

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHY**

Presence of chicoric acid and absence of echinacoside

Standard solution A: 0.2 mg/mL of USP Echinacoside RS in methanol
Standard solution B: 0.2 mg/mL of USP Caftaric Acid RS, 0.1 mg/mL of USP Chlorogenic Acid RS, and 0.2 mg/mL of USP Chicoric Acid RS in methanol
Standard solution C: 20 mg/mL of USP Powdered *Echinacea purpurea* Extract RS in methanol. Shake to disperse, sonicate for 5 min, and centrifuge. Use the supernatant.
Sample solution: Transfer 1 g of finely pulverized *Echinacea purpurea* Root to a centrifuge tube, add 10 mL of methanol, mix well, and sonicate for 10 min. Centrifuge, and use the supernatant.
Chromatographic system
(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)
Application volume: 5 µL *Standard solution C* and *Sample solution*, and 2 µL *Standard solution A* and *Standard solution B* as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
Developing solvent system: A mixture of ethyl acetate, methylethyl ketone, water, and formic acid (5:3:1:1)
Developing distance: 6 cm
Derivatization reagent: 5 mg/mL of 2-aminoethyl diphenylborinate in ethyl acetate
Analysis
Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*
Apply the *Samples* as bands to a suitable thin-layer chromatographic plate, and dry in air. Develop the chromatograms in a saturated chamber. Remove the plate from the chamber, heat at 100° for 5 min, derivatize the plate while still warm with *Derivatization reagent*, dry in air, and examine under UV light at 366 nm.
System suitability: *Standard solution A* shows one major blue band in the lower third of the chromatogram due to echinacoside. *Standard solution B* shows two major blue bands at about the middle of the chromatogram due to caftaric acid (higher R_F) and chlorogenic acid (lower R_F) that are clearly separated, and a blue band for chicoric acid in the upper third section of the chromatogram.
Acceptance criteria: The most prominent band in the *Sample solution* chromatogram is a blue band in the upper third section of the chromatogram at an R_F corresponding to the chicoric acid band in the chromatograms of *Standard solution B* and *Standard solution C* (less prominent in *Echinacea pallida* and absent or almost absent in *Echinacea angustifolia*). The second most prominent band in the *Sample solution* chromatogram is a blue band at about the middle of the chromatogram due to caftaric acid, corresponding to a band in the chromatogram of *Standard solution C* (absent in *Echinacea angustifolia* and a minor band in *Echinacea pallida*). The *Sample solution* chromatogram does not exhibit a band at the R_F of echinacoside in *Standard solution A* (difference from *Echinacea pallida* and *Echinacea angustifolia*). The *Sample solution* chromatogram may exhibit minor blue bands corresponding to similar bands in the chromatogram of *Standard solution C*. One of these is due to chlorogenic acid at an R_F corresponding to chlorogenic acid in the *Standard solution B*.

- **B. THIN-LAYER CHROMATOGRAPHY**

Presence of alkylamides

Standard solution A: 0.2 mg/mL of USP β-Sitosterol RS in methanol
Standard solution B: 100 mg/mL of USP Powdered *Echinacea purpurea* Extract RS in dichloromethane. Shake to disperse, sonicate for 5 min, and centrifuge. Use the supernatant.
Sample solution: Transfer 1 g of finely pulverized *Echinacea purpurea* Root to a centrifuge tube, add 10 mL of dichloromethane, mix well, and sonicate for 10 min. Centrifuge, and use the supernatant.

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Chromatographic system
(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plates)
Application volume: 5 µL *Standard solution B* and *Sample solution*, and 2 µL *Standard solution A* as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
Developing solvent system: A mixture of toluene, ethyl acetate, cyclohexane, and formic acid (8: 2: 1: 0.3)
Developing distance: 6 cm
Derivatization reagent: Place 85 mL of methanol in a 100-mL glass bottle, and cool it down in a water–ice cubes–salt bath or in a freezer. To the ice-cold methanol, slowly and carefully add 10 mL of acetic acid and 5 mL of sulfuric acid, and mix well. Allow the mixture to cool to room temperature, then add 0.5 mL of *p*-anisaldehyde.
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Apply the *Samples* as bands to a suitable thin-layer chromatographic plate, and dry in air. Develop the chromatograms in a saturated chamber. Remove the plate from the chamber, dry in air, derivatize with *Derivatization reagent*, heat at 100° for 3–5 min, set aside to cool, and examine under visible light.
System suitability: The chromatogram of *Standard solution B* exhibits the most prominent band as a pinkish violet band at about the middle of the chromatogram, and just below this pinkish band, a violet band at a lower R_F similar in position and color to the β-sitosterol band in the chromatograms of *Standard solution A*. These two bands are clearly separated from each other. The chromatogram of *Standard solution B* also shows a broad pink violet band close to the solvent front.
Acceptance criteria: The most prominent band of the *Sample solution* chromatogram is a pinkish violet band at about the middle of the chromatogram similar in position and color to a band in the *Standard solution B* chromatogram (much less prominent in *Echinacea angustifolia* and *Echinacea pallida*), a violet band corresponding to β-sitosterol band in the chromatograms of *Standard solution A* and *Standard solution B*, and a broad pink violet band close to the solvent front similar in position and color to the band in the chromatogram of *Standard solution B*. The *Sample solution* chromatogram does not exhibit a yellow band below the β-sitosterol band (difference from *Echinacea angustifolia*) or a prominent violet band at about two thirds of the chromatogram (difference from *Echinacea pallida*).

• **C.** The retention time of the major peak in the *Sample solution* corresponds to that of the chicoric acid peak in *Standard solution A*, and the second most prominent peak corresponds to that of the caftaric acid peak in *Standard solution B*. The *Sample solution* chromatogram shows no or a very minor peak at the retention time corresponding to the echinacoside peak in the *Standard solution D* chromatogram, all peaks as obtained in the test for *Content of Total Phenols*.

COMPOSITION

• **Content of Total Phenols**
Solution A: Phosphoric acid (0.1 in 100) in water
Solution B: Acetonitrile
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	90	10
13	78	22
14	60	40
17.5	60	40
18	90	10
30	90	10

Solvent: Alcohol and water (7:3)
Standard solution A: 30 µg/mL of USP Chicoric Acid RS in *Solvent*
Standard solution B: 20 µg/mL of USP Caftaric Acid RS in *Solvent*
Standard solution C: 20 µg/mL of USP Chlorogenic Acid RS in *Solvent*
Standard solution D: 20 µg/mL of USP Echinacoside RS in *Solvent*
Sample solution: Transfer 125 mg of finely powdered *Echinacea purpurea* Root (capable of passing through a 40-mesh sieve) to a round-bottom flask equipped with a condenser. Add 25.0 mL of *Solvent*, and heat under reflux while shaking by mechanical means for 15 min. Centrifuge, or pass through a membrane filter of 0.45-µm or finer pore size.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 330 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 35°
Flow rate: 1.5 mL/min
Injection volume: 5 µL
System suitability
Samples: *Standard solution A*
Suitability requirements
Relative standard deviation: NMT 2% for the chicoric acid peak in repeated injections, *Standard solution A*
Analysis
Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, *Standard solution D*, and *Sample solution*
Separately calculate the percentage of caftaric acid ($C_{13}H_{12}O_9$), chicoric acid ($C_{22}H_{18}O_{12}$), and chlorogenic acid ($C_{16}H_{18}O_9$) in the portion of *Echinacea purpurea* Root taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of the relevant analyte from the corresponding *Standard solution*
C_S = concentration of the relevant analyte in the corresponding *Standard solution* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of *Echinacea purpurea* taken to prepare the *Sample solution* (mg)

Calculate the percentage of total phenols in the portion of *Echinacea purpurea* Root taken by adding the individual percentages calculated.
Acceptance criteria: NLT 0.5% of total phenols on the dried basis

• **Content of Alkamides**
Mobile phase: Acetonitrile and water (55:45)

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Standard solution A: 5 mg/mL of USP Powdered *Echinacea purpurea* Extract RS in methanol. Dissolve using sonication and shaking for 10 min. After dilution, pass through a membrane filter having a 0.45-µm or finer pore size.
Standard solution B: 10 µg/mL of USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS in methanol
Sample solution: Transfer about 2.5 g of finely powdered *Echinacea purpurea* Root (capable of passing through a 40-mesh sieve) into a round-bottom flask. Add 80 mL of methanol, and reflux for 30 min. Cool to room temperature, and filter into a 100-mL volumetric flask using small portions of methanol to rinse the flask and the filter. Dilute with methanol to volume. Pass through a membrane filter having a 0.45-µm or finer pore size.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 30°
Flow rate: 1.5 mL/min
Injection volume: 25 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram from *Standard solution A* is similar to the Reference Chromatogram for alkamides provided with USP Powdered *Echinacea purpurea* Extract RS.
Resolution: NLT 1.0 between dodecatetraenoic acid isobutylamide peaks, *Standard solution A*
Tailing factor: NMT 2.0 for the 2*E*,4*E*-hexadienoic acid isobutylamide peak, *Standard solution B*
Relative standard deviation: NMT 2.5% for the 2*E*,4*E*-hexadienoic acid isobutylamide peak in repeated injections, *Standard solution B*
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Identify the peaks of the 10 major alkamides in the chromatogram from the *Sample solution* by comparison with the chromatogram from *Standard solution A*. Measure the areas for the relevant peaks.
Calculate the percentage of alkamides in the portion of *Echinacea purpurea* Root taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = sum of the peak areas of the relevant analytes from the *Sample solution*
r_S = peak area of 2*E*,4*E*-hexadienoic acid isobutylamide from *Standard solution B*
C_S = concentration of USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS in *Standard solution B* (mg/mL)
V = final volume of the *Sample solution* (mL)
W = weight of *Echinacea purpurea* root taken to prepare the *Sample solution* (mg)
F = response factor to convert 2*E*,4*E*-hexadienoic acid isobutylamide into dodecatetraenoic acid isobutylamide, 1.353

Acceptance criteria: NLT 0.025% on the dried basis

CONTAMINANTS
• **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩
Acceptance criteria
Arsenic: NMT 1.0 µg/g
Cadmium: NMT 0.5 µg/g
Lead: NMT 5.0 µg/g
Mercury: NMT 1.0 µg/g
• **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residues* ⟨561⟩**:** Meets the requirements

SPECIFIC TESTS
• **BOTANIC CHARACTERISTICS**
Macroscopic: The roots are cylindrical and irregularly branched. The outer surface is dark brown and longitudinally striated; fractures are short and tough. Transverse sections show a thin periderm and yellowish xylem with distinct rays. In older roots, the pith is spongy with a brownish center surrounded by yellow.
Microscopic: Rhizomes and roots in transverse section show a thin outer bark separated from a wide xylem by a brown vascular cambium. The cork is composed of several rows of thin-walled cells containing brown pigment. Schizogenous resin canals are present in the cortex. The rhizome contains bast fibers and stone cells. The xylem, with distinct rays, contains tracheary elements composed of reticulated vessels and tracheids (about 80 × 30 µm) with bordered pits and slanted end walls. Vessels and tracheids are surrounded by thick-walled parenchyma and fibers; fibers are elongated with narrow lumens and funnel-shaped ends (20–40 µm wide). Polygonal sclereids (about 50 µm in diameter) are also present. Xylem fibers have minimal or no phytomelanin deposits (unlike *Echinacea angustifolia* and *Echinacea pallida*). A melanogenic layer is present between adjacent xylem parenchyma cell walls. The rhizome, with pith, is composed of pitted parenchyma cells containing inulin crystals. Starch is minimal to absent, and calcium oxalate crystals are absent.
• **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩**:** NMT 3.0%
• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry a sample at 105° for 2 h.
Acceptance criteria: NMT 10.0%
• **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 7.0%
• **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 4.0%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Store in well-closed, light-resistant containers.
• **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Caftaric Acid RS
USP Chicoric Acid RS
USP Chlorogenic Acid RS
USP Powdered *Echinacea purpurea* Extract RS
USP Echinacoside RS
USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS
USP β-Sitosterol RS

Powdered *Echinacea purpurea*

DEFINITION
Powdered *Echinacea purpurea* consists of the dried rhizome and roots of *Echinacea purpurea* (L.) Moench (Fam. Asteraceae), harvested in the fall after three or more years of growth, and reduced to powder. It contains NLT 0.5% of total phenols, calculated on the dried basis as the sum of caftaric acid ($C_{13}H_{12}O_9$), chicoric acid ($C_{22}H_{18}O_{12}$), and chlorogenic acid ($C_{16}H_{18}O_9$). It contains NLT 0.025% of

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alkamides calculated as dodecatetraenoic acid isobutylamides ($C_{16}H_{25}NO$).

IDENTIFICATION

• **A. Thin-Layer Chromatography**

Presence of chicoric acid and absence of echinacoside

Standard solution A: 0.2 mg/mL of USP Echinacoside RS in methanol

Standard solution B: 0.2 mg/mL of USP Caftaric Acid RS, 0.1 mg/mL of USP Chlorogenic Acid RS, and 0.2 mg/mL of USP Chicoric Acid RS in methanol

Standard solution C: 20 mg/mL of USP Powdered *Echinacea purpurea* Extract RS in methanol. Shake to disperse, sonicate for 5 min, and centrifuge. Use the supernatant.

Sample solution: Transfer 1 g of Powdered *Echinacea purpurea* to a centrifuge tube, add 10 mL of methanol, mix well, and sonicate for 10 min. Centrifuge, and use the supernatant.

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)

Application volume: 5 µL *Standard solution C* and *Sample solution*, and 2 µL *Standard solution A* and *Standard solution B* as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

Developing solvent system: A mixture of ethyl acetate, methylethyl ketone, water, and formic acid (5:3:1:1)

Developing distance: 6 cm

Derivatization reagent: 5 mg/mL of 2-aminoethyl diphenylborinate in ethyl acetate

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Apply the *Samples* as bands to a suitable thin-layer chromatographic plate, and dry in air. Develop the chromatograms in a saturated chamber. Remove the plate from the chamber, heat at 100° for 5 min, derivatize the plate while still warm with *Derivatization reagent*, dry in air, and examine under UV light at 366 nm.

System suitability: *Standard solution A* shows one major blue band in the lower third section of the chromatogram due to echinacoside. *Standard solution B* shows two major blue bands at about the middle of the chromatogram due to caftaric acid (higher R_F) and chlorogenic acid (lower R_F) that are clearly separated, and a blue band for chicoric acid in the upper third section of the chromatogram.

Acceptance criteria: The most prominent band in the *Sample solution* chromatogram is a blue band in the upper third section of the chromatogram at an R_F corresponding to the chicoric acid band in the chromatograms of *Standard solution B* and *Standard solution C* (less prominent in *Echinacea pallida* and absent or almost absent in *Echinacea angustifolia*). The second most prominent band in the *Sample solution* chromatogram is a blue band at about the middle of the chromatogram due to caftaric acid, corresponding to a band in the chromatogram of *Standard solution C* (absent in *Echinacea angustifolia* and a minor band in *Echinacea pallida*). The *Sample solution* chromatogram does not exhibit a band at the R_F of echinacoside in *Standard solution A* (difference from *Echinacea pallida* and *Echinacea angustifolia*). The *Sample solution* chromatogram may exhibit minor blue bands corresponding to similar bands in the chromatogram of *Standard solution C*. One of these is due to chlorogenic acid at an R_F corresponding to chlorogenic acid in the *Standard solution B*.

• **B. Thin-Layer Chromatography**

Presence of alkylamides

Standard solution A: 0.2 mg/mL of USP β-Sitosterol RS in methanol

Standard solution B: 100 mg/mL of USP Powdered *Echinacea purpurea* Extract RS in dichloromethane. Shake to disperse, sonicate for 5 min, and centrifuge. Use the supernatant.

Sample solution: Transfer 1 g of Powdered *Echinacea purpurea* to a centrifuge tube, add 10 mL of dichloromethane, mix well, and sonicate for 10 min. Centrifuge, and use the supernatant.

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plates)

Application volume: 5 µL *Standard solution B* and *Sample solution*, and 2 µL *Standard solution A* as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

Developing solvent system: A mixture of toluene, ethyl acetate, cyclohexane, and formic acid (8: 2: 1: 0.3)

Developing distance: 6 cm

Derivatization reagent: Place 85 mL of methanol in a 100-mL glass bottle and cool it down in a water–ice cubes–salt bath or in a freezer. To the ice-cold methanol, slowly and carefully add 10 mL of acetic acid and 5 mL of sulfuric acid, and mix well. Allow the mixture to cool to room temperature, then add 0.5 mL of *p*-anisaldehyde.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands to a suitable thin-layer chromatographic plate, and dry in air. Condition the plate to a relative humidity of about 33% using a suitable device. Develop the chromatograms in a saturated chamber. Remove the plate from the chamber, dry in air, derivatize with *Derivatization reagent*, heat at 100° for 3–5 min, set aside to cool, and examine under visible light.

System suitability: The chromatogram of *Standard solution B* exhibits the most prominent band as a pinkish violet band at about the middle of the chromatogram, and just below this pinkish band, a violet band at a lower R_F similar in position and color to the β-sitosterol band in the chromatograms of *Standard solution A*. These two bands are clearly separated from each other. The chromatogram of *Standard solution B* also shows a broad pink violet band close to the solvent front.

Acceptance criteria: The most prominent band of the *Sample solution* chromatogram is a pinkish violet band at about the middle of the chromatogram similar in position and color to a band in the *Standard solution B* chromatogram (much less prominent in *Echinacea angustifolia* and *Echinacea pallida*), a violet band corresponding to β-sitosterol band in the chromatograms of *Standard solution A* and *Standard solution B*, and a broad pink violet band close to the solvent front similar in position and color to the band in the chromatogram of *Standard solution B*. The *Sample solution* chromatogram does not exhibit a yellow band below the β-sitosterol band (difference from *Echinacea angustifolia*) or a prominent violet band at about two thirds of the chromatogram (difference from *Echinacea pallida*).

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• **C.** The retention time of the major peak in the *Sample solution* corresponds to that of the chicoric acid peak in *Standard solution A*, and the second most prominent peak corresponds to that of the caftaric acid peak in *Standard solution B*. The *Sample solution* chromatogram shows no or a very minor peak at the retention time corresponding to the echinacoside peak in the *Standard solution D* chromatogram. All peaks as obtained in the test for *Content of Total Phenols*.

COMPOSITION

• **CONTENT OF TOTAL PHENOLS**

Solution A: Phosphoric acid (0.1 in 100) in water
Solution B: Acetonitrile
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	90	10
13	78	22
14	60	40
17.5	60	40
18	90	10
30	90	10

Solvent: Alcohol and water (7:3)
Standard solution A: 30 µg/mL of USP Chicoric Acid RS in *Solvent*
Standard solution B: 20 µg/mL of USP Caftaric Acid RS in *Solvent*
Standard solution C: 20 µg/mL of USP Chlorogenic Acid RS in *Solvent*
Standard solution D: 20 µg/mL of USP Echinacoside RS in *Solvent*
Sample solution: Transfer about 125 mg of Powdered *Echinacea purpurea* (capable of passing through a 40-mesh sieve), accurately weighed, to a round-bottom flask equipped with a condenser. Add 25.0 mL of *Solvent*, and heat under reflux while shaking by mechanical means for 15 min. Centrifuge, or pass through a membrane filter of 0.45-µm or finer pore size.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 330 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 35°
Flow rate: 1.5 mL/min
Injection volume: 5 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Relative standard deviation: NMT 2% for chicoric acid peak in repeated injections, *Standard solution A*
Analysis
Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, *Standard solution D*, and *Sample solution*
Separately calculate the percentage of caftaric acid ($C_{13}H_{12}O_9$), chicoric acid ($C_{22}H_{18}O_{12}$), and chlorogenic acid ($C_{16}H_{18}O_9$) in the portion of Powdered *Echinacea purpurea* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area for the relevant analyte from the *Sample solution*
r_S = peak area of the relevant analyte from the corresponding *Standard solution*
C_S = concentration of the relevant analyte in the corresponding *Standard solution* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Powdered *Echinacea purpurea* used to prepare the *Sample solution* (mg)

Calculate the percentage of total phenols in the portion of Powdered *Echinacea purpurea* taken by adding the individual percentages calculated.
Acceptance criteria: NLT 0.5% of total phenols on the dried basis

• **CONTENT OF ALKAMIDES**

Mobile phase: Acetonitrile and water (55:45)
Standard solution A: 5 mg/mL of USP Powdered *Echinacea purpurea* Extract RS in methanol. Dissolve using sonication and shaking for 10 min. After dilution, pass through a membrane filter having a 0.45-µm or finer pore size.
Standard solution B: 10 µg/mL of USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS in methanol
Sample solution: Transfer about 2.5 g of Powdered *Echinacea purpurea* (capable of passing through a 40-mesh sieve) into a round-bottom flask. Add 80 mL of methanol, and reflux for 30 min. Cool to room temperature, and filter into a 100-mL volumetric flask using small portions of methanol to rinse the flask and the filter. Dilute with methanol to volume. Pass through a membrane filter having a 0.45-µm or finer pore size.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 30°
Flow rate: 1.5 mL/min
Injection volume: 25 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram from *Standard solution A* is similar to the Reference Chromatogram for alkamides provided with USP Powdered *Echinacea purpurea* Extract RS.
Resolution: NLT 1.0 between dodecatetraenoic acid isobutylamide peaks, *Standard solution A*
Tailing factor: NMT 2.0 for 2*E*,4*E*-hexadienoic acid isobutylamide peak, *Standard solution B*
Relative standard deviation: NMT 2.5% for the 2*E*,4*E*-hexadienoic acid isobutylamide peak in repeated injections, *Standard solution B*
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Identify the peaks of the 10 major alkamides in the chromatogram from the *Sample solution* by comparison with the chromatogram from *Standard solution A*.
Calculate the percentage of alkamides in the portion of Powdered *Echinacea purpurea* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = sum of the peak areas of the relevant analytes from the *Sample solution*
r_S = peak area of 2*E*,4*E*-hexadienoic acid isobutylamide from *Standard solution B*

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C_S = concentration of USP *2E,4E*-Hexadienoic Acid Isobutylamide RS in *Standard solution B* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Powdered *Echinacea purpurea* used to prepare the *Sample solution* (mg)
F = response factor for *2E,4E*-hexadienoic acid isobutylamide, 1.353

Acceptance criteria: NLT 0.025% on the dried basis

CONTAMINANTS

• **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩
Acceptance criteria
Arsenic: NMT 1.0 µg/g
Cadmium: NMT 0.5 µg/g
Lead: NMT 5.0 µg/g
Mercury: NMT 1.0 µg/g

Change to read:

• **▲ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩▲ (CN 1-May-2019)**:** Meets the requirements

SPECIFIC TESTS

• **BOTANIC CHARACTERISTICS:** Under a microscope, the following characteristics are observed: vessels (80 × 30 µm) with slanted end walls and spiral or pitted secondary walls; rectangular cork cells (150 × 60 µm) with brown inclusions; rectangular parenchymatous cells (120 × 30 µm), some pitted; elongated fiber cells having a narrow lumen with funnel-shaped end (20–40 µm wide); polygonal sclereids; a melanogenic layer of variable thickness, interspersed between the cell walls of the parenchyma; and lignified sclereids, vessels, and fibers. Starch is present; calcium oxalate and inulin crystals are absent.
• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry a sample at 105° for 2 h.
Acceptance criteria: NMT 10.0%
• **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 7.0%
• **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 4.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed, light-resistant containers.
• **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Caftaric Acid RS
USP Chicoric Acid RS
USP Chlorogenic Acid RS
USP Powdered *Echinacea purpurea* Extract RS
USP Echinacoside RS
USP *2E,4E*-Hexadienoic Acid Isobutylamide RS
USP β-Sitosterol RS

Powdered *Echinacea purpurea* Extract

DEFINITION

Powdered *Echinacea purpurea* Extract is prepared from dried *Echinacea purpurea* Root, *Echinacea purpurea* Aerial Parts, or a mixture of them, by extraction with hydroalcoholic mixtures or other suitable solvents. The ratio of the starting crude plant material to Powdered Extract is between 2:1 and 8:1. It contains NLT 4.0% of total phenols, calculated as the sum of caftaric acid ($C_{13}H_{12}O_9$), chicoric acid ($C_{22}H_{18}O_{12}$), and chlorogenic acid ($C_{16}H_{18}O_9$), on the dried basis. It contains NLT 0.025% of dodecatetraenoic acid isobutylamides ($C_{16}H_{25}NO$), calculated on the dried basis.

IDENTIFICATION

• **A. THIN-LAYER CHROMATOGRAPHY**
Presence of chicoric acid and absence of echinacoside
Standard solution A: 0.2 mg/mL of USP Echinacoside RS in methanol
Standard solution B: 0.2 mg/mL of USP Caftaric Acid RS, 0.1 mg/mL of USP Chlorogenic Acid RS, and 0.2 mg/mL of USP Chicoric Acid RS in methanol
Standard solution C: 20 mg/mL of USP Powdered *Echinacea purpurea* Extract RS in methanol. Shake to disperse, sonicate for 5 min, and centrifuge. Use the supernatant.
Sample solution: 20 mg/mL of Powdered *Echinacea purpurea* Extract in methanol. Shake to disperse, sonicate for 5 min, and centrifuge. Use the supernatant.
Chromatographic system
(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)
Application volume: 5 µL *Standard solution C* and *Sample solution*, and 2 µL *Standard solution A* and *Standard solution B* as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
Developing solvent system: A mixture of ethyl acetate, methylethyl ketone, water, and formic acid (5:3:1:1)
Developing distance: 6 cm
Derivatization reagent: 5 mg/mL of 2-aminoethyl diphenylborinate in ethyl acetate
Analysis
Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*
Apply the *Samples* as bands to a suitable thin-layer chromatographic plate, and dry in air. Develop the chromatograms in a saturated chamber. Remove the plate from the chamber, heat at 100° for 5 min, derivatize the plate while still warm with *Derivatization reagent*, dry in air, and examine under UV light at 366 nm.
System suitability: *Standard solution A* shows one major blue band in the lower third section of the chromatogram due to echinacoside. *Standard solution B* shows two blue bands at about the middle of the chromatogram due to caftaric acid (higher R_F) and chlorogenic acid (lower R_F) that are clearly separated, and a blue band for chicoric acid in the upper third section of the chromatogram.
Acceptance criteria: The most prominent band in the *Sample solution* chromatogram is a blue band in the upper third section of the chromatogram at an R_F corresponding to the chicoric acid band in the chromatograms of *Standard solution B* and *Standard solution C* (less prominent in *Echinacea pallida* and absent or almost absent in *Echinacea angustifolia*). The second most prominent band in the *Sample solution* chromatogram is a blue band at about the middle of the chromatogram due to caftaric acid, corresponding to a band in the chromatograms of *Standard solution B* and *Standard solution C* (absent in *Echinacea angustifolia* and a minor band in *Echinacea pallida*). The *Sample solution* chromatogram does not exhibit a band at the R_F of echinacoside in *Standard solution A* (difference from *Echinacea pallida* and *Echinacea angustifolia*). The *Sample solution* chromatogram may exhibit minor blue bands

USP Monographs

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corresponding to similar bands in the chromatogram of *Standard solution C*. One of these is due to chlorogenic acid at an R_F corresponding to chlorogenic acid in *Standard solution B*.

• **B. THIN-LAYER CHROMATOGRAPHY**

Presence of alkylamides

Standard solution A: 0.2 mg/mL of USP β-Sitosterol RS in methanol

Standard solution B: 100 mg/mL of USP Powdered *Echinacea purpurea* Extract RS in dichloromethane. Shake to disperse, sonicate for 5 min, and centrifuge. Use the supernatant.

Sample solution: 100 mg/mL of Powdered *Echinacea purpurea* Extract in dichloromethane. Shake to disperse, sonicate for 5 min, and centrifuge. Use the supernatant.

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plates)

Application volume: 5 µL *Standard solution B* and *Sample solution*, and 2 µL *Standard solution A*, as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

Developing solvent system: A mixture of toluene, ethyl acetate, cyclohexane, and formic acid (8: 2: 1: 0.3)

Developing distance: 6 cm

Derivatization reagent: Place 85 mL of methanol in a 100-mL glass bottle, and cool it down in a water–ice cubes–salt bath or in a freezer. To the ice-cold methanol, slowly and carefully add 10 mL of acetic acid and 5 mL of sulfuric acid, and mix well. Allow the mixture to cool to room temperature, then add 0.5 mL of *p*-anisaldehyde.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands to a suitable thin-layer chromatographic plate, and dry in air. Develop the chromatograms in a saturated chamber. Remove the plate from the chamber, dry in air, derivatize with *Derivatization reagent*, heat at 100° for 3–5 min, set aside to cool, and examine under visible light.

System suitability: The chromatogram of *Standard solution B* exhibits the most prominent band as a pinkish violet band at about the middle of the chromatogram, and just below this pinkish band, a violet band at a lower R_F similar in position and color to the β-sitosterol band in the chromatograms of *Standard solution A*. These two bands are clearly separated from each other. The chromatogram of *Standard solution B* also shows a broad pink violet band close to the solvent front. The β-sitosterol band of the *Standard solution B* chromatogram and the pinkish violet band underneath are clearly separated from one another.

Acceptance criteria: The most prominent band of the *Sample solution* chromatogram is a pinkish violet band at about the middle of the chromatogram similar in position and color to a band in the *Standard solution B* chromatogram (much less prominent in *Echinacea angustifolia* and *Echinacea pallida*), a violet band corresponding to β-sitosterol band in the chromatograms of *Standard solution A* and *Standard solution B*, and a broad pink violet band close to the solvent front similar in position and color to the band in the chromatogram of *Standard solution B*. The *Sample solution* chromatogram does not exhibit a yellow band below the β-sitosterol band (difference from *Echinacea angustifolia*) or a prominent violet band at about two-thirds of the chromatogram (difference from *Echinacea pallida*).

• **C.** The retention time of the major peak in the *Sample solution* corresponds to that of the chicoric acid peak in *Standard solution A*, and the second most prominent peak corresponds to that of the caftaric acid peak in *Standard solution B*. The *Sample solution* chromatogram shows no or a very minor peak at the retention time corresponding to the echinacoside peak in the *Standard solution D* chromatogram, all peaks as obtained in the test for *Content of Total Phenols*.

COMPOSITION

• **CONTENT OF TOTAL PHENOLS**

Solvent: Alcohol and water (7:3)

Standard solution A: 30 µg/mL of USP Chicoric Acid RS in *Solvent*

Standard solution B: 20 µg/mL of USP Caftaric Acid RS in *Solvent*

Standard solution C: 20 µg/mL of USP Chlorogenic Acid RS in *Solvent*

Standard solution D: 20 µg/mL of USP Echinacoside RS in *Solvent*

Sample solution: Transfer 60 mg of Powdered *Echinacea purpurea* Extract to a round-bottom flask equipped with a condenser. Add 25 mL of *Solvent*, and heat under reflux while shaking by mechanical means for 15 min. Centrifuge, or pass through a membrane filter of 0.45-µm or finer pore size.

Solution A: Phosphoric acid (0.1 in 100) in water

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	90	10
13	78	22
14	60	40
17	60	40
17.5	90	10
22	90	10

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 330 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 35°

Flow rate: 1.5 mL/min

Injection volume: 5 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Relative standard deviation: NMT 2% for chicoric acid peak in repeated injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, *Standard solution D*, and *Sample solution*

Separately calculate the percentage of caftaric acid ($C_{13}H_{12}O_9$), chicoric acid ($C_{22}H_{18}O_{12}$), and chlorogenic acid ($C_{16}H_{18}O_9$) in the portion of Powdered *Echinacea purpurea* Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

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r_U = peak response for the relevant analyte from the *Sample solution*
r_S = peak response of the relevant analyte from the corresponding *Standard solution*
C_S = concentration of the relevant analyte in the corresponding *Standard solution* (mg/mL)
C_U = concentration of Powdered *Echinacea purpurea* Extract in the *Sample solution* (mg/mL)

Calculate the percentage of total phenols in the portion of Powdered *Echinacea purpurea* Extract taken by adding the individual percentages calculated.

Acceptance criteria: NLT 4.0% on the dried basis

• **CONTENT OF DODECATETRAENOIC ACID ISOBUTYLAMIDES**

Standard solution A: 5 mg/mL of USP Powdered *Echinacea purpurea* Extract RS in methanol. Dissolve using sonication and shaking for 10 min. After dilution, pass through a membrane filter having a 0.45-µm or finer pore size.

Standard solution B: 10 µg/mL of USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS in methanol

Sample solution: Transfer about 500 mg of Powdered *Echinacea purpurea* Extract, accurately weighed, into a 100-mL volumetric flask. Add 80 mL of methanol, and sonicate for 30 min. Cool to room temperature, and dilute with methanol to volume. Pass through a membrane filter of 0.45-µm or finer pore size.

Mobile phase: Acetonitrile and water (55:45)

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 30°
Flow rate: 1.5 mL/min
Injection volume: 25 µL

System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram from *Standard solution A* is similar to the Reference Chromatogram for alkamides provided with USP Powdered *Echinacea purpurea* Extract RS.
Resolution: NLT 1.0 between dodecatetraenoic acid isobutylamide peaks, *Standard solution A*
Tailing factor: NMT 2.0 for the 2*E*,4*E*-hexadienoic acid isobutylamide peak, *Standard solution B*
Relative standard deviation: NMT 2.5% for the 2*E*,4*E*-hexadienoic acid isobutylamide peak in repeated injections, *Standard solution B*

Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Identify the peaks due to 2*E*,4*E*,8*Z*,10*E*-dodecatetraenoic acid isobutylamide and 2*E*,4*E*,8*Z*,10*Z*-dodecatetraenoic acid isobutylamide in the chromatogram from the *Sample solution* by comparison with the chromatogram from *Standard solution A*. Measure the areas for the relevant peaks.
Calculate the percentage of dodecatetraenoic acid isobutylamides in the portion of Powdered *Echinacea purpurea* Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = sum of the peak responses of the relevant analytes from the *Sample solution*
r_S = peak response from *Standard solution B*
C_S = concentration of USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS in *Standard solution B* (mg/mL)
C_U = concentration of Powdered *Echinacea purpurea* Extract in the *Sample solution* (mg/mL)
F = response factor to convert 2*E*,4*E*-hexadienoic acid isobutylamide into dodecatetraenoic acid isobutylamides, 1.353

Acceptance criteria: NLT 0.025% on the dried basis

CONTAMINANTS

• **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩
Acceptance criteria
Arsenic: NMT 1.0 µg/g
Cadmium: NMT 0.5 µg/g
Lead: NMT 5.0 µg/g
Mercury: NMT 1.0 µg/g

Change to read:

• **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020)
: Meets the requirements
• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total bacterial count does not exceed 10^4 cfu/g. The total combined molds and yeasts count does not exceed 10^3 cfu/g.
• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.
• **BOTANICAL EXTRACTS,** *Residual Solvents* ⟨565⟩**:** Meets the requirements

SPECIFIC TESTS

• **LOSS ON DRYING** ⟨731⟩
Sample: 1 g
Analysis: Dry the *Sample* at 105° for 2 h.
Acceptance criteria: NMT 5.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, in a cool place.
• **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant from which the article was prepared. If derived from root and aerial parts, indicate the corresponding percentages. Label it to indicate the content of total phenols and dodecatetraenoic isobutylamides. The label bears a statement indicating that *Echinacea purpurea* may cause rare allergic reactions, rashes, or aggravate asthma. It meets the requirements for *Botanical Extracts* ⟨565⟩, *Labeling*.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Caftaric Acid RS
USP Chicoric Acid RS
USP Chlorogenic Acid RS
USP Powdered *Echinacea purpurea* Extract RS
USP Echinacoside RS
USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS
USP β-Sitosterol RS

Echinacea Species Dry Extract Capsules

DEFINITION

Echinacea Species Dry Extract Capsules contain one or more *Echinacea* Species (Fam. Asteraceae) Dry Extracts prepared from dried rhizome and roots of *Echinacea angustifolia* DC., dried rhizome and roots of *Echinacea pallida* (Nutt.) Nutt., dried rhizome and roots of *Echinacea purpurea* (L.) Moench,

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and dried aerial parts of *Echinacea purpurea* (L.) Moench. They contain NLT 90% and NMT 110% of the labeled amount of total phenols, calculated as the sum of caftaric acid ($C_{13}H_{12}O_9$),[1] chicoric acid ($C_{22}H_{18}O_{12}$),[2] echinacoside ($C_{35}H_{46}O_{20}$), and cynarin (1,3-di-*O*-caffeoylquinic acid) ($C_{25}H_{24}O_{12}$).[3]

IDENTIFICATION

• **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

For Capsules containing *Echinacea angustifolia* Dry Extract

Standard solution A: 0.2 mg/mL of USP Echinacoside RS and 0.2 mg/mL of cynarin in methanol

Standard solution B: 0.05 mg/mL of USP Caftaric Acid RS, 0.1 mg/mL of USP Chlorogenic Acid RS, and 0.05 mg/mL of USP Chicoric Acid RS in methanol

Standard solution C: 20 mg/mL of USP Powdered *Echinacea angustifolia* Extract RS in methanol. Shake to disperse, sonicate for 5 min, and centrifuge. Use the supernatant.

Sample solution: Transfer a portion of the Capsule contents, equivalent to 100 mg of *Echinacea angustifolia* Dry Extract, to a centrifuge tube, add 5 mL of methanol, shake to disperse, sonicate for 5 min, and centrifuge. Use the supernatant.

Chromatographic system

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plates)

Application volume: 5 µL each of *Standard solution C* and *Sample solution*, and 2 µL each of *Standard solution A* and *Standard solution B* as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33%.

Temperature: Ambient, not to exceed 30°

Developing solvent system: Ethyl acetate, methyl ethyl ketone, water, and formic acid (5:3:1:1)

Developing distance: 6 cm

Derivatization reagent: 5 mg/mL of 2-aminoethyl diphenylborinate in ethyl acetate

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Apply the *Samples* as bands and dry in air. Develop in a saturated chamber. Remove the plate from the chamber, heat at 100° for 5 min, treat while still warm with *Derivatization reagent*, dry in air, and examine under UV light at 366 nm.

System suitability: *Standard solution A* shows two major blue bands, one in the lower third section due to echinacoside, and the other band in the middle section due to dicaffeoylquinic acid (cynarin). *Standard solution B* shows two major blue bands at about the middle section due to caftaric acid (higher R_F) and chlorogenic acid (lower R_F) that are clearly separated, and a blue chicoric acid band in the upper third of the chromatogram.

Acceptance criteria: The *Sample solution* exhibits the following: the most prominent blue band in the lower third section at R_F corresponding to that of echinacoside in *Standard solution A* and *Standard solution C*; a prominent greenish-blue band in the middle section at R_F corresponding to cynarin in *Standard solution A* and *Standard solution C*; minor bands between the positions of echinacoside and cynarin. One of these is due to chlorogenic acid at R_F corresponding to that of chlorogenic acid in *Standard solution B*; very faint (or may be absent) blue bands at R_F corresponding to the caftaric acid and chicoric acid bands in *Standard solution B*.

For Capsules containing *Echinacea pallida* Dry Extract: Proceed as directed in *For Capsules containing Echinacea angustifolia Dry Extract*. For *Standard solution C* and the *Sample solution*, substitute USP Powdered *Echinacea angustifolia* Extract RS with USP Powdered *Echinacea pallida* Extract RS and *Echinacea angustifolia* Dry Extract with *Echinacea pallida* Dry Extract, respectively.

Acceptance criteria: The *Sample solution* exhibits the most prominent blue band in the lower third section at R_F corresponding to that of echinacoside in *Standard solution A* and *Standard solution C*; may exhibit bands of lesser intensity at the R_F corresponding to caftaric acid and chicoric acid in *Standard solution B* and *Standard solution C*; exhibits minor bands between the positions of echinacoside and caftaric acid. One of these is due to chlorogenic acid at an R_F corresponding to that of chlorogenic acid in *Standard solution B*. The *Sample solution* does not exhibit a blue band in the middle section at R_F corresponding to cynarin in *Standard solution A*.

For Capsules containing *Echinacea purpurea* Root Dry Extract and *Echinacea purpurea* Aerial Parts Dry Extract: Proceed as directed in *For Capsules containing Echinacea angustifolia Dry Extract*. For *Standard solution C* substitute USP Powdered *Echinacea angustifolia* Extract RS with USP Powdered *Echinacea purpurea* Extract RS. For the *Sample solution*, substitute *Echinacea angustifolia* Dry Extract with *Echinacea purpurea* Root Dry Extract and *Echinacea purpurea* Aerial Parts Dry Extract.

Acceptance criteria: The *Sample solution* exhibits the most prominent blue band in the upper third section at R_F corresponding to chicoric acid in *Standard solution B* and *Standard solution C*; exhibits the second most prominent blue band at about the middle section at R_F corresponding to that of caftaric acid in *Standard solution B* and *Standard solution C*; may exhibit minor blue bands corresponding to similar bands in *Standard solution C*. One of these is due to chlorogenic acid at an R_F corresponding to chlorogenic acid in *Standard solution B*. The *Sample solution* chromatogram does not exhibit a band at the same R_F as echinacoside in *Standard solution A*.

• **B. HPLC FOR TOTAL PHENOLS**

Analysis: Proceed as directed in the test for *Content of Total Phenols*.

Acceptance criteria: The chromatogram of the *Sample solution* prepared from Capsules labeled to contain extracts of *E. purpurea* roots or aerial parts exhibits peaks at the retention times of those due to caftaric acid, chlorogenic acid, and chicoric acid in the chromatogram of the *Standard solution*. The chromatogram of the *Sample solution* prepared from Capsules labeled to contain extract of *E. angustifolia* exhibits peaks at the retention times of those due to chlorogenic acid, cynarin, and echinacoside in the chromatogram of the *Standard solution*. The chromatogram of the *Sample solution* prepared from Capsules labeled to contain extract of *E. pallida* exhibits peaks at the retention times of those due to caftaric acid, chlorogenic acid, echinacoside, and chicoric acid in the chromatogram of the *Standard solution*.

• **C. HPLC FOR PRESENCE OF ALKYLAMIDES**

Analysis: Proceed as directed in the test for *Content of Dodecatetraenoic Acid Isobutylamides*.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks at the retention times of those due

[1] (2*R*,3*R*)-2-{[(*E*)-3-(3,4-Dihydroxyphenyl)acryloyl]oxy}-3-hydroxysuccinic acid.

[2] (2*R*,3*R*)-2,3-Bis{[(*E*)-3-(3,4-dihydroxyphenyl)acryloyl]oxy}succinic acid. Other common names for chicoric acid are cichoric acid and dicaffeoyltartaric acid.

[3] (1*R*,3*R*,4*S*,5*R*)-1,3-Bis{[(*E*)-3-(3,4-dihydroxyphenyl)acryloyl]oxy}-4,5-dihydroxycyclohexane-1-carboxylic acid.

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to dodecatetraenoic acid isobutylamides in the chromatogram of *Standard solution B* or *Standard solution C*, and the reference chromatogram provided with the lot of USP Powdered *Echinacea angustifolia* Extract RS or USP Powdered *Echinacea purpurea* Extract RS being used.

STRENGTH

• **CONTENT OF TOTAL PHENOLS**

Solution A: Phosphoric acid in water (0.1 in 100)

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	90	10
3	90	10
16	78	22
17	60	40
20	60	40
20.5	90	10
25	90	10

Solvent: Alcohol and water (7:3)

Standard solution: 20 µg/mL of USP Caftaric Acid RS, 20 µg/mL of USP Chlorogenic Acid RS, 20 µg/mL of cynarin (1,3-di-*O*-caffeoylquinic acid), 60 µg/mL of USP Echinacoside RS, and 40 µg/mL of USP Chicoric Acid RS in *Solvent*

Sample solution: Determine the total weight of 20 Capsules. Empty the Capsules, combine, and mix their contents to obtain a homogenous composite. Weigh the empty Capsule shells and calculate the average fill weight per Capsule. Transfer a portion of the Capsule contents, nominally equivalent to 60 mg of the labeled *Echinacea* Species Dry Extract, to a 100-mL round-bottom flask equipped with a condenser. Add 25.0 mL of *Solvent*, and heat under reflux for 15 min. Cool to room temperature and pass through a filter of 0.45-µm or finer pore size.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 330 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 35°

Flow rate: 1.5 mL/min

Injection volume: 5 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Resolution: NLT 3.0 between the cynarin and echinacoside peaks, and NLT 1.0 between the caftaric acid and chlorogenic acid peaks. [NOTE—Echinacoside peaks may be resolved in two components. The relative retention times for caftaric acid, chlorogenic acid, cynarin, echinacoside, and chicoric acid are 0.7, 0.75, 0.9, 1.0, and 1.4, respectively.]

Relative standard deviation: NMT 2.5% for the sum of echinacoside peaks

Analysis

Samples: *Standard solution* and *Sample solution*

Using the chromatogram of the *Standard solution*, identify and measure the areas of the peaks corresponding to caftaric acid ($C_{13}H_{12}O_9$), cynarin ($C_{25}H_{24}O_{12}$), echinacoside ($C_{35}H_{46}O_{20}$), and chicoric acid ($C_{22}H_{18}O_{12}$) in the *Sample solution* chromatogram.

Calculate the percentage of caftaric acid, cynarin, echinacoside, and chicoric acid in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of a relevant analyte from the *Sample solution*

r_S = peak area of caftaric acid, echinacoside, or chicoric acid from the *Standard solution*

C_S = concentration of a relevant analyte in the *Standard solution* (mg/mL)

V = volume of the solvent taken to prepare the *Sample solution* (mL)

W = weight of the sample taken to prepare the *Sample solution* (mg)

Calculate the percentage of the labeled amount of total phenols, calculated as the sum of determined caftaric acid, echinacoside, chicoric acid, and cynarin in the portion of Capsules taken:

$$\text{Result} = (\Sigma P_i/L) \times 100$$

ΣP_i = total combined content of caftaric acid, echinacoside, chicoric acid, and cynarin as determined above (%)

L = labeled amount of total phenols (%)

Acceptance criteria: 90%–110%

PERFORMANCE TESTS

• **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩, *Disintegration*: Meet the requirements

• **WEIGHT VARIATION** ⟨2091⟩: Meet the requirements

SPECIFIC TESTS

• **CONTENT OF DODECATETRAENOIC ACID ISOBUTYLAMIDES**

[NOTE—This test is not applicable to Capsules containing *Echinacea pallida* extract prepared from dried rhizome and roots.]

Mobile phase: Acetonitrile and water (55:45)

Standard solution A: 10 µg/mL of USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS in methanol

Standard solution B: 1 mg/mL of USP Powdered *Echinacea angustifolia* Extract RS in methanol. Sonicate to dissolve, and pass through a filter of 0.45-µm or finer pore size. [NOTE—Prepare when Capsules contain *Echinacea angustifolia* Dry Extract.]

Standard solution C: 5 mg/mL of USP Powdered *Echinacea purpurea* Extract RS in methanol. Sonicate to dissolve and pass through a filter of 0.45-µm or finer pore size. [NOTE—Prepare when Capsules do not contain *Echinacea angustifolia* Dry Extract.]

Sample solution: Transfer a portion of the Capsule contents, equivalent to 500 mg of *Echinacea* Species Dry Extract, to a 100-mL volumetric flask. Add 80 mL of methanol, and sonicate for 30 min. Dilute with methanol to volume, and pass through a membrane filter of 0.45-µm or finer pore size.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 30°

Flow rate: 1.5 mL/min

Injection volume: 25 µL

System suitability

Samples: *Standard solution A*, *Standard solution B*, or *Standard solution C*

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Suitability requirements
Resolution: NLT 1.0 between the dodecatetraenoic acid isobutylamide peaks, *Standard solution B* or *Standard solution C*
Tailing factor: NMT 2.0 for the 2*E*,4*E*-hexadienoic acid isobutylamide peak, *Standard solution A*
Relative standard deviation: NMT 2.5% for the 2*E*,4*E*-hexadienoic acid isobutylamide peak in replicate injections, *Standard solution A*
Chromatogram similarity: The chromatogram of *Standard solution B* or *Standard solution C* is similar to the reference chromatogram for alkamides provided with the USP Powdered *Echinacea angustifolia* Extract RS or USP Powdered *Echinacea purpurea* Extract RS being used.

Analysis
Samples: *Standard solution A*, *Standard solution B*, or *Standard solution C*, and *Sample solution*
Using the chromatogram of *Standard solution B* or *Standard solution C*, and the reference chromatogram provided with the lot of USP Powdered *Echinacea angustifolia* Extract RS or USP Powdered *Echinacea purpurea* Extract RS being used, identify and measure the areas of 2*E*,4*E*,8*Z*, 10*E*- and 2*E*,4*E*,8*Z*,10*Z*-dodecatetraenoic acid isobutylamide peaks in the *Sample solution*.
Calculate the percentage of dodecatetraenoic acid isobutylamides in the amount of *Echinacea* Species Dry Extract taken:

$$\text{Result} = (r_{sum}/r_S) \times (C_S \times V/W) \times (W_{av}/L) \times F \times 100$$

r_{sum} = sum of the peak areas of the relevant analytes from the *Sample solution*
r_S = peak area of 2*E*,4*E*-hexadienoic acid isobutylamide from *Standard solution A*
C_S = concentration of USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS in *Standard solution A* (mg/mL)
V = volume of the solvent taken to prepare *Sample solution* (mL)
W = weight of the sample taken to prepare *Sample solution* (mg)
W_{av} = average Capsule fill weight (mg/Capsule)
L = labeled amount of *Echinacea angustifolia* Dry Extract, *Echinacea purpurea* Root Dry Extract, or *Echinacea purpurea* Aerial Parts Dry Extract per Capsule (mg)
F = response factor of dodecatetraenoic acid isobutylamides relative to 2*E*,4*E*-hexadienoic acid isobutylamide, 1.353

Acceptance criteria
For Capsules containing *Echinacea angustifolia* Dry Extract: NLT 0.1%
For Capsules not containing *Echinacea angustifolia* Dry Extract: NLT 0.025%

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meet the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store in a cool place.
- **LABELING:** The label states the Latin binomial and the official name. The label states the amount of total phenols (as sum of presented caftaric acid, echinacoside, chicoric acid, and cynarin) and the amount of *Echinacea* Species Dry Extract in mg/Capsule.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Caftaric Acid RS
USP Chicoric Acid RS
USP Chlorogenic Acid RS
USP Powdered *Echinacea angustifolia* Extract RS
USP Powdered *Echinacea pallida* Extract RS
USP Powdered *Echinacea purpurea* Extract RS
USP Echinacoside RS
USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS

Echinacea Species Dry Extract Tablets

DEFINITION

Echinacea Species Dry Extract Tablets contain one or more *Echinacea* Species (Fam. Asteraceae) Dry Extracts prepared from dried rhizome and roots of *Echinacea angustifolia* DC., dried rhizome and roots of *Echinacea pallida* (Nutt.) Nutt., dried rhizome and roots of *Echinacea purpurea* (L.) Moench, and dried aerial parts of *Echinacea purpurea* (L.) Moench. They contain NLT 90% and NMT 110% of the labeled amount of total phenols, calculated as the sum of caftaric acid ($C_{13}H_{12}O_9$),[1] chicoric acid ($C_{22}H_{18}O_{12}$),[2] echinacoside ($C_{35}H_{46}O_{20}$), and cynarin (1,3-di-*O*-caffeoylquinic acid) ($C_{25}H_{24}O_{12}$).[3]

IDENTIFICATION

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩
For Tablets containing *Echinacea angustifolia* Dry Extract
Standard solution A: 0.2 mg/mL of USP Echinacoside RS and 0.2 mg/mL of cynarin in methanol
Standard solution B: 0.05 mg/mL of USP Caftaric Acid RS, 0.1 mg/mL of USP Chlorogenic Acid RS, and 0.05 mg/mL of USP Chicoric Acid RS in methanol
Standard solution C: 20 mg/mL of USP Powdered *Echinacea angustifolia* Extract RS in methanol. Shake to disperse, sonicate for 5 min, and centrifuge. Use the supernatant.
Sample solution: Transfer a portion of the powdered Tablets, equivalent to 100 mg of *Echinacea angustifolia* Dry Extract, to a centrifuge tube, add 5 mL of methanol, shake to disperse, sonicate for 5 min, and centrifuge. Use the supernatant.
Chromatographic system
Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plates)
Application volume: 5 µL each of *Standard solution C* and *Sample solution*, and 2 µL each of *Standard solution A* and *Standard solution B* as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33%.
Temperature: Ambient, not to exceed 30°
Developing solvent system: Ethyl acetate, methyl ethyl ketone, water, and formic acid (5:3:1:1)
Developing distance: 6 cm

[1] (2*R*,3*R*)-2-{[(*E*)-3-(3,4-Dihydroxyphenyl)acryloyl]oxy}-3-hydroxysuccinic acid.
[2] (2*R*,3*R*)-2,3-Bis{[(*E*)-3-(3,4-dihydroxyphenyl)acryloyl]oxy}succinic acid. Other common names for chicoric acid are cichoric acid and dicaffeoyltartaric acid.
[3] (1*R*,3*R*,4*S*,5*R*)-1,3-Bis{[(*E*)-3-(3,4-dihydroxyphenyl)acryloyl]oxy}-4,5-dihydroxycyclohexane-1-carboxylic acid.

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Derivatization reagent: 5 mg/mL of 2-aminoethyl diphenylborinate in ethyl acetate

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Apply the *Samples* as bands and dry in air. Develop in a saturated chamber. Remove the plate from the chamber, heat at 100° for 5 min, treat while still warm with *Derivatization reagent*, dry in air, and examine under UV light at 366 nm.

System suitability: *Standard solution A* shows two major blue bands, one in the lower third section due to echinacoside, and the other band in the middle section due to dicaffeoylquinic acid (cynarin). *Standard solution B* shows two major blue bands at about the middle section due to caftaric acid (higher R_F) and chlorogenic acid (lower R_F) that are clearly separated, and a blue chicoric acid band in the upper third of the chromatogram.

Acceptance criteria: The *Sample solution* exhibits the following: the most prominent blue band in the lower third section at R_F corresponding to that of echinacoside in *Standard solution A* and *Standard solution C*; a prominent greenish-blue band in the middle section at R_F corresponding to cynarin in *Standard solution A* and *Standard solution C*; minor bands between the positions of echinacoside and cynarin. One of these is due to chlorogenic acid at R_F corresponding to that of chlorogenic acid in *Standard solution B*; very faint (or may be absent) blue bands at R_F corresponding to the caftaric acid and chicoric acid bands in *Standard solution B*.

For Tablets containing *Echinacea pallida* Dry Extract: Proceed as directed in *For Tablets containing Echinacea angustifolia Dry Extract*. For *Standard solution C* and the *Sample solution*, substitute USP Powdered *Echinacea angustifolia* Extract RS with USP Powdered *Echinacea pallida* Extract RS and *Echinacea angustifolia* Dry Extract with *Echinacea pallida* Dry Extract, respectively.

Acceptance criteria: The *Sample solution* exhibits the most prominent blue band in the lower third section at R_F corresponding to that of echinacoside in *Standard solution A* and *Standard solution C*; may exhibit bands of lesser intensity at the R_F corresponding to caftaric acid and chicoric acid in *Standard solution B* and *Standard solution C*; exhibits minor bands between the positions of echinacoside and caftaric acid. One of these is due to chlorogenic acid at an R_F corresponding to that of chlorogenic acid in *Standard solution B*. The *Sample solution* does not exhibit a blue band in the middle section at R_F corresponding to cynarin in *Standard solution A*.

For Tablets containing *Echinacea purpurea* Root Dry Extract and *Echinacea purpurea* Aerial Parts Dry Extract: Proceed as directed in *For Tablets containing Echinacea angustifolia Dry Extract*. For *Standard solution C* substitute USP Powdered *Echinacea angustifolia* Extract RS with USP Powdered *Echinacea purpurea* Extract RS. For the *Sample solution*, substitute *Echinacea angustifolia* Dry Extract with *Echinacea purpurea* Root Dry Extract and *Echinacea purpurea* Aerial Parts Dry Extract.

Acceptance criteria: The *Sample solution* exhibits the most prominent blue band in the upper third section at R_F corresponding to chicoric acid in *Standard solution B* and *Standard solution C*; exhibits the second most prominent blue band at about the middle section at R_F corresponding to that of caftaric acid in *Standard solution B* and *Standard solution C*; may exhibit minor blue bands corresponding to similar bands in *Standard solution C*. One of these is due to chlorogenic acid at an R_F corresponding to chlorogenic acid in *Standard solution B*. The *Sample solution* does not exhibit a band at the same R_F of echinacoside in *Standard solution A*.

• **B. HPLC FOR TOTAL PHENOLS**

Analysis: Proceed as directed in the test for *Content of Total Phenols*.

Acceptance criteria: The chromatogram of the *Sample solution* prepared from Tablets labeled to contain extracts of *E. purpurea* roots or aerial parts exhibits peaks at the retention times of those due to caftaric acid, chlorogenic acid, and chicoric acid in the chromatogram of the *Standard solution*. The chromatogram of the *Sample solution* prepared from Tablets labeled to contain extract of *E. angustifolia* exhibits peaks at the retention times of those due to chlorogenic acid, cynarin, and echinacoside in the chromatogram of the *Standard solution*. The chromatogram of the *Sample solution* prepared from Tablets labeled to contain extract of *E. pallida* exhibits peaks at the retention times of those due to caftaric acid, chlorogenic acid, echinacoside, and chicoric acid in the chromatogram of the *Standard solution*.

• **C. HPLC FOR PRESENCE OF ALKYLAMIDES**

Analysis: Proceed as directed in the test for *Content of Dodecatetraenoic Acid Isobutylamides*.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks at the retention times of those due to dodecatetraenoic acid isobutylamides in *Standard solution B* or *Standard solution C*, and the reference chromatogram provided with the lot of USP Powdered *Echinacea angustifolia* Extract RS or USP Powdered *Echinacea purpurea* Extract RS being used.

STRENGTH

• **CONTENT OF TOTAL PHENOLS**

Solution A: Phosphoric acid in water (0.1 in 100)

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	90	10
3	90	10
16	78	22
17	60	40
20	60	40
20.5	90	10
25	90	10

Solvent: Alcohol and water (7:3)

Standard solution: 20 µg/mL of USP Caftaric Acid RS, 20 µg/mL of USP Chlorogenic Acid RS, 20 µg/mL of cynarin (1,3-di-*O*-caffeoylquinic acid), 60 µg/mL of USP Echinacoside RS, and 40 µg/mL of USP Chicoric Acid RS in *Solvent*

Sample solution: Weigh NLT 20 Tablets, determine the average Tablet weight, and finely powder. Transfer a portion of finely powdered Tablets, nominally equivalent to 60 mg of the labeled *Echinacea* Species Dry Extract, to a 100-mL round-bottom flask equipped with a condenser. Add 25.0 mL of *Solvent*, and heat under reflux for 15 min. Cool to room temperature, and pass through a filter of 0.45-µm or finer pore size.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 330 nm

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Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 35°
Flow rate: 1.5 mL/min
Injection volume: 5 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Resolution: NLT 3.0 between the cynarin and echinacoside peaks, and NLT 1.0 between the caftaric acid and chlorogenic acid peaks. [NOTE—Echinacoside peaks may be resolved in two components. The relative retention times for caftaric acid, chlorogenic acid, cynarin, echinacoside, and chicoric acid are 0.7, 0.75, 0.9, 1.0, and 1.4, respectively.]
Relative standard deviation: NMT 2.5% for the sum of echinacoside peaks
Analysis
Samples: *Standard solution* and *Sample solution*
Using the chromatogram of the *Standard solution*, identify and measure areas of the peaks corresponding to caftaric acid ($C_{13}H_{12}O_9$), cynarin ($C_{25}H_{24}O_{12}$), echinacoside ($C_{35}H_{46}O_{20}$), and chicoric acid ($C_{22}H_{18}O_{12}$) in the *Sample solution*.
Calculate the percentage of caftaric acid, cynarin, echinacoside, and chicoric acid in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of a relevant analyte from the *Sample solution*
r_S = peak area of caftaric acid, echinacoside, or chicoric acid from the *Standard solution*
C_S = concentration of a relevant analyte in the *Standard solution* (mg/mL)
V = volume of the solvent taken to prepare the *Sample solution* (mL)
W = weight of the sample taken to prepare the *Sample solution* (mg)

Calculate the percentage of the labeled amount of total phenols, calculated as the sum of determined caftaric acid, echinacoside, chicoric acid, and cynarin in the portion of Tablets taken:

$$\text{Result} = (\Sigma P_i/L) \times 100$$

ΣP_i = total combined content of caftaric acid, echinacoside, chicoric acid, and cynarin as determined above (%)
L = labeled amount of total phenols (%)

Acceptance criteria: 90%–110%

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩, *Disintegration*: Meet the requirements
- **WEIGHT VARIATION** ⟨2091⟩: Meet the requirements

SPECIFIC TESTS

- **CONTENT OF DODECATETRAENOIC ACID ISOBUTYLAMIDES**

[NOTE—This test is not applicable to Tablets containing *Echinacea pallida* extract prepared from dried rhizome and roots.]
Mobile phase: Acetonitrile and water (55:45)
Standard solution A: 10 µg/mL of USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS in methanol
Standard solution B: 1 mg/mL of USP Powdered *Echinacea angustifolia* Extract RS in methanol. Sonicate to dissolve, and pass through a filter of 0.45-µm or finer pore size. [NOTE—Prepare when Tablets contain *Echinacea angustifolia* Dry Extract.]
Standard solution C: 5 mg/mL of USP Powdered *Echinacea purpurea* Extract RS in methanol. Sonicate to dissolve, and pass through a filter of 0.45-µm or finer pore size. [NOTE—Prepare when Tablets do not contain *Echinacea angustifolia* Dry Extract.]
Sample solution: Weigh NLT 20 Tablets, determine the average Tablet weight, and finely powder. Transfer a portion of finely powdered Tablets, equivalent to 500 mg of *Echinacea* Species Dry Extract, to a 100-mL volumetric flask. Add 80 mL of methanol, and sonicate for 30 min. Dilute with methanol to volume, and pass through a membrane filter of 0.45-µm or finer pore size.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 30°
Flow rate: 1.5 mL/min
Injection volume: 25 µL
System suitability
Samples: *Standard solution A*, *Standard solution B* or *Standard solution C*
Suitability requirements
Resolution: NLT 1.0 between the dodecatetraenoic acid isobutylamide peaks, *Standard solution B* or *Standard solution C*
Tailing factor: NMT 2.0 for the 2*E*,4*E*-hexadienoic acid isobutylamide peak, *Standard solution A*
Relative standard deviation: NMT 2.5% for the 2*E*,4*E*-hexadienoic acid isobutylamide peak in replicate injections, *Standard solution A*
Chromatogram similarity: The chromatogram of *Standard solution B* or *Standard solution C* is similar to the reference chromatogram for alkamides provided with the USP Powdered *Echinacea angustifolia* Extract RS or USP Powdered *Echinacea purpurea* Extract RS being used.
Analysis
Samples: *Standard solution A*, *Standard solution B*, or *Standard solution C*, and *Sample solution*
Using the chromatogram of *Standard solution B* or *Standard solution C*, and the reference chromatogram provided with the lot of USP Powdered *Echinacea angustifolia* Extract RS or USP Powdered *Echinacea purpurea* Extract RS being used, identify and measure the areas of 2*E*,4*E*,8*Z*,10*E*- and 2*E*,4*E*,8*Z*,10*Z*-dodecatetraenoic acid isobutylamide peaks in the *Sample solution* chromatogram.
Calculate the percentage of dodecatetraenoic acid isobutylamides in the amount of *Echinacea* Species Dry Extract taken:

$$\text{Result} = (r_{sum}/r_S) \times (C_S \times V/W) \times (W_{av}/L) \times F \times 100$$

r_{sum} = sum of the peak areas of the relevant analytes from the *Sample solution*
r_S = peak area of 2*E*,4*E*-hexadienoic acid isobutylamide from *Standard solution A*
C_S = concentration of USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS in *Standard solution A* (mg/mL)
V = volume of the solvent taken to prepare *Sample solution* (mL)
W = weight of the sample taken to prepare *Sample solution* (mg)
W_{av} = average Tablet weight (mg/Tablet)

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L = labeled amount of *Echinacea angustifolia* Dry Extract, *Echinacea purpurea* Root Dry Extract, or *Echinacea purpurea* Aerial Parts Dry Extract per Tablet (mg)

F = response factor of dodecatetraenoic acid isobutylamides relative to 2*E*,4*E*-hexadienoic acid isobutylamide, 1.353

Acceptance criteria

For Tablets containing *Echinacea angustifolia* Dry Extract: NLT 0.1%

For Tablets not containing *Echinacea angustifolia* Dry Extract: NLT 0.025%

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meet the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store in a cool place.
- **LABELING:** The label states the Latin binomial and the official name. The label states the amount of total phenols (as sum of presented caftaric acid, echinacoside, chicoric acid, and cynarin) and the amount of *Echinacea* Species Dry Extract in mg/Tablet.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Caftaric Acid RS
USP Chicoric Acid RS
USP Chlorogenic Acid RS
USP Powdered *Echinacea angustifolia* Extract RS
USP Powdered *Echinacea pallida* Extract RS
USP Powdered *Echinacea purpurea* Extract RS
USP Echinacoside RS
USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS

Echinacea Species Powder Capsules

DEFINITION

Echinacea Species Powder Capsules contain one or more of the following *Echinacea* Species (Fam. Asteraceae) powders prepared from dried rhizome and roots of *Echinacea angustifolia* DC., dried rhizome and roots of *Echinacea pallida* (Nutt.) Nutt., dried rhizome and roots of *Echinacea purpurea* (L.) Moench, and dried aerial parts of *Echinacea purpurea* (L.) Moench. They contain NLT 0.5% of total phenols, calculated as sum of caftaric acid ($C_{13}H_{12}O_9$), chicoric acid ($C_{22}H_{18}O_{12}$),[1] echinacoside ($C_{35}H_{46}O_{20}$), cynarin (1,3-di-*O*-caffeoylquinic acid) ($C_{25}H_{24}O_{12}$), and chlorogenic acid ($C_{16}H_{18}O_9$), from the labeled amount of *Echinacea* Species Powder.

IDENTIFICATION

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

For Capsules containing *Echinacea angustifolia* powder prepared from dried rhizome and roots

Standard solution A: 0.2 mg/mL of USP Echinacoside RS and 0.2 mg/mL of cynarin in methanol

Standard solution B: 0.05 mg/mL of USP Caftaric Acid RS, 0.1 mg/mL of USP Chlorogenic Acid RS, and 0.05 mg/mL of USP Chicoric Acid RS in methanol

Standard solution C: 20 mg/mL of USP Powdered *Echinacea angustifolia* Extract RS in methanol. Shake to disperse, sonicate for 5 min, and centrifuge. Use the supernatant.

Sample solution: Transfer a portion of the Capsule contents, equivalent to 1000 mg of *Echinacea angustifolia* Powder, to a centrifuge tube, add 10 mL of methanol, shake to disperse, sonicate for 20 min, and centrifuge. Use the supernatant.

Chromatographic system

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plates)

Application volume: 5 µL each of *Standard solution C* and the *Sample solution*, and 2 µL each of *Standard solution A* and *Standard solution B* as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33%.

Temperature: Ambient, not to exceed 30°

Developing solvent system: Ethyl acetate, methyl ethyl ketone, water, and formic acid (5:3:1:1)

Developing distance: 6 cm

Derivatization reagent: 5 mg/mL of 2-aminoethyl diphenylborinate in ethyl acetate

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Apply the *Samples* as bands and dry in air. Develop in a saturated chamber. Remove the plate from the chamber, heat at 100° for 5 min, treat while still warm with the *Derivatization reagent*, dry in air, and examine under UV light at 366 nm.

System suitability: *Standard solution A* shows two major blue bands: one in the lower third section due to echinacoside, and the other band in the middle section due to dicaffeoylquinic acid (cynarin). *Standard solution B* shows two major blue bands at about the middle section due to caftaric acid (higher R_F) and chlorogenic acid (lower R_F) that are clearly separated, and a blue chicoric acid band in the upper third of the chromatogram.

Acceptance criteria: The *Sample solution* exhibits the following: the most prominent blue band in the lower third section at R_F corresponding to that of echinacoside in *Standard solution A* and *Standard solution C*; a prominent greenish-blue band in the middle section at R_F corresponding to cynarin in *Standard solution A* and *Standard solution C*; minor bands between the positions of echinacoside and cynarin. One of these is due to chlorogenic acid at R_F corresponding to that of chlorogenic acid in *Standard solution B*, and very faint (or may be absent) blue bands at R_F corresponding to the caftaric acid and chicoric acid bands in *Standard solution B*.

For Capsules containing *Echinacea pallida* powder prepared from dried rhizome and roots: Proceed as directed *For Capsules containing Echinacea angustifolia powder prepared from dried rhizome and roots*. For *Standard solution C* and the *Sample solution*, substitute USP Powdered *Echinacea angustifolia* Extract RS with USP Powdered *Echinacea pallida* Extract RS and *Echinacea angustifolia* powder with *Echinacea pallida* powder prepared from dried rhizome and roots.

Acceptance criteria: The *Sample solution* exhibits the most prominent blue band in the lower third section at R_F corresponding to that of echinacoside in *Standard solution A* and *Standard solution C*; may exhibit bands of lesser intensity at the R_F corresponding to caftaric acid and chicoric acid in *Standard solution B* and *Standard solution*

[1] Other common names for chicoric acid are cichoric acid and dicaffeoyltartaric acid.

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C; exhibits minor bands between the positions of echinacoside and caftaric acid. One of these is due to chlorogenic acid at an R_F corresponding to that of chlorogenic acid in *Standard solution B*. The *Sample solution* does not exhibit a blue band in the middle section at R_F corresponding to cynarin in *Standard solution A*.

For Capsules containing *Echinacea purpurea* powder prepared from dried rhizome and roots and *Echinacea purpurea* powder prepared from dried aerial parts: Proceed as directed *For Capsules containing Echinacea angustifolia powder prepared from dried rhizome and roots*. For *Standard solution C* and the *Sample solution*, substitute USP Powdered *Echinacea angustifolia* Extract RS with USP Powdered *Echinacea purpurea* Extract RS and *Echinacea angustifolia* powder with *Echinacea purpurea* powder prepared from rhizome and roots or *Echinacea purpurea* powder prepared from aerial parts.

Acceptance criteria: The *Sample solution* exhibits the following: the most prominent blue band in the upper third section at R_F corresponding to chicoric acid band in *Standard solution B* and *Standard solution C*; the second most prominent blue band at about the middle section at R_F corresponding to that of caftaric acid in *Standard solution B* and *Standard solution C*; may exhibit minor blue bands corresponding to similar bands in *Standard solution C*. One of these is due to chlorogenic acid at an R_F corresponding to chlorogenic acid in *Standard solution B*. The *Sample solution* chromatogram does not exhibit a band at the same R_F as echinacoside in *Standard solution A*.

• **B. HPLC FOR TOTAL PHENOLS**

Analysis: Proceed as directed in the test for *Content of Total Phenols*.

Acceptance criteria: The chromatogram of the *Sample solution* prepared from Capsules labeled to contain *E. angustifolia* exhibits peaks at the retention times of those due to chlorogenic acid, cynarin, and echinacoside in the chromatogram of the *Standard solution*. The chromatogram of the *Sample solution* prepared from Capsules labeled to contain *E. pallida* exhibits peaks at the retention times of those due to caftaric acid, chlorogenic acid, echinacoside, and chicoric acid in the chromatogram of the *Standard solution*. The chromatogram of the *Sample solution* prepared from Capsules labeled to contain *E. purpurea* exhibits peaks at the retention times of those due to caftaric acid, chlorogenic acid, and chicoric acid in the chromatogram of the *Standard solution*.

• **C. HPLC FOR PRESENCE OF ALKYLAMIDES**

Analysis: Proceed as directed in the test for *Content of Dodecatetraenoic Acid Isobutylamides*.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks at the retention times of dodecatetraenoic acid isobutylamides in the chromatogram of *Standard solution B* or *Standard solution C*, and the reference chromatogram provided with the lot of USP Powdered *Echinacea angustifolia* Extract RS or USP Powdered *Echinacea purpurea* Extract RS being used.

STRENGTH

• **CONTENT OF TOTAL PHENOLS**

Solution A: Phosphoric acid in water (0.1 in 100)
Solution B: Acetonitrile
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	90	10
3	90	10

Table 1 *(continued)*

Time (min)	Solution A (%)	Solution B (%)
16	78	22
17	60	40
20	60	40
20.5	90	10
25	90	10

Solvent: Alcohol and water (7:3)
Standard solution: 20 µg/mL of USP Caftaric Acid RS, 20 µg/mL of USP Chlorogenic Acid RS, 20 µg/mL of cynarin (1,3-di-*O*-caffeoylquinic acid), 60 µg/mL of USP Echinacoside RS, and 40 µg/mL of USP Chicoric Acid RS in *Solvent*
Sample solution: Determine the total weight of 20 Capsules. Empty the Capsules, and combine and mix their contents to obtain a homogenous composite. Weigh the empty Capsule shells and calculate the average fill weight per Capsule. Transfer a portion of the Capsule contents, equivalent to 150 mg of the labeled *Echinacea* Species Powder, to a round-bottom flask equipped with a condenser. Add 30.0 mL of *Solvent*, and heat under reflux for 20 min. Cool to room temperature and pass through a filter of 0.45-µm or finer pore size.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 330 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 35°
Flow rate: 1.5 mL/min
Injection volume: 5 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Resolution: NLT 3.0 between the cynarin and echinacoside peaks, and NLT 1.0 between the caftaric acid and chlorogenic acid peaks. [NOTE—The echinacoside peak may be resolved in two components. The relative retention times for caftaric acid, chlorogenic acid, cynarin, echinacoside, and chicoric acid are 0.7, 0.75, 0.9, 1.0, and 1.4, respectively.]
Relative standard deviation: NMT 2.5% for the sum of the respective peaks in repeated injections
Analysis
Samples: *Standard solution* and *Sample solution*
Identify and measure areas of the peaks corresponding to caftaric acid ($C_{13}H_{12}O_9$), chlorogenic acid ($C_{16}H_{18}O_9$), cynarin ($C_{25}H_{24}O_{12}$), echinacoside ($C_{35}H_{46}O_{20}$), and chicoric acid ($C_{22}H_{18}O_{12}$) in the *Sample solution* chromatogram.
Calculate the quantity, in mg, of caftaric acid, chlorogenic acid, cynarin, echinacoside, and chicoric acid in each Capsule taken:

$$\text{Result} = (r_U/r_S) \times C_S \times V \times (W_{AV}/W)$$

r_U = peak area of a relevant analyte from the *Sample solution*
r_S = peak area of caftaric acid, chlorogenic acid, cynarin, echinacoside, or chicoric acid from the *Standard solution*
C_S = concentration of a relevant analyte in the *Standard solution* (mg/mL)

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V = volume of the solvent taken to prepare the *Sample solution* (mL)
W_{AV} = average Capsule fill weight (mg)
W = weight of the sample taken to prepare the *Sample solution* (mg)

Calculate the percentage of total phenols, as the sum of caftaric acid, chlorogenic acid, echinacoside, chicoric acid, and cynarin in each Capsule taken:

$$\text{Result} = \Sigma P_i \times 100/L$$

P_i = total combined content of caftaric acid, chlorogenic acid, echinacoside, chicoric acid, and cynarin as determined above (mg)
L = labeled amount of *Echinacea* Species Powder (mg/Capsule)

Acceptance criteria: NLT 0.5%

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩, *Disintegration*: Meet the requirements
- **WEIGHT VARIATION** ⟨2091⟩: Meet the requirements

SPECIFIC TESTS

- **CONTENT OF DODECATETRAENOIC ACID ISOBUTYLAMIDES**

[NOTE—This test is not applicable to Capsules containing *Echinacea pallida* powder prepared from dried rhizome and roots.]

Mobile phase: Acetonitrile and water (55:45)

Standard solution A: 10 µg/mL of USP *2E,4E*-Hexadienoic Acid Isobutylamide RS in methanol

Standard solution B: 5 mg/mL of USP Powdered *Echinacea angustifolia* Extract RS in methanol. Sonicate to dissolve, and pass through a filter of 0.45-µm or finer pore size. [NOTE—Only prepare when Capsules contain *Echinacea angustifolia* powder prepared from dried rhizome and roots.]

Standard solution C: 5 mg/mL of USP Powdered *Echinacea purpurea* Extract RS in methanol. Sonicate to dissolve and pass through a filter of 0.45-µm or finer pore size. [NOTE—Only prepare when Capsules do not contain *Echinacea angustifolia* powder prepared from dried rhizome and roots.]

Sample solution: Transfer a portion of the Capsule contents, equivalent to 2500 mg of *Echinacea* Species Powder, to a round-bottom flask equipped with a condenser. Add 80.0 mL of methanol, and heat under reflux for 30 min. Cool to room temperature and pass through a filter of 0.45-µm or finer pore size.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 30°
Flow rate: 1.5 mL/min
Injection volume: 25 µL

System suitability
Samples: *Standard solution A*, *Standard solution B*, or *Standard solution C*
Suitability requirements
Chromatogram similarity: The chromatogram of *Standard solution B* or *Standard solution C* is similar to the reference chromatogram for alkamides provided with the lot of the USP Powdered *Echinacea angustifolia* Extract RS or USP Powdered *Echinacea purpurea* Extract RS being used.
Resolution: NLT 1.0 between the dodecatetraenoic acid isobutylamide peaks, *Standard solution B* or *Standard solution C*
Tailing factor: NMT 2.0 for the *2E,4E*-hexadienoic acid isobutylamide peak, *Standard solution A*
Relative standard deviation: NMT 2.5% for the *2E,4E*-hexadienoic acid isobutylamide peak in replicate injections, *Standard solution A*

Analysis
Samples: *Standard solution A*, *Standard solution B* or *Standard solution C*, and *Sample solution*
Using the chromatogram of *Standard solution B* or *Standard solution C*, and the reference chromatogram provided with the lot of USP Powdered *Echinacea angustifolia* Extract RS or USP Powdered *Echinacea purpurea* Extract RS being used, identify and measure the areas of the *2E,4E,8Z,10E*- and *2E,4E,8Z,10Z*-dodecatetraenoic acid isobutylamide peaks in the *Sample solution* chromatogram.
Calculate the percentage of dodecatetraenoic acid isobutylamides in the labeled amount of *Echinacea* Species Powder from the portion of Capsules taken:

$$\text{Result} = (r_T/r_S) \times (C_S \times V/W) \times (W_{AV}/L) \times F \times 100$$

r_T = sum of the peak areas of the relevant analytes from the *Sample solution*
r_S = peak area of *2E,4E*-hexadienoic acid isobutylamide from *Standard solution A*
C_S = concentration of USP *2E,4E*-Hexadienoic Acid Isobutylamide RS in *Standard solution A* (mg/mL)
V = volume of the solvent taken to prepare the *Sample solution* (mL)
W = weight of the sample taken to prepare the *Sample solution* (mg)
W_{AV} = average Capsule fill weight (mg/Capsule)
L = labeled amount of *Echinacea angustifolia* powder or *Echinacea purpurea* powder, both prepared from dried rhizome and roots (mg/Capsule)
F = response factor of dodecatetraenoic acid isobutylamides relative to *2E,4E*-hexadienoic acid isobutylamide, 1.353

Acceptance criteria
For Capsules containing *Echinacea angustifolia* powder prepared from dried rhizome and roots: NLT 0.075%
For Capsules containing *Echinacea purpurea* powder prepared from dried rhizome and roots: NLT 0.025%
For Capsules containing only *Echinacea purpurea* powder prepared from dried aerial parts: NLT 0.01%

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures*, *Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meet the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store in a cool place.
- **LABELING:** The label states the official name and the amount of *Echinacea* Species Powder in mg/Capsule.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Caftaric Acid RS
USP Chicoric Acid RS

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USP Chlorogenic Acid RS
USP Powdered *Echinacea angustifolia* Extract RS
USP Powdered *Echinacea pallida* Extract RS
USP Powdered *Echinacea purpurea* Extract RS
USP Echinacoside RS
USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS

Eleuthero Root and Rhizome

DEFINITION

Eleuthero Root and Rhizome is the dried rhizome with roots of *Eleutherococcus senticosus* (Rupr. & Maxim.) Maxim. (Fam. Araliaceae) [syn. *Acanthopanax senticosus* (Rupr. & Maxim.) Harms]. It contains NLT 0.08% of phenylpropanoid glucosides as the sum of eleutheroside B ($C_{17}H_{24}O_9$), also referred to as syringin, and eleutheroside E ($C_{34}H_{46}O_{18}$), also referred to as syringaresinol diglucoside, calculated on the dried basis.

IDENTIFICATION

• **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Solvent: Alcohol and water (1:1)

Standard solution A: 1 mg/mL of USP Eleutheroside E RS in methanol

Standard solution B: 1 mg/mL of USP Eleutheroside B RS in methanol

Standard solution C: 0.1 g of USP Powdered Eleuthero Extract RS in 5 mL of *Solvent*. Sonicate for 10 min, centrifuge, and use the supernatant.

Sample solution: Transfer about 1 g of finely powdered Eleuthero Root and Rhizome to a centrifuge tube, add 5 mL of *Solvent*, and mix well. Sonicate for 10 min. Centrifuge or filter the solution, and use the supernatant or the filtrate.

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plates)

Application volume: 10 µL, as bands

Relative humidity: Condition the plate to a relative humidity of 33%.

Temperature: Ambient, not to exceed 30°

Developing solvent system: Chloroform, methanol, and water (35:15:2)

Developing distance: 6 cm

Derivatization reagent: To 18 mL of ice-cold methanol slowly and carefully add 2 mL of sulfuric acid, and mix well. Allow the mixture to adjust to room temperature.

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Apply the *Samples* as bands and dry in air. Develop in a saturated chamber and dry in air. Treat the plate with *Derivatization reagent*. Heat the plate at 100° for 5 min, and examine under white light and under UV light (365 nm).

Acceptance criteria: Under white light, the *Sample solution* exhibits two brown bands due to eleutheroside E and eleutheroside B at R_F values of about 0.34 and 0.45, corresponding in color and R_F to the bands exhibited by *Standard solution A* and *Standard solution B*, respectively. The *Sample solution* also exhibits two additional brown bands near the application zone, corresponding in color and R_F to the bands exhibited by *Standard solution C*. Other bands may be observed in the *Sample solution* and *Standard solution C* chromatograms. Under UV light, the *Sample solution* shows a brown band due to eleutheroside E corresponding in color and R_F to the band exhibited by *Standard solution A*.

• **B. HPLC:** The *Sample solution* in the test for *Content of Eleutherosides B and E* shows a peak at the retention time corresponding to that of eleutheroside B in *Standard solution B* and a peak at the retention time corresponding to that of eleutheroside E in *Standard solution A*.

COMPOSITION

• **CONTENT OF ELEUTHEROSIDES B AND E**

Solvent: Methanol and water (1:1)

Solution A: Acetonitrile and water (5:95)

Solution B: Acetonitrile and water (60:40)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	97	3
5	97	3
30	60	40
31	5	95
45	5	95
45.1	97	3
60	97	3

Standard solution A: 0.1 mg/mL of USP Eleutheroside E RS in methanol. Transfer 2.0 mL to a 5-mL volumetric flask, and dilute with *Solvent* to volume.

Standard solution B: 0.1 mg/mL of USP Eleutheroside B RS in methanol. Transfer 2.0 mL to a 5-mL volumetric flask, and dilute with *Solvent* to volume.

Standard solution C: 5.0 mg/mL of USP Powdered Eleuthero Extract RS in *Solvent*. Sonicate for 30 min, cool to room temperature, decant, and pass through a nylon filter of 0.45-µm or finer pore size.

Sample solution: Transfer about 5.0 g of finely ground Eleuthero Root and Rhizome, accurately weighed, to a round-bottom flask equipped with a condenser. Add 50 mL of *Solvent*, and heat under reflux for 30 min. Filter the supernatant through cotton wool into a 100-mL volumetric flask. Transfer the cotton wool to the round-bottom flask, and repeat the extraction twice, using 22 mL of *Solvent* for each extraction. Filter through cotton wool into the volumetric flask, wash the residue and the cotton wool with *Solvent*, cool to room temperature, dilute with *Solvent* to volume, and mix. Before injection, pass through a nylon filter of 0.45-µm or finer pore size, discarding the first few milliliters of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 220 nm

Column: 4.0-mm × 25-cm; 5-µm packing L1

Flow rate: 1 mL/min

Injection volume: 10 µL

System suitability

Samples: *Standard solution B* and *Standard solution C*

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution C* is similar to the reference chromatogram provided with the lot of USP Powdered Eleuthero Extract RS being used.

Relative standard deviation: NMT 2.0% determined from the eleutheroside B peak in replicate injections, *Standard solution B*

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Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Identify the eleutheroside B and eleutheroside E peaks in the *Sample solution* by comparison with the chromatograms of *Standard solution B* and *Standard solution A*, respectively.
Separately calculate the percentage of eleutheroside B and eleutheroside E in the portion of Eleuthero Root and Rhizome taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of eleutheroside E or eleutheroside B from *Standard solution A* or *Standard solution B*, respectively
C_S = concentration of eleutheroside E or eleutheroside B in *Standard solution A* or *Standard solution B*, respectively (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Eleuthero Root and Rhizome taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 0.08% on the dried basis

CONTAMINANTS
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Limits of Elemental Impurities*: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Pesticide Residue Analysis*: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria do not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures*, *Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS
- **BOTANICAL CHARACTERISTICS**

Macroscopic: The rhizome is knotty and of irregular cylindrical shape with a diameter of 15–40 mm. The heartwood area is light brown, and the connecting splint wood is pale yellow. The bark is approximately 2 mm thick and is firmly affixed to the xylem. The surface is gray-brown or black-brown, coarse, and longitudinally valleculate and plicate. A broken rhizome is coarse and fibrous, particularly inside the xylem. The fractured surface of the bark shows short, thin fibers. Numerous roots spring from the underside of the rhizome. These roots are 35–150 mm long, cylindrical and knotty, with a diameter of 3–15 mm. The surface of the roots is gray-brown to black-brown, smoother than the rhizome, and has longitudinal stripes. A 0.5-mm thin bark is tightly affixed to the pale yellow xylem. A broken root is sparsely fibrous and appears yellowish-gray where the thin epidermis is flaked off.
Microscopic: The roots have five to seven rows of brown cork cells. Secretory canals with brown contents appear in groups of four or five and are NMT 20 µm in diameter. Phloem fibers with thick lignified walls occur singly or in small groups; there are cluster crystals of calcium oxalate in the phloem parenchyma. Parenchymatous cells surround the secretory cells, and medullary ray cells contain small starch granules. The xylem shows reticulately thickened and pitted vessels. The rhizome is similar to the roots except for larger secretory canals, up to 25 µm in diameter, and the presence of a pith with parenchymatous cells containing starch granules.

- **LOSS ON DRYING** ⟨731⟩
Analysis: Dry at 105° to constant weight.
Acceptance criteria: NMT 14.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Total Ash*: NMT 8.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Water-Soluble Extractives, Method 2*: NLT 4.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Foreign Organic Matter*: NMT 3.0%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed, light-resistant containers.
- **LABELING:** The label states the Latin binomial.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Powdered Eleuthero Extract RS
USP Eleutheroside B RS
β-D-Glucopyranoside, 4-(3-hydroxy-1-propenyl)-2,6-dimethoxyphenyl.
$C_{17}H_{24}O_9$ 372.37
USP Eleutheroside E RS
β-D-Glucopyranoside, (tetrahydro-1*H*,3*H*-furo(3,4-c) furan-1,4-diyl)bis(2,6-dimethoxy-4,1-phenylene)bis-.
$C_{34}H_{46}O_{18}$ 742.70

Eleuthero Root and Rhizome Dry Extract

DEFINITION
Eleuthero Root and Rhizome Dry Extract is prepared from Eleuthero Root and Rhizome using hydroalcoholic mixtures. The ratio of the starting crude plant material to Dry Extract is between 13:1 and 25:1. It contains NLT 0.8% of phenylpropanoid glucosides as eleutheroside B ($C_{17}H_{24}O_9$), also referred to as syringin, and eleutheroside E ($C_{34}H_{46}O_{18}$), also referred to as syringaresinol diglucoside, calculated on the anhydrous basis. It may contain added substances.

IDENTIFICATION
- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩
Solvent: Alcohol and water (1:1)
Standard solution A: 1 mg/mL of USP Eleutheroside E RS in methanol
Standard solution B: 1 mg/mL of USP Eleutheroside B RS in methanol
Standard solution C: 0.1 g of USP Powdered Eleuthero Extract RS in 5 mL of *Solvent*. Sonicate for 10 min, centrifuge, and use the supernatant.
Sample solution: 0.1 g of Eleuthero Root and Rhizome Dry Extract in 5 mL of *Solvent*. Sonicate for 10 min, centrifuge, and use the supernatant.
Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plates)
Application volume: 10 µL, as bands
Relative humidity: Condition the plate to a relative humidity of 33%.
Temperature: Ambient, not to exceed 30°
Developing solvent system: Chloroform, methanol, and water (35:15:2)
Developing distance: 6 cm
Derivatization reagent: To 18 mL of ice-cold methanol slowly and carefully add 2 mL of sulfuric acid, and mix well. Allow the mixture to adjust to room temperature.

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Analysis
Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*
Apply the *Samples* as bands and dry in air. Develop in a saturated chamber and dry in air. Treat the plate with *Derivatization reagent*, heat at 100° for 5 min, and examine under white light and under UV light (365 nm).
Acceptance criteria: Under white light, the *Sample solution* exhibits two brown bands due to eleutheroside E and eleutheroside B at R_F values of about 0.34 and 0.45, corresponding in color and R_F to the bands exhibited by *Standard solution A* and *Standard solution B*, respectively. The *Sample solution* also exhibits two additional brown bands near the application zone, corresponding in color and R_F to the bands exhibited by *Standard solution C*. Other bands may be observed in the *Sample solution* and *Standard solution C* chromatograms. Under UV light, the *Sample solution* shows a brown band due to eleutheroside E corresponding in color and R_F to the band exhibited by *Standard solution A*.

- **B. HPLC:** The *Sample solution* in the test for *Content of Eleutherosides B and E* shows a peak at a retention time corresponding to that of eleutheroside B in *Standard solution B* and a peak at a retention time corresponding to that of eleutheroside E in *Standard solution A*.

COMPOSITION

- **CONTENT OF ELEUTHEROSIDES B AND E**

Solvent: Methanol and water (1:1)
Solution A: Acetonitrile and water (5:95)
Solution B: Acetonitrile and water (60:40)
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	97	3
5	97	3
30	60	40
31	5	95
45	5	95
45.1	97	3
60	97	3

Standard solution A: 0.1 mg/mL of USP Eleutheroside E RS in methanol. Transfer 2.0 mL to a 5-mL volumetric flask, and dilute with *Solvent* to volume.
Standard solution B: 0.1 mg/mL of USP Eleutheroside B RS in methanol. Transfer 2.0 mL to a 5-mL volumetric flask, and dilute with *Solvent* to volume.
Standard solution C: 5.0 mg/mL of USP Powdered Eleuthero Extract RS in *Solvent*. Sonicate for 30 min, cool to room temperature, and decant. Before injection, pass through a nylon filter of 0.45-µm or finer pore size, discarding the first few milliliters of the filtrate.
Sample solution: Transfer 500 mg of Eleuthero Root and Rhizome Dry Extract, accurately weighed, to a 100-mL volumetric flask, add 80 mL of *Solvent*, and sonicate for 30 min. Cool to room temperature, dilute with *Solvent* to volume, and mix. Before injection, pass through a nylon filter of 0.45-µm or finer pore size, discarding the first few milliliters of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 220 nm
Column: 4.0-mm × 25-cm; 5-µm packing L1
Flow rate: 1 mL/min
Injection volume: 10 µL
System suitability
Samples: *Standard solution B* and *Standard solution C*
Suitability requirements
Chromatogram similarity: The chromatogram of *Standard solution C* is similar to the reference chromatogram provided with the lot of USP Powdered Eleuthero Extract RS being used.
Relative standard deviation: NMT 2.0% determined from the eleutheroside B peak in replicate injections, *Standard solution B*
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Identify the eleutheroside B and eleutheroside E peaks in the *Sample solution* by comparison with the chromatograms of *Standard solution B* and *Standard solution A*, respectively, and measure the peak areas.
Separately calculate the percentage of eleutheroside B and eleutheroside E in the portion of Eleuthero Root and Rhizome Dry Extract taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of eleutheroside E or eleutheroside B from the *Sample solution*
r_S = peak area of eleutheroside E or eleutheroside B from *Standard solution A* or *Standard solution B*, respectively
C_S = concentration of eleutheroside E or eleutheroside B in *Standard solution A* or *Standard solution B*, respectively (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Eleuthero Root and Rhizome Dry Extract used to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 0.8% on the anhydrous basis

CONTAMINANTS

Change to read:

- **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020)
: Meets the requirements
- **BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Residual Solvents*: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic microbial count does not exceed 10^4 cfu/g. The total combined yeasts and molds count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS

- **WATER DETERMINATION** ⟨921⟩, *Method I, Method Ia*: NMT 5.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Total Ash*: NMT 10.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** The label states the Latin binomial and, following the official name, the content of eleutherosides, the extracting solvent used for preparation, and the ratio of the

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starting crude plant material to Dry Extract. It meets the requirements in *Botanical Extracts* ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Labeling.*

• **USP REFERENCE STANDARDS** ⟨11⟩
USP Powdered Eleuthero Extract RS
USP Eleutheroside B RS
β-D-Glucopyranoside, 4-(3-hydroxy-1-propenyl)-2,6-dimethoxyphenyl.
$C_{17}H_{24}O_9$ 372.37
USP Eleutheroside E RS
β-D-Glucopyranoside, (tetrahydro-1*H*,3*H*-furo(3,4-c) furan-1,4-diyl)bis(2,6-dimethoxy-4,1-phenylene)bis-.
$C_{34}H_{46}O_{18}$ 742.70

Eleuthero Root and Rhizome Dry Extract Capsules

DEFINITION

Eleuthero Root and Rhizome Dry Extract Capsules contain Eleuthero Root and Rhizome Dry Extract. They contain NLT 95% of the labeled amount of phenylpropanoid glucosides as the sum of eleutheroside B ($C_{17}H_{24}O_9$), also referred to as syringin, and eleutheroside E ($C_{34}H_{46}O_{18}$), also referred to as syringaresinol diglucoside. They may contain suitable added substances.

IDENTIFICATION

Add the following:

▲• **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩
Solvent: Alcohol and water (1:1)
Standard solution A: 1 mg/mL of USP Eleutheroside E RS and 1 mg/mL of USP Eleutheroside B RS in methanol
Standard solution B: 0.1 g of USP Powdered Eleuthero Extract RS in 5 mL of *Solvent*. Sonicate for 10 min, centrifuge, and use the supernatant.
Sample solution: Open 10 Capsules, combine their contents in an appropriate container, and mix well. Transfer a portion of the Capsule contents to a centrifuge tube, and add *Solvent* to obtain a solution equivalent to 20 mg/mL of Eleuthero Root and Rhizome Dry Extract. Sonicate for 10 min, centrifuge, and use the supernatant.
Chromatographic system
Adsorbent: Chromatographic silica gel with an average particle size of 5 µm
Application volume: 10 µL, as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of 33%.
Temperature: Ambient, not to exceed 30°
Developing solvent system: Methylene chloride, methanol, and water (35:15:2)
Developing distance: 6 cm
Derivatization reagent: To 18 mL of ice-cold methanol slowly and carefully add 2 mL of sulfuric acid, and mix well. Allow the mixture to adjust to room temperature.
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Apply the *Samples* as bands and dry in air. Develop in a saturated chamber, remove the plate from the chamber, and dry in air. Treat the plate with *Derivatization reagent*, heat at 100° for 5 min, and examine under white light and under UV light at 365 nm.
System suitability: Under white light, *Standard solution A* shows two brown bands in the middle-third section of the chromatogram. The band with a higher R_F is due to eleutheroside B, and the band with a lower R_F is due to eleutheroside E. *Standard solution B* exhibits two bands corresponding in color and R_F to the bands due to eleutheroside E and eleutheroside B in *Standard solution A*. *Standard solution B* exhibits two intense brown bands in the lower-third section near the starting position.
Acceptance criteria: Under white light, the *Sample solution* exhibits two brown bands corresponding in R_F and color to eleutheroside E and eleutheroside B in *Standard solution A*. The *Sample solution* exhibits two intense bands in the lower-third section, corresponding in color and R_F to the same bands in *Standard solution B*. Multiple weak bands may be observed in the *Sample solution* corresponding in color and R_F to similar bands in *Standard solution B*. Under UV light, the *Sample solution* shows a brown band due to eleutheroside E corresponding in color and R_F to the band exhibited by *Standard solution A*. ▲ (USP 1-May-2019)

Change to read:

• **▲B.▲** (USP 1-May-2019) **HPLC**
Analysis: Proceed as directed in the test for *Content of Eleutherosides B and E*.
Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks at the retention times corresponding to the peaks due to eleutheroside B and eleutheroside E in the chromatogram of *Standard solution B*.

STRENGTH

• **CONTENT OF ELEUTHEROSIDES B AND E**
Extraction solvent: Methanol and water (6:4)
Solution A: 0.2% *o*-phosphoric acid in water
Solution B: Acetonitrile
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	90	10
2	90	10
20	70	30
25	70	30
27	90	10
37	90	10

Standard stock solution A: 0.025 mg/mL of USP Eleutheroside B RS in methanol. Sonicate for 5 min to dissolve.
Standard stock solution B: 0.1 mg/mL of USP Eleutheroside E RS in methanol. Sonicate for 5 min to dissolve.
Standard solution A: Transfer 2.0 mL of *Standard stock solution A* and 1.0 mL of *Standard stock solution B* to a 10-mL volumetric flask, dilute with water to volume, and mix well.
Standard solution B: 1 mg/mL of USP Powdered Eleuthero Extract RS in *Extraction solvent*. Sonicate for 30 min and cool to room temperature. Dilute with water to a final concentration of 0.5 mg/mL. Before injection, pass through a PVDF membrane filter of 0.45-µm or finer pore size.
Sample solution: Determine the total weight of 20 Capsules. Open the Capsules and combine their contents

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in an appropriate container. Weigh the empty Capsule shells and calculate the average fill weight per Capsule. Transfer a portion of the Capsule contents, nominally equivalent to 0.5 mg of phenylpropanoid glucosides (sum of eleutheroside B and eleutheroside E), to a 50-mL volumetric flask. Add 25 mL of *Extraction solvent* and sonicate for 30 min with occasional shaking. Shake the flask manually for 1 min, cool to room temperature, dilute with water to volume, mix well, and pass through a PVDF membrane filter of 0.45-µm or finer pore size.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 220 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 35°
Flow rate: 0.8 mL/min
Injection volume: 20 µL

System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Eleuthero Extract RS being used.
Relative standard deviation: NMT 2.0% for the eleutheroside B and eleutheroside E peaks in replicate injections, *Standard solution A*

Analysis
Samples: *Standard solution A* and *Sample solution*
Using the chromatogram from *Standard solution A* and the reference chromatogram provided with the lot of USP Powdered Eleuthero Extract RS being used, identify the peaks corresponding to eleutheroside B and eleutheroside E in the *Sample solution*. Measure the areas of the analyte peaks.
Calculate the quantity, in mg, of eleutheroside B and eleutheroside E in each Capsule taken:

$$\text{Result} = (r_U/r_S) \times C_S \times V \times (W_{AV}/W)$$

r_U = peak area of the relevant eleutheroside from the *Sample solution*
r_S = peak area of the corresponding eleutheroside from *Standard solution A*
C_S = concentration of the relevant eleutheroside in *Standard solution A* (mg/mL)
V = volume of the solvent taken for preparation of the *Sample solution* (mL)
W_{AV} = average fill weight per Capsule (mg)
W = weight of the sample taken for preparation of the *Sample solution* (mg)

Calculate the percentage of the labeled amount of phenylpropanoid glucosides, as the sum of eleutheroside B and eleutheroside E, in each Capsule taken:

$$\text{Result} = (\Sigma Q_i/L) \times 100$$

Q_i = sum of the quantities of eleutherosides as determined above (mg)
L = labeled amount of phenylpropanoid glucosides (mg)

Acceptance criteria: NLT 95%

PERFORMANCE TESTS
• **Disintegration and Dissolution** ⟨2040⟩, *Disintegration*: Meet the requirements
• **Weight Variation** ⟨2091⟩: Meet the requirements

CONTAMINANTS
• **Microbial Enumeration Tests** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
• **Absence of Specified Microorganisms** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meet the requirements

ADDITIONAL REQUIREMENTS
• **Packaging and Storage:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
• **Labeling:** The label states the Latin binomial and, following the official name, the amount of phenylpropanoid glucosides, as the sum of eleutheroside B and eleutheroside E, and the amount of Eleuthero Root and Rhizome Dry Extract in mg/Capsule.
• **USP Reference Standards** ⟨11⟩
USP Powdered Eleuthero Extract RS
USP Eleutheroside B RS
USP Eleutheroside E RS

Eleuthero Root and Rhizome Dry Extract Tablets

DEFINITION
Eleuthero Root and Rhizome Dry Extract Tablets contain Eleuthero Root and Rhizome Dry Extract. They contain NLT 95% of the labeled amount of phenylpropanoid glucosides as the sum of eleutheroside B ($C_{17}H_{24}O_9$), also referred to as syringin, and eleutheroside E ($C_{34}H_{46}O_{18}$), also referred to as syringaresinol diglucoside. They may contain added substances.

IDENTIFICATION

Add the following:

▲• **A. HPTLC for Articles of Botanical Origin** ⟨203⟩
Solvent: Alcohol and water (1:1)
Standard solution A: 1 mg/mL of USP Eleutheroside E RS and 1 mg/mL of USP Eleutheroside B RS in methanol
Standard solution B: 0.1 g of USP Powdered Eleuthero Extract RS in 5 mL of *Solvent*. Sonicate for 10 min, centrifuge, and use the supernatant.
Sample solution: Grind 10 Tablets to a powder, transfer to a suitable container, and homogenize. Transfer a portion of the homogenized powder to a centrifuge tube, and add *Solvent* to obtain a solution equivalent to 20 mg/mL of Eleuthero Root and Rhizome Dry Extract. Sonicate for 10 min, centrifuge, and use the supernatant.
Chromatographic system
Adsorbent: Chromatographic silica gel with an average particle size of 5 µm
Application volume: 10 µL, as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of 33%.
Temperature: Ambient, not to exceed 30°
Developing solvent system: Methylene chloride, methanol, and water (35:15:2)
Developing distance: 6 cm

DS Monographs

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Derivatization reagent: To 18 mL of ice-cold methanol slowly and carefully add 2 mL of sulfuric acid, and mix well. Allow the mixture to adjust to room temperature.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands and dry in air. Develop in a saturated chamber, remove the plate from the chamber, and dry in air. Treat the plate with *Derivatization reagent*, heat at 100° for 5 min, and examine under white light and under UV light at 365 nm.

System suitability: Under white light, *Standard solution A* shows two brown bands in the middle-third section of the chromatogram. The band with a higher R_F is due to eleutheroside B, and the band with a lower R_F is due to eleutheroside E. *Standard solution B* exhibits two bands corresponding in color and R_F to the bands due to eleutheroside E and eleutheroside B in *Standard solution A*. *Standard solution B* exhibits two intense brown bands in the lower-third section near the starting position.

Acceptance criteria: Under white light, the *Sample solution* exhibits two brown bands corresponding in R_F and color to eleutheroside E and eleutheroside B in *Standard solution A*. The *Sample solution* exhibits two intense bands in the lower-third section, corresponding in color and R_F to the same bands in *Standard solution B*. Multiple weak bands may be observed in the *Sample solution* corresponding in color and R_F to similar bands in *Standard solution B*. Under UV light, the *Sample solution* shows a brown band due to eleutheroside E corresponding in color and R_F to the band exhibited by *Standard solution A*. ▲ (USP 1-May-2019)

Change to read:

• **▲B.▲ (USP 1-May-2019) HPLC**

Analysis: Proceed as directed in the test for *Content of Eleutherosides B and E*.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks at the retention times corresponding to the peaks due to eleutheroside B and eleutheroside E in the chromatogram of *Standard solution B*.

STRENGTH

• **CONTENT OF ELEUTHEROSIDES B AND E**

Extraction solvent: Methanol and water (6:4)

Solution A: 0.2% *o*-phosphoric acid in water

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	90	10
2	90	10
20	70	30
25	70	30
27	90	10
37	90	10

Standard stock solution A: 0.025 mg/mL of USP Eleutheroside B RS in methanol. Sonicate for 5 min to dissolve.

Standard stock solution B: 0.1 mg/mL of USP Eleutheroside E RS in methanol. Sonicate for 5 min to dissolve.

Standard solution A: Transfer 2.0 mL of *Standard stock solution A* and 1.0 mL of *Standard stock solution B* to a 10-mL volumetric flask, dilute with water to volume, and mix well.

Standard solution B: 1 mg/mL of USP Powdered Eleuthero Extract RS in *Extraction solvent*. Sonicate for 30 min and cool to room temperature. Dilute with water to a final concentration of 0.5 mg/mL. Before injection, pass through a PVDF membrane filter of 0.45-µm or finer pore size.

Sample solution: Weigh NLT 20 Tablets, determine the average Tablet weight, and finely powder. Transfer a portion of finely powdered Tablets, nominally equivalent to 0.5 mg of phenylpropanoid glucosides (sum of eleutheroside B and eleutheroside E), to a 50-mL volumetric flask. Add 25 mL of *Extraction solvent* and sonicate for 30 min with occasional shaking. Shake the flask manually for 1 min, cool to room temperature, dilute with water to volume, mix well, and pass through a PVDF membrane filter of 0.45-µm or finer pore size.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 220 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 35°

Flow rate: 0.8 mL/min

Injection volume: 20 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Eleuthero Extract RS being used.

Relative standard deviation: NMT 2.0% for the eleutheroside B and eleutheroside E peaks in replicate injections, *Standard solution A*

Analysis

Samples: *Standard solution A* and *Sample solution*

Using the chromatogram from *Standard solution A* and the reference chromatogram provided with the lot of USP Powdered Eleuthero Extract RS being used, identify the peaks corresponding to eleutheroside B and eleutheroside E in the *Sample solution*. Measure the areas of the analyte peaks.

Calculate the quantity, in mg, of eleutheroside B and eleutheroside E in each Tablet taken:

$$\text{Result} = (r_U/r_S) \times C_S \times V \times (W_{AV}/W)$$

r_U = peak area of the relevant eleutheroside from the *Sample solution*

r_S = peak area of the corresponding eleutheroside from *Standard solution A*

C_S = concentration of the relevant eleutheroside in *Standard solution A* (mg/mL)

V = volume of the solvent taken for preparation of the *Sample solution* (mL)

W_{AV} = average Tablet weight (mg)

W = weight of the sample taken for preparation of the *Sample solution* (mg)

Calculate the percentage of the labeled amount of phenylpropanoid glucosides, as the sum of eleutheroside B and eleutheroside E, in each Tablet taken:

$$\text{Result} = (\Sigma Q_i/L) \times 100$$

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Q_i = sum of the quantities of eleutherosides as determined above (mg)
L = labeled amount of phenylpropanoid glucosides (mg)

Acceptance criteria: NLT 95%

PERFORMANCE TESTS
- **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩, *Disintegration*: Meet the requirements
- **WEIGHT VARIATION** ⟨2091⟩: Meet the requirements

CONTAMINANTS
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meet the requirements

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the amount of phenylpropanoid glucosides, as the sum of eleutheroside B and eleutheroside E, and the amount of Eleuthero Root and Rhizome Dry Extract in mg/Tablet.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Powdered Eleuthero Extract RS
  USP Eleutheroside B RS
  USP Eleutheroside E RS

Eleuthero Root and Rhizome Powder

DEFINITION
Eleuthero Root and Rhizome Powder is Eleuthero Root and Rhizome reduced to a powder or very fine powder. It contains NLT 0.08% of phenylpropanoid glucosides as the sum of eleutheroside B ($C_{17}H_{24}O_9$), also referred to as syringin, and eleutheroside E ($C_{34}H_{46}O_{18}$), also referred to as syringaresinol diglucoside, calculated on the dried basis.

IDENTIFICATION
- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩
  Solvent: Alcohol and water (1:1)
  Standard solution A: 1 mg/mL of USP Eleutheroside E RS in methanol
  Standard solution B: 1 mg/mL of USP Eleutheroside B RS in methanol
  Standard solution C: 0.1 g of USP Powdered Eleuthero Extract RS in 5 mL of *Solvent*. Sonicate for 10 min, centrifuge, and use the supernatant.
  Sample solution: Transfer about 1 g of Eleuthero Root and Rhizome Powder to a centrifuge tube, add 5 mL of *Solvent*, and mix well. Sonicate for 10 min. Centrifuge or filter the solution, and use the supernatant or the filtrate.
  Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plates)
  Application volume: 10 µL, as bands
  Relative humidity: Condition the plate to a relative humidity of 33%.
  Temperature: Ambient, not to exceed 30°
  Developing solvent system: Chloroform, methanol, and water (35:15:2)
  Developing distance: 6 cm
  Derivatization reagent: To 18 mL of ice-cold methanol slowly and carefully add 2 mL of sulfuric acid, and mix well. Allow the mixture to adjust to room temperature.
  Analysis
  Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*
  Apply the *Samples* as bands and dry in air. Develop in a saturated chamber and dry in air. Treat the plate with *Derivatization reagent*. Heat the plate at 100° for 5 min, and examine under white light and under UV light (365 nm).
  Acceptance criteria: Under white light, the *Sample solution* exhibits two brown bands due to eleutheroside E and eleutheroside B at R_F values of about 0.34 and 0.45, corresponding in color and R_F to the bands exhibited by *Standard solution A* and *Standard solution B*, respectively. The *Sample solution* also exhibits two additional brown bands near the application zone, corresponding in color and R_F to the bands exhibited by *Standard solution C*. Other bands may be observed in the *Sample solution* and *Standard solution C* chromatograms. Under UV light, the *Sample solution* shows a brown band due to eleutheroside E corresponding in color and R_F to the band exhibited by *Standard solution A*.
- **B. HPLC:** The *Sample solution* in the test for *Content of Eleutherosides B and E* shows a peak at the retention time corresponding to that of eleutheroside B in *Standard solution B* and a peak at the retention time corresponding to that of eleutheroside E in *Standard solution A*.

COMPOSITION
- **CONTENT OF ELEUTHEROSIDES B AND E**
  Solvent: Methanol and water (1:1)
  Solution A: Acetonitrile and water (5:95)
  Solution B: Acetonitrile and water (60:40)
  Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	97	3
5	97	3
30	60	40
31	5	95
45	5	95
45.1	97	3
60	97	3

Standard solution A: 0.1 mg/mL of USP Eleutheroside E RS in methanol. Transfer 2.0 mL to a 5-mL volumetric flask, and dilute with *Solvent* to volume.
Standard solution B: 0.1 mg/mL of USP Eleutheroside B RS in methanol. Transfer 2.0 mL to a 5-mL volumetric flask, and dilute with *Solvent* to volume.
Standard solution C: 5.0 mg/mL of USP Powdered Eleuthero Extract RS in *Solvent*. Sonicate for 30 min, cool to room temperature, decant, and pass through a nylon filter of 0.45-µm or finer pore size.
Sample solution: Transfer 5.0 g of Eleuthero Root and Rhizome Powder, accurately weighed, to a round-bottom flask equipped with a condenser. Add 50 mL of *Solvent*, and heat under reflux for 30 min. Filter the supernatant through cotton wool into a 100-mL volumetric flask. Transfer the cotton wool to the round-bottom flask, and repeat the extraction twice, using 22 mL of *Solvent* for each extraction. Filter through cotton wool into the volumetric flask, wash

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the residue and the cotton wool with *Solvent*, cool to room temperature, dilute with *Solvent* to volume, and mix. Before injection, pass through a nylon filter of 0.45-µm or finer pore size, discarding the first few milliliters of the filtrate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 220 nm
Column: 4.0-mm × 25-cm; 5-µm packing L1
Flow rate: 1 mL/min
Injection volume: 10 µL

System suitability
Samples: *Standard solution B* and *Standard solution C*
Suitability requirements
Chromatogram similarity: The chromatogram from *Standard solution C* is similar to the reference chromatogram provided with the lot of USP Powdered Eleuthero Extract RS being used.
Relative standard deviation: NMT 2.0%, determined from the eleutheroside B peak in replicate injections, *Standard solution B*

Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Identify the eleutheroside B and eleutheroside E peaks in the *Sample solution* by comparison with the chromatograms of *Standard solution B* and *Standard solution A*, respectively.
Separately calculate the percentage of eleutheroside B and eleutheroside E in the portion of Eleuthero Root and Rhizome Powder taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of eleutheroside E or eleutheroside B from *Standard solution A* or *Standard solution B*, respectively
C_S = concentration of eleutheroside E or eleutheroside B in *Standard solution A* or *Standard solution B*, respectively (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Eleuthero Root and Rhizome Powder taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 0.08% on the dried basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Limits of Elemental Impurities*: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Pesticide Residue Analysis*: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria do not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures*, *Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: The powder is brown with a faint aromatic odor and a slightly acrid, persistent taste.
Microscopic: Groups of secretory canals with brown contents are surrounded by parenchymatous cells containing cluster crystals of calcium oxalate. The parenchymatous cells show small starch granules, thick-walled lignified fibers, and fragments of reticulate and pitted vessels. It turns bright yellow when mounted in sodium hydroxide solution.

- **LOSS ON DRYING** ⟨731⟩

Analysis: Dry at 105° to constant weight.
Acceptance criteria: NMT 14.0%

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Total Ash*: NMT 8.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed, light-resistant containers.
- **LABELING:** The label states the Latin binomial.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Powdered Eleuthero Extract RS
USP Eleutheroside B RS
β-D-Glucopyranoside, 4-(3-hydroxy-1-propenyl)-2,6-dimethoxyphenyl.
$C_{17}H_{24}O_9$ 372.37
USP Eleutheroside E RS
β-D-Glucopyranoside, (tetrahydro-1*H*,3*H*-furo(3,4-c) furan-1,4-diyl)bis(2,6-dimethoxy-4,1-phenylene)bis-.
$C_{34}H_{46}O_{18}$ 742.70

Eleuthero Root and Rhizome Powder Capsules

DEFINITION

Eleuthero Root and Rhizome Powder Capsules contain Eleuthero Root and Rhizome Powder. They contain NLT 0.08% of phenylpropanoid glucosides as the sum of eleutheroside B ($C_{17}H_{24}O_9$), also referred to as syringin, and eleutheroside E ($C_{34}H_{46}O_{18}$), also referred to as syringaresinol diglucoside, within the labeled amount of Eleuthero Root and Rhizome Powder.

IDENTIFICATION

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 1 mg/mL of USP Eleutheroside E RS in methanol
Standard solution B: 1 mg/mL of USP Eleutheroside B RS in methanol
Standard solution C: 100 mg of USP Powdered Eleuthero Extract RS in 5 mL of aqueous ethanol 50%. Sonicate for 10 min, centrifuge, and use the supernatant.
Sample solution: Transfer a finely powdered portion of the contents of the Capsules, equivalent to 1 g of Eleuthero Root and Rhizome Powder, to a centrifuge tube. Add 5 mL of aqueous ethanol 50%, and mix well. Sonicate for 20 min. Centrifuge and use the supernatant.
Chromatographic system
Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plates)
Application volume: 10 µL, as bands
Relative humidity: Condition the plate to a relative humidity of 33%.
Temperature: Ambient, not to exceed 30°
Developing solvent system: Chloroform, methanol, and water (35:15:2)
Developing distance: 6 cm
Derivatization reagent: To 18 mL of ice-cold methanol, slowly and carefully add 2 mL of sulfuric acid, and mix well. Allow the mixture to adjust to room temperature.
Analysis
Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

USP Monographs

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Apply the *Samples* as bands and dry in air. Develop in a saturated chamber, remove the plate from the chamber, and dry in air. Treat the plate with *Derivatization reagent*, heat at 100° for 5 min, and examine under white light and UV light (365 nm).

Acceptance criteria: Under white light, the *Sample solution* exhibits two brown bands due to eleutheroside E and eleutheroside B at R_F values of about 0.34 and 0.45, corresponding in color and R_F to the bands exhibited by *Standard solution A* and *Standard solution B*, respectively. The *Sample solution* also exhibits two additional brown bands near the application zone, corresponding in color and R_F values to the bands exhibited by *Standard solution C*. Other bands may be observed in the *Sample solution* and *Standard solution C* chromatograms. Under UV light, the *Sample solution* shows a brown band due to eleutheroside E corresponding in color and R_F to the band exhibited by *Standard solution A*.

- **B. HPLC:** The chromatogram of the *Sample solution* exhibits peaks at the retention times corresponding to the peaks due to eleutheroside B and eleutheroside E in the chromatogram of *Standard solution B*.

STRENGTH

- **CONTENT OF ELEUTHEROSIDES B AND E**

Extraction solvent: Methanol and water (6:4)
Solution A: 0.2% *o*-phosphoric acid in water
Solution B: Acetonitrile
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	90	10
2	90	10
20	70	30
25	70	30
27	90	10
37	90	10

Standard stock solution A: 0.025 mg/mL of USP Eleutheroside B RS in methanol. Sonicate for 5 min to dissolve.
Standard stock solution B: 0.1 mg/mL of USP Eleutheroside E RS in methanol. Sonicate for 5 min to dissolve.
Standard solution A: Transfer 2.0 mL of *Standard stock solution A* and 1.0 mL of *Standard stock solution B* to a 10-mL volumetric flask, dilute with water to volume, and mix well.
Standard solution B: 1 mg/mL of USP Powdered Eleuthero Extract RS in *Extraction solvent*. Sonicate for 30 min and cool to room temperature. Dilute with water to a final concentration of 0.5 mg/mL. Before injection, pass through a PVDF membrane filter of 0.45-µm or finer pore size.
Sample solution: Determine the total weight of 20 Capsules. Open the Capsules and combine their contents in an appropriate container. Weigh the empty Capsule shells and calculate the average fill weight per Capsule. Transfer a portion of the Capsule contents, equivalent to 1.5 g of Eleuthero Root and Rhizome Powder, to a round-bottom flask equipped with a condenser. Add 25 mL of *Extraction solvent* and heat under reflux for 30 min. Transfer the supernatant to a 100-mL volumetric flask and repeat the extraction, using 25 mL of *Extraction solvent*. Transfer the supernatant to a volumetric flask, wash the residue with water, transfer to a volumetric flask, and cool to room temperature. Dilute with water to volume, mix well, and pass through a PVDF membrane filter of 0.45-µm or finer pore size.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 220 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 35°
Flow rate: 0.8 mL/min
Injection volume: 20 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Relative standard deviation: NMT 2.0% for the eleutheroside B and eleutheroside E peaks in replicate injections, *Standard solution A*
Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Eleuthero Extract RS being used.
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Eleuthero Extract RS being used, identify the peaks corresponding to eleutheroside B and eleutheroside E in the *Sample solution*. Measure the areas of the analyte peaks.
Calculate the quantity, in mg, of eleutheroside B and eleutheroside E in each Capsule taken:

$$\text{Result} = (r_U/r_S) \times C_S \times V \times (W_{AV}/W)$$

r_U = peak area of the relevant eleutheroside from the *Sample solution*
r_S = peak area of the corresponding eleutheroside from *Standard solution A*
C_S = concentration of the relevant eleutheroside in *Standard solution A* (mg/mL)
V = volume of the solvent taken for preparation of the *Sample solution* (mL)
W_{AV} = average Capsule fill weight (mg)
W = weight of the sample taken for preparation of the *Sample solution* (mg)

Calculate the percentage of phenylpropanoid glucosides, as the sum of eleutheroside B and eleutheroside E, within the labeled amount of Eleuthero Root and Rhizome Powder in each Capsule:

$$\text{Result} = (\Sigma Q_i/L) \times 100$$

ΣQ_i = sum of the quantities of eleutherosides as determined above (mg)
L = labeled amount of Eleuthero Root and Rhizome Powder (mg)

Acceptance criteria: NLT 0.08%

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩, *Disintegration*: Meet the requirements
- **WEIGHT VARIATION** ⟨2091⟩: Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total

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combined molds and yeasts count does not exceed 10^3 cfu/g.

- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meet the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the amount of Eleuthero Root and Rhizome Powder in mg/Capsule.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Eleutheroside B RS
  USP Eleutheroside E RS
  USP Powdered Eleuthero Extract RS

Elm—*see Elm General Monographs*

Ergocalciferol—*see Ergocalciferol General Monographs*

Ergocalciferol Capsules—*see Ergocalciferol Capsules General Monographs*

Ergocalciferol Oral Solution—*see Ergocalciferol Oral Solution General Monographs*

Ergocalciferol Tablets—*see Ergocalciferol Tablets General Monographs*

Add the following:

▲European Elder Berry Dry Extract

DEFINITION

European Elder Berry Dry Extract is prepared from the quick-frozen ripened fruits of *Sambucus nigra* L. (Family Adoxaceae/Viburnaceae) by extraction with alcohol. It contains NLT 17% of total anthocyanosides calculated as the sum of the chloride salts of cyanidin-3-*O*-sambubioside-5-*O*-glucoside, cyanidin-3,5-di-*O*-glucoside, cyanidin-3-*O*-sambubioside, and cyanidin-3-*O*-glucoside on the anhydrous basis; and NMT 0.2% of cyanidin as chloride salt on the anhydrous basis. It contains NLT 90.0% and NMT 110.0% of the labeled amount of total anthocyanosides calculated as the sum of the chloride salts of cyanidin-3-*O*-sambubioside-5-*O*-glucoside, cyanidin-3,5-di-*O*-glucoside, cyanidin-3-*O*-sambubioside, and cyanidin-3-*O*-glucoside on the anhydrous basis. It may contain suitable added substances as carriers.

IDENTIFICATION

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 0.2 mg/mL of each USP Chlorogenic Acid RS and USP Rutin RS in methanol. Sonicate to dissolve.

Standard solution B: 50 mg/mL of USP European Elder Berry Dry Extract RS in 70% alcohol. Sonicate at about 50° for 30 min, centrifuge, and use the supernatant.

Sample solution: 50 mg/mL of European Elder Berry Dry Extract in 70% alcohol. Sonicate at about 50° for 30 min, centrifuge, and use the supernatant.

Chromatographic system

Adsorbent: Chromatographic silica gel

Application volume: 5 µL

Temperature: About 25°

Developing solvent system: Ethyl acetate, glacial acetic acid, formic acid, and water (10: 0.9: 0.9: 2)

Derivatization reagent A: 10 mg/mL of 2-aminoethyl diphenylborinate in methanol

Derivatization reagent B: 50 mg/mL of polyethylene glycol 4000 in alcohol

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands and dry in air. Develop in a saturated chamber, remove the plate from the chamber, and dry at 100° for 3 min. Treat the plate with *Derivatization reagent A* and dry for 5 min in a current of cool air. Immediately treat the plate with *Derivatization reagent B*, dry for 5 min in a current of cool air, and examine under UV light at 366 nm and under white light.

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements: Under UV light at 366 nm, *Standard solution A* exhibits a yellow band due to rutin and a blue fluorescent band due to chlorogenic acid in the lower half of the chromatogram. *Standard solution B* exhibits a band corresponding in R_F and color to rutin and a band corresponding in R_F and color to chlorogenic acid in *Standard solution A*; a yellow band appears above chlorogenic acid and a weak blue band that is partially overlapped with chlorogenic acid appears between chlorogenic acid and the yellow band above. *Standard solution B* exhibits a yellow band in the upper-third section and several faint blue bands between the two aforementioned yellow bands. Under white light, *Standard solution B* exhibits a yellow band corresponding in R_F and color to rutin in *Standard solution A* and a couple of blue bands below rutin.

Acceptance criteria: Under UV light at 366 nm, the *Sample solution* exhibits a yellow band and a blue fluorescent band corresponding in R_F and color to rutin and chlorogenic acid in *Standard solution A* and *Standard solution B*. The *Sample solution* exhibits additional bands corresponding to similar bands in *Standard solution B*, including a yellow band above chlorogenic acid in the lower half of the chromatogram, a weak blue band that is partially overlapped with chlorogenic acid between chlorogenic acid and the yellow band above, a yellow band in the upper third section, and some or all the faint blue bands observed between the two aforementioned yellow bands. Under white light, the *Sample solution* exhibits a band corresponding in R_F and color to rutin in *Standard solution A*, and a couple of blue bands below rutin (the European elder berry stems do not display the blue bands).

- **B. HPLC**

Analysis: Proceed as directed in the test for *Content of Anthocyanosides and Cyanidin*.

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Acceptance criteria: The *Sample solution* exhibits a peak corresponding to cyanidin-3-*O*-glucoside in *Standard solution A*, a peak corresponding to cyanidin in *Standard solution B*, and peaks corresponding to cyanidin-3-*O*-sambubioside-5-*O*-glucoside, cyanidin-3,5-di-*O*-glucoside, and cyanidin-3-*O*-sambubioside in *Standard solution C*. The most intense peak corresponds to cyanidin-3-*O*-sambubioside. The peak that follows in intensity corresponds to cyanidin-3-*O*-glucoside. The cyanidin-3-*O*-sambubioside-5-*O*-glucoside peak is substantially smaller. Cyanidin-3,5-di-*O*-glucoside and cyanidin are minor peaks. Any other peak in the chromatogram is not more intense than the one corresponding to cyanidin-3,5-di-*O*-glucoside.

COMPOSITION

• **CONTENT OF ANTHOCYANOSIDES AND CYANIDIN**

[NOTE—Protect from light and moisture, and proceed under low-actinic light. *Standard solution A*, *Standard solution C*, and the *Sample solution* are stable for 48 h at 4°; *Standard solution B* is stable for 36 h at 4°.]

Solution A: Water, acetonitrile, and trifluoroacetic acid (19: 1: 0.06)

Solution B: Methanol, acetonitrile, water, and trifluoroacetic acid (7: 8: 5: 0.06)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	80	20
5	80	20
20	60	40
30	10	90
35	10	90
36	80	20
40	80	20

Solvent 1: 2% hydrochloric acid in methanol

Solvent 2: 10 mL of phosphoric acid diluted with water to 100 mL

Standard stock solution A: 0.30 mg/mL of USP Cyanidin-3-*O*-glucoside Chloride RS in *Solvent 1*. Sonicate to dissolve.

Standard solution A: 0.060 mg/mL of USP Cyanidin-3-*O*-glucoside Chloride RS in *Solvent 2*, prepared from *Standard stock solution A*

Standard stock solution B: 0.10 mg/mL of USP Cyanidin Chloride RS in *Solvent 1*. Sonicate to dissolve.

Standard solution B: 0.002 mg/mL of USP Cyanidin Chloride RS in *Solvent 2*, prepared from *Standard stock solution B*

Standard solution C: 1 mg/mL of USP European Elder Berry Dry Extract RS. Add 20% of final volume of *Solvent 1*, sonicate at least for 10 min to dissolve, and dilute with *Solvent 2* to volume. Before injection, pass through a membrane filter of 0.45-µm or finer pore size.

Sample solution: Transfer about 25 mg of European Elder Berry Dry Extract accurately weighed into a 25-mL volumetric flask. Add 5 mL of *Solvent 1*, sonicate to dissolve, and dilute with *Solvent 2* to volume. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, and discard the first portion of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 535 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 30°

Flow rate: 0.8 mL/min

Injection volume: 10 µL

System suitability

Samples: *Standard solution A*, *Standard solution B*, and *Standard solution C*

Suitability requirements

Resolution: NLT 1.5 between the peaks of cyanidin-3-*O*-sambubioside-5-*O*-glucoside and cyanidin-3,5-di-*O*-glucoside and between the peaks of cyanidin-3-*O*-sambubioside and cyanidin-3-*O*-glucoside, *Standard solution C*

Tailing factor: 0.8–2.0 for the cyanidin-3-*O*-glucoside peak, *Standard solution A*

Relative standard deviation: NMT 2.0% for the cyanidin-3-*O*-glucoside peak in repeated injections, *Standard solution A*

Signal-to-noise ratio: NLT 3, *Standard solution B*

Chromatogram similarity: The chromatogram of *Standard solution C* is similar to the reference chromatogram provided with the lot of USP European Elder Berry Dry Extract RS being used.

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Using the chromatograms of *Standard solution A*, *Standard solution B*, *Standard solution C*, and the reference chromatogram provided with the lot of USP European Elder Berry Dry Extract RS being used, identify the peaks corresponding to cyanidin-3-*O*-sambubioside-5-*O*-glucoside, cyanidin-3,5-di-*O*-glucoside, cyanidin-3-*O*-sambubioside, cyanidin-3-*O*-glucoside, and cyanidin in the *Sample solution*.

Separately calculate the percentage of cyanidin-3-*O*-sambubioside-5-*O*-glucoside, cyanidin-3,5-di-*O*-glucoside, cyanidin-3-*O*-sambubioside, and cyanidin-3-*O*-glucoside as chloride salts in the portion of European Elder Berry Dry Extract taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*

r_S = peak area of cyanidin-3-*O*-glucoside from *Standard solution A*

C_S = concentration of USP Cyanidin-3-*O*-glucoside Chloride RS in *Standard solution A* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of European Elder Berry Dry Extract taken to prepare the *Sample solution* (mg)

F = conversion factor for the analyte (see *Table 2*)

Table 2

Analyte	Approximate Relative Retention Time	Conversion Factor
Cyanidin-3-*O*-sambubioside-5-*O*-glucoside	0.47	1.4
Cyanidin-3,5-di-*O*-glucoside	0.52	1.1
Cyanidin-3-*O*-sambubioside	0.86	1.1
Cyanidin-3-*O*-glucoside	1.0	1.0
Cyanidin	1.97	—

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Calculate the content of total anthocyanosides as chlorides by adding the percentages of cyanidin-3-*O*-sambubioside-5-*O*-glucoside, cyanidin-3,5-di-*O*-glucoside, cyanidin-3-*O*-sambubioside, and cyanidin-3-*O*-glucoside.

Calculate the percentage of cyanidin as cyanidin chloride in the portion of European Elder Berry Dry Extract taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of cyanidin from the *Sample solution*
r_S = peak area of cyanidin from *Standard solution B*
C_S = concentration of USP Cyanidin Chloride RS in *Standard solution B* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of European Elder Berry Dry Extract taken to prepare the *Sample solution* (mg)

Calculate the percentage of the labeled amount of total anthocyanosides as chlorides in the portion of European Elder Berry Dry Extract taken:

$$\text{Result} = (P/L) \times 100$$

P = content of total anthocyanosides, determined previously (%)
L = labeled amount of total anthocyanosides (%)

Acceptance criteria

Total anthocyanosides: NLT 17% as chloride salts on the anhydrous basis; 90.0%–110.0% of the labeled amount as chloride salts on the anhydrous basis

Cyanidin: NMT 0.2% as chloride salt on the anhydrous basis

CONTAMINANTS

- **BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS

- **WATER DETERMINATION** ⟨921⟩, *Method I*: NMT 5.0%, determined on 0.5 g
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Total Ash*: NMT 3.0%
- **BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Residual Solvents*: Meets the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light and moisture, and store in a cool place.
- **LABELING:** The label states the Latin binomial following the official name of the plant from which the article was prepared. It meets other labeling requirements in *Botanical Extracts* ⟨565⟩.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Chlorogenic Acid RS
  USP Cyanidin-3-*O*-glucoside Chloride RS
  USP Cyanidin Chloride RS
  USP European Elder Berry Dry Extract RS
  USP Rutin RS▲ 2S (USP41)

Evening Primrose Oil

[90028-66-3].

DEFINITION

Evening Primrose Oil is derived from seeds of *Oenothera biennis* L. The oil is extracted by cold press, where seeds are squeezed at very high pressure. It can be extracted using hexane as a solvent. It is then refined. A suitable antioxidant may be added.

IDENTIFICATION

- **A.** It meets the requirements in *Specific Tests* for *Fats and Fixed Oils* ⟨401⟩, *Fatty Acid Composition*.
- **B. IDENTIFICATION OF FIXED OILS BY THIN-LAYER CHROMATOGRAPHY** ⟨202⟩: The R_F values of the principal spots of the *Sample solution* correspond to those of the *Standard solution*.

SPECIFIC TESTS

- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩: NMT 1.0
- **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩: NMT 5.0
- **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩: 185–195
- **FATS AND FIXED OILS,** *Unsaponifiable Matter* ⟨401⟩: NMT 2.0%
- **FATS AND FIXED OILS,** *Fatty Acid Composition* ⟨401⟩: Evening Primrose Oil exhibits the composition profile of fatty acids in *Table 1*.

Table 1

Fatty Acid	Shorthand Notation	Percentage (%)
Palmitic acid	16:0	4.0–10.0
Stearic acid	18:0	1.0–4.0
Oleic acid	18:1	5.0–12.0
Gamma-linolenic acid	18:3	7.0–14.0
Linoleic acid	18:2	65.0–85.0

- **REFRACTIVE INDEX** ⟨831⟩: 1.477–1.479 at 20°

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, preferably under an atmosphere of an inert gas, protected from light.
- **LABELING:** Where Evening Primrose Oil is intended for use in the manufacture of dosage forms, it is so labeled.

Evening Primrose Oil Capsules

DEFINITION

Evening Primrose Oil Capsules are prepared with Evening Primrose Oil and contain NLT 95.0% of the labeled amounts of each, γ-linolenic (C18:3 n–6), linoleic (C18:2 n–6), and oleic (C18:1 n–9) acids.

IDENTIFICATION

- **A. FATTY ACID PROFILE**
  System suitability solution and **Chromatographic system:** Proceed as directed in *Strength*.
  Sample solution: Proceed as directed for the *Sample solution* in *Strength*, beginning with "Transfer 80 mg" but without the addition of the *Internal standard*.
  Analysis: Identify the specified fatty acid methyl ester peaks by comparing them to the reference chromatogram provided with the lot of USP Evening Primrose Oil RS being

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used. Determine the percentage of each constituent relative to the total integrated area.

Acceptance criteria: The *Sample solution* conforms to the fatty acids composition profile in *Table 1*.

Table 1

Fatty Acid	Shorthand Notation	Percentage (%)
Palmitic acid	16:0	4.0–10.0
Stearic acid	18:0	1.0–4.0
Oleic acid	18:1 n–9	5.0–12.0
Linoleic acid	18:2 n–6	65.0–85.0
γ-Linolenic acid	18:3 n–6	7.0–14.0

STRENGTH

• **CONTENT OF γ-LINOLENIC, LINOLEIC, AND OLEIC ACIDS**

[NOTE—γ-Linolenic acid is quantitated against USP Methyl Linolenate RS.]

0.5 N methanolic sodium hydroxide solution: Dissolve 2 g of sodium hydroxide in 100 mL of methanol.

Internal standard: Methyl pentadecanoic acid

Sample solution: Weigh NLT 10 Capsules. With a sharp blade, carefully slice open the Capsules, avoiding loss of shell material. Combine the Capsule contents in a suitable container and mix well. Remove any adhering substance from the emptied Capsules by washing with several portions of diethyl ether, and discard the washings. Allow the empty Capsule shells to air-dry over a period of NMT 30 min, taking precautions to avoid uptake or loss of moisture. Weigh the empty Capsule shells and calculate the average fill weight/Capsule (A_F). Transfer 80 mg of the combined Capsule contents directly into a tared 30-mL screw-top glass centrifuge tube and weigh accurately. Re-tare and accurately weigh about 40 mg of the *Internal standard* into the same tube. Add 2 mL of *0.5 N methanolic sodium hydroxide solution*, tightly cap, and transfer to a heating block or another appropriate heating device. Reflux the solution until fat globules disappear (usually 5–10 min). Add 2 mL of 0.14 g/mL boron trifluoride in methanol, cap, and reflux for 2 min. Add 4 mL of chromatographic *n*-heptane, cap, and reflux for 1 min. Cool, add about 8 mL of saturated sodium chloride solution, shake, and centrifuge to separate the layers. Dilute an aliquot of the upper (heptane) layer with chromatographic *n*-heptane (1:8) and mix well.

System suitability solution: Using about 80 mg of USP Evening Primrose Oil RS, proceed as directed for the *Sample solution*, beginning with "Transfer 80 mg" but without the addition of the *Internal standard*.

Standard solution: Accurately weigh about 20 mg of USP Methyl Oleate RS, 70 mg of USP Methyl Linoleate RS, and 20 mg of USP Methyl Linolenate RS directly into a tared 30-mL screw-top glass centrifuge tube. Proceed as directed for the *Sample solution*, beginning with "Re-tare".

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.53-mm × 30-m fused silica capillary; coated with a 1.0-µm film of G16

Temperatures

Injection port: 220°

Detector: 260°

Column: See *Table 2*.

Table 2

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	0	70	2
70	5	240	5

Carrier gas: Helium

Linear velocity: 50 cm/s

Split mode: Splitless

Injection volume: 1 µL

System suitability

Samples: *System suitability solution* and *Standard solution*

Suitability requirements

Chromatogram similarity: The *System suitability solution* chromatogram is similar to the reference chromatogram provided with the lot of USP Evening Primrose Oil RS being used.

Resolution: NLT 1.5 between methyl stearate and methyl oleate, *System suitability solution*

Relative standard deviation: NMT 2% for the peak area ratios of specified analytes to the internal standard, *Standard solution*

Analysis

Samples: *Sample solution* and *Standard solution*

[NOTE—The relative retention times for γ-methyl linolenate and α-methyl linolenate are about 0.98 and 1.0, respectively.]

Identify the retention times of the relevant fatty acid methyl esters by comparing the peaks in the chromatogram of the *System suitability solution* with those in the reference chromatogram. Identify the locus for the internal standard peak by comparison of the chromatograms of the *Standard solution* and the *System suitability solution*.

Calculate the content, in mg/g, of γ-linolenic, linoleic, and oleic acids in the portion of Capsule content taken:

$$\text{Result} = (R_U/R_S) \times (A_U/A_S) \times (m_S/W) \times (M_{r1}/M_{r2})$$

R_U = peak area ratio of the relevant methyl ester peak to the internal standard peak from the *Sample solution*

R_S = peak area ratio of the relevant methyl ester peak to the internal standard peak from the *Standard solution*

A_U = weight of the *Internal standard* in the *Sample solution* (mg)

A_S = weight of the *Internal standard* in the *Standard solution* (mg)

m_S = weight of the relevant USP Methyl Ester RS in the *Standard solution* (mg)

W = sample weight used to prepare the *Sample solution* (g)

M_{r1} = molecular weight of the relevant fatty acid (g/mol)

M_{r2} = molecular weight of the relevant fatty acid methyl ester (g/mol)

Calculate the percentage of the labeled amounts of each (γ-linolenic, linoleic, and oleic) acid in the portion of Capsules taken:

$$\text{Result} = A \times A_F \times 100/L$$

A = content of the relevant fatty acid in the portion of Capsule content taken (mg/g)

A_F = average fill weight (g/Capsule)

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L = labeled content of the relevant fatty acid (mg/Capsule)

Acceptance criteria
γ-Linolenic, linoleic, and oleic acids: NLT 95.0% of the labeled amount of each

PERFORMANCE TESTS
- **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩**:** Meet the requirements in *Rupture Test for Soft Shell Capsules*
- **WEIGHT VARIATION** ⟨2091⟩**:** Meet the requirements

SPECIFIC TESTS
- **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩**:** NMT 10.0

CONTAMINANTS
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 1×10^3 cfu/g, and the combined molds and yeasts count does not exceed 3×10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meet the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tightly-closed, light-resistant containers.
- **LABELING:** The label states the article that the Capsules were prepared with and the content of γ-linolenic, linoleic, and oleic acids in mg/Capsule.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Evening Primrose Oil RS
  USP Methyl Linoleate RS
  USP Methyl Linolenate RS
  USP Methyl Oleate RS

Fenugreek Seed

DEFINITION
Fenugreek Seed consists of the dried ripe seeds of *Trigonella foenum-graecum* L. (Fam. Fabaceae). It contains NLT 0.2% of 4-hydroxyisoleucine, calculated on the dried basis.

IDENTIFICATION
- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩**—AMINO ACID PROFILE**

Standard solution A: 0.5 mg/mL of USP 4-Hydroxyisoleucine RS in an ethanol and water (7:3) mixture

Standard solution B: 50 mg/mL of USP Trigonella Foenum-graecum Seed Dry Extract RS in an ethanol and water (7:3) mixture. Sonicate for 10 min, centrifuge, and use the supernatant.

Sample solution: Suspend about 1 g of Fenugreek Seed, finely powdered, in 5 mL of an ethanol and water (7:3) mixture, and incubate at 50° for 15 min. Centrifuge, and use the supernatant.

[NOTE—*Standard solution B* and the *Sample solution* may also be used for *Identification* test *B* and for *Specific Tests, Presence of Trigonelline.*]

Chromatographic system

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plate)[1]

Application volume: 2 µL each of *Standard solution A* and *Standard solution B,* and 4 µL of the *Sample solution,* as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of 33% using a suitable device.

Temperature: Ambient, not to exceed 30°

Developing solvent system: A mixture of *n*-butanol, acetic acid, and water (7:2:1)

Derivatization reagent: A solution of 0.3% ninhydrin in a mixture of isopropanol and glacial acetic acid (19:1)

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements: Under white light, the derivatized chromatogram of *Standard solution B* displays, in its lower half, five or six brown bands; the darkest band corresponding to the 4-hydroxyisoleucine band in the chromatogram of *Standard solution A.* Under long-wave UV (365 nm), the derivatized chromatogram of *Standard solution B* exhibits, in its lower half, four or five brown bands; the darkest band corresponding to the 4-hydroxyisoleucine band in the chromatogram of *Standard solution A.* In the upper half of the chromatogram, three diffuse yellow to orange-yellow bands are seen, the middle one being the most intense.

Analysis

Samples: *Standard solution A, Standard solution B,* and *Sample solution*

Apply the samples as bands, and dry in air. Condition at relative humidity at about 33%. Develop in a saturated chamber, until the solvent front has migrated over a path of 6 cm. Air-dry, treat with *Derivatization reagent,* heat for 3 min at 105°, and immediately examine under white light and under long-wave UV light (365 nm).

Acceptance criteria: Under white light, the derivatized chromatogram of the *Sample solution* appears monochromatic, with the bands differing in intensity, but not the color, which is uniformly reddish-brown. In the lower third of the plate, two or three thin bands are seen, proximate to the origin, followed by a very intense band due to 4-hydroxyisoleucine, coincident with the corresponding bands in *Standard solution A* and *Standard solution B,* and comparable in intensity to the band in the *Standard solution B* chromatogram. Two lightly colored bands, one just above the 4-hydroxyisoleucine band, another further upwards, are seen. The upper half of the plate is devoid of discernible features. Under long-wave UV (365 nm), the derivatized chromatogram of the *Sample solution* exhibits, in its lower half, two or three light reddish-brown bands followed by the darker-brown intense band due to 4-hydroxyisoleucine, coincident with the corresponding bands in *Standard solution A* and *Standard solution B.* Above it, two lighter-brown and somewhat diffuse bands appear. In the upper third of the plate, three yellowish-orange bands are seen, corresponding in position and color to those observed in *Standard solution B.*

- **B. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩**—STEROIDAL SAPONINS PROFILE**

Standard solution: 50 mg/mL of USP Trigonella Foenum-graecum Seed Dry Extract RS in an ethanol and water (7:3) mixure. Sonicate for 10 min, centrifuge, and use the supernatant.

Sample solution: Suspend about 1 g of Fenugreek Seed, finely powdered, in 5 mL of an ethanol and water (7:3) mixture, and incubate at 50° for 15 min. Centrifuge, and use the supernatant.

[NOTE—The *Standard solution* and *Sample solution* may also be used for *Identification* test *A* and for *Specific Tests, Presence of Trigonelline.*]

Chromatographic system

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plate)

Application volume: 2 µL, as 8-mm bands

[1] Suitable commercially available plates are HPTLC Silica Gel 60 F_{254} from EMD Millipore (e.g., Part No. 1.05642.0001).

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Relative humidity: Condition the plate to a relative humidity of 33% using a suitable device.
Temperature: Ambient, not to exceed 30°
Developing solvent system: A mixture of dichloromethane, methanol, and water (18:8:1)
Derivatization reagent: A mixture of methanol, glacial acetic acid, sulfuric acid, and *p*-anisaldehyde (170:20:10:1). Prepare on an ice bath, and mix well.
System suitability
Sample: *Standard solution*
Suitability requirements: Under white light, the derivatized chromatogram of the *Standard solution* exhibits, in its lower half, three or four lightly-shaded bands. Under long-wave UV (365 nm), the derivatized chromatogram of the *Standard solution* exhibits, in its lower third, two diffuse bands of blue fluorescence, and another blue fluorescent band in the middle of the plate.
Analysis
Samples: *Standard solution* and *Sample solution*
Apply the *Samples* as bands, and dry in air. Condition at relative humidity at about 33%. Develop in a saturated chamber, until the solvent front has migrated approximately 6 cm. Air-dry, treat with *Derivatization reagent*, heat for 3 min at 105°, and immediately examine under white light and under long-wave UV light (365 nm).
Acceptance criteria: Under white light, the chromatogram of the *Sample solution*, in its lower third, shows two medium-intensity bands immediately next to the application line. Further upwards, two closely spaced, less intense bands are followed by a more prominent band, and another pair of closely spaced lighter bands, which may merge. In the middle third of the chromatogram, a medium-intensity band is followed by a faint diffuse band. In the upper third of the chromatogram, two well-defined bands of medium intensity are observed. Under UV light (365 nm), the lower half of the chromatogram features three or four deep-blue fluorescent bands of varying intensity interspersed with greyish-brown zones.

COMPOSITION

• **CONTENT OF 4-HYDROXYISOLEUCINE**
Solution A: 0.1% Phosphoric acid in water
Solution B: Acetonitrile
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	80.0	20.0
20	40.0	60.0
21	80.0	20.0
25	80.0	20.0

Diluent: Methanol and water (1:1)
Reagent: A mixture of acetonitrile, water, and triethylamine (10:3:2)
Standard solution: Transfer about 4.0 mg of USP 4-Hydroxyisoleucine RS, accurately weighed, into a 50-mL volumetric flask, and dissolve in 5 mL of the *Diluent*. Add 10 mL of *Reagent* and 0.5 mL of phenyl isothiocyanate, and shake for 5 min. Add 30 mL of methanol, adjust with water to volume, and mix well.
Sample stock solution: Transfer about 2.0 g of Fenugreek Seed, finely powdered and accurately weighed, into a centrifuge tube. Add 8 mL of *Diluent*, place on a water bath at 65° for 5 min, sonicate for 5 min, and centrifuge. Retain the supernatant, and repeat extraction with 8 mL of *Diluent* two more times. Combine all three extracts in the 25-mL volumetric flask, dilute with *Diluent* to volume, and mix well.
Sample solution: Transfer 5.0 mL of *Sample stock solution* into a 50-mL volumetric flask, add 10 mL of *Reagent* and 0.5 mL of phenyl isothiocyanate, and shake for 5 min. Add 30 mL of methanol, dilute with water to volume, and mix well. Pass through a nylon filter having a 0.45-µm or finer pore size, discarding the initial few mL of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: HPLC
Detector: UV 254 nm
Column: 4.6-mm × 15-cm; 5-µm packing L1
Column temperature: Ambient
Flow rate: 1.5 mL/min
Injection volume: 20 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Tailing factor: NMT 2.0 for the 4-hydroxyisoleucine peak, *Standard solution*
Relative standard deviation: NMT 2.0% determined for the 4-hydroxyisoleucine peak in replicate injections, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Using the chromatogram of the *Standard solution*, identify the retention time of the peak corresponding to 4-hydroxyisoleucine in the *Sample solution* chromatogram.
Calculate the percentage of 4-hydroxyisoleucine in the portion of Fenugreek Seed taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times 100$$

r_U = peak area of 4-hydroxyisoleucine from the *Sample solution*
r_S = peak area of 4-hydroxyisoleucine from the *Standard solution*
C_S = concentration of USP 4-Hydroxyisoleucine RS in the *Standard solution* (mg/mL)
V = volume of the *Sample stock solution* (mL)
W = weight of Fenugreek Seed taken to prepare the *Sample stock solution* (mg)
D = dilution factor to prepare the *Sample solution* from the *Sample stock solution*, 10

Acceptance criteria: NLT 0.2% on the dried basis

CONTAMINANTS

• **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩
Acceptance criteria
Arsenic: NMT 2.0 µg/g
Cadmium: NMT 1.0 µg/g
Lead: NMT 10.0 µg/g
Mercury: NMT 1.0 µg/g
• **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements
• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacterial count does not exceed 10^3 cfu/g.
• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

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SPECIFIC TESTS

• **HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩—**PRESENCE OF TRIGONELLINE**

Standard solution A: 1.5 mg/mL of USP Trigonelline RS in an ethanol and water (7:3) mixture

Standard solution B: 50 mg/mL of USP Trigonella Foenum-graecum Seed Dry Extract RS in an ethanol and water (7:3) mixture. Sonicate for 10 min, centrifuge, and use the supernatant.

Sample solution: Suspend about 1 g of Fenugreek Seed, finely powdered, in 5 mL of an ethanol and water (7:3) mixture, and incubate at 50° for 15 min. Centrifuge, and use the supernatant.

[NOTE—*Standard solution B* and the *Sample solution* may also be used for *Identification* test *A* and for *Identification* test *B*.]

Chromatographic system

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plate)

Application volume: 5 µL, as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of 33% using a suitable device.

Temperature: Ambient, not to exceed 30°

Developing solvent system: A mixture of isopropyl alcohol, methanol, and water (4:1:4)

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements: Under short-wave UV light (254 nm), the chromatogram of *Standard solution B* displays, in its lower half, a quenching band coincident with that of the trigonelline band in the chromatogram of *Standard solution A*.

Analysis

Samples: *Standard solution A, Standard solution B,* and *Sample solution*

Apply the *Samples* as bands, and dry in air. Condition at relative humidity at about 33%. Develop in a saturated chamber, until the solvent front has migrated over a path of 6 cm. Air-dry, and examine under short-wave UV light (254 nm).

Acceptance criteria: Under short-wave UV light, the chromatogram of the *Sample solution* displays a quenching band corresponding to the trigonelline band in the chromatograms of *Standard solution A* and *Standard solution B*.

• **BOTANICAL CHARACTERISTICS**

Macroscopic: The seeds are oblong, 3–5 mm long, 2–3 mm wide, 1.5–2.0 mm thick, with rounded corners, smooth, dull yellowish-brown to reddish-brown. They are flattened and have a very characteristic rhomboidal outline. Nearly in the center of one of the long, narrow sides is a small depression in which both hilum and micropyle are situated, the former being distinctly visible as a whitish point. This depression is continued in the form of a furrow running diagonally across part of each of the adjoining sides, dividing the seed into two unequal lobes. A cut made transversely to pass through both lobes reveals two accumbent cotyledons in the larger lobe, and the radicle in the smaller lobe. Both are yellowish in color, and surrounded by a darker, horny, translucent endosperm, which also separates the radicle from the cotyledons.

Microscopic: Transverse section shows an epidermis of palisade cells, one layer, with thick cuticle and thick lamellated walls, and a relatively large lumen at the lower part. Longitudinal pit-canals fine and close. Subepidermal layer of basket-like cells, with bar-like thickening on the radial walls, followed by a parenchymatous layer. Endosperm consists of a layer of thick-walled cells containing aleurone grains, several layers of polyhedral cells with stratified mucilaginous contents and thickened walls. Cotyledons of parenchymatous cells containing fixed oil globules and aleurone grains up to 15 µm in diameter. The parenchyma of the testa composed of several layers of thin-walled cells which appear similar in sectional view but in surface view the layers show structural differences: some are composed of elongated rectangular cells with slightly thickened and beaded walls; other layers include thin-walled polygonal cells which may be very irregular in size or may enclose irregular intercellular spaces.

• **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩: NMT 2.0%

• **LOSS ON DRYING** ⟨731⟩

Sample: 1 g

Analysis: Dry the *Sample* at 105° for 2 h.

Acceptance criteria: NMT 12.0%

• **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩: NMT 5.0%

• **ARTICLES OF BOTANICAL ORIGIN,** *Alcohol-Soluble Extractives, Method 1* ⟨561⟩: NLT 5.0%

• **ARTICLES OF BOTANICAL ORIGIN,** *Water-Soluble Extractives, Method 1* ⟨561⟩: NLT 9.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.

• **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP 4-Hydroxyisoleucine RS

USP Trigonella Foenum-graecum Seed Dry Extract RS

USP Trigonelline RS

Fenugreek Seed Powder

DEFINITION

Fenugreek Seed Powder consists of the dried ripe seeds of *Trigonella foenum-graecum* L. (Fam. Fabaceae), reduced to powder or very fine powder. It contains NLT 0.2% of 4-hydroxyisoleucine, calculated on the dried basis.

IDENTIFICATION

• **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 0.5 mg/mL of USP 4-Hydroxyisoleucine RS in an ethanol and water (7:3) mixture

Standard solution B: 50 mg/mL of USP Trigonella Foenum-graecum Seed Dry Extract RS in an ethanol and water (7:3) mixture. Sonicate for 10 min, centrifuge, and use the supernatant.

Sample solution: Suspend about 1 g of Powder in 5 mL of an ethanol and water (7:3) mixture, and incubate at 50° for 15 min. Centrifuge, and use the supernatant.

[NOTE—*Standard solution B* and the *Sample solution* may also be used for *Identification* test *B* and for *Specific Tests, Presence of Trigonelline*.]

Chromatographic system

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plate)[1]

Application volume: 2 µL each of *Standard solution A* and *Standard solution B*, and 4 µL of the *Sample solution*, as 8-mm bands

[1] Suitable commercially available plates are HPTLC Silica Gel 60 F_{254} from EMD Millipore (e.g., Part No. 1.05642.0001).

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Relative humidity: Condition the plate to a relative humidity of 33% using a suitable device.
Temperature: Ambient, not to exceed 30°
Developing solvent system: A mixture of *n*-butanol, acetic acid, and water (7:2:1)
Derivatization reagent: A solution of 0.3% ninhydrin in a mixture of isopropanol and glacial acetic acid (19:1)
System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements: Under white light, the derivatized chromatogram of *Standard solution B* displays, in its lower half, five or six brown bands; the darkest band corresponding to the 4-hydroxyisoleucine band in the chromatogram of *Standard solution A*. Under long-wave UV (365 nm), the derivatized chromatogram of *Standard solution B* exhibits, in its lower half, four or five brown bands; the darkest band corresponding to the 4-hydroxyisoleucine band in the chromatogram of *Standard solution A*. In the upper half of the chromatogram, three diffuse yellow to orange-yellow bands are seen, the middle one being the most intense.
Analysis
Samples: *Standard solution A, Standard solution B,* and *Sample solution*
Apply the *Samples* as bands, and dry in air. Condition at relative humidity at about 33%. Develop in a saturated chamber, until the solvent front has migrated over a path of 6 cm. Air-dry, treat with *Derivatization reagent*, heat for 3 min at 105°, and immediately examine under white light and under long-wave UV light (365 nm).
Acceptance criteria: Under white light, the derivatized chromatogram of the *Sample solution* appears monochromatic, with the bands differing in intensity, but not the color, which is uniformly reddish-brown. In the lower third of the plate, two or three thin bands are seen, proximate to the origin, followed by a very intense band due to 4-hydroxyisoleucine, coincident with the corresponding bands in *Standard solution A* and *Standard solution B*, and comparable in intensity to the band in the *Standard solution B* chromatogram. Two lightly colored bands, one just above the 4-hydroxyisoleucine band, another further upwards, are seen. The upper half of the plate is devoid of discernible features. Under long-wave UV (365 nm), the derivatized chromatogram of the *Sample solution* exhibits, in its lower half, two or three light reddish-brown bands followed by the darker-brown intense band due to 4-hydroxyisoleucine, coincident with the corresponding bands in *Standard solution A* and *Standard solution B*. Above it, two lighter brown and somewhat diffuse bands appear. In the upper third of the plate, three yellowish-orange bands are seen, corresponding in position and color to those observed in *Standard solution B*.

• **B. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩
Standard solution: 50 mg/mL of USP Trigonella Foenum-graecum Seed Dry Extract RS in an ethanol and water (7:3) mixture. Sonicate for 10 min, centrifuge, and use the supernatant.
Sample solution: Suspend about 1 g of Powder in 5 mL of an ethanol and water (7:3) mixture, and incubate at 50° for 15 min. Centrifuge, and use the supernatant.
[NOTE—The *Standard solution* and *Sample solution* may also be used for *Identification* test *A* and for *Specific Tests, Presence of Trigonelline*.]
Chromatographic system
Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plate)
Application volume: 2 µL each of the *Standard solution* and the *Sample solution*, as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of 33% using a suitable device.
Temperature: Ambient, not to exceed 30°
Developing solvent system: A mixture of dichloromethane, methanol, and water (18:8:1)
Derivatization reagent: A mixture of methanol, glacial acetic acid, sulfuric acid, and *p*-anisaldehyde (170:20:10:1). Prepare on an ice bath, and mix well.
System suitability
Sample: *Standard solution*
Suitability requirements: Under white light, the derivatized chromatogram of the *Standard solution* exhibits, in its lower half, three or four lightly shaded bands. Under long-wave UV (365 nm), the derivatized chromatogram of the *Standard solution* exhibits, in its lower third, two diffuse bands of blue fluorescence, and another blue fluorescent band in the middle of the plate.
Analysis
Samples: *Standard solution* and *Sample solution*
Apply the *Samples* as bands, and dry in air. Condition at relative humidity at about 33%. Develop in a saturated chamber, until the solvent front has migrated over a path of 6 cm. Air-dry, treat with *Derivatization reagent*, heat for 3 min at 105°, and immediately examine under white light and under the long-wave UV light (365 nm).
Acceptance criteria: Under white light, the chromatogram of the *Sample solution*, in its lower third, shows two medium-intensity bands immediately next to the application line. Further upwards, two closely spaced, less intense, bands are followed by a more prominent band, and another pair of closely spaced lighter bands, which may merge. In the middle third of the chromatogram, a medium-intensity band is followed by a faint diffuse band. In the upper third of the chromatogram, two well-defined bands of medium intensity are observed. Under UV light (365 nm), the lower half of the chromatogram features three or four deep-blue fluorescent bands of varying intensity interspersed with greyish-brown zones.

COMPOSITION

• **CONTENT OF 4-HYDROXYISOLEUCINE**
Solution A: 0.1% Phosphoric acid in water
Solution B: Acetonitrile
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	80.0	20.0
20	40.0	60.0
21	80.0	20.0
25	80.0	20.0

Diluent: Methanol and water (1:1)
Reagent: A mixture of acetonitrile, water, and triethylamine (10:3:2)
Standard solution: Transfer about 4.0 mg of USP 4-Hydroxyisoleucine RS, accurately weighed, into a 50-mL volumetric flask, and dissolve in 5 mL of the *Diluent*. Add 10 mL of *Reagent* and 0.5 mL of phenyl isothiocyanate, and shake for 5 min. Add 30 mL of methanol, adjust with water to volume, and mix well.
Sample stock solution: Transfer about 2.0 g of accurately weighed Powder into a centrifuge tube. Add 8 mL of *Diluent*, place on a water bath at 65° for 5 min, sonicate for 5 min, and centrifuge. Retain the supernatant, and repeat extraction with 8 mL of *Diluent* two more times. Combine

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all three extracts in the 25-mL volumetric flask, dilute with *Diluent* to volume, and mix well.

Sample solution: Transfer 5.0 mL of *Sample stock solution* into a 50-mL volumetric flask, add 10 mL of *Reagent* and 0.5 mL of phenyl isothiocyanate, and shake for 5 min. Add 30 mL of methanol, dilute with water to volume, and mix well. Pass through a nylon filter having a 0.45-µm or finer pore size, discarding the initial few mL of the filtrate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: HPLC
Detector: UV 254 nm
Column: 4.6-mm × 15-cm; 5-µm packing L1
Column temperature: Ambient
Flow rate: 1.5 mL/min
Injection volume: 20 µL

System suitability
Sample: *Standard solution*
Suitability requirements
Tailing factor: NMT 2.0 for the 4-hydroxyisoleucine peak, *Standard solution*
Relative standard deviation: NMT 2.0% determined for the 4-hydroxyisoleucine peak in replicate injections, *Standard solution*

Analysis
Samples: *Standard solution* and *Sample solution*
Using the chromatogram of the *Standard solution*, identify the retention time of the peak corresponding to 4-hydroxyisoleucine in the *Sample solution* chromatogram.
Calculate the percentage of 4-hydroxyisoleucine in the portion of Powder taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times 100$$

r_U = peak area of 4-hydroxyisoleucine from the *Sample solution*
r_S = peak area of 4-hydroxyisoleucine from the *Standard solution*
C_S = concentration of USP 4-Hydroxyisoleucine RS in the *Standard solution* (mg/mL)
V = volume of the *Sample stock solution* (mL)
W = weight of Powder taken to prepare the *Sample stock solution* (mg)
D = dilution factor to prepare the *Sample solution* from the *Sample stock solution*, 10

Acceptance criteria: NLT 0.2% on the dried basis

CONTAMINANTS

- **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩
  Acceptance criteria
  Arsenic: NMT 2.0 µg/g
  Cadmium: NMT 1.0 µg/g
  Lead: NMT 10.0 µg/g
  Mercury: NMT 1.0 µg/g
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria do not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

- **PRESENCE OF TRIGONELLINE**
  Standard solution A: 1.5 mg/mL of USP Trigonelline RS in an ethanol and water (7:3) mixture
  Standard solution B: 50 mg/mL of USP Trigonella Foenum-graecum Seed Dry Extract RS in an ethanol and water (7:3) mixture. Sonicate for 10 min, centrifuge, and use the supernatant.
  Sample solution: Suspend about 1 g of Powder in 5 mL of an ethanol and water (7:3) mixture, and incubate at 50° for 15 min. Centrifuge, and use the supernatant.
  [NOTE—*Standard solution B* and the *Sample solution* may also be used for *Identification* test *A* and for *Identification* test *B*.]
  Chromatographic system
  (See *HPTLC for Articles of Botanical Origin* ⟨203⟩.)
  Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plate)
  Application volume: 5 µL, as 8-mm bands
  Relative humidity: Condition the plate to a relative humidity of 33% using a suitable device.
  Temperature: Ambient, not to exceed 30°
  Developing solvent system: A mixture of isopropyl alcohol, methanol, and water (4:1:4)
  System suitability
  Samples: *Standard solution A* and *Standard solution B*
  Suitability requirements: Under short-wave UV light (254 nm), the chromatogram of *Standard solution B* displays, in its lower half, a quenching band coincident with that of the trigonelline band in the chromatogram of *Standard solution A*.
  Analysis
  Samples: *Standard solution A, Standard solution B,* and *Sample solution*
  Apply the *Samples* as bands, and dry in air. Condition at relative humidity at about 33%. Develop in a saturated chamber, until the solvent front has migrated over a path of 6 cm. Air-dry, and examine under short-wave UV light (254 nm).
  Acceptance criteria: Under short-wave UV light, the chromatogram of the *Sample solution* displays a quenching band corresponding to the trigonelline band in the chromatograms of *Standard solution A* and *Standard solution B*.
- **BOTANICAL CHARACTERISTICS**
  Macroscopic: Yellow-brown powder
  Microscopic: Fragments of the test in sectional view with thick cuticle covering lageniform epidermal cells, with an underlying hypodermis of large cells, narrower at the upper end and constricted in the middle, with bar-like thickenings of the radial walls; yellowish-brown fragments of the epidermis in surface view, composed of small, polygonal cells with thickened and pitted walls, frequently associated with the hypodermal cells, circular in outline with thickened and closely beaded walls; fragments of the hypodermis viewed from below, composed of polygonal cells whose bar-like thickenings extend to the upper and lower walls; parenchyma of the testa with elongated, rectangular cells with slightly thickened and beaded walls; fragments of endosperm with irregularly thickened, sometimes elongated cells, containing mucilage
- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩**:** NMT 2.0%
- **LOSS ON DRYING** ⟨731⟩
  Sample: 1 g
  Analysis: Dry the *Sample* at 105° for 2 h.
  Acceptance criteria: NMT 12.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 5.0%

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- **ARTICLES OF BOTANICAL ORIGIN,** *Alcohol-Soluble Extractives, Method 1* ⟨561⟩: NLT 5.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Water-Soluble Extractives, Method 1* ⟨561⟩: NLT 9.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP 4-Hydroxyisoleucine RS
  USP Trigonella Foenum-graecum Seed Dry Extract RS
  USP Trigonelline RS

Fenugreek Seed Powdered Extract

DEFINITION

Fenugreek Seed Powdered Extract is prepared from the dried ripe seeds of *Trigonella foenum-graecum* L. (Fam. Fabaceae) by extraction with hydroalcoholic mixtures. It contains NLT 90.0% and NMT 110.0% of the labeled amount of 4-hydroxyisoleucine, calculated on the dried basis.

IDENTIFICATION

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩**—AMINO ACID PROFILE**

Standard solution A: 0.5 mg/mL of USP 4-Hydroxyisoleucine RS in an ethanol and water (7:3) mixture

Standard solution B: 50 mg/mL of USP Trigonella Foenum-graecum Seed Dry Extract RS in an ethanol and water (7:3) mixture. Sonicate for 10 min, centrifuge, and use the supernatant.

Sample solution: 50 mg/mL of Powdered Extract in an ethanol and water (7:3) mixture. Sonicate for 10 min, centrifuge, and use the supernatant.

[NOTE—*Standard solution B* and the *Sample solution* may also be used for *Identification* test *B* and for *Specific Tests, Presence of Trigonelline*.]

Chromatographic system

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plate)[1]

Application volume: 2 µL, as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of 33% using a suitable device.

Temperature: Ambient, not to exceed 30°

Developing solvent system: A mixture of *n*-butanol, acetic acid, and water (7:2:1)

Derivatization reagent: A solution of 0.3% ninhydrin in a mixture of isopropanol and glacial acetic acid (19:1)

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements: Under white light, the derivatized chromatogram of *Standard solution B* displays, in its lower half, five or six brown bands; the darkest band corresponding to the 4-hydroxyisoleucine band in the chromatogram of *Standard solution A*. Under long-wave UV (365 nm), the derivatized chromatogram of *Standard solution B* exhibits, in its lower half, four or five brown bands; the darkest band corresponding to the 4-hydroxyisoleucine band in the chromatogram of *Standard solution A*. In the upper half of the chromatogram, three diffuse yellow to orange-yellow bands are seen, the middle one being the most intense.

Analysis

Samples: *Standard solution A, Standard solution B,* and *Sample solution*

Apply the *Samples* as bands, and dry in air. Condition at relative humidity at about 33%. Develop in a saturated chamber until the solvent front has migrated over a path of 6 cm. Air-dry, treat with *Derivatization reagent*, heat for 3 min at 105°, and immediately examine under white light and under the long-wave UV light (365 nm).

Acceptance criteria: Under white light, and under long-wave UV light (365 nm), the chromatogram of the *Sample solution* displays the bands generally similar in pattern and color to those observed with *Standard solution B*, in particular, the prominent band corresponding to the 4-hydroxyisoleucine band in the chromatograms of *Standard solution A* and *Standard solution B*. However, depending on the procedures and excipients used in preparation of Powdered Extract, the number, position, and coloration of the bands in the *Sample solution* may differ from those observed in the *Standard solution B* chromatogram.

- **B. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩**—STEROIDAL SAPONINS PROFILE**

Standard solution: 50 mg/mL of USP Trigonella Foenum-graecum Seed Dry Extract RS in an ethanol and water (7:3) mixture. Sonicate for 10 min, centrifuge, and use the supernatant.

Sample solution: 50 mg/mL of Powdered Extract in an ethanol and water (7:3) mixture. Sonicate for 10 min, centrifuge, and use the supernatant.

[NOTE—The *Standard solution* and *Sample solution* may also be used for *Identification* test *A* and for *Specific Tests, Presence of Trigonelline*.]

Chromatographic system

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plate)

Application volume: 2 µL, as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of 33% using a suitable device.

Temperature: Ambient, not to exceed 30°

Developing solvent system: A mixture of dichloromethane, methanol, and water (18:8:1)

Derivatization reagent: A mixture of methanol, glacial acetic acid, sulfuric acid, and *p*-anisaldehyde (170:20:10:1). Prepare on an ice bath, and mix well.

System suitability

Sample: *Standard solution*

Suitability requirements: Under white light, the derivatized chromatogram of the *Standard solution* exhibits, in its lower half, three or four lightly shaded bands. Under long-wave UV (365 nm), the derivatized chromatogram of the *Standard solution* exhibits, in its lower third, two diffuse bands of blue fluorescence, and another blue fluorescent band in the middle of the plate.

Analysis

Samples: *Standard solution* and *Sample solution*

Apply the *Samples* as bands, and dry in air. Condition at relative humidity at about 33%. Develop in a saturated chamber, until the solvent front has migrated over a path of 6 cm. Air-dry, treat with *Derivatization reagent*, heat for 3 min at 105°, and immediately examine under white light and under the long-wave UV light (365 nm).

Acceptance criteria: Under white light, and under long-wave UV light (365 nm), the chromatogram of the *Sample solution* displays the bands similar in pattern and color to those observed with the *Standard solution*.

[1] Suitable commercially available plates are HPTLC Silica Gel 60 F_{254} from EMD Millipore (e.g., Part No. 1.05642.0001).

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However, depending on the procedures and excipients used in preparation of Powdered Extract, the number, position, and coloration of the bands in the *Sample solution* may differ from those observed in the *Standard solution* chromatogram.

COMPOSITION

• **CONTENT OF 4-HYDROXYISOLEUCINE**

Solution A: 0.1% Phosphoric acid in water

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	80.0	20.0
20	40.0	60.0
21	80.0	20.0
25	80.0	20.0

Diluent: Methanol and water (1:1)

Reagent: A mixture of acetonitrile, water, and triethylamine (10:3:2)

Standard solution: Transfer about 4.0 mg of USP 4-Hydroxyisoleucine RS, accurately weighed, into a 50-mL volumetric flask, and dissolve in 5 mL of the *Diluent*. Add 10 mL of *Reagent* and 0.5 mL of phenyl isothiocyanate, and shake for 5 min. Add 30 mL of methanol, adjust with water to volume, and mix well.

Sample solution: Transfer the amount of accurately weighed Powdered Extract calculated to contain about 4 mg of 4-hydroxyisoleucine into a 50-mL volumetric flask, and dissolve in 5 mL of *Diluent*. Add 10 mL of *Reagent* and 0.5 mL of phenyl isothiocyanate, and shake for 5 min. Add 30 mL of methanol, adjust with water to volume, and mix well.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: HPLC

Detector: UV 254 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Column temperature: Ambient

Flow rate: 1.5 mL/min

Injection volume: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Tailing factor: NMT 2.0 for the 4-hydroxyisoleucine peak, *Standard solution*

Relative standard deviation: NMT 2.0% determined for the 4-hydroxyisoleucine peak in replicate injections, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Using the chromatogram of the *Standard solution*, identify the retention time of the peak corresponding to 4-hydroxyisoleucine in the *Sample solution* chromatogram.

Calculate the percentage of 4-hydroxyisoleucine in the portion of Powdered Extract taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of 4-hydroxyisoleucine from the *Sample solution*

r_S = peak area of 4-hydroxyisoleucine from the *Standard solution*

C_S = concentration of USP 4-Hydroxyisoleucine RS in the *Standard solution* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Powdered Extract taken to prepare the *Sample solution* (mg)

Acceptance criteria: 90.0%–110.0% of the labeled amount of 4-hydroxyisoleucine, on the dried basis

CONTAMINANTS

• **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩

Acceptance criteria

Arsenic: NMT 2.0 µg/g

Cadmium: NMT 1.0 µg/g

Lead: NMT 10.0 µg/g

Mercury: NMT 1.0 µg/g

Change to read:

• **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020)**:** Meets the requirements

• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

• **HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩**—PRESENCE OF TRIGONELLINE**

Standard solution A: 1.5 mg/mL of USP Trigonelline RS in an ethanol and water (7:3) mixture

Standard solution B: 50 mg/mL of USP Trigonella Foenum-graecum Seed Dry Extract RS in an ethanol and water (7:3) mixture. Sonicate for 10 min, centrifuge, and use the supernatant.

Sample solution: 50 mg/mL of Powdered Extract in methanol. Sonicate for 10 min, centrifuge, and use the supernatant.

[NOTE—*Standard solution B* and the *Sample solution* may also be used for *Identification* test *A* and for *Identification* test *B*.]

Chromatographic system

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plate)

Application volume: 5 µL, as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of 33% using a suitable device.

Temperature: Ambient, not to exceed 30°

Developing solvent system: A mixture of isopropyl alcohol, methanol, and water (4:1:4)

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements: Under short-wave UV light (254 nm), the chromatogram of *Standard solution B* displays, in its lower half, a quenching band coincident with that of the trigonelline band in the chromatogram of *Standard solution A*.

Analysis

Samples: *Standard solution A, Standard solution B,* and *Sample solution*

Apply the *Samples* as bands, and dry in air. Condition at relative humidity at about 33%. Develop in a saturated chamber, until the solvent front has migrated over a path of 6 cm. Air-dry, and examine under short-wave UV light (254 nm).

Supplement 2

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Acceptance criteria: Under short-wave UV light, the chromatogram of the *Sample solution* displays a quenching band corresponding to the trigonelline band in the chromatograms of *Standard solution A* and *Standard solution B*.

- **WATER DETERMINATION,** *Method Ia* ⟨921⟩**:** NMT 9.0%
- **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 5.0%
- **RESIDUAL SOLVENTS** ⟨467⟩**:** Meets the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the extract was derived. It also meets the requirements for *Labeling* in *Botanical Extracts* ⟨565⟩.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP 4-Hydroxyisoleucine RS
  USP Trigonella Foenum-graecum Seed Dry Extract RS
  USP Trigonelline RS

Ferrous Fumarate—*see Ferrous Fumarate General Monographs*

Ferrous Fumarate Tablets—*see Ferrous Fumarate Tablets General Monographs*

Ferrous Gluconate—*see Ferrous Gluconate General Monographs*

Ferrous Gluconate Capsules—*see Ferrous Gluconate Capsules General Monographs*

Ferrous Gluconate Oral Solution—*see Ferrous Gluconate Oral Solution General Monographs*

Ferrous Gluconate Tablets—*see Ferrous Gluconate Tablets General Monographs*

Ferrous Sulfate—*see Ferrous Sulfate General Monographs*

Ferrous Sulfate Oral Solution—*see Ferrous Sulfate Oral Solution General Monographs*

Ferrous Sulfate Syrup—*see Ferrous Sulfate Syrup General Monographs*

Ferrous Sulfate Tablets—*see Ferrous Sulfate Tablets General Monographs*

Ferrous Sulfate, Dried—*see Dried Ferrous Sulfate General Monographs*

Feverfew

DEFINITION

Feverfew consists of the dried leaves of *Tanacetum parthenium* (L.) Sch. Bip. (Fam. Asteraceae), collected when the plant is in flower.

IDENTIFICATION

- **A.** The retention time of the parthenolide peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Parthenolide*.
- **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

  Standard solution: 1.0 mg/mL of USP Parthenolide RS in methanol

  Sample solution: Transfer 1.0 g of finely powdered Feverfew to a suitable flask. Add 20 mL of methanol, heat the flask over a water bath at 60° for 15 min, cool, and filter. Evaporate the filtrate under reduced pressure to dryness, and dissolve the residue in 2.0 mL of methanol.

  Adsorbent: 0.5-mm layer of chromatographic silica gel, typically 20 cm long (TLC plates)

  Application volume: 20 µL

  Developing solvent system: Toluene and acetone (85:15)

  Spray reagent: 0.5% Solution of vanillin in a mixture of sulfuric acid and alcohol (4:1)

  Analysis

  Samples: *Standard solution* and *Sample solution*

  Develop the chromatograms until the solvent front has moved three-fourths of the length of the plate. Remove the plate from the chromatographic chamber, mark the solvent front, and allow it to air-dry. Spray the plate with *Spray reagent*. After 5 min, examine the plate under white light.

  Acceptance criteria: A blue spot in the middle portion of the chromatogram of the *Sample solution* that corresponds in color and R_F value to the principal spot obtained in the chromatogram of the *Standard solution* indicates the presence of parthenolide. The lower one-third of the chromatogram of the *Sample solution* may exhibit two pink spots, and the upper one-third may exhibit one pink spot.
- **C. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

  Standard solution: 0.25 mg/mL of USP Rutin RS in methanol

  Sample solution: To 1 g of finely powdered Feverfew, add 10 mL of methanol, and heat on a water bath at 60° for 15 min. Cool, and filter.

  Adsorbent: 0.25-mm layer of chromatographic silica gel, typically 20 cm long (TLC plates)

  Application volume: 20 µL

  Developing solvent system: Ethyl acetate, anhydrous formic acid, glacial acetic acid, and water (10: 1.1: 1.1: 2.7)

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Spray reagent A: 1% Solution of 2-aminoethyl diphenylborinate in methanol
Spray reagent B: 5% (w/v) Solution of polyethylene glycol 4000 in alcohol
Analysis
Samples: *Standard solution* and *Sample solution*
Develop the chromatogram until the solvent front has moved three-fourths of the length of the plate. Remove the plate from the chromatographic chamber, and allow it to air-dry. Spray the plate with *Spray reagent A* followed by *Spray reagent B*, and examine the plate under UV light at 366 nm.
Acceptance criteria: Relative to the R_F value of the principal spot of the *Standard solution*, the chromatogram of the *Sample solution* exhibits no blue spot at R_F 1.1 (distinction from Roman chamomile) but exhibits a green spot at R_F 2.3 (distinction from *Matricaria*), and colored spots at the R_F values indicated are as follows: 1.5 (yellowish orange), 1.65 (yellowish green), 2.0 (greenish blue), and 2.25 (whitish blue).

COMPOSITION

• **CONTENT OF PARTHENOLIDE**
Mobile phase: Acetonitrile and water (9:11)
Standard solution: 0.04 mg/mL of USP Parthenolide RS in methanol
Sample stock solution: Reduce 100 g of Feverfew to a fine powder. Transfer about 1.0 g of the finely powdered Feverfew, accurately weighed, to a suitable flask. Add 100 mL of methanol, and heat on a water bath at 60° for 10 min. Remove the flask from the water bath, cool, and filter. Rinse the flask with three 5-mL portions of methanol, and filter, adding the rinsings to the filtrate. Transfer the residue left within the filter to the same flask. Add 50 mL of methanol, and continue the rinse procedure as described above. Evaporate the combined filtrates under reduced pressure to dryness, and dissolve the residue in 20.0 mL of methanol.
Sample solution: Transfer 10 mL of the *Sample stock solution* to a 25-mL volumetric flask, and dilute with methanol to volume.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 210 nm
Column: 4.6-mm × 25-cm; packing L1
Flow rate: 2 mL/min
Injection volume: 10 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Tailing factor: NMT 2.0 for the parthenolide peak
Relative standard deviation: NMT 2.0% for the parthenolide peak in repeated injections
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of parthenolide in the portion of Feverfew taken to prepare the *Sample solution*:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times 100$$

r_U = peak area of the parthenolide peak in the *Sample solution*
r_S = peak area of the parthenolide peak in the *Standard solution*
C_S = concentration of USP Parthenolide RS in the *Standard solution* (mg/mL)
V = final volume of the *Sample stock solution* (mL)
W = weight of Feverfew used to prepare the *Sample stock solution* (mg)
D = dilution factor to prepare the *Sample solution* from the *Sample stock solution*

Acceptance criteria: NLT 0.2% on the dried basis

CONTAMINANTS

• **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
• **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements
• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.
• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩ It meets the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

• **BOTANICAL CHARACTERISTICS**
Macroscopic: Yellowish green, petiolate, usually 2–5 cm in length but sometimes up to 10 cm, ovate, deeply divided into 5 or occasionally 7 segments, each with a coarsely crenate margin and obtuse apex; both surfaces downy and the mid-rib prominent on the lower surface
Microscopic: Upper and lower epidermal cells with wavy anticlinical walls, striated cuticle and anomocytic stomata, more frequent on the lower epidermis; trichomes, more abundant on the lower epidermis, of two types; covering trichomes uniseriate with up to 6 small isodiametric basal cells and elongated, tapering apical cells, often at right angles to the axis of the basal cells; glandular trichomes slightly sunken, composed of a short, biseriate, 2- or 4-celled stalk and a biseriate head of 4 cells, around which the cuticle forms a bladder-like covering
• **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩**:** NMT 10.0%, including the stalk
• **ARTICLES OF BOTANICAL ORIGIN,** *Water-Soluble Extractives,Method 2* ⟨561⟩**:** NLT 15.0%
• **LOSS ON DRYING** ⟨731⟩
Sample: 1.0 g of finely powdered Feverfew
Analysis: Dry the *Sample* at 105° for 1 h.
Acceptance criteria: NMT 10.0%
• **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 12.0%
• **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 3.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store in a dry place, protected from light.
• **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Parthenolide RS
USP Rutin RS

Powdered Feverfew

DEFINITION

Powdered Feverfew is Feverfew pulverized to a fine or very fine powder.

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IDENTIFICATION

• **A.** The retention time of the parthenolide peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Parthenolide*.

• **B. THIN-LAYER CHROMATOGRAPHY**

Standard solution: 1.0 mg/mL of USP Parthenolide RS in methanol

Sample solution: Transfer 1.0 g of Powdered Feverfew to a suitable flask. Add 20 mL of methanol, heat the flask over a water bath at 60° for 15 min, cool, and filter. Evaporate the filtrate under reduced pressure to dryness, and dissolve the residue in 2.0 mL of methanol.

Adsorbent: 0.5-mm layer of chromatographic silica gel, typically 20 cm long (TLC plates)

Application volume: 20 µL

Developing solvent system: Toluene and acetone (85:15)

Derivatization reagent: 0.5% Solution of vanillin in a mixture of sulfuric acid and alcohol (4:1)

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the chromatograms until the solvent front has moved three-fourths of the length of the plate. Remove the plate from the chromatographic chamber, mark the solvent front, and allow it to air-dry. Spray the plate with *Derivatization reagent*. After 5 min, examine the plate under white light.

Acceptance criteria: A blue spot in the middle portion of the chromatogram of the *Sample solution* that corresponds in color and R_F value to the principal spot obtained in the chromatogram of the *Standard solution* indicates the presence of parthenolide. The lower one-third of the chromatogram of the *Sample solution* may exhibit two pink spots, and the upper one-third may exhibit one pink spot.

• **C. THIN-LAYER CHROMATOGRAPHY**

Standard solution: 0.25 mg/mL of USP Rutin RS in methanol

Sample solution: To 1 g of Powdered Feverfew, add 10 mL of methanol, and heat on a water bath at 60° for 15 min. Cool, and filter.

Adsorbent: 0.25-mm layer of chromatographic silica gel, typically 20 cm long (TLC plates)

Application volume: 20 µL

Developing solvent system: Ethyl acetate, anhydrous formic acid, glacial acetic acid, and water (10: 1.1: 1.1: 2.7)

Derivatization reagent A: 1% Solution of 2-aminoethyl diphenylborinate in methanol

Derivatization reagent B: 5% (w/v) Solution of polyethylene glycol 4000 in alcohol

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the chromatogram until the solvent front has moved three-fourths of the length of the plate. Remove the plate from the chromatographic chamber, and allow it to air-dry. Treat the plate with *Derivatization reagent A* followed by *Derivatization reagent B*, and examine the plate under UV light at 366 nm.

Acceptance criteria: Relative to the R_F value of the principal spot of the *Standard solution*, the chromatogram of the *Sample solution* exhibits no blue spot at R_F 1.1 (distinction from Roman chamomile) but exhibits a green spot at R_F 2.3 (distinction from *Matricaria*), and colored spots at the R_F values indicated are as follows: 1.5 (yellowish orange), 1.65 (yellowish green), 2.0 (greenish blue), and 2.25 (whitish blue).

COMPOSITION

• **CONTENT OF PARTHENOLIDE**

Mobile phase: Acetonitrile and water (9:11)

Standard solution: 0.04 mg/mL of USP Parthenolide RS in methanol

Sample stock solution: Transfer about 1.0 g of the Powdered Feverfew, accurately weighed, to a suitable flask. Add 100 mL of methanol, and heat on a water bath at 60° for 10 min. Remove the flask from the water bath, cool, and filter. Rinse the flask with three 5-mL portions of methanol, and filter, adding the rinsings to the filtrate. Transfer the residue left within the filter to the same flask. Add 50 mL of methanol, and continue the rinse procedure as described above. Evaporate the combined filtrates under reduced pressure to dryness, and dissolve the residue in 20.0 mL of methanol.

Sample solution: Transfer 10 mL of the *Sample stock solution* to a 25-mL volumetric flask, and dilute with methanol to volume.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 210 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 2 mL/min

Injection volume: 10 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Tailing factor: NMT 2.0 for the parthenolide peak

Relative standard deviation: NMT 2.0% for the parthenolide peak in repeated injections

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of parthenolide in the portion of Powdered Feverfew taken to prepare the *Sample solution*:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times 100$$

r_U = area of the parthenolide peak in the *Sample solution*

r_S = area of the parthenolide peak in the *Standard solution*

C_S = concentration of USP Parthenolide RS in the *Standard solution* (mg/mL)

V = final volume of the *Sample stock solution* (mL)

W = weight of Powdered Feverfew used to prepare the *Sample stock solution* (mg)

D = dilution factor to prepare the *Sample solution* from the *Sample stock solution*

Acceptance criteria: NLT 0.2% on the dried basis

CONTAMINANTS

• **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements

• **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements

• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

• **ARTICLES OF BOTANICAL ORIGIN,** *Water-Soluble Extractives, Method 2* ⟨561⟩**:** NLT 15.0%

• **LOSS ON DRYING** ⟨731⟩

Sample: 1.0 g of Powdered Feverfew

Analysis: Dry the *Sample* at 105° for 1 h.

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Acceptance criteria: NMT 10.0%

- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩: NMT 12.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩: NMT 3.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant source from which the article was derived.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Parthenolide RS
  USP Rutin RS

Fish Oil Containing Omega-3 Acids

Change to read:

DEFINITION

Fish Oil Containing Omega-3 Acids is the purified, winterized, and deodorized fatty oil obtained from fish of the families Engraulidae, Carangidae, Clupeidae, Osmeridae, ▲Scombridae,▲ (ERR 1-Aug-2018) and Ammodytidae. The omega-3 acids are defined as the following: alpha-linolenic acid (C18:3 n–3), moroctic acid (C18:4 n–3), eicosatetraenoic acid (C20:4 n–3), eicosapentaenoic acid (EPA) (C20:5 n–3), heneicosapentaenoic acid (C21:5 n–3), docosapentaenoic acid (C22:5 n–3), and docosahexaenoic acid (DHA) (C22:6 n –3). It contains NLT 28.0% (w/w) of total omega-3 acids, expressed as free acids, consisting of NLT 13.0% of EPA and NLT 9.0% of DHA. Suitable antioxidants in appropriate concentrations may be added.

IDENTIFICATION

- **A.** The retention times of the docosahexaenoic acid methyl ester and eicosapentanoic acid methyl ester peaks from *Test solution 1* in *Content of EPA and DHA* correspond to those of the docosahexaenoic acid methyl ester and eicosapentanoic acid methyl ester peaks from *Standard solution 2a* and *Standard solution 2b*, respectively, in *Fats and Fixed Oils* ⟨401⟩, *Content of EPA and DHA*. The sum of the area for EPA and DHA methyl esters is NLT 22% of the total detected area for the methyl esters, and no other peak has an area higher than 20% of the total detected area for the methyl esters. In addition to the EPA and DHA peaks, *Test solution 1* exhibits at least 15 more peaks with retention times similar to those of the *Fish oil standard solution*, as obtained in the test for *Content of EPA and DHA*.

COMPOSITION

- **CONTENT OF EPA AND DHA**

(See *Fats and Fixed Oils* ⟨401⟩, *Omega-3 Fatty Acids Determination and Profile*.)

Standard solution 1a, Standard solution 1b, Standard solution 2a, Standard solution 2b, Test solution 1, Test solution 2, System suitability solution 1, System suitability solution 2, Chromatographic system, System suitability, and **Analysis:** Proceed as directed in *Fats and Fixed Oils* ⟨401⟩, *Content of EPA and DHA* for triglycerides.

Fish oil standard solution: Transfer 300 mg of USP Fish Oil RS into a 10-mL volumetric flask, and dissolve in and dilute with *Antioxidant Solution* to volume. Proceed as directed for *Test Solution 1 (for triglycerides)* in *Fats and Fixed Oils* ⟨401⟩, *Content of EPA and DHA*, starting with "Transfer 2.0 mL".

Identify the relevant fatty acid methyl esters in the *Fish oil standard solution* by comparing their retention times with those in the reference chromatogram supplied with the USP Fish Oil RS.

Acceptance criteria: NLT 13.0% (w/w) of EPA and NLT 9.0% (w/w) of DHA

- **CONTENT OF TOTAL OMEGA-3 ACIDS**

(See *Fats and Fixed Oils* ⟨401⟩, *Omega-3 Fatty Acids Determination and Profile*.)

Analysis: Proceed as directed in *Fats and Fixed Oils* ⟨401⟩, *Content of Total Omega-3 Acids (for triglycerides)*.

Acceptance criteria: NLT 28.0% (w/w) of total omega-3 acids, expressed as free acids

CONTAMINANTS

- **LIMIT OF ARSENIC**

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytef, and plastic vessels before use, use water that has been passed first through a strong-acid, strong-base, mixed-bed ion-exchange resin. Select all reagents to have as low a content of arsenic as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytef, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

1% Palladium stock solution: Transfer 1 g of ultrapure palladium metal into a Teflon beaker. Add 20 mL of water and 10 mL of nitric acid, and warm on a hot plate to dissolve. Allow the solution to cool to room temperature, transfer into a 100-mL volumetric flask, and dilute with deionized water to volume.

1% Magnesium nitrate stock solution: Transfer 1 g of ultrapure magnesium nitrate into a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer into a 100-mL volumetric flask, and dilute with deionized water to volume.

Modifier working solution: *1% Palladium stock solution*, *1% Magnesium nitrate stock solution*, and 2% nitric acid (3:2:5). A volume of 5 µL provides 0.015 mg of palladium and 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (5 in 100)

Standard stock solution: Transfer 10.0 mL of *Standard Arsenic Solution*, prepared as directed in *Arsenic* ⟨211⟩, to a 100-mL volumetric flask. Add 40 mL of water and 5 mL of nitric acid, and dilute with water to volume. This solution contains 0.10 µg/mL of arsenic.

Standard solutions: Dilute the *Standard stock solution* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 µg/mL of arsenic.

Sample solution: For preparation of the *Sample solution*, use a microwave oven with a magnetron frequency of 2455 MHz and a selectable output power of 0–950 watts in 1% increments, equipped with advanced composite vessels with 100-mL polytef liners. Use rupture membranes to vent vessels should the pressure exceed 125 psi. The vessels fit into a turntable, and each vessel can be vented into an overflow container. Equip the microwave oven with an exhaust tube to ventilate fumes. [**CAUTION**—Wear proper eye protection and protective clothing and gloves.] Transfer approximately 500 mg of Fish Oil Containing Omega-3 Acids, weighed to the nearest 0.1 mg, into a Teflon digestion vessel liner. Prepare samples in duplicate. Add 15 mL of nitric acid, and swirl gently. Cover the vessels with lids, leaving the vent fitting off. Predigest overnight under a hood. Place the rupture membrane in the vent fitting, and tighten the lid. Place all vessels on the microwave oven turntable. Connect the vent tubes to the vent trap, and connect the pressure-sensing line to the

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appropriate vessel. Initiate a two-stage digestion procedure by heating the microwave at 15% power for 15 min, followed by 25% power for 45 min. Remove the turntable of vessels from the oven, and allow the vessels to cool to room temperature. [NOTE—A cool water bath may be used to speed the cooling process.] Vent the vessels when they reach room temperature. Remove the lids, and slowly add 2 mL of 30% hydrogen peroxide to each. Allow the reactions to subside, and seal the vessels. Return the vessels on the turntable to the microwave oven, and heat for an additional 15 min at 30% power. Remove the vessels from the oven, and allow them to cool to room temperature. Transfer the cooled digests into 25-mL volumetric flasks, and dilute with water to volume.

Analysis: Program the graphite furnace as follows. Dry at 115° using a 1-s ramp, a 65-s hold, and an argon flow of 300 mL/min. Char the sample at 1000° using a 1-s ramp, a 20-s hold, and an airflow of 300 mL/min. Cool down, and purge the air from the furnace for 10 s using a 20° set temperature and an argon flow of 300 mL/min. Atomize at 2400° using a 0-s ramp and a 5-s hold with the argon flow stopped. Clean out at 2600° using a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 µL) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by a 5-µL injection of the *Modifier working solution* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for arsenic. Determine the peak area at the arsenic emission line at 193.7 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of arsenic, in µg/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in µg/mL, of arsenic in each mL of the *Sample solution* by interpolation from the regression line.

Calculate the content of arsenic in the portion of Fish Oil Containing Omega-3 Acids taken:

$$\text{Result} = (C/W) \times 25$$

C = concentration of arsenic in each mL of the *Sample solution* (µg/mL)

W = weight of Fish Oil Containing Omega-3 Acids taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g

• **LIMIT OF LEAD**

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytef, and plastic vessels before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin before use. Select all reagents to have as low a content of lead as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytef, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

10% Monobasic ammonium phosphate solution: 10 g of ultrapure monobasic ammonium phosphate in 1 mL of nitric acid and 40 mL of water to dissolve the phosphate. Dilute with deionized water to 100 mL.

1% Magnesium nitrate solution: Transfer 1 g of ultrapure magnesium nitrate to a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer to a 100-mL volumetric flask, and dilute with deionized water to volume.

Modifier working solution: *10% Monobasic ammonium phosphate solution*, *1% Magnesium nitrate solution*, and 2% nitric acid (2:1:2). A volume of 5 µL provides 0.2 mg of phosphate plus 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (5 in 100)

Standard stock solution: Transfer 10.0 mL of lead nitrate stock solution TS to a 100-mL volumetric flask. Add 40 mL of water and 5 mL of nitric acid, and dilute with water to volume. Transfer 1.0 mL of this solution to a second 100-mL volumetric flask. Add 50 mL of water and 1 mL of nitric acid, and dilute with water to volume. This solution contains 0.10 µg/mL of lead.

Standard solutions: Dilute the *Standard stock solution* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 µg/mL of lead.

Sample solution: Prepare as directed in *Limit of Arsenic*.

Analysis: Program the graphite furnace as follows. Dry at 120° using a 1-s ramp, a 55-s hold, and an argon flow of 300 mL/min. Char the sample at 850° using a 1-s ramp, a 30-s hold, and an airflow of 300 mL/min. Cool down, and purge the air from the furnace for 10 s using a 20° set temperature and an argon flow of 300 mL/min. Atomize at 2100° using a 0-s ramp and a 5-s hold with the argon flow stopped. Clean out at 2600° using a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 µL) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by a 5-µL injection of the *Modifier working solution* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for lead. Determine the peak area at the lead emission line at 283.3 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of lead, in µg/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in µg/mL, of lead in each mL of the *Sample solution* by interpolation from the regression line.

Calculate the content of lead in the portion of Fish Oil Containing Omega-3 Acids taken:

$$\text{Result} = (C/W) \times 25$$

C = concentration of lead in each mL of the *Sample solution* (µg/mL)

W = weight of Fish Oil Containing Omega-3 Acids taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g

• **LIMIT OF CADMIUM**

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytef, and plastic vessels before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin before use. Select all reagents to have as low a content of cadmium as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytef, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

10% Monobasic ammonium phosphate solution: 10 g of ultrapure monobasic ammonium phosphate in 40 mL of water and 1 mL of nitric acid to dissolve the phosphate. Dilute with deionized water to 100 mL.

1% Magnesium nitrate solution: Transfer 1 g of ultrapure magnesium nitrate to a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer to a 100-mL volumetric flask, and dilute with deionized water to volume.

Modifier working solution: *10% Monobasic ammonium phosphate solution*, *1% Magnesium nitrate solution*, and 2%

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nitric acid (2:1:2). A volume of 5 µL provides 0.2 mg of phosphate and 0.01 mg of magnesium nitrate.
Blank: Nitric acid and water (5 in 100)
Standard stock solution A: 0.1372 mg/mL of cadmium nitrate in water
Standard stock solution B: *Standard stock solution A*, nitric acid, and water (2:1:97). This solution contains 0.10 µg/mL of cadmium. [NOTE—Before diluting to final volume, dissolve in a portion of water and nitric acid.]
Standard solutions: Dilute *Standard stock solution B* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 µg/mL of cadmium.
Sample solution: Prepare as directed in *Limit of Arsenic*.
Analysis: Program the graphite furnace as follows. Dry at 120° using a 1-s ramp, a 55-s hold, and an argon flow of 300 mL/min. Char the sample at 850° using a 1-s ramp, a 30-s hold, and an airflow of 300 mL/min. Cool down, and purge the air from the furnace for 10 s using a 20° set temperature and an argon flow of 300 mL/min. Atomize at 2400° using a 0-s ramp and a 5-s hold with the argon flow stopped. Clean out at 2600° using a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 µL) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by a 5-µL injection of the *Modifier working solution* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for cadmium. Determine the peak area at the cadmium emission line at 228.8 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of cadmium, in µg/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in µg/mL, of cadmium in each mL of the *Sample solution* by interpolation from the regression line.
Calculate the content of cadmium in the Fish Oil Containing Omega-3 Acids taken:

$$\text{Result} = (C/W) \times 25$$

C = concentration of cadmium in each mL of the *Sample solution* (µg/mL)
W = weight of Fish Oil Containing Omega-3 Acids taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g
• **LIMIT OF MERCURY:** Proceed as directed in *Mercury* ⟨261⟩, *Method IIa*, except use a *Standard Mercury Solution* having the equivalent of 0.1 µg/mL of mercury.
Sample solution: Prepare as directed for the *Sample solution* in *Limit of Arsenic*, combining the two duplicate cooled digests into 1.0 mL of *Potassium Permanganate Solution*.
Acceptance criteria: NMT 0.1 µg/g
• **LIMIT OF DIOXINS, FURANS, AND POLYCHLORINATED BIPHENYLS**
Analysis: Determine the content of polychlorinated dibenzo-para-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) by Method No. 1613, Revision B, of the Environmental Protection Agency. Determine the content of polychlorinated biphenyls (PCBs) by Method No. 1668, Revision A of the Environmental Protection Agency.
Acceptance criteria: The sum of PCDDs and PCDFs is NMT 2.0 pg/g of World Health Organization (WHO) toxic equivalents. The sum of PCDDs, PCDFs, and dioxin-like PCBs (polychlorinated biphenyls, nonortho IUPAC congeners PCB-77, PCB-81, PCB-126, and PCB-169, and mono-ortho IUPAC congeners PCB-105, PCB-114, PCB-118, PCB-123, PCB-156, PCB-157, PCB-167, and PCB-189) is NMT 10.0 pg/g of WHO toxic equivalents.

SPECIFIC TESTS
• **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩**:** NMT 3
• **FATS AND FIXED OILS,** *Anisidine Value* ⟨401⟩**:** NMT 20.0
• **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩**:** NMT 5.0
• **FATS AND FIXED OILS,** *Total Oxidation Value (TOTOX)* ⟨401⟩
Analysis: Calculate TOTOX as follows:

$$\text{Result} = (2 \times PV) + AV$$

PV = peroxide value
AV = anisidine value

Acceptance criteria: NMT 26
• **FATS AND FIXED OILS,** *Unsaponifiable Matter* ⟨401⟩**:** NMT 1.5%
• **STEARIN**
Sample: 10 mL
Analysis: Cool the *Sample* at 0° for 3 h.
Acceptance criteria: The *Sample* remains clear.
• **ABSORBANCE**
Sample solution: 0.24 mg/mL in isooctane
Acceptance criteria: The absorbance is NMT 0.70, determined at 233 nm.

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at controlled room temperature. It may be bottled or otherwise packaged in containers from which air has been expelled by the production of a vacuum or by an inert gas.
• **LABELING:** The label states the average content of DHA and EPA in mg/g. It also states the name and concentration of any added antioxidant.

Change to read:

• **USP REFERENCE STANDARDS** ⟨11⟩
▲▲ (CN 1-May-2018)
USP Fish Oil RS
▲▲ (CN 1-May-2018)

Fish Oil Containing Omega-3 Acids Capsules

Change to read:

DEFINITION
Fish Oil Containing Omega-3 Acids Capsules contain NLT 95.0% and NMT 105.0% of the labeled amount of Fish Oil Containing Omega-3 Acids where Fish Oil Containing Omega-3 Acids is the purified, winterized, and deodorized fatty oil obtained from fish of the families Engraulidae, Carangidae, Clupeidae, Osmeridae, ▲Scombridae,▲ (ERR 1-Sep-2018) and Ammodytidae. The omega-3 acids are defined as the following: alpha-linolenic acid (C18:3 n–3), moroctic acid (C18:4 n–3), eicosatetraenoic acid (C20:4 n–3), eicosapentaenoic acid (EPA) (C20:5 n–3), heneicosapentaenoic acid (C21:5 n–3), docosapentaenoic acid (C22:5 n–3), and docosahexaenoic acid (DHA) (C22:6 n–3). It contains NLT 28.0% (w/w) of total omega-3 acids, expressed as free acids, consisting of NLT 13.0% of EPA and NLT 9.0% of DHA. Suitable antioxidants in appropriate concentrations may be added.

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IDENTIFICATION

• **A.** The oil contained in the Capsules meet the requirements for the following test. The retention times of the docosahexaenoic acid methyl ester and eicosapentanoic acid methyl ester peaks from *Test solution 1* in *Content of EPA and DHA* correspond to those of the docosahexaenoic acid methyl ester and eicosapentanoic acid methyl ester peaks from *Standard solution 2a* and *Standard solution 2b*, respectively, in *Fats and Fixed Oils* ⟨401⟩, *Content of EPA and DHA*. The sum of the areas for EPA and DHA methyl esters is NLT 22% of the total detected area for the methyl esters, and no other peak in the chromatogram has an area higher than 20% of the total detected area for the methyl esters. In addition to the EPA and DHA peaks, *Test solution 1* exhibits at least 15 more peaks with retention times similar to those of the *Fish oil standard solution*, as obtained in the test for *Content of EPA and DHA*.

STRENGTH

• **CONTENT OF FISH OIL**

Analysis: Weigh NLT 10 Capsules in a tared weighing bottle; carefully open the Capsules, without loss of shell material; and transfer the combined Capsule contents to a 100-mL beaker. Remove any adhering substance from the emptied Capsules by washing with several small portions of 2,2,4-trimethylpentane. Discard the washings, and allow the empty Capsules to dry in a current of dry air until the 2,2,4-trimethylpentane is completely evaporated. Weigh the empty Capsules in the original tared weighing bottle, and calculate the average net weight per Capsule.

Acceptance criteria: 95.0%–105.0% of the labeled amount

• **CONTENT OF EPA AND DHA**

(See *Fats and Fixed Oils* ⟨401⟩, *Omega-3 Fatty Acids Determination and Profile*.)

System suitability solution 1, System suitability solution 2, Standard solution 1a, Standard solution 1b, Standard solution 2a, Standard solution 2b, Test solution 1, Test solution 2, Chromatographic system, System suitability, and **Analysis:** Proceed as directed in *Fats and Fixed Oils* ⟨401⟩, *Content of EPA and DHA*, for triglycerides.

Fish oil standard solution: Transfer 300 mg of USP Fish Oil RS into a 10-mL volumetric flask, and dissolve in and dilute with *Antioxidant Solution* to volume. Proceed as directed for *Test Solution 1 (for triglycerides)* in *Fats and Fixed Oils* ⟨401⟩, *Content of EPA and DHA*, starting with "Transfer 2.0 mL".

Identify the relevant fatty acid methyl esters in the *Fish oil standard solution* by comparing their retention times with those in the reference chromatogram supplied with the USP Fish Oil RS.

Calculate the percentage of EPA and DHA in the portion of fish oil containing omega-3 acids taken from the Capsules.

Acceptance criteria: NLT 13.0% (w/w) of EPA and NLT 9.0% (w/w) of DHA

• **CONTENT OF TOTAL OMEGA-3 ACIDS**

(See *Fats and Fixed Oils* ⟨401⟩, *Omega-3 Fatty Acids Determination and Profile*.)

Analysis: Proceed as directed in *Fats and Fixed Oils* ⟨401⟩, *Content of Total Omega-3 Acids (for triglycerides)*.

Acceptance criteria: NLT 28.0% (w/w) of total omega-3 acids, expressed as free acids

PERFORMANCE TESTS

• **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** ⟨2040⟩**:** Meet the requirements for *Rupture Test for Soft Shell Capsules*

• **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩**:** Meet the requirements

CONTAMINANTS

• **LIMIT OF ARSENIC**

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytef, and plastic vessels before use, use water that has first been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin. Select all reagents to have as low a content of arsenic as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytef, and plastic vessels before use by soaking them in warm 8 N nitric acid for 30 min and by rinsing them with deionized water.]

1% Palladium stock solution: Transfer 1 g of ultrapure palladium metal into a Teflon beaker. Add 20 mL of water and 10 mL of nitric acid, and warm on a hot plate to dissolve. Allow the solution to cool to room temperature, transfer into a 100-mL volumetric flask, and dilute with deionized water to volume.

1% Magnesium nitrate stock solution: Transfer 1 g of ultrapure magnesium nitrate to a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer to a 100-mL volumetric flask, and dilute with deionized water to volume.

Modifier working solution: *1% Palladium stock solution*, *1% Magnesium nitrate stock solution*, and 2% nitric acid (3:2:5). A volume of 5 µL provides 0.015 mg of palladium and 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (5 in 100)

Standard stock solution: Transfer 10.0 mL of *Standard Arsenic Solution*, prepared as directed in the test for *Arsenic* ⟨211⟩, to a 100-mL volumetric flask. Add 40 mL of water and 5 mL of nitric acid, and dilute with water to volume. This solution contains 0.10 µg/mL of arsenic.

Standard solutions: Dilute the *Standard stock solution* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 µg/mL of arsenic.

Sample solution: For the preparation of the *Sample solution*, use a microwave oven with a magnetron frequency of 2455 MHz and a selectable output power of 0–950 W in 1% increments, equipped with advanced composite vessels with 100-mL polytef liners. Use rupture membranes to vent vessels should the pressure exceed 125 psi. The vessels fit into a turntable, and each vessel can be vented into an overflow container. Equip the microwave oven with an exhaust tube to ventilate fumes. [CAUTION—Wear proper eye protection and protective clothing and gloves.] Transfer approximately 500 mg from content of Capsules, weighed to the nearest 0.1 mg, into a Teflon digestion vessel liner. Prepare samples in duplicate. Add 15 mL of nitric acid, and swirl gently. Cover the vessels with lids, leaving the vent fitting off. Predigest overnight under a hood. Place the rupture membrane in the vent fitting, and tighten the lid. Place all vessels on the microwave oven turntable. Connect the vent tubes to the vent trap, and connect the pressure-sensing line to the appropriate vessel. Initiate a two-stage digestion procedure by heating the microwave at 15% power for 15 min, followed by 25% power for 45 min. Remove the turntable of vessels from the oven, and allow the vessels to cool to room temperature. [NOTE—A cool water bath may be used to speed the cooling process.] Vent the vessels when they reach room temperature. Remove the lids, and slowly add 2 mL of 30% hydrogen peroxide to each. Allow the reactions to subside, and seal the vessels. Return the vessels on the turntable to the microwave oven, and heat for an additional 15 min at 30% power. Remove the vessels from the oven, and allow them to cool to room temperature.

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Transfer the cooled digests into 25-mL volumetric flasks, and dilute with water to volume.

Analysis: Program the graphite furnace as follows. Dry at 115° using a 1-s ramp, a 65-s hold, and an argon flow of 300 mL/min. Char the sample at 1000° using a 1-s ramp, a 20-s hold, and an airflow of 300 mL/min. Cool down, and purge the air from the furnace for 10 s by using a 20° set temperature and an argon flow of 300 mL/min. Atomize at 2400° using a 0-s ramp and a 5-s hold with the argon flow stopped. Clean out at 2600° using a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 µL) of the *Blank*, the *Standard solutions*, and the *Sample solution*, followed by a 5-µL injection of the *Modifier working solution* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for arsenic. Determine the peak area at the arsenic emission line at 193.7 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of arsenic, in µg/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in µg/mL, of arsenic in each mL of the *Sample solution* by interpolation from the regression line.

Calculate the content of arsenic in the portion of Capsules taken:

$$\text{Result} = (C/W) \times 25$$

C = concentration of arsenic in each mL of the *Sample solution* (µg/mL)
W = weight of fish oil containing omega-3 acids taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g

• **LIMIT OF LEAD**

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytef, and plastic vessels before use, use water that has first been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin. Select all reagents to have as low a content of lead as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytef, and plastic vessels before use by soaking them in warm 8 N nitric acid for 30 min and by rinsing them with deionized water.]

10% Monobasic ammonium phosphate solution: 10 g of ultrapure monobasic ammonium phosphate in 1 mL of nitric acid and 40 mL of water to dissolve the phosphate. Dilute with deionized water to 100 mL.

1% Magnesium nitrate solution: Transfer 1 g of ultrapure magnesium nitrate to a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer to a 100-mL volumetric flask, and dilute with deionized water to volume.

Modifier working solution: *10% Monobasic ammonium phosphate solution*, *1% Magnesium nitrate solution*, and 2% nitric acid (2:1:2). A volume of 5 µL provides 0.2 mg of phosphate plus 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (5 in 100)

Standard stock solution: Transfer 10.0 mL of lead nitrate stock solution TS to a 100-mL volumetric flask. Add 40 mL of water and 5 mL of nitric acid, and dilute with water to volume. Transfer 1.0 mL of this solution to a second 100-mL volumetric flask. Add 50 mL of water and 1 mL of nitric acid, and dilute with water to volume. This solution contains 0.10 µg/mL of lead.

Standard solutions: Dilute the *Standard stock solution* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 µg/mL of lead.

Sample solution: Prepare as directed in *Limit of Arsenic*.

Analysis: Program the graphite furnace as follows. Dry at 120° using a 1-s ramp, a 55-s hold, and an argon flow of 300 mL/min. Char the sample at 850° using a 1-s ramp, a 30-s hold, and an airflow of 300 mL/min. Cool down, and purge the air from the furnace for 10 s by using a 20° set temperature and an argon flow of 300 mL/min. Atomize at 2100° using a 0-s ramp and a 5-s hold with the argon flow stopped. Clean out at 2600° using a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 µL) of the *Blank*, the *Standard solutions*, and the *Sample solution*, followed by a 5-µL injection of the *Modifier working solution* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for lead. Determine the peak area at the lead emission line at 283.3 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of lead, in µg/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in µg/mL, of lead in each mL of the *Sample solution* by interpolation from the regression line.

Calculate the content of lead in the portion of Capsules taken:

$$\text{Result} = (C/W) \times 25$$

C = concentration of lead in each mL of the *Sample solution* (µg/mL)
W = weight of fish oil containing omega-3 acids taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g

• **LIMIT FOR CADMIUM**

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytef, and plastic vessels before use, use water that has first been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin. Select all reagents to have as low a content of cadmium as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytef, and plastic vessels before use by soaking them in warm 8 N nitric acid for 30 min and by rinsing them with deionized water.]

10% Monobasic ammonium phosphate solution: 10 g of ultrapure monobasic ammonium phosphate in 40 mL of water and 1 mL of nitric acid to dissolve the phosphate. Dilute with deionized water to 100 mL.

1% Magnesium nitrate solution: Transfer 1 g of ultrapure magnesium nitrate to a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer to a 100-mL volumetric flask, and dilute with deionized water to volume.

Modifier working solution: *10% Monobasic ammonium phosphate solution*, *1% Magnesium nitrate solution*, and 2% nitric acid to volume (2:1:2). A volume of 5 µL provides 0.2 mg of phosphate and 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (5 in 100)

Standard stock solution A: 0.1372 mg/mL of cadmium nitrate in water

Standard stock solution B: *Standard stock solution A*, nitric acid, and water (2:1:97). This solution contains 0.10 µg/mL of cadmium. [NOTE—Before diluting to final volume, dissolve in a portion of water and nitric acid.]

Standard solutions: Dilute *Standard stock solution B* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 µg/mL of cadmium.

Sample solution: Prepare as directed in *Limit of Arsenic*.

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Analysis: Program the graphite furnace as follows. Dry at 120° using a 1-s ramp, a 55-s hold, and an argon flow of 300 mL/min. Char the sample at 850° using a 1-s ramp, a 30-s hold, and an airflow of 300 mL/min. Cool down and purge the air from the furnace for 10 s by using a 20° set temperature and an argon flow of 300 mL/min. Atomize at 2400° using a 0-s ramp and a 5-s hold with the argon flow stopped. Clean out at 2600° using a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 µL) of the *Blank*, the *Standard solutions*, and the *Sample solution*, followed by a 5-µL injection of the *Modifier working solution* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for cadmium. Determine the peak area at the cadmium emission line at 228.8 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of cadmium, in µg/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in µg/mL, of cadmium in each mL of the *Sample solution* by interpolation from the regression line.

Calculate the content of cadmium in the portion of Capsules taken:

$$\text{Result} = (C/W) \times 25$$

C = concentration of cadmium in each mL of the *Sample solution* (µg/mL)
W = weight of fish oil containing omega-3 acids taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g

- **LIMIT OF MERCURY**

Sample solution: Prepare as directed in *Limit of Arsenic*, combining the two duplicate cooled digests into 1.0 mL of *Potassium Permanganate Solution* in *Mercury* ⟨261⟩, *Method IIa and IIb, Reagents*.

Analysis: Proceed as directed for *Mercury* ⟨261⟩, *Method IIa*, except use a *Standard Mercury Solution* having the equivalent of 0.1 µg/mL of mercury.

Acceptance criteria: NMT 0.1 µg/g

- **LIMIT OF DIOXINS, FURANS, AND POLYCHLORINATED BIPHENYLS**

Analysis: Determine the content of polychlorinated dibenzo-para-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) by Method No. 1613, Revision B, of the Environmental Protection Agency. Determine the content of polychlorinated biphenyls (PCBs) by Method No. 1668, Revision A, of the Environmental Protection Agency.

Acceptance criteria: The sum of PCDDs and PCDFs is NMT 2.0 pg/g of World Health Organization (WHO) toxic equivalents. The sum of PCDDs, PCDFs, and dioxin-like PCBs (polychlorinated biphenyls, nonortho IUPAC congeners PCB-77, PCB-81, PCB-126, and PCB-169, and mono-ortho IUPAC congeners PCB-105, PCB-114, PCB-118, PCB-123, PCB-156, PCB-157, PCB-167, and PCB-189) is NMT 10.0 pg/g of WHO toxic equivalents.

SPECIFIC TESTS

- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩**:** NMT 3
- **FATS AND FIXED OILS,** *Anisidine Value* ⟨401⟩**:** NMT 20.0
- **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩**:** NMT 5.0
- **FATS AND FIXED OILS,** *Total Oxidation Value (TOTOX)* ⟨401⟩**:** NMT 26, calculated:

$$\text{Result} = (2 \times PV) + AV$$

PV = peroxide value
AV = anisidine value

- **FATS AND FIXED OILS,** *Unsaponifiable Matter* ⟨401⟩**:** NMT 1.5%
- **STEARIN:** 10 mL remains clear after cooling at 0° for 3 h.
- **ABSORBANCE**

Sample solution: 0.24 mg/mL in isooctane

Acceptance criteria: The absorbance is NMT 0.70, determined at 233 nm.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and store at room temperature, protected from light.
- **LABELING:** The label states the amount of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) in mg per Capsule.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Docosahexaenoic Acid Ethyl Ester RS
All *cis*-4,7,10,13,16,19-docosahexaenoic ethyl ester.
$C_{24}H_{36}O_2$ 356.55

USP Eicosapentaenoic Acid Ethyl Ester RS
All *cis*-5,8,11,14,17-eicosapentaenoic ethyl ester.
$C_{22}H_{34}O_2$ 330.51

USP Fish Oil RS

USP Methyl Tricosanoate RS
Tricosanoic acid methyl ester.
$C_{24}H_{48}O_2$ 368.64

Fish Oil Containing Omega-3 Acids Delayed-Release Capsules

Change to read:

DEFINITION

Fish Oil Containing Omega-3 Acids Delayed-Release Capsules are enteric-coated Capsules that contain NLT 95.0% and NMT 105.0% of the labeled amount of Fish Oil Containing Omega-3 Acids where Fish Oil Containing Omega-3 Acids is the purified, winterized, and deodorized fatty oil obtained from fish of the families Engraulidae, Carangidae, Clupeidae, Osmeridae, ▲Scombridae,▲ (ERR 1-Sep-2018) and Ammodytidae. The omega-3 acids are defined as the following: alpha-linolenic acid (C18:3 n–3), moroctic acid (C18:4 n–3), eicosatetraenoic acid (C20:4 n–3), eicosapentaenoic acid (EPA) (C20:5 n–3), heneicosapentaenoic acid (C21:5 n–3), docosapentaenoic acid (C22:5 n–3), and docosahexaenoic acid (DHA) (C22:6 n–3). It contains NLT 28.0% (w/w) of total omega-3 acids, expressed as free acids, consisting of NLT 13.0% of EPA and NLT 9.0% of DHA. Suitable antioxidants in appropriate concentrations may be added.

IDENTIFICATION

- **A.** The oil contained in the Capsules meets the requirements for the following test. The retention times of the docosahexaenoic acid methyl ester and eicosapentanoic acid methyl ester peaks of *Test solution 1* in *Content of EPA and DHA* correspond to those of the docosahexaenoic acid methyl ester and eicosapentanoic acid methyl ester peaks of *Standard solution 2a* and *Standard solution 2b*, respectively, in *Fats and Fixed Oils* ⟨401⟩, *Omega-3 Fatty Acids Determination and Profile, Content of EPA and DHA*. The sum of the area for EPA and DHA methyl esters is NLT 22% of the total detected area for the methyl esters, and no other peak has an area higher than 20% of the total detected area for the methyl esters. In addition to the EPA and DHA peaks, *Test solution 1* exhibits at least 15 more peaks with retention times similar to those of the *Fish oil standard solution*, as obtained in *Content of EPA and DHA*.

DS Monographs

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STRENGTH

• **CONTENT OF FISH OIL**

Analysis: Weigh NLT 10 Capsules in a tared weighing bottle, carefully open the Capsules, without loss of shell material, and transfer the combined Capsule contents to a 100-mL beaker. Remove any adhering substance from the emptied Capsules by washing with several small portions of 2,2,4-trimethylpentane. Discard the washings, and allow the empty Capsules to dry in a current of dry air until the 2,2,4-trimethylpentane is completely evaporated. Weigh the empty Capsules in the original tared weighing bottle, and calculate the average net weight per Capsule.

Acceptance criteria: 95.0%–105.0% of the labeled amount

• **CONTENT OF EPA AND DHA**

(See *Fats and Fixed Oils* ⟨401⟩, *Omega-3 Fatty Acids Determination and Profile.*)

System suitability solution 1, System suitability solution 2, Standard solution 1a, Standard solution 1b, Standard solution 2a, Standard solution 2b, Test solution 1, Test solution 2, Chromatographic system, System suitability, and **Analysis:** Proceed as directed in *Fats and Fixed Oils* ⟨401⟩, *Content of EPA and DHA* for triglycerides.

Fish oil standard solution: Transfer 300 mg of USP Fish Oil RS to a 10-mL volumetric flask, and dissolve in and dilute with *Antioxidant Solution* to volume. Proceed as directed for *Test Solution 1 (for triglycerides)* in *Fats and Fixed Oils* ⟨401⟩, *Content of EPA and DHA*, starting with "Transfer 2.0 mL".

Identify the relevant fatty acid methyl esters in the *Fish oil standard solution* by comparing their retention times with those in the reference chromatogram supplied with the USP Fish Oil RS.

Calculate the percentage of EPA and DHA in the portion of fish oil containing omega-3 acids taken from the Capsules.

Acceptance criteria: NLT 13.0% (w/w) of EPA and NLT 9.0% (w/w) of DHA

• **CONTENT OF TOTAL OMEGA-3 ACIDS**

(See *Fats and Fixed Oils* ⟨401⟩, *Omega-3 Fatty Acids Determination and Profile.*)

Analysis: Proceed as directed in *Fats and Fixed Oils* ⟨401⟩, *Content of Total Omega-3-Acids* (for triglycerides).

Acceptance criteria: NLT 28.0% (w/w) of total omega-3 acids, expressed as free acids

PERFORMANCE TESTS

• **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩**:** Meet the requirements for *Disintegration, Delayed-Release (Enteric-Coated) Soft Shell Capsules*

• **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩**:** Meet the requirements

CONTAMINANTS

• **FATS AND FIXED OILS,** *Trace Metals* ⟨401⟩**:** NMT 0.1 ppm each of Pb, Cd, As, and Hg

• **LIMIT OF DIOXINS, FURANS, AND POLYCHLORINATED BIPHENYLS**

Analysis: Determine the content of polychlorinated dibenzo-para-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) by Method 1613, Revision B, of the Environmental Protection Agency. Determine the content of polychlorinated biphenyls (PCBs) by Method 1668, Revision A, of the Environmental Protection Agency.

Acceptance criteria: The sum of PCDDs and PCDFs is NMT 2.0 pg/g of World Health Organization (WHO) toxic equivalents. The sum of PCDDs, PCDFs, and dioxin-like PCBs (polychlorinated biphenyls, non-ortho IUPAC congeners PCB-77, PCB-81, PCB-126, and PCB-169, and mono-ortho IUPAC congeners PCB-105, PCB-114, PCB-118, PCB-123, PCB-156, PCB-157, PCB-167, and PCB-189) is NMT 10.0 pg/g of WHO toxic equivalents.

SPECIFIC TESTS

• **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩**:** NMT 3

• **FATS AND FIXED OILS,** *Anisidine Value* ⟨401⟩**:** NMT 20.0

• **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩**:** NMT 5.0

• **FATS AND FIXED OILS,** *Total Oxidation Value (TOTOX)* ⟨401⟩**:** NMT 26, calculated:

$$\text{Result} = (2 \times PV) + AV$$

PV = peroxide value
AV = anisidine value

• **FATS AND FIXED OILS,** *Unsaponifiable Matter* ⟨401⟩**:** NMT 1.5%

• **STEARIN:** 10 mL remains clear after cooling at 0° for 3 h.

• **ABSORBANCE**

Sample solution: 0.24 mg/mL in isooctane

Acceptance criteria: NMT 0.70, determined at 233 nm

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers, and store at room temperature, protected from light.

• **LABELING:** The label states the amount of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) in mg per Capsule.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Docosahexaenoic Acid Ethyl Ester RS
All *cis*-4,7,10,13,16,19-docosahexaenoic ethyl ester.
$C_{24}H_{36}O_2$ 356.55

USP Eicosapentaenoic Acid Ethyl Ester RS
All *cis*-5,8,11,14,17-eicosapentaenoic ethyl ester.
$C_{22}H_{34}O_2$ 330.51

USP Fish Oil RS

USP Methyl Tricosanoate RS

Flax Seed Oil

[8001-26-1].

DEFINITION

Flax Seed Oil is derived from flaxseed or linseed (*Linum usitatissimum* L.). The oil is extracted from the seeds by cold pressing. No solvents or external heat are employed in the extraction process. It contains no added substances.

IDENTIFICATION

• **A.** It meets the requirements in *Specific Tests* for *Fats and Fixed Oils* ⟨401⟩, *Procedures, Fatty Acid Composition.*

• **B. IDENTIFICATION OF FIXED OILS BY THIN-LAYER CHROMATOGRAPHY** ⟨202⟩**:** The R_F values of the principal spots of the *Sample solution* correspond to those of the *Standard solution.*

SPECIFIC TESTS

• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Acid Value***:** NMT 2.0

• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Peroxide Value***:** NMT 10.0

• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Iodine Value***:** 160–200

• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Saponification Value***:** 180–195

• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Unsaponifiable Matter***:** NMT 1.5

• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Fatty Acid Composition***:** Flax Seed Oil exhibits the composition profile of fatty acids in *Table 1.*

Supplements

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Table 1

Fatty Acid	Shorthand Notation	Percentage (%)
Palmitic acid	16:0	2.0–7.5
Stearic acid	18:0	1.0–6.0
Oleic acid	18:1	12.0–24.0
Linoleic acid	18:2	11.0–23.0
Alpha-linolenic acid	18:3	50.0–65.0

• **REFRACTIVE INDEX** ⟨831⟩: 1.460–1.490 at 20°

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed, tight, light-resistant containers.

• **LABELING:** Where Flax Seed Oil is intended for use in the manufacture of dosage forms, it is so labeled.

Flax Seed Oil Capsules

DEFINITION

Flax Seed Oil Capsules are prepared with Flax Oil derived from cold-pressed flax seeds and contain NLT 95.0% of the labeled amounts of α-linolenic (C18:3 n–3), linoleic (C18:2 n–6), and oleic (C18:1 n–9) acids.

IDENTIFICATION

• **A.**

Sample: A portion of oil, about 2 mL, from NLT 10 Capsules

Analysis: Transfer the *Sample* to a suitable test tube. Add 2 mL of glacial acetic acid, and warm the test tube to about 50°, while swirling, for 5 min. Cool, and add 1 drop of sulfuric acid.

Acceptance criteria: A greenish color develops.

• **B. FATTY ACID PROFILE**

System suitability solution and **Chromatographic system:** Proceed as directed in *Strength*.

Sample solution: Proceed as directed for the *Sample solution* in *Strength*, beginning with "Transfer 80 mg" without the addition of the *Internal standard*.

Analysis: Identify the specified fatty acid methyl ester peaks by comparing them to the reference chromatogram provided with the lot of USP Flax Seed Oil RS being used. Determine the percentage of each constituent relative to the total integrated area.

Acceptance criteria: The *Sample solution* conforms to the fatty acids composition profile in *Table 1*.

Table 1

Fatty Acid	Shorthand Notation	Percentage (%)
Palmitic acid	16:0	2.0–7.5
Stearic acid	18:0	1.0–6.0
Oleic acid	18:1 n–9	12.0–24.0
Linoleic acid	18:2 n–6	11.0–23.0
α-Linolenic acid	18:3 n–3	50.0–65.0

STRENGTH

• **CONTENT OF α-LINOLENIC, LINOLEIC, AND OLEIC ACIDS**

0.5 N methanolic sodium hydroxide solution: Dissolve 2 g of sodium hydroxide in 100 mL of methanol.

Internal standard: Methyl nonadecanoate

Sample solution: Weigh NLT 10 Capsules. With a sharp blade, carefully slice open the Capsules, avoiding loss of shell material. Combine the Capsule contents in a suitable container, and mix well. Remove any adhering substance from the emptied Capsules by washing with several portions of diethyl ether, and discard the washings. Allow the empty Capsule shells to air-dry over a period of NMT 30 min, taking precautions to avoid uptake or loss of moisture. Weigh the empty Capsule shells, and calculate the average fill weight/Capsule (A_p). Transfer 80 mg of the accurately weighed combined Capsule contents directly into a tared 30-mL screw-top glass centrifuge tube. Re-tare, and accurately weigh about 50 mg of *Internal standard*. Add 2 mL of *0.5 N methanolic sodium hydroxide solution*, tightly cap, and transfer to a heating block, or another appropriate heating device. Reflux the solution until fat globules disappear (usually 5–10 min). Add 2 mL of 0.14 g/mL boron trifluoride in methanol, cap, and reflux for 2 min. Add 4 mL of chromatographic *n*-heptane, cap, and reflux for 1 min. Cool, add about 8 mL of saturated sodium chloride solution, shake, and centrifuge to separate layers. Dilute an aliquot of the upper (heptane) layer 1:8 with chromatographic *n*-heptane, and mix well.

System suitability solution: Using about 80 mg of USP Flax Seed Oil RS, proceed as directed for the *Sample solution*, beginning with "Transfer 80 mg" without the addition of the *Internal standard*.

Standard solution: Directly into a tared 30-mL screw-top glass centrifuge tube accurately weigh about 50 mg of USP Methyl Linolenate RS, 20 mg of USP Methyl Linoleate RS, and 20 mg of USP Methyl Oleate RS. Proceed as directed for the *Sample solution*, beginning with "Re-tare".

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.53-mm × 30-m fused silica capillary; coated with a 1.0-µm film of G16

Temperatures

Injection port: 220°

Detector: 260°

Column: See *Table 2*.

Table 2

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	0	70	2
70	5	240	5

Carrier gas: Helium

Linear velocity: 50 cm/s

Split mode: Splitless

Injection volume: 1 µL

System suitability

Samples: *System suitability solution* and *Standard solution*

Suitability requirements

Chromatogram similarity: The *System suitability solution* chromatogram is similar to the reference chromatogram provided with the lot of USP Flax Seed Oil RS being used.

Resolution: NLT 1.5 between methyl oleate and methyl stearate, *System suitability solution*

Relative standard deviation: NMT 2% for the peak area ratios of analytes to internal standard, *Standard solution*

Analysis

Samples: *Sample solution* and *Standard solution*

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Identify the retention times of the relevant fatty acid methyl esters by comparing the peaks in the chromatogram of the *System suitability solution* with those in the reference chromatogram. Identify the locus for the internal standard peak by comparison of the chromatograms of the *Standard solution* and *System suitability solution*.

Calculate the content, in mg/g, of α-linolenic, linoleic, and oleic acids in the portion of the Capsule contents taken:

$$\text{Result} = (R_U/R_S) \times (A_U/A_S) \times (m_S/W) \times (M_{r1}/M_{r2})$$

R_U = peak area ratio of the relevant methyl ester to the internal standard in the *Sample solution*
R_S = peak area ratio of the relevant methyl ester to the internal standard in the *Standard solution*
A_U = weight of the *Internal standard* in the *Sample solution* (mg)
A_S = weight of the *Internal standard* in the *Standard solution* (mg)
m_S = weight of the relevant USP Methyl Ester RS in the *Standard solution* (mg)
W = weight of sample used to prepare the *Sample solution* (g)
M_{r1} = molecular weight of the relevant fatty acid (g/mol)
M_{r2} = molecular weight of the relevant fatty acid methyl ester (g/mol)

Calculate the percentage of the labeled amounts of each individual α-linolenic, linoleic, and oleic acid in the portion of Capsules taken:

$$\text{Result} = A \times A_F \times (100/L)$$

A = content of the relevant fatty acid in the portion of Capsule contents taken (mg/g)
A_F = average fill weight (g)
L = labeled content of the relevant fatty acid (mg/Capsule)

Acceptance criteria: 95.0% of the labeled amounts of α-linolenic, linoleic, and oleic acids

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩**:** Meet the requirements for *Rupture Test for Soft Shell Capsules*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩ **:** Meet the requirements

SPECIFIC TESTS

- **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩**:** NMT 10.0

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 1×10^3 cfu/g, and the combined molds and yeasts count does not exceed 3×10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meet the requirements of the tests for absence of *Escherichia coli*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tightly-closed, light-resistant containers.
- **LABELING:** The label states the article from which the Capsules were prepared and the content of α-linolenic, linoleic, and oleic acids in mg/Capsule.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Flax Seed Oil RS
  USP Methyl Linoleate RS
  USP Methyl Linolenate RS
  USP Methyl Oleate RS

Folic Acid—*see Folic Acid General Monographs*

Folic Acid Tablets—*see Folic Acid Tablets General Monographs*

Forskohlii

DEFINITION

Forskohlii consists of the dried roots of *Plectranthus barbatus* Andrews, also known as *Coleus barbatus* (Andrews) Benth. and *Coleus forskohlii* Briq. (Fam. Lamiaceae). It contains NLT 0.4% of forskolin, calculated on the dried basis.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩
  Standard solution A: 50 µg/mL of USP Forskolin RS in acetonitrile. Sonicate for about 10 min.
  Standard solution B: 5 mg/mL of USP Powdered Forskohlii Extract RS in acetonitrile. Sonicate for about 15 min, centrifuge, and use the supernatant.
  Sample stock solution: Use the *Sample solution*, prepared as directed in the test for *Content of Forskolin*.
  Sample solution: Dilute 10 mL of the *Sample stock solution* with acetonitrile to 25 mL.
  Adsorbent: Chromatographic silica gel with an average particle size of 10–15 µm (TLC plates)
  Application volume: 10 µL, as 4-mm bands
  Developing solvent system: Toluene and ethyl acetate (85:15)
  Spray reagent: 5% vanillin in glacial acetic acid and 10% sulfuric acid in water (1:1)
  Analysis
  Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
  Apply the *Samples* as bands. Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about 90% of the plate. Remove the plate from the chamber, dry, spray with the *Spray reagent*, heat for 5–10 min at 105°, and examine under white light.
  Acceptance criteria: The chromatogram of the *Sample solution* exhibits a violet zone due to forskolin at an R_F value of approximately 0.3, corresponding in color and R_F to that in the chromatogram of *Standard solution A*; a minor violet zone, a pink zone, and a brick-red zone at R_F values of approximately 0.1, 0.62, and 0.69, due to isoforskolin, 1,9-dideoxyforskolin, and crocetindialdehyde, respectively. Zones detected in the *Sample solution* chromatogram correspond in position and color to those in *Standard solution B*. Other minor zones may be observed in the *Sample solution* and *Standard solution B* chromatograms.
- **B.** The chromatogram of the *Sample solution* from the test for *Content of Forskolin* shows a main peak at a retention time corresponding to that of forskolin in the chromatogram of *Standard solution A*. Identify other diterpene peaks in the *Sample solution* chromatogram by comparison with the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Forskohlii Extract RS. The *Sample solution* chromatogram shows an additional peak corresponding to isoforskolin.

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COMPOSITION

• **CONTENT OF FORSKOLIN**

Solution A: Acetonitrile, filtered and degassed

Solution B: Water, filtered and degassed

Standard solution A: Sonicate a quantity of USP Forskolin RS in acetonitrile to obtain a solution having a known concentration of about 1.0 mg/mL.

Standard solution B: 5 mg/mL of USP Powdered Forskohlii Extract RS in acetonitrile. Sonicate for about 15 min, centrifuge, and use the supernatant. Before injection, pass through a membrane filter of 0.45-µm or finer pore size.

Sample solution: Transfer about 3.0 g of Forskohlii, finely powdered and accurately weighed, to a 100-mL round-bottom flask fitted with a reflux condenser. Add 50 mL of acetonitrile, reflux for 20 min, cool to room temperature, and decant the supernatant. Repeat until the extract is colorless. Combine the extracts, filter, concentrate under vacuum, and adjust the volume with acetonitrile to 100 mL. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first 5 mL of the filtrate.

System suitability solution: *Standard solution A* and 0.01% toluene in acetonitrile (1:1)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	45	55
25	45	55
28	95	5
35	95	5
36	45	55
45	45	55

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 220 nm

Column: 4.6-mm × 25-cm; 5-µm, 100 Å

Column temperature: 30°

Flow rate: 1.8 mL/min

Injection volume: 20 µL

System suitability

Samples: *Standard solution A*, *Standard solution B*, and *System suitability solution*

[NOTE—The approximate relative retention times for isoforskolin and forskolin are 0.51 and 1.00, respectively.]

Suitability requirements: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Forskohlii Extract RS being used.

Resolution: NLT 1.5 between the forskolin and toluene peaks, *System suitability solution*

Relative standard deviation: NMT 2% determined from the forskolin peak for replicate injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatogram of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Forskohlii Extract RS being used, identify the retention times of the peaks corresponding to isoforskolin and forskolin.

Calculate the percentage of forskolin in the portion of Forskohlii taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of forskolin from the *Sample solution*

r_S = peak area of forskolin from *Standard solution A*

C_S = concentration of USP Forskolin RS in *Standard solution A* (mg/mL)

C_U = concentration of Forskohlii in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 0.4% on the dried basis

IMPURITIES

• **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 2%

• **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements

• **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩**:** NMT 2.0%

• **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements

SPECIFIC TESTS

• **BOTANICAL CHARACTERISTICS**

Macroscopic: Fresh root, pale pinkish yellow, cylindrical to subcylindrical, with tapering ends, 5–12 in length, 1–2 cm in diameter; surface rough, shows lateral rootlets or scars of rootlets and transversely running lenticels. Pharmacopeial article is dark brown; surface rough, irregularly cylindrical, longitudinally wrinkled, showing irregular grooves and prominent ridges; fracture short; cut surface yellowish-white; characteristic and pleasant aromatic odor, and slightly bitter to pungent taste.

Microscopic

Transverse section of roots: Irregular circular in outline; showing narrow cork, 10–15 rows of tangentially elongated radially arranged cork cells; cortex composed of 10–15 rows of thin-walled parenchyma cells showing sclereids and crystals of calcium oxalate; vascular cambium in the form of a continuous ring; xylem showing narrow rays of vessels, few lignified fibers are present in older roots, 3–8 cell-wide medullary rays, and parenchyma showing few sclereids, oleoresin canals and simple starch grains; pith composed of parenchyma cells in young roots and replaced by compactly arranged vessels, fibers, and tracheids in mature roots

• **LOSS ON DRYING** ⟨731⟩

Sample: 1.0 g of finely powdered Forskohlii

Analysis: Dry the *Sample* at 105° for 3 h.

Acceptance criteria: NMT 12.0%

• **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩

Sample: 1.0 g of finely powdered Forskohlii

Acceptance criteria: NMT 6%

• **ARTICLES OF BOTANICAL ORIGIN,** *Alcohol-Soluble Extractives, Method 2* ⟨561⟩**:** NLT 25.0%

• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacterial count does not exceed 10^3 cfu/g.

• **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

• **ARTICLES OF BOTANICAL ORIGIN,** *Test for Aflatoxins* ⟨561⟩**:** Meets the requirements

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ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Forskolin RS
  USP Powdered Forskohlii Extract RS

Powdered Forskohlii

DEFINITION

Powdered Forskohlii is Forskohlii reduced to a powder or very fine powder. It contains NLT 0.4% of forskolin, calculated on the dried basis.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩

Standard solution A: 50 µg/mL of USP Forskolin RS in acetonitrile. Sonicate for about 10 min.

Standard solution B: 5 mg/mL of USP Powdered Forskohlii Extract RS in acetonitrile. Sonicate for about 15 min, centrifuge, and use the supernatant.

Sample stock solution: Use the *Sample solution*, prepared as directed in the test for *Content of Forskolin*.

Sample solution: Dilute 10 mL of the *Sample stock solution* with acetonitrile to 25 mL.

Adsorbent: Chromatographic silica gel with an average particle size of 10–15 µm (TLC plates)

Application volume: 10 µL, as 4-mm bands

Developing solvent system: Toluene and ethyl acetate (85:15)

Spray reagent: 5% vanillin in glacial acetic acid and 10% sulfuric acid in water (1:1)

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands. Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about 90% of the plate. Remove the plate from the chamber, dry, spray with the *Spray reagent*, heat for 5–10 min at 105°, and examine under white light.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits a violet zone due to forskolin at an R_F value of approximately 0.3, corresponding in color and R_F to that in the chromatogram of *Standard solution A*; a minor violet zone, a pink zone, and a brick-red zone at R_F values of approximately 0.1, 0.62, and 0.69, due to isoforskolin, 1,9-dideoxyforskolin, and crocetindialdehyde, respectively. Zones detected in the *Sample solution* correspond in position and color to those in *Standard solution B*. Other minor zones may be observed in the *Sample solution* and *Standard solution B*.

- **B.** The *Sample solution* from the test for *Content of Forskolin* shows a main peak at a retention time corresponding to that of forskolin in the chromatogram of *Standard solution A*. Identify other diterpene peaks in the *Sample solution* by comparison with *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Forskohlii Extract RS being used. The *Sample solution* chromatogram shows an additional peak corresponding to isoforskolin.

COMPOSITION

- **CONTENT OF FORSKOLIN**

Solution A: Acetonitrile, filtered and degassed

Solution B: Water, filtered and degassed

Standard solution A: Sonicate a quantity of USP Forskolin RS in acetonitrile to obtain a solution having a known concentration of about 1.0 mg/mL.

Standard solution B: 5 mg/mL of USP Powdered Forskohlii Extract RS in acetonitrile. Sonicate for about 15 min, centrifuge, and use the supernatant. Before injection, pass through a membrane filter having a 0.45-µm or finer pore size.

Sample solution: Transfer about 3.0 g of accurately weighed Powdered Forskohlii to a 100-mL round-bottom flask fitted with a reflux condenser. Add 50 mL of acetonitrile, reflux for 20 min, cool to room temperature, and decant the supernatant. Repeat until the extract is colorless. Combine the extracts, filter, concentrate under vacuum, and adjust the volume with acetonitrile to 100 mL. Before injection, pass through a membrane filter having a 0.45-µm or finer pore size, discarding the first 5 mL of the filtrate.

System suitability solution: *Standard solution A* and 0.01% toluene in acetonitrile (1:1)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	45	55
25	45	55
28	95	5
35	95	5
36	45	55
45	45	55

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 220 nm
Column: 4.6-mm × 25-cm; 5-µm, 100 Å
Column temperature: 30°
Flow rate: 1.8 mL/min
Injection volume: 20 µL

System suitability
Samples: *Standard solution A*, *Standard solution B*, and *System suitability solution*
[NOTE—The relative retention times for isoforskolin and forskolin are 0.51 and 1.00, respectively.]
Suitability requirements: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Forskohlii Extract RS being used.
Relative standard deviation: NMT 2% determined from the forskolin peak in repeated injections, *Standard solution A*
Resolution: NLT 1.5 between the forskolin and toluene peaks, *System suitability solution*

Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Using the chromatogram of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Forskohlii Extract RS being used, identify the retention times of the peaks corresponding to isoforskolin and forskolin.

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Calculate the percentage of forskolin in the portion of Powdered Forskohlii taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of forskolin from the *Sample solution*
r_S = peak area of forskolin from *Standard solution A*
C_S = concentration of USP Forskolin RS in *Standard solution A* (mg/mL)
C_U = concentration of Powdered Forskohlii in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 0.4% on the dried basis

IMPURITIES

- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 2%
- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS:** Yellowish brown powder; characteristic and pleasant aromatic odor; and slightly bitter to pungent taste. Under a microscope, it shows the presence of parenchyma cells with oleoresin canals, starch grains and prisms of calcium oxalate; oil globules; simple starch grains, cork cells; sclereids; stone cells; pitted vessels; and thin-walled fibers.
- **LOSS ON DRYING** ⟨731⟩
  Sample: 1.0 g of Powdered Forskohlii
  Analysis: Dry the *Sample* at 105° for 3 h.
  Acceptance criteria: NMT 12.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩
  Sample: 1.0 g of Powdered Forskohlii
  Acceptance criteria: NMT 6%
- **ARTICLES OF BOTANICAL ORIGIN,** *Alcohol-Soluble Extractives, Method 2* ⟨561⟩**:** NLT 25.0%
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^5 cfu/g; the total combined molds and yeasts count does not exceed 10^3 cfu/g; and the bile-tolerant Gram-negative bacterial count does not exceed 10^3 cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*
- **ARTICLES OF BOTANICAL ORIGIN,** *Test for Aflatoxins* ⟨561⟩**:** Meets the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Forskolin RS
  USP Powdered Forskohlii Extract RS

Powdered Forskohlii Extract

DEFINITION

Powdered Forskohlii Extract is prepared from Forskohlii using suitable solvents such as methanol, ethyl acetate, hexane or a mixture of these solvents. The ratio of plant material to extract is between 65:1 and 35:1. It contains NLT 90.0% and NMT 110.0% of the labeled amount of forskolin, calculated on the dried basis. It contains suitable added substances as carriers.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩
  Standard solution A: 50 µg/mL of USP Forskolin RS in acetonitrile. Sonicate for about 10 min.
  Standard solution B: 5 mg/mL of USP Powdered Forskohlii Extract RS in acetonitrile. Sonicate for about 15 min, centrifuge, and use the supernatant.
  Sample solution: 5 mg/mL of Powdered Forskohlii Extract in acetonitrile. Sonicate for about 15 min, centrifuge, and use the supernatant.
  Adsorbent: Chromatographic silica gel with an average particle size of 10–15 µm (TLC plates)
  Application volume: 10 µL, as 4-mm bands
  Developing solvent system: A mixture of toluene and ethyl acetate (85:15)
  Spray reagent: A mixture of 5% vanillin in glacial acetic acid and 10% sulfuric acid in water (1:1)
  Analysis
  Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
  Apply the samples as bands to a suitable thin-layer chromatographic plate (see *Chromatography* ⟨621⟩). Use saturated chamber. Develop the chromatograms until the solvent front has moved up about 90% of the plate. Remove the plate from the chamber, dry, spray with the *Spray reagent*, heat for 5–10 min at 105°, and examine under visible light.
  Acceptance criteria: The *Sample solution* exhibits a violet zone due to forskolin at an R_F value of approximately 0.3, corresponding in color and R_F to that from *Standard solution A*; and a minor violet zone, a pink zone, and a brick red zone at R_F values of approximately 0.1, 0.62, and 0.69, due to isoforskolin, 1,9-dideoxyforskolin, and crocetindialdehyde, respectively. Zones detected from the *Sample solution* correspond in position and color to zones from *Standard solution B*. Other minor zones may be observed from the *Sample solution* and *Standard solution B*.
- **B.** The *Sample solution* from the test for *Content of Forskolin* shows a main peak at a retention time corresponding to that of forskolin from *Standard solution A*. Identify other diterpene peaks in the *Sample solution* by comparison with *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Forskohlii Extract RS being used. The *Sample solution* shows an additional peak corresponding to isoforskolin.

COMPOSITION

- **CONTENT OF FORSKOLIN**
  Solution A: Use filtered and degassed acetonitrile.
  Solution B: Use filtered and degassed water.
  Standard solution A: Sonicate a quantity of USP Forskolin RS in acetonitrile to obtain a solution having a known concentration of about 1.0 mg/mL.
  Standard solution B: 5 mg/mL of USP Powdered Forskohlii Extract RS in acetonitrile. Sonicate for about 15 min, centrifuge, and use the supernatant. Before injection, pass through a membrane filter having a 0.45-µm or finer pore size.
  Sample solution: Transfer an amount of Powdered Forskohlii Extract equivalent to about 25 mg of forskolin to a 25-mL volumetric flask, and add 15 mL of acetonitrile. Sonicate and heat in a water bath for about 10 min, cool, dilute with acetonitrile to volume, and mix. Before injection, filter through a membrane filter having a 0.45-µm or finer pore size, discarding the first 5 mL of the filtrate.

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System suitability solution: A mixture of *Standard solution A* and 0.01% toluene in acetonitrile (1:1)
Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	45	55
25	45	55
28	95	5
35	95	5
36	45	55
45	45	55

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 220 nm
Column: 4.6-mm × 25-cm; 5-µm, 100 Å
Column temperature: 30 ± 2°
Flow rate: 1.8 mL/min
Injection size: 20 µL
System suitability
Samples: *Standard solution A*, *Standard solution B*, and *System suitability solution*
[NOTE—The relative retention times for isoforskolin and forskolin are 0.51 and 1.00, respectively.]
Suitability requirements: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Forskohlii Extract RS being used.
Relative standard deviation: NMT 2% determined from the forskolin peak in repeated injections, *Standard solution A*
Resolution: NLT 1.5 between the forskolin and toluene peaks, *System suitability solution*
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Using the chromatogram of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Forskohlii Extract RS being used, identify the retention times of the peaks corresponding to isoforskolin and forskolin.
Calculate the percentage of forskolin in the portion of Powdered Forskohlii Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of forskolin from the *Sample solution*
r_S = peak response of forskolin from *Standard solution A*
C_S = concentration of USP Forskolin RS in *Standard solution A* (mg/mL)
C_U = concentration of Powdered Forskohlii Extract in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 90.0% and NMT 110.0% of the labeled amount of forskolin on the dried basis

IMPURITIES

Change to read:

ORGANIC IMPURITIES
- **Procedure:** ▲Botanical Extracts ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues:*▲ (CN 1-May-2020) Meets the requirements

SPECIFIC TESTS
- **LOSS ON DRYING** ⟨731⟩**:** Dry 1.0 g of Powdered Forskohlii Extract at 105° for 3 h: it loses NMT 5.0% of its weight.
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^4 cfu/g. The total combined yeasts and molds count does not exceed 10^3 cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.
- **ARTICLES OF BOTANICAL ORIGIN,** *Test for Aflatoxins* ⟨561⟩**:** Meets the requirements

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived. It meets other labeling requirements under *Botanical Extracts* ⟨565⟩.
- **OTHER REQUIREMENTS:** It meets the requirements of the test for *Residual Solvents* under *Botanical Extracts* ⟨565⟩.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Forskolin RS
  USP Powdered Forskohlii Extract RS

Ganoderma Lucidum Fruiting Body

DEFINITION
Ganoderma Lucidum Fruiting Body consists of the dried fruiting body of *Ganoderma lucidum* (W. Curt.:Fr.) P. Karst. (Fam. Ganodermataceae). It contains NLT 0.3% of triterpenoic acids, calculated on the dried basis as a sum of ganoderic acids A, B, C_2, D, F, G, and H and ganoderenic acids B, C, and D.

IDENTIFICATION
- **A. THIN-LAYER CHROMATOGRAPHY**
  Standard solution A: 1.0 mg/mL of USP Ganoderic Acid A RS in alcohol
  Standard solution B: 0.3 mg/mL of USP Ergosterol RS in alcohol
  Standard solution C: 50 mg/mL of USP Ganoderma Lucidum Fruiting Body Powdered Extract RS in alcohol. Sonicate for about 10 min, centrifuge, and use the supernatant.
  Sample solution: Sonicate about 1 g of Ganoderma Lucidum Fruiting Body, finely powdered, in 50 mL of alcohol for 15 min. Centrifuge, withdraw the supernatant, and evaporate to dryness under reduced pressure at 50°. Dissolve the residue in 2.0 mL of alcohol, centrifuge, and use the supernatant.
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
  Mode: HPTLC

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Adsorbent: Chromatographic silica gel with an average particle size of 5-µm (HPTLC plate).[1] Predevelop the plate in methanol, and dry at 105° for 30 min.
Application volume: 2 µL each of *Standard solution A* and *Standard solution B*, and 4 µL each of *Standard solution C* and the *Sample solution* as 8-mm bands
Column temperature: Ambient, not to exceed 30°
Developing solvent system: Toluene, ethyl formate, and formic acid (5: 5: 0.2)
Developing distance: 6 cm
Derivatization reagent: A solution of 10% sulfuric acid in alcohol. [NOTE—Prepare fresh. Slowly and gradually add sulfuric acid to ice-cold alcohol, and mix well.]

System suitability
Samples: *Standard solution A*, *Standard solution B*, and *Standard solution C*
Suitability requirements
Chromatographic pattern: Under long-wave UV light (365 nm), the chromatogram of *Standard solution C* displays, in the bottom third of the plate, the following bands in the order of increasing R_F: a yellowish or orange band (sometimes, two orange bands are seen); a bluish-green band corresponding to the light-blue ganoderic acid A band in *Standard solution A*; an intense yellow band corresponding to ganoderic acid B, ganoderic acid G, ganoderic acid H, and ganoderenic acid B; and a bluish-green band coincident with ganoderic acid D and ganoderenic acid D. In the middle third of the chromatogram, a variable number of blue-green bands appear. At the top of the middle third of the *Standard solution C* chromatogram, a somewhat diffuse band coincident with the ergosterol band in *Standard solution B* is seen. In the upper third of the chromatogram, three or four diffuse bands of varying colors appear. Under white light, *Standard solution C* exhibits, in its lower third, two brownish-red bands, the upper of them coincident with the ganoderic acid A band in *Standard solution A*, followed by a more intense brown band; and a lighter brown band corresponding to ganoderic acid D and ganoderenic acid D. In the middle third of the chromatogram, five or six light-brown bands are seen; one of those, deepest in color and relatively diffuse, corresponds to the ergosterol band in *Standard solution B*. Two or three light-brown bands are seen under white light in the upper third of the chromatogram of *Standard solution C*. [NOTE—The *Standard solutions* are stable for 72 h at room temperature.]

Analysis
Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*
Apply the samples as bands and dry in air. Develop in a saturated chamber, remove the plate, air-dry, treat with *Derivatization reagent*, and heat at 105°–110° for 5 min. Immediately examine under white light and under the long-wave UV light (365 nm).
Acceptance criteria: Under long-wave UV light (365 nm) and under white light, the chromatogram of the *Sample solution* exhibits bands corresponding in color and R_F to similar bands in the chromatogram of *Standard solution C*, at the R_F values listed for *System suitability*. Under white light, the chromatogram of the *Sample solution* exhibits an additional violet band above the ergosterol band. [NOTE—The *Sample solution* is stable for 72 h at room temperature.]

[1] A suitable commercially available plate is the HPTLC Silica Gel 60 F_{254} from EMD Millipore (e.g., Part No. 1.05642.0001).

• **B. HPLC**
Analysis: Proceed as directed in the test for *Content of Triterpenoic Acids*.
Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks at the retention times corresponding to those of ganoderenic acid C, ganoderic acid C_2, ganoderic acid G, ganoderenic acid B, ganoderic acid B, ganoderic acid A, ganoderic acid H, ganoderenic acid D, ganoderic acid D, and ganoderic acid F in the chromatogram of *Standard solution B*.

• **C. HPLC**
Analysis: Proceed as directed in the test for *Content of Water-Soluble Polysaccharides*.
Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks at the retention times corresponding to the peaks due to mannose, glucuronic acid, dextrose, galactose, and L-fucose in the chromatogram of the *Standard solution*.

COMPOSITION

• **CONTENT OF TRITERPENOIC ACIDS**
Solution A: 0.075% Phosphoric acid in water
Solution B: Acetonitrile
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	80.0	20.0
3	73.5	26.5
34	73.5	26.5
52	61.5	38.5
53	80.0	20.0
58	80.0	20.0

[NOTE—Maintain the *Mobile phase* at 73.5% of *Solution A* for the period sufficient for complete elution of ganoderic acid A.]
Standard solution A: 0.1 mg/mL of USP Ganoderic Acid A RS in methanol. Sonicate to dissolve if necessary.
Standard solution B: Sonicate 40 mg of USP Ganoderma Lucidum Fruiting Body Powdered Extract RS in 5 mL of alcohol, and centrifuge. Pass through a nylon filter of 0.2-µm pore size, and discard the initial 1 mL of the filtrate.
Sample solution: Transfer 2.0 g of Ganoderma Lucidum Fruiting Body, finely powdered and accurately weighed, to a 200-mL round-bottom flask, add 75 mL of alcohol, attach a condenser, reflux for 45 min, cool, and filter. Rinse the flask with two 10-mL portions of alcohol and filter, combining the rinsates and the filtrate. Evaporate to dryness under reduced pressure, and dissolve the residue in about 20 mL of alcohol. Transfer the solution to a 25-mL volumetric flask, dilute with alcohol to volume, and mix well. Pass through a nylon filter of 0.2-µm pore size, and discard the initial 1 mL of the filtrate. [NOTE—To facilitate the chromatographic column longevity, the following solid phase extraction procedure may be employed. Condition the solid phase extraction column containing about 200 mg of L1 packing with 5 mL of methanol followed by 3 mL of water; do not allow the column to dry. Transfer 2.0 mL of Ganoderma Lucidum Fruiting Body solution in alcohol into a 20-mL volumetric flask, dilute with water to volume, and mix well. Apply the entire volume onto the column, and elute at the rate of approximately 1 drop/s, employing vacuum. Rinse the column with 3 mL of water, and discard the rinsate. Elute with 2.0 mL of methanol and

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collect the eluate into the 2.0-mL volumetric flask. Adjust with methanol to volume, and mix well.]
[NOTE—This method may result in coelution of ganoderenic acid A and ganoderic acid K.]

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 257 nm
Column: 2.1-mm × 15-cm; 1.8-µm packing L1
Column temperature: 25°
Flow rate: 0.4 mL/min
Injection volume: 5 µL

System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatographic similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Ganoderma Lucidum Fruiting Body Powdered Extract RS being used.
Resolution: NLT 1.0 between ganoderic acid A and ganoderic acid H peaks, *Standard solution B*
Tailing factor: NMT 2.0 for the ganoderic acid A peak, *Standard solution A*
Relative standard deviation: NMT 2.0% determined from the ganoderic acid A peak in replicate injections, *Standard solution A*

Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
[NOTE—*Standard solution A*, *Standard solution B*, and the *Sample solution* are stable for 24 h at room temperature.]
Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Ganoderma Lucidum Fruiting Body Powdered Extract RS being used, identify all specified ganoderic and ganoderenic acids in the *Sample solution* chromatogram. The approximate relative retention times, with respect to ganoderic acid A, are provided in *Table 2*.

Table 2

Analyte	Relative Retention Time	Relative Response Factor
Ganoderenic acid C	0.36	0.51
Ganoderic acid C_2	0.42	1.05
Ganoderic acid G	0.56	1.18
Ganoderenic acid B	0.60	0.45
Ganoderic acid B	0.66	1.10
Ganoderic acid A	1.00	1.00
Ganoderic acid H	1.05	1.54
Ganoderenic acid D	1.25	0.51
Ganoderic acid D	1.33	1.08
Ganoderic acid F	1.54	1.45

Separately calculate the percentages of each triterpenoic acid in the portion of Ganoderma Lucidum Fruiting Body taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of ganoderic acid A in *Standard solution A*
C_S = concentration of USP Ganoderic Acid A RS in *Standard solution A* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Ganoderma Lucidum Fruiting Body taken to prepare the *Sample solution* (mg)
F = relative response factor, with respect to ganoderic acid A (see *Table 2*)

Calculate the sum of the percentages of all specified triterpenoic acids.
Acceptance criteria
Sum of triterpenoic acids: NLT 0.3% on the dried basis

CONTAMINANTS

- **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩
Acceptance criteria
Arsenic: NMT 2.0 µg/g
Cadmium: NMT 1.0 µg/g
Lead: NMT 5.0 µg/g
Mercury: NMT 1.0 µg/g
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^5 cfu/g, and the bile-tolerant Gram-negative bacteria count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

- **CONTENT OF WATER-SOLUBLE POLYSACCHARIDES**
Solution A: 0.05 M phosphate buffer, pH 6.0
Solution B: Acetonitrile
Mobile phase: See *Table 3*.

Table 3

Time (min)	Solution A (%)	Solution B (%)
0	84.0	16.0
30	82.5	17.5
55	81.0	19.0
60	81.0	19.0
61	84.0	16.0

Reagent: 0.1 M solution of 1-phenyl-3-methyl-5-pyrazolone in methanol
Internal standard solution: 0.5 mg/mL of D-lyxose in water
Standard stock solution: Composite solution containing 0.20 mg/mL each of USP Mannose RS, USP D-Glucuronic Acid RS, and USP Galactose RS; 2.0 mg/mL of USP Dextrose RS; and 0.10 mg/mL of USP L-Fucose RS in water
Standard solution: Combine 0.125 mL of *Standard stock solution* with 0.125 mL of *Internal standard solution*, 0.300 mL of 0.15 M sodium hydroxide solution, and 0.50 mL of *Reagent* in a capped reaction vial. Seal the vial, heat at 70° for 30 min, and cool to room temperature. Add to the vial 0.300 mL of 0.15 M hydrochloric acid and 0.65 mL of water, mix well, and pass through a nylon filter of 0.45-µm or finer pore size.
[NOTE—The amounts of individual analytes (A_S) in the 0.125-mL aliquot of the *Standard solution* submitted to derivatization are approximately 0.25 mg for dextrose and 0.025 mg for mannose, galactose, and D-glucuronic acid.]

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Sample solution: Transfer 2.0 g of Ganoderma Lucidum Fruiting Body, finely powdered and accurately weighed, into a 200-mL round-bottom flask, add 60 mL of water, and allow to stand for 1 h. Attach a condenser, heat under reflux for 4 h, and filter immediately. Transfer the residue and the filter to the same 200-mL round-bottom flask. Add 60 mL of water, heat under reflux for 3 h, and filter immediately. Rinse the flask with three 5-mL portions of water, and filter. Combine the filtrates and the rinsates in a 250-mL beaker, and evaporate on the water bath to dryness. Dissolve the residue in 5 mL of water, add 75 mL of alcohol, mix well, allow to stand at 4° for 12 h, and centrifuge at 4000 rpm for 30 min. Discard the supernatant, and dry the precipitate on a water bath. Dissolve the residue in hot water and quantitatively transfer into a 10-mL volumetric flask. Cool to room temperature, dilute with water to volume, and mix well. Centrifuge at 4000 rpm for 10 min. Accurately transfer 0.250 mL of the supernatant into a reaction vial, and add about 0.25 mL of 4 M trifluoroacetic acid. Seal the vial, heat at 110° for 4 h, cool to room temperature, add 0.5 mL of methanol, and evaporate to dryness at 60° under vacuum. Repeat the addition of 0.5 mL of methanol and subsequent evaporation three times. Add to the residue 0.125 mL of water, 0.125 mL of the *Internal standard solution*, 0.300 mL of 0.15 M sodium hydroxide solution, and 0.50 mL of the *Reagent*. Seal the vial, heat at 70° for 30 min, and cool to room temperature. Add to the vial 0.300 mL of 0.15 M hydrochloric acid and 0.65 mL of water, mix well, and pass through a nylon filter of 0.45-µm or finer pore size.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 250 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 35°

Flow rate: 1.0 mL/min

Injection volume: 10 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Resolution: NLT 1.5 between the D-lyxose peak and the closest subsequent peak, and NLT 1.5 between the glucuronic acid peak and the closest preceding peak

Tailing factor: NMT 2.0 for the dextrose peak

Relative standard deviation: NMT 2.0% determined for the dextrose peak in replicate injections

Analysis

Samples: *Standard solution* and *Sample solution*

[NOTE—*Standard solution* and *Sample solution* are stable for 24 h at room temperature.]

Using the chromatograms of the *Standard solution* and the reference chromatogram provided with the lot of USP Ganoderma Lucidum Fruiting Body Powdered Extract RS being used, identify the individual derivatized monosaccharides at about the following relative retention times, with respect to dextrose: 0.48 for mannose, 0.58 for lyxose, 0.82 for D-glucuronic acid, 1.09 for galactose, and 1.35 for L-fucose.

Separately calculate the percentages of derivatized monosaccharides in the portion of Ganoderma Lucidum Fruiting Body taken:

$$\text{Result} = (R_U/R_S) \times A_S \times (F/W) \times 100$$

R_U = peak response ratio of the relevant analyte to the internal standard from the *Sample solution*

R_S = peak response ratio of the relevant analyte to the internal standard from the *Standard solution*

A_S = amount of the relevant analyte in the aliquot of the *Standard solution* subjected to derivatization (mg)

F = dilution factor to account for the sample aliquot submitted to derivatization (0.250 mL) relative to the volume of the *Sample solution* (10.0 mL), 40

W = weight of Ganoderma Lucidum Fruiting Body taken to prepare the *Sample solution* (mg)

Calculate the sum of the percentages of mannose, D-glucuronic acid, dextrose, galactose, and L-fucose.

Acceptance criteria

Sum of monosaccharides: NLT 0.7% on the dried basis

• **BOTANICAL CHARACTERISTICS**

Macroscopic: Basidiocarp (fruiting body) morphology is highly variable. Shape of pileus (cap) ranges from reniform to subcircular, convex or concave, 15 cm or more broad, single to multiple layers thick (up to 3 cm); margin generally thick and blunt, sometimes acute. Pileus surface radially rugose (wrinkled) and concentrically sulcate; shiny, yellowish-red to reddish-black. Stipe (stem) attachment predominantly lateral; stipe length varies from very short to 10–12 cm long, 1–3 cm thick, cylindrical, reddish to almost black, laccate (lacquered). Hymenophore (pore surface) yellowish-white to tawny. Pores small, circular to irregular, 4–7 per mm, 6–200 µm in diameter, distance between axes of pores about 260 µm.

Microscopic: Hyphal system trimitic with hyaline, thin-walled, clamped, septate generative hyphae, 1–4 µm in diameter, septa restricted to clamps, scantily branched, abundant at the growth margin of pileus and dissepiments (partitions). Skeletal hyphae are arboriform, aseptate, clampless, very long, 3–6 µm in diameter, scantily branched, branches with limited growth at distal end, with thick walls; they compose most of the context (flesh) and dissepiments, originating immediately behind the growth margin from generative hyphae. Binding hyphae of the "Bovista" type are aseptate, clampless, profusely branched, generally thinner and lighter than the skeletal, 1–3 µm in diameter. Basidiospores ovoid, double-walled, truncated at apex. Epispore thin, ovoid, hyaline, 9.0–11.5 × 6.0–8.0 µm; endospore thick, ovoid, 6.5–8.5 × 5.0–6.5 µm, bearing relatively few long and thick echinules that support the epispore, sometimes fused into a short crest.

• **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩**:** NMT 2.0%

• **LOSS ON DRYING** ⟨731⟩

Sample: 1.0 g of powdered Ganoderma Lucidum Fruiting Body

Analysis: Dry at 105° for 4 h.

Acceptance criteria: NMT 17.0%

• **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩

Sample: 1.0 g of powdered Ganoderma Lucidum Fruiting Body

Acceptance criteria: NMT 4.0%

• **ARTICLES OF BOTANICAL ORIGIN,** *Alcohol-Soluble Extractives, Method 1* ⟨561⟩

Sample: 2–4 g of powdered Ganoderma Lucidum Fruiting Body

Acceptance criteria: NLT 2.0%

• **ARTICLES OF BOTANICAL ORIGIN,** *Water-Soluble Extractives, Method 1* ⟨561⟩

Sample: 2–4 g of powdered Ganoderma Lucidum Fruiting Body

Acceptance criteria: NLT 3.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.

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- **LABELING:** The label states the Latin binomial and, following the official name, the part of the fungus from which the article was derived.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Dextrose RS
  USP Ergosterol RS
  USP L-Fucose RS
  USP Galactose RS
  USP Ganoderic Acid A RS
  USP Ganoderma Lucidum Fruiting Body Powdered Extract RS
  USP D-Glucuronic Acid RS
  USP Mannose RS

Ganoderma Lucidum Fruiting Body Powder

DEFINITION

Ganoderma Lucidum Fruiting Body Powder is dried Ganoderma Lucidum Fruiting Body reduced to a powder or a very fine powder. It contains NLT 0.3% of triterpenoic acids, calculated on the dried basis as a sum of ganoderic acids A, B, C_2, D, F, G, and H and ganoderenic acids B, C, and D.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHY**

Standard solution A: 1.0 mg/mL of USP Ganoderic Acid A RS in alcohol

Standard solution B: 0.3 mg/mL of USP Ergosterol RS in alcohol

Standard solution C: 50 mg/mL of USP Ganoderma Lucidum Fruiting Body Powdered Extract RS in alcohol. Sonicate for about 10 min, centrifuge, and use the supernatant.

Sample solution: Sonicate about 1 g of Powder in 50 mL of alcohol for 15 min, centrifuge, withdraw the supernatant, and evaporate to dryness under reduced pressure at 50°. Dissolve the residue in 2.0 mL of alcohol, centrifuge, and use the supernatant.

Chromatographic system
(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
Mode: HPTLC
Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plate).[1] Predevelop the plate in methanol and dry at 105° for 30 min.
Application volume: 2 µL each of *Standard solution A* and *Standard solution B*, and 4 µL each of *Standard solution C* and *Sample solution* as 8-mm bands
Column temperature: Ambient, not to exceed 30°
Developing solvent system: Toluene, ethyl formate, and formic acid (5: 5: 0.2)
Developing distance: 6 cm
Derivatization reagent: A solution of 10% sulfuric acid in alcohol. [NOTE—Prepare fresh. Slowly and gradually add sulfuric acid to ice-cold alcohol, and mix well.]

System suitability
Samples: *Standard solution A*, *Standard solution B*, and *Standard solution C*
Suitability requirements
Chromatographic pattern: Under long-wave UV (365 nm), the chromatogram of *Standard solution C* displays, in the bottom third of the plate, the following bands in the order of increasing R_F: a yellowish or orange band (sometimes, two orange bands are seen); a bluish-green band corresponding to the light-blue ganoderic acid A band in *Standard solution A*; an intense yellow band corresponding to ganoderic acid B, ganoderic acid G, ganoderic acid H, and ganoderenic acid B; and a bluish-green band coincident with ganoderic acid D and ganoderenic acid D. In the middle third of the chromatogram, a variable number of blue-green bands appear. At the top of the middle third of the *Standard solution C* chromatogram, a somewhat diffuse band coincident with the ergosterol band in *Standard solution B* is seen. In the upper third of the chromatogram, three or four diffuse bands of varying colors appear. Under white light, *Standard solution C* exhibits, in its lower third, two brownish-red bands, the upper of them coincident with the ganoderic acid A band in *Standard solution A*, followed by a more intense brown band; and a lighter brown band corresponding to ganoderic acid D and ganoderenic acid D. In the middle third of the chromatogram, five or six light-brown bands are seen; one of those, deepest in color and relatively diffuse, corresponds to the ergosterol band in *Standard solution B*. Two or three light-brown bands are seen under white light in the upper third of the chromatogram of *Standard solution C*. [NOTE—The *Standard solutions* are stable for 72 h at room temperature.]

Analysis
Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*
Apply the samples as bands and dry in air. Develop in a saturated chamber, remove the plate, air-dry, treat with *Derivatization reagent*, and heat for 5 min at 105°–110°. Immediately examine under white light and under the long-wave UV light (365 nm).

Acceptance criteria: Under the long-wave UV light (365 nm) and under white light, the chromatogram of the *Sample solution* exhibits the bands corresponding in color and R_F to similar bands in the chromatogram of *Standard solution C*. Under white light, the chromatogram of the *Sample solution* exhibits an additional violet band above the ergosterol band. [NOTE—The *Sample solution* is stable for 72 h at room temperature.]

- **B. HPLC**

Analysis: Proceed as directed in the test for *Content of Triterpenoic Acids*.
Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks at the retention times corresponding to those of ganoderenic acid C, ganoderic acid C_2, ganoderic acid G, ganoderenic acid B, ganoderic acid B, ganoderic acid A, ganoderic acid H, ganoderenic acid D, ganoderic acid D, and ganoderic acid F in the chromatogram of *Standard solution B*.

- **C. HPLC**

Analysis: Proceed as directed in the test for *Content of Water-Soluble Polysaccharides*.
Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks at the retention times corresponding to the peaks due to mannose, glucuronic acid, dextrose, galactose, and L-fucose in the chromatogram of the *Standard solution*.

COMPOSITION

- **CONTENT OF TRITERPENOIC ACIDS**

Solution A: 0.075% Phosphoric acid in water
Solution B: Acetonitrile
Mobile phase: See *Table 1*.

[1] A suitable commercially available plate is the HPTLC Silica Gel 60 F_{254} from EMD Millipore (e.g., Part No. 1.05642.0001).

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Table 1

Time (min)	Solution A (%)	Solution B (%)
0	80.0	20.0
3	73.5	26.5
34	73.5	26.5
52	61.5	38.5
53	80.0	20.0
58	80.0	20.0

[NOTE—Maintain the *Mobile phase* at 73.5% of *Solution A* for the period sufficient for the complete elution of ganoderic acid A.]

Standard solution A: 0.1 mg/mL of USP Ganoderic Acid A RS in methanol. Sonicate to dissolve if necessary.

Standard solution B: Sonicate 40 mg of USP Ganoderma Lucidum Fruiting Body Powdered Extract RS in 5 mL of alcohol and centrifuge. Pass through a nylon filter of 0.2-µm pore size, and discard the initial 1 mL of the filtrate.

Sample solution: Transfer 2.0 g of Powder, accurately weighed, to a 200-mL round-bottom flask, and add 75 mL of alcohol. Attach a condenser, reflux for 45 min, cool, and filter. Rinse the flask with two 10-mL portions of alcohol, and filter, combining the rinsates and the filtrate. Evaporate to dryness under reduced pressure, and dissolve the residue in about 20 mL of alcohol. Transfer the solution to a 25-mL volumetric flask, dilute with alcohol to volume, and mix well. Pass through a nylon filter of 0.2-µm pore size, and discard the initial 1 mL of the filtrate. [NOTE—To facilitate the chromatographic column longevity, the following solid phase extraction procedure may be employed. Condition the solid phase extraction column containing about 200 mg of L1 packing with 5 mL of methanol followed by 3 mL of water; do not allow the column to dry. Transfer 2.0 mL of Powder solution in alcohol to a 20-mL volumetric flask, dilute with water to volume, and mix well. Apply the entire volume onto the column, and elute at the rate of approximately 1 drop/s, employing a vacuum. Rinse the column with 3 mL of water, and discard the rinsate. Elute with 2.0 mL of methanol and collect the eluate into the 2.0-mL volumetric flask. Adjust with methanol to volume, and mix well.]

[NOTE—This method may result in coelution of ganoderenic acid A and ganoderic acid K.]

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 257 nm

Column: 2.1-mm × 15-cm; 1.8-µm packing L1

Column temperature: 25°

Flow rate: 0.4 mL/min

Injection volume: 5 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatographic similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Ganoderma Lucidum Fruiting Body Powdered Extract RS being used.

Resolution: NLT 1.0 between ganoderic acid A and ganoderic acid H peaks, *Standard solution B*

Tailing factor: NMT 2.0 for the ganoderic acid A peak, *Standard solution A*

Relative standard deviation: NMT 2.0% determined from the ganoderic acid A peak in replicate injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

[NOTE—*Standard solution A*, *Standard solution B*, and the *Sample solution* are stable for 24 h at room temperature.]

Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Ganoderma Lucidum Fruiting Body Powdered Extract RS being used, identify all specified ganoderic and ganoderenic acids in the *Sample solution* chromatogram. The approximate relative retention times, with respect to ganoderic acid A, are provided in *Table 2*.

Table 2

Analyte	Relative Retention Time	Relative Response Factor
Ganoderenic acid C	0.36	0.51
Ganoderic acid C_2	0.42	1.05
Ganoderic acid G	0.56	1.18
Ganoderenic acid B	0.60	0.45
Ganoderic acid B	0.66	1.10
Ganoderic acid A	1.00	1.00
Ganoderic acid H	1.05	1.54
Ganoderenic acid D	1.25	0.51
Ganoderic acid D	1.33	1.08
Ganoderic acid F	1.54	1.45

Separately calculate the percentages of each triterpenoic acid in the portion of Powder taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*

r_S = peak area of ganoderic acid A in *Standard solution A*

C_S = concentration of USP Ganoderic Acid A RS in *Standard solution A* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Powder taken to prepare the *Sample solution* (mg)

F = relative response factor, with respect to ganoderic acid A (see *Table 2*)

Calculate the sum of the percentages of all specified triterpenoic acids.

Acceptance criteria

Sum of triterpenoic acids: NLT 0.3% on the dried basis

CONTAMINANTS

- **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩

Acceptance criteria

Arsenic: NMT 2.0 µg/g

Cadmium: NMT 1.0 µg/g

Lead: NMT 5.0 µg/g

Mercury: NMT 1.0 µg/g

- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^5 cfu/g, and the bile-tolerant Gram-negative bacteria count does not exceed 10^3 cfu/g.

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• **Absence of Specified Microorganisms** ⟨2022⟩: Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

• **Content of Water-Soluble Polysaccharides**

Solution A: 0.05 M phosphate buffer, pH 6.0
Solution B: Acetonitrile
Mobile phase: See *Table 3*.

Table 3

Time (min)	Solution A (%)	Solution B (%)
0	84.0	16.0
30	82.5	17.5
55	81.0	19.0
60	81.0	19.0
61	84.0	16.0

Reagent: 0.1 M solution of 1-phenyl-3-methyl-5-pyrazolone in methanol
Internal standard solution: 0.5 mg/mL of D-lyxose in water
Standard stock solution: Composite solution containing 0.20 mg/mL each of USP Mannose RS, USP D-Glucuronic Acid RS, and USP Galactose RS; 2.0 mg/mL of USP Dextrose RS; and 0.10 mg/mL of USP L-Fucose RS in water
Standard solution: Combine 0.125 mL of *Standard stock solution* with 0.125 mL of *Internal standard solution*, 0.300 mL of 0.15 M sodium hydroxide solution, and 0.50 mL of *Reagent* in a capped reaction vial. Seal the vial, heat at 70° for 30 min, and cool to room temperature. Add to the vial 0.300 mL of 0.15 M hydrochloric acid and 0.65 mL of water, mix well, and pass through a nylon filter of 0.45-µm or finer pore size.
[Note—The amounts of individual analytes (A_S) in the 0.125 mL aliquot of the *Standard solution* submitted to derivatization are approximately 0.25 mg for dextrose and 0.025 mg for mannose, galactose, and D-glucuronic acid.]
Sample solution: Transfer 2.0 g of Powder, accurately weighed, to a 200-mL round-bottom flask, add 60 mL of water, and allow to stand for 1 h. Attach a condenser, heat under reflux for 4 h, and filter immediately. Transfer the residue and the filter to the same 200-mL round-bottom flask. Add 60 mL of water, heat under reflux for 3 h, and filter immediately. Rinse the flask with three 5-mL portions of water, and filter. Combine the filtrates and the rinsates in a 250-mL beaker, and evaporate on the water bath to dryness. Dissolve the residue in 5 mL of water, add 75 mL of alcohol, mix well, allow to stand at 4° for 12 h, and centrifuge at 4000 rpm for 30 min. Discard the supernatant, and dry the precipitate on a water bath. Dissolve the residue in hot water and quantitatively transfer to a 10-mL volumetric flask. Cool to room temperature, dilute with water to volume, and mix well. Centrifuge at 4000 rpm for 10 min. Accurately transfer 0.250 mL of the supernatant to a reaction vial, and add about 0.25 mL of 4 M trifluoroacetic acid. Seal the vial, and heat at 110° for 4 h. Cool to room temperature, add 0.5 mL of methanol, and evaporate to dryness at 60° under vacuum. Repeat the addition of 0.5 mL of methanol and subsequent evaporation three times. Add to the residue 0.125 mL of water, 0.125 mL of the *Internal standard solution*, 0.300 mL of 0.15 M sodium hydroxide solution, and 0.50 mL of *Reagent*. Seal the vial, heat at 70° for 30 min, and cool to room temperature. Add to the vial 0.300 mL of 0.15 M hydrochloric acid and 0.65 mL of water, mix well, and pass through a nylon filter of 0.45-µm or finer pore size.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 250 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 35°
Flow rate: 1.0 mL/min
Injection volume: 10 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Resolution: NLT 1.5 between the D-lyxose peak and the closest subsequent peak, and NLT 1.5 between the glucuronic acid peak and the closest preceding peak
Tailing factor: NMT 2.0 for the dextrose peak
Relative standard deviation: NMT 2.0% determined for the dextrose peak in replicate injections
Analysis
Samples: *Standard solution* and *Sample solution*
[Note—The *Standard solution* and *Sample solution* are stable for 24 h at room temperature.]
Using the chromatograms of the *Standard solution* and the reference chromatogram provided with the lot of USP Ganoderma Lucidum Fruiting Body Powdered Extract RS being used, identify the individual derivatized monosaccharides at about the following relative retention times, with respect to dextrose: 0.48 for mannose, 0.58 for lyxose, 0.82 for D-glucuronic acid, 1.09 for galactose, and 1.35 for L-fucose.
Separately calculate the percentages of derivatized monosaccharides in the portion of Powder taken:

$$\text{Result} = (R_U/R_S) \times A_S \times (F/W) \times 100$$

R_U = peak response ratio of the relevant analyte to the internal standard from the *Sample solution*
R_S = peak response ratio of the relevant analyte to the internal standard from the *Standard solution*
A_S = amount of the relevant analyte in the aliquot of the *Standard solution* subjected to derivatization (mg)
F = dilution factor to account for the sample aliquot submitted to derivatization (0.250 mL) relative to the volume of the *Sample solution* (10.0 mL), 40
W = weight of Powder taken to prepare the *Sample solution* (mg)

Calculate the sum of the percentages of mannose, D-glucuronic acid, dextrose, galactose, and L-fucose.
Acceptance criteria
Sum of monosaccharides: NLT 0.7% on the dried basis

• **Botanical Characteristics:** When milled, the fruiting body typically grinds into a fibrous mass or fractures into tiny strips rather than a fine powder. Hyphal system trimitic with hyaline, thin-walled, clamped, septate generative hyphae, 1–4 µm in diameter, septa restricted to clamps, scantily branched, abundant at the growth margin of pileus and dissepiments (partitions). Skeletal hyphae are arboriform, aseptate, clampless, very long, 3–6 µm in diameter, scantily branched, branches with limited growth at distal end, with thick walls; they compose most of the context (flesh) and dissepiments, originating immediately behind the growth margin from generative hyphae. Binding hyphae of the "Bovista" type are aseptate, clampless, profusely branched, generally thinner and lighter than the skeletal, 1–3 µm in diameter. Basidiospores ovoid, double-walled, truncated at apex. Epispore thin, ovoid, hyaline, 9.0–11.5 × 6.0–8.0 µm; endospore thick,

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ovoid, 6.5–8.5 × 5.0–6.5 µm, bearing relatively few long and thick echinules that support the epispore, sometimes fused into a short crest.

- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩: NMT 2.0%
- **LOSS ON DRYING** ⟨731⟩
  Sample: 1.0 g of Powder
  Analysis: Dry at 105° for 4 h.
  Acceptance criteria: NMT 17.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩
  Sample: 1.0 g of Powder
  Acceptance criteria: NMT 4.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Alcohol-Soluble Extractives, Method 1* ⟨561⟩
  Sample: 2–4 g of Powder
  Acceptance criteria: NLT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Water-Soluble Extractives, Method 1* ⟨561⟩
  Sample: 2–4 g of Powder
  Acceptance criteria: NLT 3.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the fungus from which the article was derived.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Dextrose RS
  USP Ergosterol RS
  USP L-Fucose RS
  USP Galactose RS
  USP Ganoderic Acid A RS
  USP Ganoderma Lucidum Fruiting Body Powdered Extract RS
  USP D-Glucuronic Acid RS
  USP Mannose RS

Garcinia cambogia

DEFINITION

Garcinia cambogia consists of the dried pericarp of the fruits of *Garcinia gummi-gutta* (L.) N. Robson, also known as *Garcinia cambogia* (Gaertn.) Desr. (Fam. Clusiaceae). It contains NLT 12% of the sum of (–)-hydroxycitric acid and (–)-hydroxycitric acid lactone, on the dried basis.

IDENTIFICATION

- **A.** *Garcinia cambogia* meets the requirements under *Specific Tests, Botanical Characteristics.*
- **B. HPLC:** The *Sample solution* chromatogram exhibits a peak for hydroxycitric acid at a retention time corresponding to that of *Standard solution A*, as obtained in the test for *Content of (–)-Hydroxycitric Acid and (–)-Hydroxycitric Acid Lactone*. The *Sample solution* also exhibits a peak for hydroxycitric acid lactone. The hydroxycitric acid and the hydroxycitric acid lactone peaks are the main peaks in the *Sample solution* chromatogram.

COMPOSITION

- **CONTENT OF (–)-HYDROXYCITRIC ACID AND (–)-HYDROXYCITRIC ACID LACTONE**

Solution A: 30% Phosphoric acid in water
Mobile phase: Dissolve 1.36 g of anhydrous potassium dihydrogen phosphate in 900 mL of water, adjust with *Solution A* to a pH of 2.5, dilute to 1000 mL with water, mix, filter, and degas.
Solvent: A mixture of *Solution A* and water (1:9)
Standard solution A: A solution of USP Calcium (–)-Hydroxycitrate RS equivalent to about 2.5 mg/mL of (–)-hydroxycitric acid in *Solvent*. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.
Standard solution B: 5 mg/mL of USP Powdered Garcinia Hydroxycitrate Extract RS in *Solvent*. Before injection, pass through a membrane filter of 0.45-µm or finer pore size.
Sample solution: Transfer about 5 g of *Garcinia cambogia*, finely powdered and accurately weighed, to a 250-mL round-bottom flask fitted with a reflux condenser. Add 50 mL of *Solvent*, reflux while stirring for 30 min, set aside to settle, and decant the supernatant. Repeat the extraction using four 50-mL portions of water, combine all extracts, cool, filter into a 250-mL volumetric flask, and dilute with water to volume. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability.*)
Mode: LC
Detector: UV 215 nm
Column: 4.6-mm × 25-cm; packing L1
Column temperature: 25°
Flow rate: 1.0 mL/min
Injection volume: 20 µL
System suitability
Samples: *Standard solution A, Standard solution B,* and *Sample solution*
[NOTE—The relative retention times for the hydroxycitric acid lactone and hydroxycitric acid peaks are about 0.9 and 1.0, respectively.]
Suitability requirements
Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Garcinia Hydroxycitrate Extract RS being used.
Resolution: NLT 1.0 between hydroxycitric acid lactone and hydroxycitric acid, *Sample solution*
Tailing factor: NMT 2.0 for the hydroxycitric acid peak, *Standard solution A*
Relative standard deviation: NMT 2.0%, determined from the hydroxycitric acid peak in repeated injections, *Standard solution A*
Analysis
Samples: *Standard solution A, Standard solution B,* and *Sample solution*
[NOTE—*Standard solution A, Standard solution B,* and *Sample solution* are stable for 6 h.]
Calculate the percentages of (–)-hydroxycitric acid and (–)-hydroxycitric acid lactone in the portion of *Garcinia cambogia* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of hydroxycitric acid from *Standard solution A*
C_S = concentration of (–)-hydroxycitric acid in *Standard solution A* (mg/mL)
V = final volume of the *Sample solution* (mL)
W = weight of *Garcinia cambogia* used to prepare the *Sample solution* (mg)
F = conversion factor: 2.17 for (–)-hydroxycitric acid lactone, and 1.00 for (–)-hydroxycitric acid

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Acceptance criteria: The sum of percentages of (–)-hydroxycitric acid and (–)-hydroxycitric acid lactone: NLT 12% on the dried basis

IMPURITIES

- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩: NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩: NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩: Meets the requirements

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: Fresh fruits are spherical to oval in shape, 4–8 cm in height, 3–6 cm in width, yellowish to pale pinkish when ripe, resemble a miniature pumpkin, with 7–13 deep longitudinal grooves, extending up to a circular elevated base of stigma with blackish tip, situated in the depressed end of the fruit, containing 6–8 seeds surrounded by a succulent aril. Compendial article consists of dried pieces of pericarp, longitudinal, of variable size and shape, strongly curved inward; leathery; externally rough, irregularly wrinkled, longitudinally grooved; internally smooth, longitudinally faintly striated and ridged. Dark brown to blackish-brown odor characteristic; taste sour, astringent, and slightly bitter.

Microscopic: Transverse section of the pericarp shows a layer of epicarp, composed of rectangular to tangentially elongated cells covered with thin cuticle; wide mesocarp, composed of 100–150 rows of parenchyma cells of variable size and shape, the outer rows composed of relatively larger cells with wide intercellular spaces; vascular bundles appear throughout the mesocarp, more toward the inner zone; dark brown gummy exudates, simple and compound starch granules and prisms of calcium oxalate are present in the parenchyma cells throughout the mesocarp.

- **LIMIT OF CITRIC ACID**

Solvent and **Chromatographic system:** Prepare as directed in the test for *Content of (–)-Hydroxycitric Acid and (–)-Hydroxycitric Acid Lactone*.

Standard solution: 0.5 mg/mL of USP Citric Acid RS in *Solvent*. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.

Analysis

Sample: *Standard solution*

Calculate the percentage of citric acid in the portion of *Garcinia cambogia* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of citric acid from the *Sample solution* in the test for *Content of (–)-Hydroxycitric Acid and (–)-Hydroxycitric Acid Lactone* (mg/mL)
r_S = peak area of citric acid from the *Standard solution*
C_S = concentration of USP Citric Acid RS in the *Standard solution* (mg/mL)
V = final volume of the *Sample solution* (mL)
W = weight of *Garcinia cambogia* used to prepare the *Sample solution* in the test for *Content of (–)-Hydroxycitric Acid and (–)-Hydroxycitric Acid Lactone* (mg)

Acceptance criteria: NMT 2% of citric acid on the dried basis

- **LOSS ON DRYING** ⟨731⟩

Sample: 2.0 g of *Garcinia cambogia*, finely powdered

Analysis: Dry the *Sample* at 105° for 3 h.

Acceptance criteria: NMT 12.0%

- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩: Determined on 1.0 g of finely powdered *Garcinia cambogia*: NMT 3.0%; NMT 8.0% if sodium chloride was added as a preservative during collection of the fruits
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria do not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩: Meets the requirements of the tests for absence of *Salmonella species* and *Escherichia coli*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Calcium (–)-Hydroxycitrate RS
USP Citric Acid RS
USP Powdered Garcinia Hydroxycitrate Extract RS

Powdered *Garcinia cambogia*

DEFINITION

Powdered *Garcinia cambogia* is *Garcinia cambogia* reduced to a powder or very fine powder. It contains NLT 12% of the sum of (–)-hydroxycitric acid and (–)-hydroxycitric acid lactone, on the dried basis.

IDENTIFICATION

- **A.** Powdered *Garcinia cambogia* meets the requirements under *Specific Tests, Botanical Characteristics*.
- **B. HPLC:** The *Sample solution* chromatogram exhibits a peak for hydroxycitric acid at a retention time corresponding to that of *Standard solution A*, as obtained in the test for *Content of (–)-Hydroxycitric Acid and (–)-Hydroxycitric Acid Lactone*. The *Sample solution* also exhibits a peak for hydroxycitric acid lactone. The hydroxycitric acid and the hydroxycitric acid lactone peaks are the main peaks in the *Sample solution* chromatogram.

COMPOSITION

- **CONTENT OF (–)-HYDROXYCITRIC ACID AND (–)-HYDROXYCITRIC ACID LACTONE**

Solution A: 30% Phosphoric acid in water

Mobile phase: Dissolve 1.36 g of anhydrous potassium dihydrogen phosphate in 900 mL of water, adjust with *Solution A* to a pH of 2.5, dilute with water to 1000 mL, mix, filter, and degas.

Solvent: *Solution A* and water (1:9)

Standard solution A: A solution of USP Calcium (–)-Hydroxycitrate RS equivalent to about 2.5 mg/mL of (–)-hydroxycitric acid in *Solvent*. Before injection, pass through a membrane filter of 0.45-µm or finer pore size.

Standard solution B: 5 mg/mL of USP Powdered Garcinia Hydroxycitrate Extract RS in *Solvent*. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of filtrate.

Sample solution: Transfer about 5 g of Powdered *Garcinia cambogia*, accurately weighed, to a 250-mL round-bottom flask fitted with a reflux condenser. Add 50 mL of *Solvent*, reflux while stirring for 30 min, set aside to settle, and

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decant the supernatant. Repeat the extraction using four 50-mL portions of water, combine all extracts, Cool, filter into a 250-mL volumetric flask, and dilute with water to volume. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of filtrate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 215 nm
Column: 4.6-mm × 25-cm; packing L1
Column temperature: 25°
Flow rate: 1.0 mL/min
Injection volume: 20 µL

System suitability
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
[NOTE—The relative retention times for the hydroxycitric acid lactone and hydroxycitric acid peaks are about 0.9 and 1.0, respectively.]
Suitability requirements
Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Garcinia Hydroxycitrate Extract RS being used.
Resolution: NLT 1.0 between the hydroxycitric acid lactone and hydroxycitric acid peaks, *Sample solution*
Tailing factor: NMT 2.0 for the hydroxycitric acid peak, *Standard solution A*
Relative standard deviation: NMT 2.0%, determined from the hydroxycitric acid peak for replicate injections, *Standard solution A*

Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
[NOTE—*Standard solution A*, *Standard solution B*, and the *Sample solution* are stable for 6 h.]
Calculate the percentages of (–)-hydroxycitric acid and (–)-hydroxycitric acid lactone in the portion of Powdered *Garcinia cambogia* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of hydroxycitric acid from *Standard solution A*
C_S = concentration of (–)-hydroxycitric acid in *Standard solution A* (mg/mL)
V = final volume of the *Sample solution* (mL)
W = weight of Powdered *Garcinia cambogia* used to prepare the *Sample solution* (mg)
F = conversion factor: 2.17 for (–)-hydroxycitric acid lactone, and 1.00 for (–)-hydroxycitric acid

Acceptance criteria: The sum of percentages of (–)-hydroxycitric acid and (–)-hydroxycitric acid lactone: NLT 12% on the dried basis

IMPURITIES
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements

SPECIFIC TESTS
- **BOTANICAL CHARACTERISTICS:** Dark brown powder; odor characteristic; taste sour, astringent and slightly bitter. Under a microscope, it shows parenchyma cells containing dark reddish-brown gummy exudates, parenchyma cells containing simple and compound starch granules, prisms of calcium oxalate, and fragments of spiral and annular vessels.
- **LIMIT OF CITRIC ACID**
Solvent: Prepare as directed in the test for *Content of (–)-Hydroxycitric Acid and (–)-Hydroxycitric Acid Lactone*.
Standard solution: 0.5 mg/mL of USP Citric Acid RS in *Solvent*. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of filtrate.
Analysis
Sample: *Standard solution*
Calculate the percentage of citric acid in the portion of Powdered *Garcinia cambogia* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of citric acid from the *Sample solution* in the test for *Content of (–)-Hydroxycitric Acid and (–)-Hydroxycitric Acid Lactone*
r_S = peak area of citric acid from the *Standard solution*
C_S = concentration of USP Citric Acid RS in the *Standard solution* (mg/mL)
V = final volume of the *Sample solution* (mL)
W = weight of Powdered *Garcinia cambogia* used to prepare the *Sample solution* in the test for *Content of (–)-Hydroxycitric Acid and (–)-Hydroxycitric Acid Lactone* (mg)

Acceptance criteria: NMT 2% of citric acid on the dried basis
- **LOSS ON DRYING** ⟨731⟩
Sample: 2.0 g of Powdered *Garcinia cambogia*
Analysis: Dry the *Sample* at 105° for 3 h.
Acceptance criteria: NMT 12.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** Determined on 1.0 g of Powdered *Garcinia cambogia*: NMT 3.0%; NMT 8.0% if sodium chloride was added as a preservative during collection of the fruits
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria do not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Calcium (–)-Hydroxycitrate RS
USP Citric Acid RS
USP Powdered Garcinia Hydroxycitrate Extract RS

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Powdered Garcinia Hydroxycitrate Extract

DEFINITION

Powdered Garcinia Hydroxycitrate Extract is prepared from *Garcinia cambogia* or *Garcinia indica* by extraction with water, alcohol, or mixtures of these solvents, followed by stabilization of the (–)-hydroxycitric acid content in the form of a calcium, potassium, magnesium, and/or sodium salt. The ratio of plant material to extract is about 5:1 to 10:1. It contains NLT 40% of (–)-hydroxycitric acid, calculated on the dried basis. It may contain suitable added substances.

IDENTIFICATION

• **A. HPLC IDENTIFICATION TEST:** The *Sample solution* chromatogram exhibits a peak for hydroxycitric acid at a retention time corresponding to that of *Standard solution A*, as obtained in the test for *Content of (–)-Hydroxycitric Acid and Limit of (–)-Hydroxycitric Acid Lactone*.

COMPOSITION

• **CONTENT OF (–)-HYDROXYCITRIC ACID AND LIMIT OF (–)-HYDROXYCITRIC ACID LACTONE**

Solution A: 30% phosphoric acid in water

Mobile phase: Dissolve 1.36 g of anhydrous potassium dihydrogen phosphate in 900 mL of water, adjust with *Solution A* to a pH of 2.5, complete to 1000 mL with water, mix, filter, and degas.

Solvent: A mixture of *Solution A* and water (1:9)

Standard solution A: A solution of USP Calcium (–)-Hydroxycitrate RS equivalent to about 2.5 mg/mL of (–)-hydroxycitric acid in *Solvent*. Before injection, pass through a membrane filter of 0.45-µm or finer pore size.

Standard solution B: 5 mg/mL of USP Powdered Garcinia Hydroxycitrate Extract RS in *Solvent*. Before injection, pass through a membrane filter of 0.45-µm or finer pore size.

Sample solution: 5 mg/mL of Powdered Garcinia Hydroxycitrate Extract in *Solvent*. Before injection, pass through a membrane filter of 0.45-µm or finer pore size.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 215 nm

Column: 4.6-mm × 25-cm; packing L1

Column temperature: 25 ± 1°

Flow rate: 1.0 mL/min

Injection size: 20 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Garcinia Hydroxycitrate Extract RS being used.

Tailing factor: NMT 2.0 for the hydroxycitric acid peak, *Standard solution A*

Relative standard deviation: NMT 2.0%, determined from the hydroxycitric acid peak, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*. [NOTE—*Standard solution A*, *Standard solution B*, and the *Sample solution* are stable for 6 h.]

Calculate the percentage of (–)-hydroxycitric acid and the limit of (–)-hydroxycitric acid lactone, if present, in the portion of Powdered Garcinia Hydroxycitrate Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area for the relevant analyte from the *Sample solution*

r_S = peak area of hydroxycitric acid from *Standard solution A*

C_S = concentration of (–)-hydroxycitric acid in *Standard solution A* (mg/mL)

C_U = concentration of Powdered Garcinia Hydroxycitrate Extract in the *Sample solution* (mg/mL)

F = conversion factor for each analyte: 2.17 for (–)-hydroxycitric acid lactone, and 1.00 for (–)-hydroxycitric acid

Acceptance criteria: NLT 40% of (–)-hydroxycitric acid and NMT 8% of (–)-hydroxycitric acid lactone on the dried basis

IMPURITIES

INORGANIC IMPURITIES

• **Articles of Botanical Origin,** *Acid-Insoluble Ash* ⟨561⟩: NMT 3.0%

Change to read:

ORGANIC IMPURITIES

• **Procedure:** ▲**Botanical Extracts** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues:*▲ (CN 1-May-2020) Meets the requirements

SPECIFIC TESTS

• **LIMIT OF CITRIC ACID**

Solvent: Prepare as directed in the test for *Content of (–)-Hydroxycitric Acid and Limit of (–)-Hydroxycitric Acid Lactone*.

Standard solution: 0.5 mg/mL of USP Citric Acid RS in *Solvent*. Before injection, pass through a membrane filter of 0.45-µm or finer pore size.

Analysis

Sample: *Standard solution*

Calculate the percentage of citric acid in the portion of Powdered Garcinia Hydroxycitrate Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of citric acid, using the peak area of citric acid from the *Sample solution* in the test for *Content of (–)-Hydroxycitric Acid and Limit of (–)-Hydroxycitric Acid Lactone*

r_S = peak area of citric acid from the *Standard solution*

C_S = concentration of USP Citric Acid RS in the *Standard solution* (mg/mL)

C_U = concentration of Powdered Garcinia Hydroxycitrate Extract in the *Sample solution* in the test for *Content of (–)-Hydroxycitric Acid and Limit of (–)-Hydroxycitric Acid Lactone* (mg/mL)

Acceptance criteria: NMT 5% of citric acid on the dried basis

• **IDENTIFICATION TESTS—GENERAL** ⟨191⟩**:** Test for the presence of calcium, magnesium, potassium, and/or sodium.

• **LOSS ON DRYING** ⟨731⟩**:** Dry 2.0 g of Powdered Extract at 105° for 3 h: Powdered Extract containing calcium hydroxycitrate loses NMT 5.0% of its weight; Powdered Extract containing other salts loses NMT 9.0% of its weight.

• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.

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- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩: Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.
- **OTHER REQUIREMENTS:** It meets the requirements of the test for *Residual Solvents* under *Botanical Extracts* ⟨565⟩.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. It meets other *Labeling* requirements under *Botanical Extracts* ⟨565⟩.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Calcium (–)-Hydroxycitrate RS
  USP Citric Acid RS
  USP Powdered Garcinia Hydroxycitrate Extract RS

Garcinia indica

DEFINITION

Garcinia indica consists of the dried pericarp of the fruits of *Garcinia indica* (Thouars) Choisy (Fam. Clusiaceae). It contains NLT 12% of the sum of (–)-hydroxycitric acid and (–)-hydroxycitric acid lactone, on the dried basis.

IDENTIFICATION

- **A.** *Garcinia indica* meets the requirements under *Specific Tests, Botanical Characteristics*.
- **B. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩
  Standard solution: 0.5 mg/mL of garcinol in alcohol
  Sample solution: Transfer about 2.0 g of *Garcinia indica*, finely powdered, to a Soxhlet apparatus, add 100 mL of alcohol, and extract for 6 h. Filter and concentrate under vacuum to about 10 mL. [NOTE—Use a thimble of suitable size such that the volume of alcohol used in the Soxhlet extraction is at least twice the volume of the thimble.]
  Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plates)
  Application volume: 5 µL, as 8-mm bands
  Developing solvent system: Toluene, ethyl acetate, and formic acid (4: 1: 0.5)
  Developing distance: 6 cm
  Derivatization reagent: A mixture of 1% vanillin in alcohol and 10% sulfuric acid in alcohol (1:1)
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Apply the *Samples* as bands. Develop in a saturated chamber. Remove the plate from the chamber, dry, treat with *Derivatization reagent*, heat for 5–10 min at 105°, and examine under white light.
  Acceptance criteria: The *Sample solution* chromatogram exhibits a main greenish-gray band due to garcinol at an R_F value of approximately 0.6, which corresponds in position and color to the main band in the chromatogram of the *Standard solution*. The *Sample solution* exhibits the following additional bands: two purple bands, two greenish-gray bands, two blue bands and a purple band at R_F values of approximately 0.31, 0.34, 0.37, 0.47, 0.54, 0.83, and 0.93, respectively. Other bands may be observed in the *Sample solution*.
- **C. HPLC:** The *Sample solution* chromatogram exhibits a peak for hydroxycitric acid at a retention time corresponding to that of *Standard solution A*, as obtained in the test for *Content of (–)-Hydroxycitric Acid and (–)-Hydroxycitric Acid Lactone*. The *Sample solution* also exhibits a peak for hydroxycitric acid lactone. The hydroxycitric acid and the hydroxycitric acid lactone peaks are the main peaks in the *Sample solution* chromatogram.

COMPOSITION

- **CONTENT OF (–)-HYDROXYCITRIC ACID AND (–)-HYDROXYCITRIC ACID LACTONE**
  Solution A: 30% Phosphoric acid in water
  Mobile phase: Dissolve 1.36 g of anhydrous potassium dihydrogen phosphate in 900 mL of water, adjust with *Solution A* to a pH of 2.5, dilute with water to 1000 mL, mix, filter, and degas.
  Solvent: A mixture of *Solution A* and water (1:9)
  Standard solution A: A solution of USP Calcium (–)-Hydroxycitrate RS equivalent to about 4 mg/mL of (–)-hydroxycitric acid in *Solvent*. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.
  Standard solution B: 8 mg/mL of USP Powdered Garcinia Hydroxycitrate Extract RS in *Solvent*. Before injection, pass through a membrane filter of 0.45-µm or finer pore size.
  Sample solution: Transfer about 5 g of *Garcinia indica*, finely powdered and accurately weighed, to a 250-mL round-bottom flask fitted with a reflux condenser. Add 50 mL of *Solvent*, reflux while stirring for 30 min, set aside to settle, and decant the supernatant. Repeat the extraction using four 50-mL portions of water, combine all extracts, cool, filter into a 250-mL volumetric flask, and dilute with water to volume. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: LC
  Detector: UV 215 nm
  Column: 4.6-mm × 25-cm; packing L1
  Column temperature: 25°
  Flow rate: 1.0 mL/min
  Injection volume: 20 µL
  System suitability
  Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
  [NOTE—The relative retention times for the hydroxycitric acid lactone and hydroxycitric acid peaks are about 0.9 and 1.0, respectively.]
  Suitability requirements
  Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Garcinia Hydroxycitrate Extract RS being used.
  Resolution: NLT 1.0 between the hydroxycitric acid lactone and hydroxycitric acid peaks, *Sample solution*
  Tailing factor: NMT 2.0 for the hydroxycitric acid peak, *Standard solution A*
  Relative standard deviation: NMT 2.0%, determined from the hydroxycitric acid peak for replicate injections, *Standard solution A*
  Analysis
  Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*. [NOTE—*Standard solution A*, *Standard solution B*, and the *Sample solution* are stable for 6 h.]
  Calculate the percentages of (–)-hydroxycitric acid and (–)-hydroxycitric acid lactone in the portion of *Garcinia indica* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*

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r_S = peak area of hydroxycitric acid from *Standard solution A*
C_S = concentration of (–)-hydroxycitric acid in *Standard solution A* (mg/mL)
V = final volume of the *Sample solution* (mL)
W = weight of *Garcinia indica* used to prepare the *Sample solution* (mg)
F = conversion factor: 2.17 for (–)-hydroxycitric acid lactone, and 1.00 for (–)-hydroxycitric acid

Acceptance criteria: The sum of percentages of (–)-hydroxycitric acid and (–)-hydroxycitric acid lactone is NLT 12% on the dried basis.

IMPURITIES

- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 0.5%
- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩**:** NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: Fresh fruits are globular in shape, 3–4 cm in diameter, purplish to pinkish orange when ripe, with persistant calyx lobes at the base and flattened radiating sessile stigma at the apex; containing 5–8 seeds surrounded by a succulant aril. Compendial article consists of dried pieces of pericarp, bluish black, of various size and shapes, flattened, flexible; remains of pedicels, calyx, and stigma may be present; odor characteristic; taste sour.

Microscopic: Transverse section of the pericarp shows a layer of epicarp, composed of isodiametric cells, with thin cuticle and stomata; hypodermis consisting of several rows of compactly arranged, tangentially elongated, thick-walled cells, containing dark brown contents, showing narrow irregular elongated cavities; outer mesocarp consisting of loosely arranged, tangentially elongated, parenchyma cells, few are full of starch grains, traversed by narrow bands of collapsed cells and oleoresin ducts; inner mesocarp consisting of collapsed and compactly arranged cells, showing rows of fibrovascular bundles; endocarp is not distinct, consisting of thin-walled collapsed cells, with dark brown content.

- **LIMIT OF CITRIC ACID**

Solvent and **Chromatographic system:** Prepare as directed in the test for *Content of (–)-Hydroxycitric Acid and (–)-Hydroxycitric Acid Lactone.*

Standard solution: 0.5 mg/mL of USP Citric Acid RS in *Solvent.* Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.

Analysis

Sample: *Standard solution*

Calculate the percentage of citric acid in the portion of *Garcinia indica* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of citric acid from the *Sample solution* in the test for *Content of(–)-Hydroxycitric Acid and (–)-Hydroxycitric Acid Lactone*
r_S = peak area of citric acid from the *Standard solution*
C_S = concentration of USP Citric Acid RS in the *Standard solution* (mg/mL)
V = final volume of the *Sample solution* (mL)
W = weight of *Garcinia indica* used to prepare the *Sample solution* in the test for *Content of(–)-Hydroxycitric Acid and Limit of(–)-Hydroxycitric Acid Lactone* (mg)

Acceptance criteria: NMT 2% of citric acid on the dried basis

- **LOSS ON DRYING** ⟨731⟩

Sample: 2.0 g of Powdered *Garcinia indica*
Analysis: Dry the *Sample* at 105° for 3 h.
Acceptance criteria: NMT 12.0%

- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** Determined on 1.0 g of finely powdered *Garcinia indica*: NMT 3.0%; NMT 8.0% if sodium chloride was added as a preservative during collection of the fruits
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria do not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella species* and *Escherichia coli*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Calcium (–)-Hydroxycitrate RS
USP Citric Acid RS
USP Powdered Garcinia Hydroxycitrate Extract RS

Powdered *Garcinia indica*

DEFINITION

Powdered *Garcinia indica* is *Garcinia indica* reduced to a fine or very fine powder. It contains NLT 12% of the sum of (–)-hydroxycitric acid and (–)-hydroxycitric acid lactone, on the dried basis.

IDENTIFICATION

- **A.** Powdered *Garcinia indica* meets the requirements under *Specific Tests, Botanical Characteristics.*
- **B. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution: 0.5 mg/mL of garcinol in alcohol
Sample solution: Transfer about 2.0 g of Powdered *Garcinia indica* to a Soxhlet apparatus, add 100 mL of alcohol, and extract for 6 h. Filter and concentrate under vacuum to about 10 mL. [NOTE—Use a thimble of a suitable size such that the volume of alcohol used in the Soxhlet extraction is at least twice the volume of the thimble.]
Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plates)
Application volume: 5 µL, as 8-mm bands
Developing solvent system: Toluene, ethyl acetate, and formic acid (4: 1: 0.5)
Developing distance: 6 cm
Derivatization reagent: A mixture of 1% vanillin in alcohol and 10% sulfuric acid in alcohol (1:1)
Analysis
Samples: *Standard solution* and *Sample solution*
Apply the *Samples* as bands. Develop in a saturated chamber. Remove the plate from the chamber, dry,

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treat with *Derivatization reagent*, heat for 5–10 min at 105°, and examine under white light.

Acceptance criteria: The *Sample solution* chromatogram exhibits a main greenish-gray band due to garcinol at an R_F value of approximately 0.6, which corresponds in position and color to the main band in the chromatogram of the *Standard solution*. The *Sample solution* exhibits the following additional bands: two purple bands, two greenish-gray bands, two blue bands, and a purple band at R_F values of approximately 0.31, 0.34, 0.37, 0.47, 0.54, 0.83, and 0.93, respectively. Other bands may be observed for the *Sample solution*.

- **C. HPLC:** The *Sample solution* chromatogram exhibits a peak for hydroxycitric acid at a retention time corresponding to that in the chromatogram of *Standard solution A*, as obtained in the test for *Content of (–)-Hydroxycitric Acid and (–)-Hydroxycitric Acid Lactone*. The *Sample solution* also exhibits a peak for hydroxycitric acid lactone. The hydroxycitric acid and the hydroxycitric acid lactone peaks are the main peaks in the *Sample solution* chromatogram.

COMPOSITION

- **CONTENT OF (–)-HYDROXYCITRIC ACID AND (–)-HYDROXYCITRIC ACID LACTONE**

Solution A: 30% Phosphoric acid in water

Mobile phase: Dissolve 1.36 g of anhydrous potassium dihydrogen phosphate in 900 mL of water, adjust with *Solution A* to a pH of 2.5, dilute with water to 1000 mL, mix, filter, and degas.

Solvent: A mixture of *Solution A* and water (1:9)

Standard solution A: A solution of USP Calcium (–)-Hydroxycitrate RS equivalent to about 4 mg/mL of (–)-hydroxycitric acid in *Solvent*. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.

Standard solution B: 8 mg/mL of USP Powdered Garcinia Hydroxycitrate Extract RS in *Solvent*. Before injection, pass through a membrane filter of 0.45-µm or finer pore size.

Sample solution: Transfer about 5 g of Powdered *Garcinia indica*, accurately weighed, to a 250-mL round-bottom flask fitted with a reflux condenser. Add 50 mL of *Solvent*, reflux while stirring for 30 min, set aside to settle, and decant the supernatant. Repeat the extraction using four 50-mL portions of water, combine all extracts, cool, filter into a 250-mL volumetric flask, and dilute with water to volume. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 215 nm

Column: 4.6-mm × 25-cm; packing L1

Column temperature: 25°

Flow rate: 1.0 mL/min

Injection volume: 20 µL

System suitability

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

[NOTE—The relative retention times for the hydroxycitric acid lactone and hydroxycitric acid peaks are about 0.9 and 1.0, respectively.]

Suitability requirements

Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Garcinia Hydroxycitrate Extract RS being used.

Resolution: NLT 1.0 between the hydroxycitric acid lactone and hydroxycitric acid peaks, *Sample solution*

Tailing factor: NMT 2.0 for the hydroxycitric acid peak, *Standard solution A*

Relative standard deviation: NMT 2.0%, determined from the hydroxycitric acid peak for replicate injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*. [NOTE—*Standard solution A*, *Standard solution B*, and the *Sample solution* are stable for 6 h.]

Calculate the percentages of (–)-hydroxycitric acid and (–)-hydroxycitric acid lactone in the portion of Powdered *Garcinia indica* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*

r_S = peak area of hydroxycitric acid from *Standard solution A*

C_S = concentration of (–)-hydroxycitric acid in *Standard solution A* (mg/mL)

V = final volume of the *Sample solution* (mL)

W = weight of Powdered *Garcinia indica* used to prepare the *Sample solution* (mg)

F = conversion factor: 2.17 for (–)-hydroxycitric acid lactone, and 1.00 for (–)-hydroxycitric acid

Acceptance criteria: The sum of percentages of (–)-hydroxycitric acid and (–)-hydroxycitric acid lactone is NLT 12% on the dried basis.

IMPURITIES

- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 0.5%
- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: Dark brown powder; odor characteristic; taste sour.

Microscopic: It shows cells containing dark brown content; cells containing yellow content, parenchyma cells containing simple and compound starch granules; fragments of epicarp cells containing stomata; and fragments of spiral and annular vessels.

- **LIMIT OF CITRIC ACID**

Solvent and **Chromatographic system:** Prepare as directed in the test for *Content of (–)-Hydroxycitric Acid and (–)-Hydroxycitric Acid Lactone*.

Standard solution: 0.5 mg/mL of USP Citric Acid RS in *Solvent*. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.

Analysis

Sample: *Standard solution*

Calculate the percentage of citric acid in the portion of Powdered *Garcinia indica* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of citric acid from the *Sample solution* in the test for *Content of (–)-Hydroxycitric Acid and (–)-Hydroxycitric Acid Lactone*

r_S = peak area of citric acid from the *Standard solution*

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C_S = concentration of USP Citric Acid RS in the *Standard solution* (mg/mL)
V = final volume of the *Sample solution* (mL)
W = weight of Powdered *Garcinia indica* used to prepare the *Sample solution* in the test for *Content of (–)-Hydroxycitric Acid and (–)-Hydroxycitric Acid Lactone* (mg)

Acceptance criteria: NMT 2% of citric acid on the dried basis

• **Loss on Drying** ⟨731⟩
Sample: 2.0 g of Powdered *Garcinia indica*
Analysis: Dry the *Sample* at 105° for 3 h.
Acceptance criteria: NMT 12.0%

• **Articles of Botanical Origin,** *Total Ash* ⟨561⟩**:** Determined on 1.0 g of Powdered *Garcinia indica*: NMT 3.0%; and NMT 8.0% if sodium chloride was added as a preservative during collection of the fruits

• **Microbial Enumeration Tests** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria do not exceed 10^3 cfu/g.

• **Absence of Specified Microorganisms** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella species* and *Escherichia coli*

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.

• **Labeling:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.

• **USP Reference Standards** ⟨11⟩
USP Calcium (–)-Hydroxycitrate RS
USP Citric Acid RS
USP Powdered Garcinia Hydroxycitrate Extract RS

Garlic

DEFINITION

Garlic consists of the fresh or dried compound bulbs of *Allium sativum* L. (Fam. Liliaceae). It contains NLT 0.5% of alliin and NLT 0.2% of γ-glutamyl-(*S*)-allyl-L-cysteine, calculated on the dried basis.

IDENTIFICATION

• **A. Thin-Layer Chromatography**
Standard solution A: 0.5 mg/mL of USP L-Methionine RS in a mixture of methanol and water (1:1)
Standard solution B: 0.5 mg/mL of USP Alliin RS in a mixture of methanol and water (1:1)
Sample solution: Cut a freeze-dried garlic bulb into small pieces, transfer 1 g of the cut pieces to an extractor, and extract with two 20-mL portions of a mixture of methanol and water (1:1), combining the extracts. Concentrate to a small volume (about 5 mL), using a rotary evaporator.
Chromatographic system
Adsorbent: 0.25-mm layer of chromatographic silica gel, typically 20-cm long (TLC plates)
Application volume: 20 µL, applied separately as 10-mm bands
Developing solvent system: Butyl alcohol, *n*-propyl alcohol, glacial acetic acid, and water (3:1:1:1)
Derivatization reagent: 0.2% Solution of ninhydrin in a mixture of butyl alcohol and 2 N acetic acid (19:1)
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Develop the chromatograms until the solvent front has moved up about three-fourths of the plate, in a saturated chamber. Remove the plate, and allow the solvent to evaporate. Spray with the *Derivatization reagent*, heat at 100°–105° for 10 min, and immediately examine the plate under white light.
Acceptance criteria: The chromatogram of the *Sample solution* shows the following zones: a violet zone having an R_F value of about 0.89; a pink zone having an R_F value of about 0.5 and corresponding in color and R_F value to that obtained from the chromatogram of *Standard solution A*; a pinkish zone having an R_F value of about 0.43; a strong orange zone having an R_F value of about 0.38; a pinkish violet zone having an R_F value of about 0.3 and corresponding in color and R_F value to that of the chromatogram of *Standard solution B*; and additional pinkish orange zones situated very close to each other just below the zone corresponding to alliin in the chromatogram of *Standard solution B*.

• **B.**
Sample: About 10 g of garlic bulbs that have been cut into small pieces
Analysis: Transfer to a suitable flask. Add 10 mL of 1 N sodium hydroxide and 10 mL of water, heat the flask in boiling water for 10 min, cool, and filter. Add a few drops of freshly prepared sodium nitroferricyanide TS to 2 mL of the filtrate.
Acceptance criteria: The appearance of a red or orange-red color indicates the presence of sulfur-containing compounds in the *Sample*.

• **C.** The retention time of the major peak in the *Sample solution* corresponds to that of one of the alliin diastereomer peaks in the *Standard solution*, as obtained in the test for *Content of Alliin*.

• **D. Thin-Layer Chromatography**
Extraction column: 1-cm × 5-cm solid-phase extraction column containing styrene-divinylbenzene copolymer packing with a 75- to 150-µm diameter and a 400- to 600-Å pore size. Condition column before use by washing with 50 mL of methanol and with 50 mL of a mixture of methanol and water (3:7). [Note—Do not allow the column to dry.]
Standard solution: 0.2 mg/mL each of USP β-Chlorogenin RS and USP Agigenin RS in methanol
Sample solution: Transfer about 10 g of freshly peeled garlic cloves to a 37-mL homogenizing cup, and homogenize with 25 mL of methanol at the highest speed for 1 min. Centrifuge the mixture, and decant the supernatant to a flask. Add 70 mL of water. Transfer to the *Extraction column*, allow to drain, and discard the eluate. Wash the column with 50 mL of a mixture of methanol and water (3:7), allow the solvent mixture to drain, and discard the eluate. Finally, elute the crude saponin fraction off the column with 20 mL of methanol, collect the eluate, and evaporate to dryness. Dissolve the residue in 4 mL of a mixture of 8% sulfuric acid and alcohol (1:1), transfer the solution to a screw-capped test tube, and heat on a boiling water bath for 5 h. Cool the test tube, add 20 mL of water, and transfer the solution to a freshly conditioned *Extraction column*, allow to drain, and discard the eluate. Wash the column with 30 mL of a mixture of methanol and water (7:3), and discard the eluate. Finally, elute the column with 50 mL of methanol. Collect the eluate, evaporate it to dryness, and dissolve the residue in 0.5 mL of methanol.

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Chromatographic system
Adsorbent: 0.25-mm layer of chromatographic silica gel, typically 20-cm long (TLC plates)
Application volume: 20 µL, as 7-mm bands
Developing solvent system: Methylene chloride and methanol (15:2)
Derivatization reagent: Dissolve 0.5 mL of 4-methoxybenzaldehyde and 0.5 mL of sulfuric acid in sufficient alcohol to make 10 mL.
Analysis
Samples: *Standard solution* and *Sample solution*
Develop the chromatograms until the solvent front has moved up about three-fourths of the plate, in a saturated chamber. Remove the plate, and allow the solvent to evaporate. Spray the plate with *Derivatization reagent*, heat the plate at 100°–105° for 5 min, and examine the plate under white light.
Acceptance criteria: The chromatogram of the *Sample solution* exhibits, among several yellowish and grayish green spots, a grayish green spot at an R_F value of about 0.4, corresponding to the grayish green spot due to β-chlorogenin of the *Standard solution*. The chromatogram of the *Sample solution* does not exhibit a spot at an R_F value of about 0.2, corresponding to agigenin of the *Standard solution*.

COMPOSITION

• **CONTENT OF ALLIIN**
Allinase inhibitor solution: Dissolve 109 mg of carboxymethoxylamine hemihydrochloride in 100.0 mL of water.
Solution A: Monobasic sodium phosphate 0.045 M in water, adjusted with 0.2 M sodium hydroxide to a pH of 7.1
Buffer: Monobasic sodium phosphate 0.05 M in water, adjusted with 0.2 M sodium hydroxide to a pH of 9.5
Derivatization reagent: Dissolve 140 mg of *o*-phthaldialdehyde in 5 mL of methanol, add 100 µL of *t*-butylthiol, and dilute with *Buffer* to 50 mL. [NOTE—This reagent may occasionally become opaque during preparation. Store at room temperature, and use within 1 week.]
Mobile phase: Acetonitrile, 1,4-dioxane, tetrahydrofuran, and *Solution A* (25: 2.9: 2.2: 69.9)
Standard solution: 0.05 mg/mL of USP Alliin RS in a mixture of methanol and water (1:1)
Sample stock solution: Transfer about 10.0 g of freshly peeled garlic cloves, accurately weighed, to a 110-mL homogenizing cup. Add 70.0 mL of *Allinase inhibitor solution*, and blend at the highest speed for 30 s. Centrifuge, and decant the supernatant into a 100-mL volumetric flask. Mix the remaining solids in the cup with 20 mL of *Allinase inhibitor solution*, centrifuge, and add the supernatant to the volumetric flask. Dilute the contents of the flask with *Allinase inhibitor solution* to volume.
Sample solution: Dilute a portion of the *Sample stock solution* 1 in 10 with a mixture of methanol and water (1:1).
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 337 nm
Column: 4-mm × 10-cm; packing L1
Flow rate: 1 mL/min
Injection volume: 10 µL
System suitability
Sample: *Standard solution*
[NOTE—Alliin exhibits two major peaks representing its diastereomers.]
Suitability requirements
Relative standard deviation: NMT 2.0% for each of the major peaks, in repeated injections
Analysis
Samples: *Standard solution* and *Sample solution*
Using a syringe, transfer 0.1 mL of the *Standard solution* or *Sample solution* to separate septum-capped vials, and add 0.5 mL of the *Derivatization reagent* to each vial. Allow a reaction time of NLT 2 min before injection into the chromatograph. Record the chromatograms, and measure the areas of the alliin diastereomer peaks.
Calculate the percentage of alliin in the portion of Garlic taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times 100$$

r_U = peak area of alliin from the *Sample solution*
r_S = peak areas of alliin diastereomers from the *Standard solution*
C_S = concentration of USP Alliin RS in the *Standard solution* (mg/mL)
V = volume of the *Sample stock solution* (mL)
W = weight of Garlic used to prepare the *Sample stock solution* (mg)
D = dilution factor to prepare the *Sample solution* from the *Sample stock solution*, 10

Acceptance criteria: NLT 0.5% on the dried basis

• **CONTENT OF γ-GLUTAMYL-(*S*)-ALLYL-L-CYSTEINE**
Solution A: Dissolve 6.80 g of monobasic potassium phosphate in 900 mL of water, and adjust with phosphoric acid to a pH of 2.6. Dilute with water to 1000.0 mL, and mix.
Mobile phase: Methanol and *Solution A* (3:17)
Standard solution: 0.08 mg/mL of USP γ-Glutamyl-(*S*)-allyl-L-cysteine RS in a mixture of methanol and water (1:1)
Sample solution: Transfer about 10 g of freshly peeled garlic cloves, accurately weighed, to a 110-mL homogenizing cup. Add 80 mL of a mixture of methanol and water (1:1), and homogenize at the highest speed for 1 min. Centrifuge the mixture, and decant the supernatant into a 250-mL volumetric flask. Mix the remaining solids with two 70-mL portions of a mixture of methanol and water (1:1), centrifuge, and transfer the supernatants to the volumetric flask. Dilute the contents of the flask with a mixture of methanol and water (1:1) to volume.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 205 nm
Column: 4.6-mm × 15-cm; packing L1
Flow rate: 0.8 mL/min
Injection volume: 10 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 2.0% for the γ-glutamyl-(*S*)-allyl-L-cysteine peak in repeated injections
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of γ-glutamyl-(*S*)-allyl-L-cysteine in the portion of Garlic taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak response of γ-glutamyl-(*S*)-allyl-L-cysteine from the *Sample solution*

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r_S = peak response of γ-glutamyl-(*S*)-allyl-L-cysteine from the *Standard solution*
C_S = concentration of USP γ-Glutamyl-(*S*)-allyl-L-cysteine RS in the *Standard solution* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Garlic used to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 0.2% on the dried basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: Subglobular compound bulbs, 3–5 cm in width, consisting of 8–20 cloves, the whole surrounded by 2–5 layers of white scale leaves attached to a flattened, circular base; cloves ovoid and 3- to 4-sided, summit acute, narrowed into a threadlike portion of fiber base, truncate, each clove covered with a white scale leaf and a pinkish white epidermis, easily separated from the solid portion, consisting of two flaky scale leaves and two yellowish green conduplicate foliage leaves

Microscopic: The protective leaf contains an epidermis enclosing a mesophyll free from chlorophyll. The outer epidermis consists of lignified sclereid cells of thick, pitted walls, elongated, covered with thin cuticle, long fibers up to 500 µm in length and 30 µm in width.

The cortical cells are thick-walled, nonlignified, tending to collapse on maturity, isodiametric, and contain purple pigments. The vascular bundles consist of lignified spiral and annular vessels. The storage leaves show an outer epidermis of thin, delicate cells of variable shape, arranged in somewhat irregular rows, 60 µm in length and 30 µm in width. Stomata are present on the outer epidermis only at the extreme tip near the base of the foliage leaves.

The mesophyll consists of swollen storage parenchyma cells filled with fine granular reserve material; scattered in the cortex are 20 laticiferous tubes, 500–1000 µm in length. Two series of vascular bundles consisting of narrow lignified spiral and annular vessels are arranged in the mesophyll.

- **ARTICLES OF BOTANICAL ORIGIN,** *Water Content* ⟨561⟩**:** NMT 65.0% for fresh bulbs, and NMT 7.0% for dried bulbs
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 5.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Store in well-closed containers in a cool, dry place, protected from light.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Agigenin RS
USP Alliin RS
USP β-Chlorogenin RS
USP γ-Glutamyl-(*S*)-allyl-L-cysteine RS
USP L-Methionine RS

Powdered Garlic

DEFINITION

Powdered Garlic is produced from Garlic that has been cut, freeze-dried or dried at a temperature not exceeding 65°, and powdered. It contains NLT 0.3% of alliin and NLT 0.1% of γ-glutamyl-(*S*)-allyl-L-cysteine, calculated on the dried basis.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHY**

Standard solution A: 0.5 mg/mL of USP L-Methionine RS in a mixture of methanol and water (1:1)
Standard solution B: 0.5 mg/mL of USP Alliin RS in a mixture of methanol and water (1:1)
Sample solution: Transfer 1 g of the Powdered Garlic to an extractor, and extract with two 20-mL portions of a mixture of methanol and water (1:1), combining the extracts. Concentrate to a small volume (about 5 mL), using a rotary evaporator.
Chromatographic system
Adsorbent: 0.25-mm layer of chromatographic silica gel, typically 20 cm long (TLC plates)
Application volume: 20 µL, applied separately as 10-mm bands
Developing solvent system: Butyl alcohol, *n*-propyl alcohol, glacial acetic acid, and water (3:1:1:1)
Derivatization reagent: 0.2 in 100 solution of ninhydrin in a mixture of butyl alcohol and 2 N acetic acid (19:1)
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Develop the chromatograms until the solvent front has moved up about three-fourths of the plate, in a saturated chamber. Remove the plate, and allow the solvent to evaporate. Spray with *Derivatization reagent*, heat at 100°–105° for 10 min, and immediately examine the plate under white light.
Acceptance criteria: The chromatogram of the *Sample solution* shows the following zones: a violet zone having an R_F value of about 0.89; a pink zone having an R_F value of about 0.5 and corresponding in color and R_F value to that obtained from the chromatogram of *Standard solution A*; a pinkish zone having an R_F value of about 0.43; a strong orange zone having an R_F value of about 0.38; a pinkish violet zone having an R_F value of about 0.3 and corresponding in color and R_F value to that of the chromatogram of *Standard solution B*; and additional pinkish orange zones situated very close to each other just below the zone corresponding to alliin in the chromatogram of *Standard solution B*.

- **B.**

Sample: About 10 g of Powdered Garlic
Analysis: Transfer to a suitable flask. Add 10 mL of 1 N sodium hydroxide and 10 mL of water, heat the flask in boiling water for 10 min, cool, and filter. Add a few drops of freshly prepared sodium nitroferricyanide TS to 2 mL of the filtrate.
Acceptance criteria: The appearance of a red or orange-red color indicates the presence of sulfur-containing compounds in the *Sample*.

- **C.** The retention time of the major peak in the *Sample solution* corresponds to that of one of the alliin diastereomer peaks in the *Standard solution*, as obtained in the test for *Content of Alliin*.
- **D. THIN-LAYER CHROMATOGRAPHY**

Extraction column: 1-cm × 5-cm solid-phase extraction column containing styrene-divinylbenzene copolymer packing with a 75- to 150-µm diameter and a 400- to

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600-Å pore size. Condition column before use by washing with 50 mL of methanol and with 50 mL of a mixture of methanol and water (3:7). [NOTE—Do not allow the column to dry.]

Standard solution: 0.2 mg/mL each of USP β-Chlorogenin RS and USP Agigenin RS in methanol

Sample solution: Transfer about 10 g of Powdered Garlic to a 37-mL homogenizing cup, and homogenize with 25 mL of methanol at the highest speed for 1 min. Centrifuge the mixture, and decant the supernatant to a flask. Add 70 mL of water. Transfer to the *Extraction column*, allow to drain, and discard the eluate. Wash the column with 50 mL of a mixture of methanol and water (3:7), allow the solvent mixture to drain, and discard the eluate. Finally, elute the crude saponin fraction off the column with 20 mL of methanol, collect the eluate, and evaporate to dryness. Dissolve the residue in 4 mL of a mixture of 8% sulfuric acid and alcohol (1:1), transfer the solution to a screw-capped test tube, and heat on a boiling water bath for 5 h. Cool the test tube, add 20 mL of water, and transfer the solution to a freshly conditioned *Extraction column*, allow to drain, and discard the eluate. Wash the column with 30 mL of a mixture of methanol and water (7:3), and discard the eluate. Finally, elute the column with 50 mL of methanol. Collect the eluate, evaporate it to dryness, and dissolve the residue in 0.5 mL of methanol.

Chromatographic system

Adsorbent: 0.25-mm layer of chromatographic silica gel, typically 20 cm long (TLC plates)

Application volume: 20 µL, as 7-mm bands

Developing solvent system: Methylene chloride and methanol (15:2)

Derivatization reagent: Dissolve 0.5 mL of 4-methoxybenzaldehyde and 0.5 mL of sulfuric acid in sufficient alcohol to make 10 mL.

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the chromatograms until the solvent front has moved up about three-fourths of the plate, in a saturated chamber. Remove the plate, and allow the solvent to evaporate. Spray the plate with *Derivatization reagent*, heat the plate at 100°–105° for 5 min, and examine the plate under white light.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits, among several yellowish and grayish green spots, a grayish green spot at an R_F value of about 0.4, corresponding to the grayish green spot due to β-chlorogenin of the *Standard solution*. The chromatogram of the *Sample solution* does not exhibit a spot at an R_F value of about 0.2, corresponding to agigenin of the *Standard solution*.

COMPOSITION

• **CONTENT OF ALLIIN**

Alliinase inhibitor solution: Dissolve 109 mg of carboxymethoxylamine hemihydrochloride in 100.0 mL of water.

Solution A: 0.045 M monobasic sodium phosphate in water. Adjust with 0.2 M sodium hydroxide to a pH of 7.1.

Buffer: 0.05 M monobasic sodium phosphate in water. Adjust with 0.2 M sodium hydroxide to a pH of 9.5.

Derivatization reagent: Dissolve 140 mg of *o*-phthaldialdehyde in 5 mL of methanol, add 100 µL of *t*-butylthiol, and dilute with *Buffer* to 50 mL. [NOTE—This reagent may occasionally become opaque during preparation. Store at room temperature, and use within 1 week.]

Mobile phase: Acetonitrile, 1,4-dioxane, tetrahydrofuran, and *Solution A* (25: 2.9: 2.2: 69.9)

Standard solution: 0.05 mg/mL of USP Alliin RS in a mixture of methanol and water (1:1)

Sample stock solution: Transfer about 1.0 g of Powdered Garlic, accurately weighed, to a flask. Add 30.0 mL of *Alliinase inhibitor solution*, and shake vigorously until the powder is fully dispersed. Centrifuge to obtain a clear solution.

Sample solution: Transfer 5.0 mL of the *Sample stock solution* to a 10-mL volumetric flask, and dilute with *Alliinase inhibitor solution* to volume.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 337 nm

Column: 4-mm × 10-cm; packing L1

Flow rate: 1 mL/min

Injection volume: 10 µL

System suitability

Sample: *Standard solution*

Suitability requirements

[NOTE—Alliin exhibits two major peaks representing its diastereomers.]

Relative standard deviation: NMT 2.0% for each of the major peaks, in repeated injections

Analysis

Samples: *Standard solution* and *Sample solution*

Using a syringe, transfer 0.1 mL of the *Standard solution* or the *Sample solution* to separate septum-capped vials, add 0.5 mL of the *Derivatization reagent* to each vial, and mix. Allow a reaction time of NLT 2 min before injection into the chromatograph. Record the chromatograms, and measure the areas of the alliin diastereomer peaks.

Calculate the percentage of alliin in the portion of Powdered Garlic taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times 100$$

r_U = peak area of alliin from the *Sample solution*

r_S = sum of the peak areas of alliin diastereomers from the *Standard solution*

C_S = concentration of USP Alliin RS in the *Standard solution* (mg/mL)

V = volume of the *Sample stock solution* (mL)

W = weight of Powdered Garlic used to prepare the *Sample stock solution* (mg)

D = dilution factor to prepare the *Sample solution* from the *Sample stock solution*, 2

Acceptance criteria: NLT 0.3% on the dried basis

• **CONTENT OF γ-GLUTAMYL-(*S*)-ALLYL-L-CYSTEINE**

Solution A: Dissolve 6.80 g of monobasic potassium phosphate in 900 mL of water, and adjust with phosphoric acid to a pH of 2.6. Dilute with water to 1000.0 mL, and mix.

Mobile phase: Methanol and *Solution A* (3:17)

Standard solution: 0.08 mg/mL of USP γ-Glutamyl-(*S*)-allyl-L-cysteine RS in a mixture of methanol and water (1:1)

Sample solution: Transfer about 1.0 g of Powdered Garlic, accurately weighed, to a 50-mL volumetric flask. Add 30 mL of methanol and water (1:1), and shake vigorously until the powder is fully dispersed. Dilute the contents of the flask with a mixture of methanol and water (1:1) to volume. Centrifuge to obtain a clear solution.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 205 nm

Column: 4.6-mm × 15-cm; packing L1

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Flow rate: 0.8 mL/min
Injection volume: 10 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 2.0% for the γ-glutamyl-(*S*)-allyl-L-cysteine peak in repeated injections
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of γ-glutamyl-(*S*)-allyl-L-cysteine in the portion of Powdered Garlic taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak response for γ-glutamyl-(*S*)-allyl-L-cysteine from the *Sample solution*
r_S = peak response for γ-glutamyl-(*S*)-allyl-L-cysteine from the *Standard solution*
C_S = concentration of USP γ-Glutamyl-(*S*)-allyl-L-cysteine RS in the *Standard solution* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Powdered Garlic used to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 0.1% on the dried basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS:** Under a microscope, Powdered Garlic shows numerous fragments of parenchyma with large cells containing crystals of calcium oxalate and small triangular or quadrangular intercellular spaces at the corners; spiral vessels accompanied by subquadratic cells; elongated epidermal cells with thick, pitted walls.
- **ABSENCE OF STARCH:** A water slurry of Powdered Garlic shows no blue color when iodine TS is added.
- **LOSS ON DRYING** ⟨731⟩
  Sample: 1.0 g of Powdered Garlic
  Analysis: Dry the *Sample* at 105° for 2 h.
  Acceptance criteria: NMT 7.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 5.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Store in well-closed containers in a cool, dry place, protected from light.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Agigenin RS
  USP Alliin RS
  USP β-Chlorogenin RS
  USP γ-Glutamyl-(*S*)-allyl-L-cysteine RS
  USP L-Methionine RS

Powdered Garlic Extract

DEFINITION

Powdered Garlic Extract is prepared from fresh Garlic bulbs by extraction with alcohol. The ratio of the starting crude plant material to Powdered Extract is 9.5:1–13.5:1. It contains NLT 4.0% of alliin ($C_6H_{11}NO_3S$). It may contain added Powdered Garlic or other suitable substances.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**
  Standard solution A: 0.5 mg/mL of USP L-Methionine RS in a mixture of methanol and water (1:1)
  Standard solution B: 0.5 mg/mL of USP Alliin RS in a mixture of methanol and water (1:1)
  Sample solution: Transfer a quantity of Powdered Extract, equivalent to about 5 mg of alliin, to a suitable container. Add 40 mL of a mixture of methanol and water (1:1), and shake until the powder is fully dispersed. Centrifuge, and decant the supernatant into a round-bottomed flask. Concentrate to a small volume (about 5 mL) using a rotary evaporator.
  Adsorbent: 0.25-mm layer of chromatographic silica gel, typically 20 cm long (TLC plates).
  Application volume: 20 µL, applied separately as 10-mm bands
  Developing solvent system: Butyl alcohol, *n*-propyl alcohol, glacial acetic acid, and water (3:1:1:1)
  Spray reagent: 0.2% solution of ninhydrin in a mixture of butyl alcohol and 2 N acetic acid (19:1)
  Analysis
  Samples: *Standard solutions* and *Sample solution*
  Develop the chromatograms until the solvent front has moved up about three-fourths of the plate, in a saturated chamber. Remove the plate, and allow the solvent to evaporate. Spray with the *Spray reagent*, heat at 100°–105° for 10 min, and immediately examine the plate.
  Acceptance criteria: The chromatogram of the *Sample solution* shows the following orange and pinkish violet zones: a violet zone having an R_F value of about 0.89; a pink zone having an R_F value of about 0.5 and corresponding in color and R_F value to that of the chromatogram of *Standard solution A*; a pinkish zone having an R_F value of about 0.43; a strong orange zone having an R_F value of about 0.38; a pinkish violet zone having an R_F value of about 0.3 and corresponding in color and R_F value to that of the chromatogram of *Standard solution B*; and additional pinkish orange zones situated very close to each other just below the zone attributed to alliin in the chromatogram of *Standard solution B*.
- **B.** The retention time of the alliin peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Alliin*.

COMPOSITION

- **CONTENT OF ALLIIN**
  Allinase inhibitor solution: 1.09 mg/mL of carboxymethoxylamine hemihydrochloride
  Solution A: Monobasic sodium phosphate 0.045 M in water adjusted with 0.2 M sodium hydroxide to a pH of 7.1
  Buffer: Monobasic sodium phosphate 0.05 M in water adjusted with 0.2 M sodium hydroxide to a pH of 9.5
  Derivatization reagent: Dissolve 140 mg of *o*-phthaldialdehyde in 5 mL of methanol. Add 100 µL of *t*-butyl thiol, dilute with *Buffer* to 50 mL, and mix. [NOTE—This reagent may occasionally become opaque during preparation. Store at room temperature, and use within 1 week.]

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Mobile phase: Acetonitrile, 1,4-dioxane, tetrahydrofuran, and *Solution A* (25: 2.9: 2.2: 69.9)
Standard solution: 0.05 mg/mL of USP Alliin RS in a mixture of methanol and water (1:1)
Sample solution: Transfer about 0.10 g of Powdered Extract, accurately weighed, to a 50-mL volumetric flask. Add 30 mL of *Allinase inhibitor solution*, and shake until the Powdered Extract is fully dispersed. Dilute with *Allinase inhibitor solution* to volume. Centrifuge, transfer 5 mL of the clear supernatant to a 10-mL volumetric flask, and dilute with *Allinase inhibitor solution* to volume.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 337 nm
Column: 4-mm × 10-cm; packing L1
Flow rate: 1 mL/min
Injection size: 10 µL
System suitability
Sample: *Standard solution*
Suitability requirements
[NOTE—Alliin exhibits two major peaks representing its diastereomers.]
Relative standard deviation: NMT 2.0% for each of the major peaks
Analysis
Samples: *Standard solution* and *Sample solution*
Using a volumetric syringe, transfer 0.1 mL of the *Sample solution* or the *Standard solution* to separate septum-capped vials, and add 0.5 mL of the *Derivatization reagent* to each vial. Allow a reaction time of NLT 2 min before injection into the chromatograph. Record the chromatograms, and measure the areas of the alliin diastereomer peaks.
Calculate the percentage of alliin in the portion of Powdered Extract taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of alliin from the *Sample solution*
r_S = peak areas of alliin diastereomers from the *Standard solution*
C_S = concentration of USP Alliin RS in the *Standard solution* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Powdered Garlic Extract used to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 4.0% on the dried basis

CONTAMINANTS
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS
- **ALLIINASE ACTIVITY**

Allinase inhibitor solution, Solution A, Buffer, Derivatization reagent, Mobile phase, Standard solution, Chromatographic system, and **Analysis:** Proceed as directed in the test for *Content of Alliin*.
Sample solution: Incubate 200 mg of Powdered Extract with 20 mL of water at room temperature for 5 min. Immediately after incubation, add 80.0 mL of *Allinase inhibitor solution*, mix, and centrifuge.
Acceptance criteria: The area of the alliin peak of the *Sample solution* is NMT 1% of the area of the alliin peak of the *Standard solution*.
- **ALCOHOL DETERMINATION,** *Method II* ⟨611⟩**:** NMT 0.5%
- **ARTICLES OF BOTANICAL ORIGIN,** *Water Content* ⟨561⟩**:** NMT 5.0%
- **OTHER REQUIREMENTS:** Meets the requirements under *Botanical Extracts* ⟨565⟩, *Packaging and Storage* and *Pesticide Residues*

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers, in a cool place, protected from light.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. The label also indicates the content of alliin, the extracting solvent or solvent mixture used for preparation, and the ratio of the starting crude plant material to Powdered Extract. It meets the requirements under *Botanical Extracts* ⟨565⟩, *Labeling*.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Alliin RS
USP L-Methionine RS

Garlic Fluidextract

DEFINITION
Garlic Fluidextract is prepared as follows. Soak 1000 g of Garlic, whole or sliced, in a volume of a mixture of water and alcohol (between 80:20 and 50:50) sufficient to cover the cloves. Store in a suitable container for a length of time sufficient to extract the constituents, avoiding any contamination, and then filter. Concentrate the filtrate, if necessary, at the lowest possible temperature, and add sufficient water or alcohol to make the product measure 1000 mL. [NOTE—Complete extraction may require 30 days.]

IDENTIFICATION
- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Extraction column: Use a solid-phase extraction column that contains benzenesulfonylpropyl bonded to silica gel in the hydrogen form having a sorbent mass-to-column volume ratio of 1 g per 6 mL, or equivalent. Condition the column before use by washing with 10 mL of methanol and 10 mL of water. [NOTE—Do not allow the column to dry.]
Standard solution: 0.5 mg/mL of USP *S*-Allyl-l-cysteine RS in a mixture of methanol and water (1:1)
Sample solution: Mix 1 mL of Fluidextract and 5 mL of water, and transfer to the *Extraction column*. Allow to drain, and discard the eluate. Wash the column with 10 mL of water and 10 mL of methanol, discarding the eluates. Elute the amino acid fraction with 3 mL of ammonium hydroxide solution in methanol (7 in 100), and collect the eluate.
Adsorbent: 0.25-mm layer of chromatographic silica gel, typically 20 cm long (TLC plates)
Application volume: 5 µL
Developing solvent system: Ethyl acetate, methanol, acetone, glacial acetic acid, and water (10:4:3:1:3)
Spray reagent: Iodoplatinate TS
Analysis
Samples: *Standard solution* and *Sample solution*
Develop the chromatograms until the solvent front has moved up about three-fourths of the plate, in a saturated chamber. Remove the plate, and allow the solvent to evaporate. Spray with the *Spray reagent*, and examine the plate.

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Acceptance criteria: The chromatogram of the *Sample solution* exhibits, among several yellow spots on the purple plate, a yellow spot at an R_F value of about 0.4 corresponding to that of the yellow spot obtained in the chromatogram of the *Standard solution* (presence of *S*-allyl-L-cysteine).

COMPOSITION

• **CONTENT OF *S*-ALLYL- L-CYSTEINE**

Mobile phase: Transfer 15.8 g of sodium citrate dihydrate to 250 mL of water, and carefully add 10.5 mL of hydrochloric acid. Using a pH meter, adjust with 6 N sodium hydroxide to a pH of 4.0. Dilute with water to 1000 mL, and mix.

Derivatizing reagent: Dissolve 0.8 g of *o*-phthalal dehyde in 2 mL of 2-mercaptoethanol. Add to a solution containing 24.70 g of boric acid and 22.35 g of potassium hydroxide in 1000 mL of water, and mix.

Reactivating solution: 0.2 N sodium hydroxide. Prepare by dissolving 0.8 g of sodium hydroxide in 100 mL of water.

Standard solution: 0.01 mg/mL of USP *S*-Allyl-l-cysteine RS in water

Sample solution: Transfer about 2.0 g of Fluidextract, accurately weighed, to a 100-mL volumetric flask, dilute with trichloroacetic acid solution (5 in 100) to volume, and mix. Centrifuge for 5 min, and filter the supernatant.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Fluorometric detector; excitation wavelength of 340 nm and emission wavelength of 455 nm

Column: 4.6-mm × 12-cm; packing L17

Column temperature: 40°

Injection size: 10 µL

[NOTE—The *Mobile phase* and the *Reactivating solution* are pumped separately, each at the rate of 0.4 mL/min, by pumps connected to the opposing arms of a tee. The outlet of the tee is connected to the injector and the chromatographic column. The outlet of the column is attached to a tee, the opposing arm of which is attached to a tube from which the *Derivatizing reagent* is constantly pumped through the system at a rate of 0.6 mL/min. The outlet of the tee is connected to a 0.5-mm × 2.0-m postcolumn polytef reaction coil maintained at 40°. The outlet of the reaction coil is connected to the detector. The system is programmed to deliver the *Mobile phase* for 10 min, the *Reactivating solution* for the next 6 min, and the *Mobile phase* for the 24 min remaining before the next injection.]

System suitability

Sample: *Standard solution*

Suitability requirements

Capacity factor (k′): 2.5–4.5

Tailing factor: NMT 2.0 for the *S*-allyl-L-cysteine peak

Relative standard deviation: NMT 2.0% for the *S*-allyl-L-cysteine peak in repeated injections

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of *S*-allyl-L-cysteine ($C_6H_{11}SN$) in the portion of Fluidextract taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak height of *S*-allyl-L-cysteine from the *Sample solution*

r_S = peak height of *S*-allyl-L-cysteine from the *Standard solution*

C_S = concentration of the USP *S*-Allyl-l-cysteine RS in the *Standard solution* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Fluidextract used to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 0.05% on the dried basis

CONTAMINANTS

• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^3 cfu/mL, and the total combined molds and yeasts count does not exceed 10^2 cfu/mL.

• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

• **RESIDUE ON EVAPORATION:** Proceed as directed under *Botanical Extracts* ⟨565⟩: NLT 20% of the Fluidextract portion taken remains as residue.

• **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 3.0%

• **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩ NMT 0.2%

• **pH** ⟨791⟩**:** 4.5–6.5

• **OTHER REQUIREMENTS:** Meets the requirements under *Botanical Extracts* ⟨565⟩, *General Pharmacopeial Requirements*, sections for *Packaging and Storage*, *Labeling*, *Pesticide Residues*, and *Alcohol Content* for Fluidextracts

ADDITIONAL REQUIREMENTS

• **USP REFERENCE STANDARDS** ⟨11⟩

USP *S*-Allyl-l-cysteine RS

Garlic Delayed-Release Tablets

DEFINITION

Garlic Delayed-Release Tablets are prepared from Powdered Garlic or Powdered Garlic Extract and contain NLT 90.0% and NMT 140.0% of the labeled amount of alliin ($C_6H_{11}NO_3S$) and NLT 90.0% and NMT 140.0% of the labeled amount of potential allicin ($C_6H_{10}OS_2$).

IDENTIFICATION

• **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution A: 0.5 mg/mL of USP L-Methionine RS

Standard solution B: 0.5 mg/mL of USP Alliin RS, in a mixture of methanol and water (1:1)

Sample solution: Transfer an amount of pulverized Tablets, equivalent to 30 mg of alliin, to a 100-mL volumetric flask. Add 70 mL of a mixture of methanol and water (1:1), shake, and centrifuge. Concentrate to a small volume (about 5 mL) using a rotary evaporator.

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Application volume: 20 µL, applied separately as 10-mm bands

Developing solvent system: Butyl alcohol, *n*-propyl alcohol, glacial acetic acid, and water (3:1:1:1)

Spray reagent: 2 mg/mL of ninhydrin, in a mixture of butyl alcohol and 2 N acetic acid (19:1)

Analysis

Samples: *Standard solutions* and *Sample solution*

Proceed as directed in the chapter. Spray with the *Spray reagent*, heat at 100°–105° for 10 min, and immediately examine the plate.

Acceptance criteria: The chromatogram of the *Sample solution* shows the following orange and pinkish violet zones: a violet zone having an R_F value of 0.89; a pink zone

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having an R_F value of 0.5 and corresponding in color and R_F value to that of the chromatogram of *Standard solution A*; a pinkish zone having an R_F value of 0.43; a strong orange zone having an R_F value of 0.38; a pinkish violet zone having an R_F value of 0.3 and corresponding in color and R_F value to that of the chromatogram of *Standard solution B*; and additional pinkish orange zones situated very close to each other, just below the zone attributed to alliin in the chromatogram of *Standard solution B*.

• **B. HPLC IDENTIFICATION TEST**

Analysis: Proceed as directed in the test for *Content of Alliin*.

Acceptance criteria: The *Sample solution* exibits a peak for alliin corresponding to one of the diasteroisomer pairs of peaks in the *Standard solution*.

STRENGTH

• **CONTENT OF ALLIIN**

Alliinase inhibitor solution: Dissolve 109 mg of carboxymethoxylamine hemihydrochloride in 100.0 mL of water.

Solution A: Dissolve 1.24 g of monobasic sodium phosphate in 100 mL of water, adjust with 0.2 M sodium hydroxide to a pH of 7.1, and dilute with water to 200.0 mL.

Buffer: Dissolve 1.38 g of monobasic sodium phosphate in 100 mL of water, adjust with 0.2 M sodium hydroxide to a pH of 9.5, and dilute with water to 200.0 mL.

Derivatization reagent: Dissolve 140 mg of *o*-phthaldialdehyde in 5 mL of methanol, add 100 µL of *t*-butylthiol, and dilute with *Buffer* to 50 mL. [NOTE—This reagent may occasionally become opaque during preparation. Store at room temperature, and use within 1 week.]

Mobile phase: Acetonitrile, 1,4-dioxane, tetrahydrofuran, and *Solution A* (25: 2.9: 2.2: 69.9)

Standard solution: 0.05 mg/mL of USP Alliin RS in a mixture of methanol and water (1:1). Use a syringe to transfer 0.1 mL of this solution to a septum-capped vial; and add 0.5 mL of the *Derivatization reagent*. Allow a reaction time of NLT 2 min before injection into the chromatograph.

Sample solution: Pulverize a counted number of Tablets, equivalent to 50 mg of alliin, with a mortar and pestle. Transfer a quantity of powder equivalent to 5 mg of alliin to a 100-mL volumetric flask. Add 70 mL of *Alliinase inhibitor solution*, and shake for 1 min. Dilute with *Alliinase inhibitor solution* to volume. Use a volumetric syringe to transfer 0.1 mL of this solution to a septum-capped vial, and add 0.5 mL of the *Derivatization reagent*. Allow a reaction time of NLT 2 min before injection into the chromatograph.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 337 nm

Column: 4-mm × 10-cm; packing L1

Flow rate: 1 mL/min

[NOTE—Alliin exhibits two major peaks, representing its diastereomers.]

Injection size: 10 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0% for each of the major peaks

Analysis

Samples: *Standard solution* and *Sample solution*

[NOTE—Record the chromatograms, and measure the areas of the responses of the alliin diastereomer peaks.]

Calculate the percentage of alliin in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area for alliin from the *Sample solution*

r_S = sum of the peak area for alliin diastereomers from the *Standard solution*

C_S = concentration of USP Alliin RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of alliin in the *Sample solution* (µg/mL)

Acceptance criteria: 90%–140.0%

• **CONTENT OF POTENTIAL ALLICIN**

Alliinase inhibitor solution: Dissolve 109 mg of carboxymethoxylamine hemihydrochloride in 100.0 mL of water.

Crude alliinase solution: Homogenize 5 g of raw garlic cloves with 25 mL of water. Filter, and extract three times with 50 mL of *tert*-butyl methyl ether. Discard the organic phase, and remove the residual solvent from the aqueous phase by rotary evaporation in vacuum for 5 min. Filter, and store frozen in small vials. [NOTE—This solution is stable for 6 months when stored as directed. Thaw at room temperature just before use.]

Mobile phase: Methanol and water (3:2)

Standard stock solution: 50 µg/mL of USP Alliin RS

Standard solution: Transfer 1.0 mL of the *Standard stock solution* to a 5-mL volumetric flask containing 100 µL of *Crude alliinase solution*, and allow to stand for 5 min at room temperature. Dilute with water to volume, and pass through a filter having a 0.45-µm or finer pore size.

Sample solution: Transfer an equivalent to 5 mg of potential allicin, from finely powdered Tablets (NLT 20), to a 200-mL volumetric flask, and add 25 mL of water. Incubate at room temperature for exactly 30 min. Stop the enzymatic reaction by diluting with *Alliinase inhibitor solution* to volume. Centrifuge a portion of this solution, transfer 1.0 mL of the supernatant to a 5-mL volumetric flask, and dilute with water to volume.

Blank solution: 100 µL of *Crude alliinase solution* diluted with water to 1 mL

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 240 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection size: 100 µL

System suitability

Samples: *Standard solution*, *Sample solution*, and *Blank solution*

[NOTE—The allicin peak is identified by comparing the chromatograms of the *Blank solution* and the *Standard solution*.]

Suitability requirements

Resolution: NLT 2.0 between the allicin peak and the preceding peak at a relative retention time of 0.80 (allyl methyl thiosulfinates), *Sample solution*

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution*, *Sample solution*, and *Blank solution*

Calculate the percentage of potential allicin in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of allicin, corrected by the response of the *Blank solution*, from the *Sample solution*

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r_S = peak area of allicin, corrected by the response of the *Blank solution*, from the *Standard solution*
C_S = concentration of USP Alliin RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of potential allicin in the *Sample solution* (µg/mL)
M_{r1} = molecular weight of allicin, 162.26
M_{r2} = twice the molecular weight of alliin, 354.42

Acceptance criteria: 90.0%–140.0%

PERFORMANCE TESTS

• **ALLICIN RELEASE:** Proceed as directed in *Dissolution* ⟨711⟩ for *Method A* in *Apparatus 1 and Apparatus 2, Delayed-Release Dosage Forms*. Place a number of Tablets, equivalent to 5 mg of potential allicin, in each vessel.

Apparatus 2: 100 rpm

Time: 60 min for the *Buffer stage*

Crude alliinase solution, Mobile phase, Blank solution, and **Chromatographic system:** Proceed as directed in the test for *Content of Potential Allicin*.

Standard stock solution: 50 µg/mL of USP Alliin RS

Standard solution: Transfer 1.0 mL of the *Standard stock solution* to a 5-mL volumetric flask containing 100 µL of *Crude alliinase solution*, and allow to stand for 5 min at room temperature. Dilute with water to volume, and pass through a membrane filter having a 0.45-µm or finer pore size.

Sample solution: Transfer 1.0 mL of the solution under test to a test tube containing 50 µL of 0.21 M carboxymethoxylamine hemihydrochloride solution. [NOTE—The solution must be transferred immediately upon removal from the dissolution vessel to inhibit the alliinase enzyme.]

Injection size: 100 µL

Analysis

[NOTE—Do not perform the allicin determination in the *Acid stage*.]

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of potential allicin released in the *Buffer stage*:

$$\text{Result} = (r_U/r_S) \times (C_S \times D \times V/L) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of allicin from the *Sample solution*
r_S = peak area of allicin from the *Standard solution*
C_S = concentration of USP Alliin RS in the *Standard solution* (µg/mL)
D = dilution factor for the *Sample solution*, 1.050 (1 mL of the *Sample solution* + 50 µL of 0.21 M carboxymethoxylamine hemihydrochloride solution)
V = volume of final medium, 1000 mL
L = labeled amount of potential allicin (µg/Tablet)
M_{r1} = molecular weight of allicin, 162.26
M_{r2} = twice the molecular weight of alliin, 354.42

Tolerances: It meets the requirements of *Acceptance Table 4* in *Dissolution* ⟨711⟩. [NOTE—*Q* is the percentage of the labeled amount of potential allicin released only in the *Buffer stage*.]

• **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩ **:** Meet the requirements

SPECIFIC TESTS

• **ALLIINASE ACTIVITY**

Alliinase inhibitor solution, Solution A, Buffer, Derivatization reagent, Mobile phase, Standard solution, and **Chromatographic system:** Proceed as directed in the test for *Content of Alliin*.

Sample solution: Transfer an equivalent to 5 mg of alliin, from finely powdered Tablets (NLT 20), to a 100-mL volumetric flask, and add 25 mL of water. Incubate at room temperature for exactly 5 min. Stop the enzymatic reaction by diluting with *Alliinase inhibitor solution* to volume. Centrifuge a portion of this solution, and use a volumetric syringe to transfer 0.1 mL of the supernatant to a septum-capped vial. Add 0.5 mL of the *Derivatization reagent*, and allow a reaction time of NLT 2 min before injection into the chromatograph.

Analysis

Samples: *Standard solution* and *Sample solution*

Proceed as directed in the test for *Content of Alliin*.

Acceptance criteria: The area of the alliin peak from the *Sample solution* is NMT 1% of the area of the alliin peak from the *Standard solution*.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers.

• **LABELING:** The label states the Latin binomial and, following the official name, the article from which the Tablets were prepared. Label it to indicate the amount of total alliin, in µg/Tablet, and the amount of potential allicin, in µg/Tablet.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Alliin RS
USP L-Methionine RS

Ginger

DEFINITION

Ginger is the dried rhizome of *Zingiber officinale* Roscoe (Fam. Zingiberaceae), scraped, partially scraped, or unscraped. It is known in commerce as unbleached ginger.

IDENTIFICATION

• **A.**

Analysis: Pulverize 5 g of Ginger. To 1 g of the pulverized Ginger add 5 mL of dilute acetic acid, prepared by diluting 1 part of glacial acetic acid with 1 part of water, and shake for 15 min. Filter, and add a few drops of ammonium oxalate TS to the filtrate.

Acceptance criteria: NMT a slight turbidity is produced.

• **B.**

Sample: 50 mg of the residue obtained in the test for *Articles of Botanical Origin, Alcohol-Soluble Extractives, Method 2*

Analysis: Dissolve the *Sample* in 25 mL of water, and extract this solution with two 15-mL portions of ether. Combine the ether extracts, and evaporate in a porcelain dish. To the residue add 5 mL of sulfuric acid solution (7.5 in 10.0) and 5 mg of vanillin. Allow to stand for 15 min, and add an equal volume of water.

Acceptance criteria: The solution turns azure blue.

• **C. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 0.2 mg/mL of USP Ginger Constituent Mixture RS in methanol

Standard solution B: 100 mg/mL of USP Powdered Ginger RS in methanol. Sonicate for 10 min, and centrifuge or filter. Use supernatant or filtrate.

Sample solution: Pulverize 5 g of Ginger. Prepare a 100-mg/mL dispersion of Ginger in methanol. Sonicate for 10 min, and centrifuge or filter. Use supernatant or filtrate.

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plate)[1]

[1] Suitable commercially available plates are HPTLC Silica Gel 60 F_{254} from EMD Millipore (e.g., Part No. 1.05642.0001).

USP Monographs

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Application volume: 6 µL of *Standard solution A* and 2 µL each of *Standard solution B* and *Sample solution* as 8-mm bands
Temperature: Ambient, not to exceed 30°
Developing solvent system: Toluene and ethyl acetate (3:1)
Derivatization reagent: To 170 mL of ice-cold methanol add 20 mL of glacial acetic acid, 10 mL of sulfuric acid, and 1 mL of anisaldehyde. Mix well.
System suitability
Samples: *Standard solution A* and *Standard solution B*
Apply the *Samples* and dry in air. Condition at relative humidity of about 33%. Develop in a saturated chamber until the solvent front has migrated over a path of 6 cm. Dry in a current of cold air, and immerse into *Derivatization reagent* for 1 s. Heat for 3 min at 100°, and examine under white light and under long-wave UV (365 nm).
Suitability requirements
Chromatographic pattern: Under white light, the derivatized chromatogram of *Standard solution A* displays two prominent bands: the lower due to 6-gingerol, the upper due to 6-shogaol. Under white light, the derivatized chromatogram of *Standard solution B* shows a succession of dark-violet bands between the origin and the intense dark-brown band corresponding to that of the 6-gingerol in *Standard solution A*. Immediately proximate to the 6-gingerol band in the *Standard solution B* chromatogram, less intense bands due to 8-gingerol and 10-gingerol are observed. A variable number of low-intensity dark-gray bands appear between 10-gingerol and the second prominent band corresponding to 6-shogaol in *Standard solution A*. In the distal part of the chromatogram, a dark-purple somewhat diffuse band is observed. Under long-wave UV (365 nm), the chromatograms of the *Standard solutions* exhibit patterns similar to those observed under white light. The bands due to gingerols and shogaols are bright orange; the bands between the origin and the 6-gingerol band are dark-red to brown, somewhat less prominent than when observed in white light. The bands between 10-gingerol and 6-shogaol are variably colored; frequently, a light-gray band appears halfway between them, with a light-purple diffuse band between it and the orange band due to 6-shogaol. The distal diffuse band assumes a purple-pink hue.
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Treat and examine the *Samples* as described under *System suitability*.
Acceptance criteria: Under white light and under long-wave UV (365 nm), the chromatogram of the *Sample solution* exhibits the band pattern similar to that observed with *Standard solution B*. The band in the distal part of the chromatogram, however, has no diagnostic significance. Its color may range from purple-pink to muddy yellow, or the band may be altogether absent. Potential adulterants lack the band pattern characteristic of the gingerol–shogaol succession. *Kaempferia galanga* L. rhizome shows no diagnostic bands under UV, but under white light a purple band is seen at about two-thirds from the application line. Lesser galangal (*Alpinia officinarum* Hance) rhizome presents a yellow band at an R_F just below the 6-gingerol band, followed by a continuous broad blue smudge, and a distinct tandem of light-orange and yellow bands close to the middle of the plate.

COMPOSITION

• **Content of Gingerols and Gingerdiones**
Solution A: Acetonitrile, dilute phosphoric acid (1 in 1000), and methanol (55:44:1)
Solution B: Acetonitrile
Mobile phase: Use *Solution A* for NLT 7 times the retention time of capsaicin.
Column washing: After each chromatographic run, wash the column, using *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
2	0	100
12	0	100
14	100	0
29	100	0

Standard solution: 0.1 mg/mL of USP Capsaicin RS in methanol
System suitability solution: Reconstitute the content of one vial of USP Ginger Constituent Mixture RS in 1 mL of the *Standard solution*.
Sample solution: Use the filtrate retained from the test for *Articles of Botanical Origin, Alcohol-Soluble Extractives, Method 2*.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 282 nm
Column: 4.6-mm × 25-cm; packing L1
Flow rate: 1 mL/min
Injection volume: 25 µL
System suitability
Samples: *Standard solution* and *System suitability solution*
[Note—The relative retention times for 6-gingerol, capsaicin, and 6-shogaol are about 0.8, 1.0, and 1.9, respectively, *System suitability solution*.]
Suitability requirements
Resolution: NLT 3.0 between the 6-gingerol and capsaicin peaks; NLT 10.0 between the capsaicin and 6-shogaol peaks, *System suitability solution*
Tailing factor: NMT 2.0 for the 6-gingerol, capsaicin, and 6-shogaol peaks, *System suitability solution*
Relative standard deviation: NMT 2.5%, *Standard solution*
Analysis
Samples: *Standard solution*, *System suitability solution*, and *Sample solution*
Calculate the sum of the peak responses due to gingerols and gingerdiones occurring at about the following retention times, relative to 1.0 for capsaicin: 0.8 for 6-gingerol, 1.5 for 8-gingerol A, 2.2 for 8-gingerol B, 2.5 for 6-gingerdiol, 2.6 for 6-gingerdione, 3.4 for 10-gingerol, and 5.2 for 8-gingerdione.
Calculate the percentage of gingerols and gingerdiones in the portion of Ginger taken:

$$\text{Result} = (r_T/r_S) \times (C_S/W) \times 10$$

r_T = sum of the peak responses for gingerols and gingerdiones from the *Sample solution*
r_S = peak response of capsaicin from the *Standard solution*
C_S = concentration of USP Capsaicin RS in the *Standard solution* (mg/mL)

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W = weight of Ginger used in the test for *Articles of Botanical Origin, Alcohol-Soluble Extractives, Method 2* (g)

Acceptance criteria: NLT 0.8%

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli.*

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: Ginger occurs in horizontal, laterally flattened, sympodially branching pieces. Whole rhizomes are 5–15 cm long, 1.5–6 cm wide, and up to 2 cm thick, sometimes split longitudinally, pale yellowish buff or light brown externally, longitudinally striated, somewhat fibrous; branches are flattish, obovate, short, about 2 cm long, each ending with a depressed stem scar; fracture is short with projecting fibers, or sometimes resinous; internally it is yellowish brown, showing a yellow endodermis separating the narrow cortex from the wide stele, and numerous yellowish points, secretion cells and numerous bigger grayish points, and vascular bundles are scattered on the whole surface. The unscraped rhizome shows in addition an outer layer of dark-brown cork. Morphological characteristics of different varieties and forms of Ginger from different geographical areas are listed in *Table 1* of *Supplemental Information for Articles of Botanical Origin* ⟨2030⟩.

Microscopic: The scraped rhizome in transverse section shows a cortex composed of multiple layers of parenchyma cells rich in simple, large, flattened, ovoid or sack-shaped starch granules, 5–15 µm wide and 30–60 µm long having an eccentric hilum, some showing faint transverse striations. The cortex also shows numerous oleoresin cells with a yellow or yellowish-brown content and scattered collateral vascular bundles; a single layer of endodermal cells free from starch; a wide central stele composed of parenchyma cells rich in starch and oleoresin cells similar to those of the cortex, and containing scattered collateral vascular bundles, some enclosed in a sheath of septate nonlignified fibers with wide lumen. In addition to the above, the unscraped rhizome shows an outer zone of dark-brown cork cells.

- **LIMIT OF SHOGAOLS**

Analysis: From the chromatograms obtained in the test for *Content of Gingerols and Gingerdiones*, calculate the sum of the peak responses due to shogaols, occurring at the following retention times, relative to 1.0 for capsaicin: 1.9 for 6-shogaol, 4.2 for 8-shogaol, and 5.8 for 10-shogaol.

Calculate the percentage of shogaols in the portion of Ginger taken:

$$\text{Result} = (r_T/r_S) \times (C_S/W) \times 10$$

r_T = sum of the peak responses of shogaols from the *Sample solution*

r_S = peak response of capsaicin from the *Standard solution*

C_S = concentration of USP Capsaicin RS in the *Standard solution*, prepared as directed in the test for *Content of Gingerols and Gingerdiones* (mg/mL)

W = weight of Ginger used in the test for *Articles of Botanical Origin, Alcohol-Soluble Extractives, Method 2* (g)

Acceptance criteria: NMT 0.18%

- **ARTICLES OF BOTANICAL ORIGIN,** *Alcohol-Soluble Extractives, Method 2* ⟨561⟩

Analysis: Collect the filtrate in a 100-mL volumetric flask, and dilute with alcohol to volume. Evaporate 50 mL of the filtrate at a temperature not exceeding 90°. [NOTE—Save the residue for use in *Identification* test *B* and the remaining volume of the filtrate for the tests for *Limit of Shogaols* and *Content of Gingerols and Gingerdiones.*]

Acceptance criteria: NLT 4.5% residue

- **ARTICLES OF BOTANICAL ORIGIN,** *Starch Content, Method 1* ⟨561⟩**:** NLT 42%, *Method 1A* of the *General Procedure* being used
- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩**:** NMT 1.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 8.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Volatile Oil Determination* ⟨561⟩**:** NLT 1.8 mL/100 g
- **ARTICLES OF BOTANICAL ORIGIN,** *Water-Soluble Ash* ⟨561⟩**:** NLT 1.9%
- **ARTICLES OF BOTANICAL ORIGIN,** *Water-Soluble Extractives, Method 2* ⟨561⟩**:** NLT 10.0%
- **WATER DETERMINATION,** *Method Ia* ⟨921⟩**:** NMT 10%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store in a cool area.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article. This article is exempted from the requirements of *Labeling* ⟨7⟩, *Labels and Labeling for Products and Other Categories, Botanicals*, with respect to the pregnancy and lactation statement.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Capsaicin RS
USP Ginger Constituent Mixture RS
USP Powdered Ginger RS

Powdered Ginger

DEFINITION

Powdered Ginger is Ginger reduced to a fine or a very fine powder.

IDENTIFICATION

- **A.**

Analysis: To 1 g of the Powdered Ginger add 5 mL of dilute acetic acid, prepared by diluting 1 part of glacial acetic acid with 1 part of water, and shake for 15 min. Filter, and add a few drops of ammonium oxalate TS to the filtrate.

Acceptance criteria: NMT a slight turbidity is produced.

- **B.**

Sample: 50 mg of the residue obtained in the test for *Articles of Botanical Origin, Alcohol-Soluble Extractives, Method 2*

USP Monographs

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Analysis: Dissolve the *Sample* in 25 mL of water, and extract this solution with two 15-mL portions of ether. Combine the ether extracts, and evaporate in a porcelain dish. To the residue add 5 mL of sulfuric acid solution (7.5 in 10.0) and 5 mg of vanillin. Allow to stand for 15 min, and add an equal volume of water.

Acceptance criteria: The solution turns azure blue.

• **C. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 0.2 mg/mL of USP Ginger Constituent Mixture RS in methanol

Standard solution B: 100 mg/mL of USP Powdered Ginger RS in methanol. Sonicate for 10 min, and centrifuge or filter. Use supernatant or filtrate.

Sample solution: 100 mg/mL of Powdered Ginger in methanol. Sonicate for 10 min, and centrifuge or filter. Use supernatant or filtrate.

Mode: HPTLC

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plate)[1]

Application volume: 6 µL of *Standard solution A* and 2 µL each of *Standard solution B* and *Sample solution* as 8-mm bands

Temperature: Ambient, not to exceed 30°

Developing solvent system: Toluene and ethyl acetate (3:1)

Derivatization reagent: To 170 mL of ice-cold methanol add 20 mL of glacial acetic acid, 10 mL of sulfuric acid, and 1 mL of anisaldehyde. Mix well.

System suitability

Samples: *Standard solution A* and *Standard solution B*

Apply the *Samples* and dry in air. Condition at relative humidity of about 33%. Develop in a saturated chamber until the solvent front has migrated over a path of 6 cm. Dry in a current of cold air, and immerse into *Derivatization reagent* for 1 s. Heat for 3 min at 100°, and examine under white light and under long-wave UV (365 nm).

Suitability requirements

Chromatographic pattern: Under white light, the derivatized chromatogram of *Standard solution A* displays two prominent bands: the lower due to 6-gingerol, the upper due to 6-shogaol. Under white light, the derivatized chromatogram of *Standard solution B* shows a succession of dark-violet bands between the origin and the intense dark-brown band corresponding to that of the 6-gingerol in *Standard solution A*. Immediately proximate to the 6-gingerol band in the *Standard solution B* chromatogram, less intense bands due to 8-gingerol and 10-gingerol are observed. A variable number of low-intensity dark-gray bands appear between 10-gingerol and the second prominent band corresponding to 6-shogaol in *Standard solution A*. In the distal part of the chromatogram, a dark-purple somewhat diffuse band is observed. Under long-wave UV (365 nm), the chromatograms of the *Standard solutions* exhibit patterns similar to those observed under white light. The bands due to gingerols and shogaols are bright orange; the bands between the origin and the 6-gingerol band are dark-red to brown, somewhat less prominent than when observed in white light. The bands between 10-gingerol and 6-shogaol are variably colored; frequently, a light-gray band appears halfway between them with a light-purple diffuse band between it and the orange band due to 6-shogaol. The distal diffuse band assumes a purple-pink hue.

[1] Suitable commercially available plates are HPTLC Silica Gel 60 F_{254} from EMD Millipore (e.g., Part No. 1.05642.0001).

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Treat and examine the *Samples* as described under *System suitability.*

Acceptance criteria: Under white light and under long-wave UV (365 nm), the chromatogram of the *Sample solution* exhibits the band pattern similar to that observed with *Standard solution B*. The band in the distal part of the chromatogram, however, has no diagnostic significance. Its color may range from purple-pink to muddy yellow, or the band may be altogether absent. Potential adulterants lack the band pattern characteristic of the gingerol–shogaol succession. Katsumada's galangal (*Alpinia hainanensis* K. Schum.) seed displays a green band close to the origin and a reddish-orange band below it due to 6-gingerol. Sharpleaf galangal (*Alpinia oxyphylla* Miq.) fruit shows a prominent greenish band in the middle of the chromatogram. *Kaempferia galangal* L. rhizome shows no diagnostic bands under UV, but under white light, a purple band is seen at about two-thirds from the application line. Lesser galangal (*Alpinia officinarum* Hance) rhizome presents a yellow band at an R_F just below the 6-gingerol band, followed by a continuous broad blue smudge and a distinct tandem of light-orange and yellow bands close to the middle of the plate.

COMPOSITION

• **CONTENT OF GINGEROLS AND GINGERDIONES**

Solution A: Acetonitrile, dilute phosphoric acid (1 in 1000), and methanol (55:44:1)

Solution B: Acetonitrile

Mobile phase: Use *Solution A* for NLT 7 times the retention time of capsaicin.

Column washing: After each chromatographic run, wash the column, using *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
2	0	100
12	0	100
14	100	0
29	100	0

Standard solution: 0.1 mg/mL of USP Capsaicin RS in methanol

System suitability solution: Reconstitute the content of one vial of USP Ginger Constituent Mixture RS in 1 mL of the *Standard solution.*

Sample solution: Use the filtrate retained from the test for *Articles of Botanical Origin, Alcohol-Soluble Extractives, Method 2.*

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability.*)

Mode: LC

Detector: UV 282 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection volume: 25 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

[NOTE—The relative retention times for 6-gingerol, capsaicin, and 6-shogaol are about 0.8, 1.0, and 1.9, respectively, *System suitability solution.*]

DS Monographs

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Suitability requirements
Resolution: NLT 3.0 between the 6-gingerol and capsaicin peaks; NLT 10.0 between the capsaicin and 6-shogaol peaks, *System suitability solution*
Tailing factors: NMT 2.0 for the 6-gingerol, capsaicin, and 6-shogaol peaks, *System suitability solution*
Relative standard deviation: NMT 2.5%, *Standard solution*
Analysis
Samples: *Standard solution, System suitability solution, Sample solution*
Calculate the sum of the peak responses due to gingerols and gingerdiones, occurring at about the following retention times, relative to 1.0 for capsaicin: 0.8 for 6-gingerol, 1.5 for 8-gingerol A, 2.2 for 8-gingerol B, 2.5 for 6-gingerdiol, 2.6 for 6-gingerdione, 3.4 for 10-gingerol, and 5.2 for 8-gingerdione.
Calculate the percentage of gingerols and gingerdiones in the the portion of Powdered Ginger taken:

$$\text{Result} = (r_T/r_S) \times (C_S/W) \times 10$$

r_T = sum of the peak responses of gingerols and gingerdiones from the *Sample solution*
r_S = peak response of capsaicin from the *Standard solution*
C_S = concentration of USP Capsaicin RS in the *Standard solution* (mg/mL)
W = weight of Powdered Ginger used in the test for *Articles of Botanical Origin, Alcohol-Soluble Extractives, Method 2* (g)

Acceptance criteria: NLT 0.8%

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total bacterial count does not exceed 10^5 cfu/g; the total combined molds and yeasts count does not exceed 10^3 cfu/g; the bile-tolerant Gram-negative bacteria count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli.*

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS:** Under a microscope, Powdered Ginger reveals mainly starch granules and parenchyma cells containing them; simple, large, flattened, ovoid or sack-shaped starch granules, 5–15 µm wide and 30–60 µm long having an eccentric hilum, some showing faint transverse striations; parenchyma cells containing yellow-brown to dark-brown resinous substances; groups of large, thin-walled nonlignified septate fibers with wide lumen; portions of septate fibers with attached vessels; large vessels with annular, spiral, or reticulate thickening and often accompanied by parenchyma cells containing brown content; oleoresin in fragments or droplets, staining with iodine TS and potassium iodide TS; and, rarely, fragments of brown cork tissue, usually seen in surface view. Sclerenchymatous cells, trichomes, and calcium oxalate are absent.
- **LIMIT OF SHOGAOLS**
Analysis: From the chromatograms obtained in the test for *Content of Gingerols and Gingerdiones*, calculate the sum of the peak responses due to shogaols, occurring at the following retention times, relative to 1.0 for capsaicin: 1.9 for 6-shogaol, 4.2 for 8-shogaol, and 5.8 for 10-shogaol.
Calculate the percentage of shogaols in the portion of Powdered Ginger taken:

$$\text{Result} = (r_T/r_S) \times (C_S/W) \times 10$$

r_T = sum of the peak responses of shogaols from the *Sample solution*
r_S = peak response of capsaicin from the *Standard solution*
C_S = concentration of USP Capsaicin RS in the *Standard solution*, prepared as directed in the test for *Content of Gingerols and Gingerdiones* (mg/mL)
W = weight of Powdered Ginger used in the test for *Articles of Botanical Origin, Alcohol-Soluble Extractives, Method 2* (g)

Acceptance criteria: NMT 0.18%

- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Alcohol-Soluble Extractives, Method 2* ⟨561⟩
Analysis: Collect the filtrate in a 100-mL volumetric flask, and dilute with alcohol to volume. Evaporate 50 mL of the filtrate at a temperature not exceeding 90°.
Acceptance criteria: NLT 4.5% residue. [NOTE—Save the residue for use in *Identification* test *B* and the remaining volume of the filtrate for the tests for *Limit of Shogaols* and *Content of Gingerols and Gingerdiones.*]
- **ARTICLES OF BOTANICAL ORIGIN,** *Starch Content, Method 1* ⟨561⟩**:** NLT 42%, *Method 1A* of the *General Procedure* being used
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 8.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Volatile Oil Determination* ⟨561⟩**:** NLT 1.8 mL/100 g
- **ARTICLES OF BOTANICAL ORIGIN,** *Water-Soluble Ash* ⟨561⟩**:** NLT 1.9%
- **ARTICLES OF BOTANICAL ORIGIN,** *Water-Soluble Extractives, Method 2* ⟨561⟩**:** NLT 10.0%
- **WATER DETERMINATION,** *Method Ia* ⟨921⟩**:** NMT 10%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store in a cool area.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived. This article is exempted from the requirements of *Labeling* ⟨7⟩, *Labels and Labeling for Products and Other Categories, Botanicals*, with respect to the pregnancy and lactation statement.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Capsaicin RS
USP Ginger Constituent Mixture RS
USP Powdered Ginger RS

Ginger Tincture

DEFINITION

Ginger Tincture is prepared as follows.

Ginger	200 g
A mixture of Alcohol and Water (7:3), a sufficient quantity to make	1000 mL

DS Monographs

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Prepare the Tincture as directed in *Botanical Extracts* ⟨565⟩, *Preparations, Tinctures, Maceration Process*. It contains NLT 0.10% of gingerols.

IDENTIFICATION

• **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 0.2 mg/mL of USP Ginger Constituent Mixture RS in methanol

Standard solution B: 100 mg/mL of USP Powdered Ginger RS in methanol. Sonicate for 10 min and centrifuge or filter. Use supernatant or filtrate.

Sample solution: Tincture

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plate)[1]

Application volume: 6 µL of *Standard solution A* and 2 µL each of *Standard solution B* and *Sample solution* as 8-mm bands

Temperature: Ambient, not to exceed 30°

Developing solvent system: Toluene and ethyl acetate (3:1)

Derivatization reagent: To 170 mL of ice-cold methanol add 20 mL of glacial acetic acid, 10 mL of sulfuric acid, and 1 mL of anisaldehyde. Mix well.

System suitability

Samples: *Standard solution A* and *Standard solution B*

Apply the *Samples* and dry in air. Condition at a relative humidity of about 33%. Develop in a saturated chamber until the solvent front has migrated over a path of 6 cm. Dry in a current of cold air, and immerse into *Derivatization reagent* for 1 s. Heat for 3 min at 100°, and examine under white light and under long-wave UV (365 nm).

Suitability requirements

Chromatographic pattern: Under white light, the derivatized chromatogram of *Standard solution A* displays two prominent bands: the lower due to 6-gingerol, the upper due to 6-shogaol. Under white light, the derivatized chromatogram of *Standard solution B* shows a succession of dark-violet bands between the origin and the intense dark-brown band corresponding to that of the 6-gingerol in *Standard solution A*. Immediately proximate to the 6-gingerol band in the *Standard solution B* chromatogram, less intense bands due to 8-gingerol and 10-gingerol are observed. A variable number of low-intensity dark-gray bands appear between 10-gingerol and the second prominent band corresponding to 6-shogaol in *Standard solution A*. In the distal part of the chromatogram, a dark-purple somewhat diffuse band is observed. Under long-wave UV (365 nm), the chromatograms of the *Standard solutions* exhibit patterns similar to those observed under white light. The bands due to gingerols and shogaols are bright orange; the bands between the origin and the 6-gingerol band are dark-red to brown, somewhat less prominent than when observed in white light. The bands between 10-gingerol and 6-shogaol are variably colored; frequently, a light-gray band appears halfway between them, with a light-purple diffuse band between it and the orange band due to 6-shogaol. The distal diffuse band assumes a purple-pink hue.

Analysis

Samples: *Standard solution A, Standard solution B,* and *Sample solution.*

Treat and examine the *Samples* as described in *System suitability.*

Acceptance criteria: Under white light and under long-wave UV (365 nm), the chromatogram of the *Sample solution* exhibits the band pattern similar to that observed with *Standard solution B*. The band in the distal part of the chromatogram, however, has no diagnostic significance. Its color may range from purple-pink to muddy yellow, or the band may be altogether absent. Potential adulterants lack the band pattern characteristic of the gingerol–shogaol succession. *Kaempferia galanga* L. rhizome shows no diagnostic bands under UV, but under white light a purple band is seen at about two-thirds from the application line. Lesser galangal (*Alpinia officinarum* Hance) rhizome presents a yellow band at an R_F just below the 6-gingerol band, followed by a continuous broad blue smudge, and a distinct tandem of light-orange and yellow bands close to the middle of the plate.

STRENGTH

• **CONTENT OF GINGEROLS**

Solution A: Acetonitrile, dilute phosphoric acid (1 in 1000), and methanol (55:44:1)

Solution B: Acetonitrile

Mobile phase: Use *Solution A* for NLT 7 times the retention time of capsaicin.

Column washing: After each chromatographic run, wash the column, using *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
2	0	100
12	0	100
14	100	0
29	100	0

Standard solution: 0.1 mg/mL of USP Capsaicin RS in methanol

System suitability solution: Reconstitute the content of one vial of USP Ginger Constituent Mixture RS in 1 mL of *Standard solution.*

Sample solution: Tincture

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability.*)

Mode: LC

Detector: UV 282 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection volume: 25 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

[NOTE—The relative retention times for 6-gingerol, capsaicin, and 6-shogaol are about 0.8, 1.0, and 1.9, respectively, *System suitability solution.*]

Suitability requirements

Resolution: NLT 3.0 between the 6-gingerol and capsaicin peaks; NLT 10.0 between the capsaicin and 6-shogaol peaks, *System suitability solution*

Tailing factors: NMT 2.0 for the 6-gingerol, capsaicin, and 6-shogaol peaks, *System suitability solution*

Relative standard deviation: NMT 2.5%, *Standard solution*

Analysis

Samples: *Standard solution, System suitability solution,* and *Sample solution*

Calculate the percentage of gingerols in the portion of Tincture taken:

$$\text{Result} = (r_T/r_S) \times C_S \times 0.1$$

[1] Suitable commercially available plates are HPTLC Silica Gel 60 F_{254} from EMD Millipore (e.g., Part No. 1.05642.0001).

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r_T = sum of the peak responses of gingerols from the *Sample solution*
r_S = peak response of capsaicin from the *Standard solution*
C_S = concentration of USP Capsaicin RS in the *Standard solution* (mg/mL)

Acceptance criteria: NLT 0.10%

OTHER COMPONENTS

- **ALCOHOL DETERMINATION,** *Method I* ⟨611⟩**:** 90.0%–110.0% of the labeled amount of alcohol (C_2H_5OH)

Change to read:

CONTAMINANTS

- **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020) **:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.

SPECIFIC TESTS

- **LIMIT OF 6-SHOGAOL**

Analysis: Using the chromatograms from the test for *Content of Gingerols*, calculate the percentage of 6-shogaol in the portion of Tincture taken:

$$\text{Result} = (r_U/r_S) \times C_S \times 0.1$$

r_U = peak response of 6-shogaol from the *Sample solution*
r_S = peak response of capsaicin from the *Standard solution*
C_S = concentration of USP Capsaicin RS in the *Standard solution* (mg/mL)

Acceptance criteria: NMT 0.034%

- **LIMIT OF NONVOLATILE RESIDUE**

Sample: 10-mL portion
Analysis: Evaporate the *Sample* in a tared platinum or porcelain dish, and dry at 105° for 6 h.
Acceptance criteria: The weight of the residue is 80–120 mg.

- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 0.5%
- **SPECIFIC GRAVITY** ⟨841⟩**:** 0.90–0.95
- **OTHER REQUIREMENTS:** Meets the requirements in *Botanical Extracts* ⟨565⟩, *Preparations, Tinctures, Packaging and Storage*

ADDITIONAL REQUIREMENTS

- **LABELING:** Label it to indicate that it is for manufacturing purposes only, in addition to the information specified in *Botanical Extracts* ⟨565⟩, *Preparations, Tinctures, Labeling.* This article is exempted from the requirements of *Labeling* ⟨7⟩, *Labels and Labeling for Products and Other Categories, Botanicals,* with respect to the pregnancy and lactation statement.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Capsaicin RS
USP Ginger Constituent Mixture RS
USP Powdered Ginger RS

Ginger Capsules

DEFINITION

Ginger Capsules are prepared from Powdered Ginger and contain NLT 90.0% and NMT 110.0% of the labeled amount of gingerols, gingerdiones, and shogaols, and NLT 90.0% of the labeled amount of volatile oil.

IDENTIFICATION

- **A.**

Analysis: Pulverize an amount of the contents of Capsules equivalent to 5 g of ginger. To an amount equivalent to 1 g of ginger add 5 mL of dilute acetic acid, prepared by diluting 1 part of glacial acetic acid with 1 part of water, and shake for 15 min. Filter, and add a few drops of ammonium oxalate TS to the filtrate.
Acceptance criteria: NMT a slight turbidity is produced.

- **B.**

Sample (see *Articles of Botanical Origin* ⟨561⟩, *Alcohol-Soluble Extractives, Method 2*)**:** Collect the filtrate in a 100-mL volumetric flask, and dilute with alcohol to volume. Evaporate 50 mL of the filtrate at a temperature not exceeding 90°. Use 50 mg of the residue for the test.
Analysis: Dissolve the *Sample* in 25 mL of water, and extract with two 15-mL portions of ether. Combine the ether extracts, and evaporate in a porcelain dish. To the residue add 5 mL of sulfuric acid solution (7.5 in 10.0) and 5 mg of vanillin. Allow to stand for 15 min, and add an equal volume of water.
Acceptance criteria: The solution turns azure blue.

- **C. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution A: Proceed as directed for the *Sample solution*, except to use 0.2 g of USP Powdered Ginger RS.
Standard solution B: Use the *System suitability solution*, prepared as directed in the test for *Content of Gingerols, Gingerdiones, and Shogaols.*
Sample solution: Pulverize an amount of the contents of Capsules equivalent to 5 g of ginger. Transfer an amount equivalent to 0.2 g of ginger to a test tube, add 5 mL of methanol, shake for 30 min, and centrifuge. Apply the supernatant to the plate.
Adsorbent: 0.50-mm layer of chromatographic silica gel mixture
Application volume: 20 µL for *Standard solution A* and the *Sample solution*; 40 µL for *Standard solution B*
Developing solvent system: Ether and hexanes (7:3)
Spray reagent: 10% sulfuric acid in alcohol
Analysis
Samples: *Standard solution A, Standard solution B,* and *Sample solution*
Proceed as directed in the chapter. Examine the plate under UV light at 254 nm. Spray the plate with *Spray reagent,* heat at 100°–105° for 10 min, and examine under daylight.
Acceptance criteria: The chromatogram of the *Sample solution* exhibits a spot due to gingerols that occurs at an R_F value of 0.2, and a spot of shogaols may occur at an R_F value of 0.4, corresponding to those shown in the chromatogram of *Standard solution B.* [NOTE—The chromatograms of *Standard solution A* and the *Sample solution* may exhibit other spots in the upper region and at the origin of the plate.]

STRENGTH

- **CONTENT OF GINGEROLS, GINGERDIONES, AND SHOGAOLS**

Solution A: Acetonitrile, dilute phosphoric acid (1 in 1000), and methanol (55:44:1)
Solution B: Acetonitrile
Mobile phase: Use *Solution A* for NLT seven times the retention time of capsaicin.

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Column washing: After each chromatographic run, wash the column, using *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
2	0	100
12	0	100
14	100	0
29	100	0

Standard solution: 0.1 mg/mL of USP Capsaicin RS in methanol

System suitability solution: Reconstitute the content of 1 vial of USP Ginger Constituent Mixture RS in 1 mL of the *Standard solution*.

Sample solution: Mix and finely powder the contents of NLT 20 Capsules, and transfer an amount equivalent to 2.0 g of powdered ginger to a glass-stoppered conical flask. Add 50 mL of alcohol, insert a stopper into the flask, and macerate for 24 h, shaking frequently during the first 8 h, and then allowing to stand for 16 h. Filter, and use the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 282 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection size: 25 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

[NOTE—The relative retention times for 6-gingerol, capsaicin, and 6-shogaol are about 0.8, 1.0, and 1.9, respectively, *System suitability solution*.]

Suitability requirements

Resolution: NLT 3.0 between the 6-gingerol and capsaicin peaks and NLT 10.0 between the capsaicin and 6-shogaol peaks, *System suitability solution*

Tailing factors: NMT 2.0 for the 6-gingerol, capsaicin, and 6-shogaol peaks, *System suitability solution*

Relative standard deviation: NMT 2.5% for the capsaicin peak for replicate injections, *Standard solution*

Analysis

Samples: *Standard solution*, *Sample solution*, and *System suitability solution*

Calculate the sum of the peak responses due to gingerols and gingerdiones occurring at about the following retention times relative to 1.0 for capsaicin: 0.8 for 6-gingerol, 1.5 for 8-gingerol A, 2.2 for 8-gingerol B, 2.5 for 6-gingerdiol, 2.6 for 6-gingerdione, 3.4 for 10-gingerol, and 5.2 for 8-gingerdione.

Calculate the sum of the peak responses due to shogaols, occurring at about the following retention times, relative to 1.0 for capsaicin: 1.9 for 6-shogaol, 4.2 for 8-shogaol, and 5.8 for 10-shogaol.

Calculate the percentage of the labeled amount of gingerols, gingerdiones, and shogaols in the portion of Capsules taken:

$$\text{Result} = (r_T/r_S) \times C_S \times (V/W_U) \times (A_{WC}/L) \times 100$$

r_T = sum of the peak responses for gingerols, gingerdiones, and shogaols from the *Sample solution*

r_S = peak response of capsaicin from the *Standard solution*

C_S = concentration of USP Capsaicin RS in the *Standard solution* (mg/mL)

V = final volume of the *Sample solution* (mL)

W_U = weight of the portion of Capsules taken (mg)

A_{WC} = average weight of the Capsule content (mg)

L = labeled amount of gingerols, gingerdiones, and shogaols (mg/Capsule)

Acceptance criteria: 90.0%–110.0% of the labeled amount of gingerols, gingerdiones, and shogaols

Calculate the amount (G_0), in mg, of 6-gingerol in each Capsule taken:

$$G_0 = (r_U/r_S) \times (C_S/W) \times V \times A$$

r_U = peak response of 6-gingerol from the *Sample solution*

r_S = peak response of capsaicin from the *Standard solution*

C_S = concentration of USP Capsaicin RS in the *Standard solution* (mg/mL)

W = weight of powdered ginger used in the preparation of the *Sample solution* (g)

V = final volume of the *Sample solution* (mL)

A = average Capsule fill weight (g)

• **ARTICLES OF BOTANICAL ORIGIN,** *Volatile Oil Determination* ⟨561⟩

Sample: Finely powder a quantity of Capsules, equivalent to 100 g of powdered ginger.

Acceptance criteria: NLT 1.4 mL/100 g (NLT 90.0% of the labeled amount of volatile oil)

PERFORMANCE TESTS

• **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩**:** Meet the requirements for *Dissolution*

Medium: 0.1 N hydrochloric acid; 500 mL

Apparatus 2: 75 rpm

Time: 60 min

[NOTE—In each dissolution vessel, place a number of Capsules equivalent to 20 mg of the labeled amounts of gingerols, gingerdiones, and shogaols.]

Solution A, Solution B, Mobile phase, Column washing, System suitability solution, Chromatographic system, and **System suitability:**

Proceed as directed in the test for *Content of Gingerols, Gingerdiones, and Shogaols*.

Standard stock solution: Use the *Standard solution* prepared in the test for *Content of Gingerols, Gingerdiones, and Shogaols*.

Standard solution: 0.025 mg/mL of USP Capsaicin RS from *Standard stock solution* in *Medium*

Sample solution: Transfer an aliquot of solution from each dissolution vial to a suitable vial. Allow to stand for 5 min so that the powder settles into the suspension, or centrifuge to obtain a clear supernatant. Pass through a membrane filter of 0.45-µm or finer pore size.

Analysis

Samples: *Standard solution* and *Sample solution*

[NOTE—Allow the *Sample solution* to elute for NLT three times the retention time of capsaicin.]

Calculate the quantity, G, in mg, of 6-gingerol dissolved from each Capsule taken:

$$G = (r_U/r_S) \times (C/N) \times V$$

r_U = peak response of 6-gingerol from the *Sample solution*

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r_S = peak response of capsaicin from the *Standard solution*
C = concentration of USP Capsaicin RS in the *Standard solution* (mg/mL)
N = number of Capsules in each vessel
V = volume of *Medium*; 500 mL

Calculate the percentage of the relative amount of 6-gingerol dissolved:

$$\text{Result} = (G/G_0) \times 100$$

G_0 = content of 6-gingerol in each Capsule, as determined in the test for *Content of Gingerols, Gingerdiones, and Shogaols* (mg)

Tolerances: NLT 60% of the content of 6-gingerol ($C_{17}H_{26}O_4$) is dissolved.

- **Weight Variation of Dietary Supplements** ⟨2091⟩: Meet the requirements

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in well-closed containers, and store at controlled room temperature.
- **Labeling:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. The label also indicates the content of gingerols, gingerdiones, and shogaols, in mg per Capsule, and the content of volatile oil, in µL per Capsule. This article is exempt from the requirements of the *Labeling* ⟨7⟩, *Labels and Labeling for Products and Other Categories, Botanicals,* with respect to the pregnancy and lactation statement.
- **USP Reference Standards** ⟨11⟩
  USP Capsaicin RS
  USP Ginger Constituent Mixture RS
  USP Powdered Ginger RS

Ginkgo

DEFINITION

Ginkgo consists of the dried leaf of *Ginkgo biloba* L. (Fam. Ginkgoaceae). It contains NLT 0.5% of flavonoids, calculated as flavonol glycosides, and NLT 0.1% of terpene lactones, calculated as the sum of bilobalide ($C_{15}H_{18}O_8$), ginkgolide A ($C_{20}H_{24}O_9$), ginkgolide B ($C_{20}H_{24}O_{10}$), and ginkgolide C ($C_{20}H_{24}O_{11}$), both on the dried basis.

IDENTIFICATION

- **A. HPTLC for Articles of Botanical Origin** ⟨203⟩

Test for flavonoids

Standard solution: A solution of 0.6 mg/mL of USP Rutin RS, 0.2 mg/mL of USP Chlorogenic Acid RS, and 0.2 mg/mL of USP Quercetin RS in methanol

Sample solution: Transfer 1.0 g of finely powdered Ginkgo to a 50-mL round-bottom flask fitted with a reflux condenser. Add 10 mL of methanol, reflux on a water bath for 10 min, allow to cool to room temperature, and filter. [Note—Reserve some of the *Sample solution* for use in the *Test for terpene lactones*.]

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plates)

Application volume: 5 µL

Developing solvent system: Ethyl acetate, water, anhydrous formic acid, and glacial acetic acid (100:26:11:11)

Derivatization reagent A: 5 mg/mL of 2-aminoethyl diphenylborinate in methanol

Derivatization reagent B: 50 mg/mL of polyethylene glycol 400 in alcohol

Analysis

Samples: *Standard solution* and *Sample solution*

Before development of the chromatograms, saturate the chamber for 20 min with *Developing solvent system*. If the relative humidity in the laboratory exceeds 50%, condition the plate to about 35% relative humidity using a suitable device. Apply the samples separately as bands, and allow to dry. Develop the plate over a path of 6 cm, remove from the chromatographic chamber, and dry in a circulating air oven at 105° for 5 min. Immediately treat the hot plate with *Derivatization reagent A*, then with *Derivatization reagent B*, dry, and examine under long-wave UV light (365 nm).

Acceptance criteria: The *Standard solution* shows in its lower part with increasing R_F values a yellowish-brown fluorescent zone due to rutin (R_F 0.28), a light blue fluorescent zone due to chlorogenic acid (R_F 0.36), and a yellow fluorescent zone due to quercetin (R_F 0.92). The *Sample solution* shows a yellowish-brown fluorescent zone, a light blue fluorescent zone, and a yellowish-brown fluorescent zone at R_F similar to those of rutin, chlorogenic acid, and quercetin, respectively, in the *Standard solution*. Additional yellowish to yellowish-green zones due to flavonoids detected in the *Sample solution* chromatogram include one zone below the rutin zone, two zones between the rutin and chlorogenic acid zones, and two zones above the chlorogenic acid zone. Other zones may be seen in the *Sample solution* chromatogram.

Test for terpene lactones

Standard solution: Dissolve an amount of USP Ginkgo Terpene Lactones RS in methanol to obtain a solution containing in each mL about 1.0, 0.9, 0.6, 0.7, and 0.2 mg of bilobalide, ginkgolide A, ginkgolide B, ginkgolide C, and ginkgolide J, respectively.

Sample solution: Use the *Sample solution* prepared in the *Test for flavonoids*.

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plates)

Application volume: 5 µL

Developing solvent system: Toluene, ethyl acetate, acetone, and methanol (20: 10: 10: 1.2)

Derivatization reagent: Acetic anhydride

Analysis

Samples: *Standard solution* and *Sample solution*

Immerse the plate for 2 s in an 8 g/200-mL solution of sodium acetate in methanol. Allow the excess liquid to drip from the plate, dry in a forced-air oven at 70° for 30 min, and cool in a desiccator. Apply the samples separately as bands to the impregnated plate, and allow to air-dry. Before development of the chromatograms, saturate the chamber for 20 min with *Developing solvent system*. If the relative humidity in the laboratory exceeds 50%, condition the plate to about 35% relative humidity, using a suitable device. Develop the plate over a path of 6 cm, remove from the chromatographic chamber, and dry in cold air. Treat the plate with *Derivatization reagent*, heat at 180° for 10 min, cool, and examine under short-wave UV light (254 nm).

Acceptance criteria: The *Standard solution* chromatogram shows five distinct quenching zones corresponding to the different ginkgo terpene lactones: ginkgolide C, ginkgolide J, ginkgolide B, ginkgolide A, and bilobalide at R_F values of about 0.13, 0.18, 0.32, 0.38, and 0.45, respectively. The *Sample solution* chromatogram shows a strong quenching zone at the application position, a broad quenching zone near the solvent front, and five distinct quenching zones corresponding to different

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ginkgo terpene lactones at R_F similar to those detected in the *Standard solution* chromatogram.
[NOTE—R_F values may differ from one plate to another due to the impregnation step.]

COMPOSITION

• **CONTENT OF FLAVONOL GLYCOSIDES**

Extraction solvent: Alcohol, hydrochloric acid, and water (25:4:10)

Mobile phase: Methanol, water, and phosphoric acid (100:100:1)

Standard solution A: 0.02 mg/mL of USP Quercetin RS in methanol

Standard solution B: 0.02 mg/mL of USP Kaempferol RS in methanol

Standard solution C: 0.005 mg/mL of USP Isorhamnetin RS in methanol

Sample solution: Transfer about 1.0 g of Ginkgo, finely powdered, to a 250-mL flask fitted with a reflux condenser. Add 78 mL of *Extraction solvent*, and reflux on a water bath for 135 min. [NOTE—The solution will turn deep red. The color of the solution is not a definitive indication of reaction completeness.] Allow to cool to room temperature. Decant into a 100-mL volumetric flask. Add 20 mL of methanol to the 250-mL flask, and sonicate for 30 min. Filter, transfer the filtrate into the 100-mL volumetric flask, wash the residue on the filter with a small amount of methanol, transfer the rinsate into the same 100-mL volumetric flask, dilute with methanol to volume, and mix.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 370 nm
Column: 4.6-mm × 25-cm; packing L1
Flow rate: 1.5 mL/min
Injection volume: 20 µL

System suitability
Samples: *Standard solution A*, *Standard solution B*, and *Standard solution C*
[NOTE—The relative retention times for quercetin, kaempferol, and isorhamnetin are about 1.0, 1.8, and 2.0, respectively, *Standard solution A*, *Standard solution B*, and *Standard solution C*.]
Suitability requirements
Relative standard deviation: NMT 2.0% determined from the quercetin peak in repeated injections, *Standard solution A*

Analysis
Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*
Calculate the percentage of each flavonol glycoside in the portion of Ginkgo taken:

$$\text{Result} = (r_U/r_S) \times (C_S/W) \times F \times 10$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of the relevant analyte from *Standard solution A*, *Standard solution B*, or *Standard solution C*
C_S = concentration of the relevant analyte in *Standard solution A*, *Standard solution B*, or *Standard solution C* (mg/mL)
W = weight of Ginkgo taken to prepare the *Sample solution* (g)
F = factor to convert each flavonol aglycone into its respective flavonol glycoside: 2.504 for quercetin, 2.437 for isorhamnetin, and 2.588 for kaempferol

Calculate the total percentage of flavonol glycosides by adding the individual percentages calculated.

Acceptance criteria: NLT 0.5% of flavonoids, as flavonol glycosides, on the dried basis

• **CONTENT OF TERPENE LACTONES**

Solvent: Methanol and water (9:1)

Buffer solution: Dissolve 1.19 g of dibasic sodium phosphate and 8.25 g of monobasic potassium phosphate in 1000 mL of water, and adjust to a pH of 5.8.

Diluent: Methanol and water (1:1)

Solution A: Water

Solution B: Methanol

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	75	25
23	52	48
28	52	48
30	25	75
35	10	90
40	75	25
50	75	25

Standard solutions: Using the labeled content of the individual terpene lactones, prepare five solutions of the USP Ginkgo Terpene Lactones RS in *Diluent* within the range of 5–500 µg/mL for each of the relevant terpene lactones. Use sonication to dissolve the analytes if necessary. Pass through a filter of 0.45-µm or finer pore size.

Sample solution: Transfer about 2.5 g of Ginkgo, accurately weighed, to a 30-mL glass centrifuge tube with a screw cap and a PTFE gasket. Add 10 mL of *Solvent*, seal the tube, and mix well on a vortex mixer. Heat on a water bath at 90° for 30 min. Mix the hot suspension on a vortex mixer, and repeat the heating at 90° for 30 min. Cool, centrifuge, transfer the supernatant into a round-bottom flask, and keep the residue in the glass tube. Repeat the extraction two more times, each time using 10 mL of *Solvent*. Combine the extracts, and evaporate to dryness under vacuum on a water bath maintained at 50°. Add 10 mL of *Buffer solution* to the residue, and sonicate for 5 min. Quantitatively transfer the solution to a glass chromatographic tube filled with chromatographic siliceous earth capable of holding 20 mL of aqueous phase.[1] Rinse the beaker with two 5-mL portions of *Buffer solution*, and transfer the rinsates to the column. [NOTE—Do not exceed 20 mL of total aqueous phase or the holding capacity of the chromatographic tube.]
Allow the *Buffer solution* to be absorbed into the column. After 15 min, elute the column with 100 mL of ethyl acetate, collect the eluate, and evaporate to dryness under vacuum on a water bath maintained at 50°. Dissolve the residue in 10.0 mL of *Diluent*.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Evaporative light-scattering detector.
[NOTE—The parameters of the detector are adjusted to achieve the best signal-to-noise ratio, according to manufacturer recommendations.]
Column: 4.6-mm × 25-cm; packing L1

[1] Suitable commercially available material is Extrelut® NT 20 from E Merck Science.

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Column temperature: 25°
Flow rate: 1 mL/min
Injection volume: 15 µL
System suitability
Samples: *Standard solutions*
Suitability requirements
Chromatogram similarity: The chromatograms of the *Standard solutions* are similar to the reference chromatogram provided with the lot of USP Ginkgo Terpene Lactones RS being used.
Relative standard deviation: NMT 2.0% determined from the bilobalide peak in replicate injections
Correlation coefficient: NLT 0.995 for the regression line as determined in *Analysis*
Analysis
Samples: *Standard solutions* and *Sample solution*
Record the chromatograms, and identify the peaks of the relevant analytes in the chromatograms of the *Standard solutions* by comparison with the reference chromatogram of the USP Ginkgo Terpene Lactones RS lot being used. Plot the logarithms of the relevant peak areas against the logarithms of respective concentrations, in mg/mL, of each analyte from the *Standard solutions*, and establish the regression lines by least-squares regression.
From the graphs, determine the concentrations, *C*, in mg/mL, of each relevant analyte in the *Sample solution*.
Separately calculate the percentages of bilobalide ($C_{15}H_{18}O_8$), ginkgolide A ($C_{20}H_{24}O_9$), ginkgolide B ($C_{20}H_{24}O_{10}$), and ginkgolide C ($C_{20}H_{24}O_{11}$) in the portion of Ginkgo taken:

$$\text{Result} = (C/W) \times 1000$$

C = concentration of the relevant analyte in the *Sample solution* (mg/mL)
W = weight of Ginkgo taken to prepare the *Sample solution* (mg)

Calculate the total percentage of terpene lactones in the portion of Ginkgo taken by adding the percentages calculated for each analyte.
Acceptance criteria: NLT 0.1% of terpene lactones, calculated as the sum of bilobalide, ginkgolide A, ginkgolide B, and ginkgolide C, on the dried basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria do not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: Dried whole, folded, or fragmented leaves, with or without attached petiole, vary from khaki green to greenish brown in color; often more brown at the apical edge, and darker on the adaxial surface. Laminae are broadly obcuneate (fan-shaped), 2–12 cm in width and 2–9.5 cm in length from petiole to apical margin; mostly 1.5–2 times wider than long. The base margins are entire, concave; apical margin sinuate, usually truncate or centrally cleft, and rarely multiply cleft. The surface is glabrous, with wrinkled appearance due to prominent dichotomous venation appearing parallel and extending from the lamina base to the apical margin. Petioles, similar in color to leaf, are channeled on the adaxial surface, and 2–8 cm in length.
Microscopic
Transverse section of lamina: A thin but marked cuticle occurs over a single layer of epidermal cells on both surfaces. Stomata are present on the lower surface only, with guard cells sunken with respect to adjacent epidermal cells. Palisade elements, elongated, at right angles to the surface and often irregular in appearance, occur just below the upper epidermis. Vascular bundles occur at intervals along the width of the blade, with adjacent cluster crystals of calcium oxalate. Cells of the mesophyll are smaller than the palisade cells, elongated, parallel to the leaf surface, and separated by large intercellular spaces.
Powdered lamina and petiole: Under the microscope, transverse fragments of the leaf display a smooth cuticle, present on both leaf surfaces and staining pinkish orange with sudan III TS. In surface view, cells of the upper epidermis are elongated and wavy-walled, with abundant yellow droplets 2–12 µm in diameter visible in mature and old leaves but not in young leaves. Cells of the lower epidermis are similar in shape but have straighter walls and are interrupted by anisocytic stomata. Numerous lignified elements derived from the lamina and petiole are present, including xylem vessels with annular thickening, tracheids, and vessels with bordered pits. The extent of lignification, particularly in the petiole, increases with age of leaf. Calcium oxalate crystals are numerous, scattered or associated with vessels, ranging in size from 5 to 50 µm in young leaves and 15 to 100 µm in mature leaves. Under crossed polarizers, numerous smaller prism- or tear-shaped shiny features of indeterminate nature may be present. Very occasional, highly elongated, uniseriate, covering trichomes with no obvious cross walls and smooth or warty surfaces may be seen. Mature leaves may show the presence of very rare, polygonal to circular starch granules approximately 20 µm in diameter, with a central hilum and exhibiting a marked Maltese cross under crossed polarizers.

- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩**:** NMT 3.0% of stems and NMT 2.0% of other foreign organic matter
- **LOSS ON DRYING** ⟨731⟩
Sample: 1.0 g of finely powdered Ginkgo
Analysis: Dry the *Sample* at 105° for 2 h.
Acceptance criteria: NMT 11.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩
Sample: 1.0 g of finely powdered Ginkgo
Acceptance criteria: NMT 11.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Chlorogenic Acid RS
USP Ginkgo Terpene Lactones RS
USP Isorhamnetin RS
USP Kaempferol RS
USP Quercetin RS
USP Rutin RS

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Ginkgo Capsules

DEFINITION

Ginkgo Capsules are prepared with Powdered Ginkgo Extract and contain, in the labeled amount of Powdered Ginkgo Extract, NLT 22.0% and NMT 27.0% of flavonol glycosides and NLT 5.4% and NMT 12.0% of terpene lactones, calculated as the sum of bilobalide ($C_{15}H_{18}O_8$), ginkgolide A ($C_{20}H_{24}O_9$), ginkgolide B ($C_{20}H_{24}O_{10}$), and ginkgolide C ($C_{20}H_{24}O_{11}$).

IDENTIFICATION

- **A. HPLC:** The retention times of the quercetin, isorhamnetin, and kaempferol peaks of the *Sample solution* correspond to those of the *Standard solution*, as obtained in the test for *Content of Flavonol Glycosides*. In the chromatogram of the *Sample solution*, the ratio of the kaempferol peak to the quercetin peak is NLT 0.7, and the isorhamnetin peak is NLT 0.1 times the size of the quercetin peak.
- **B. HPLC:** The retention times of the bilobalide, ginkgolide A, ginkgolide B, and ginkgolide C peaks of the *Sample solution* correspond to those of the *Standard solutions*, as obtained in the test for *Content of Terpene Lactones*.

STRENGTH

Change to read:

- **CONTENT OF FLAVONOL GLYCOSIDES**

Mobile phase: Methanol, water, and phosphoric acid (100:100:1)

▲Standard solution: 0.2 mg/mL of USP Quercetin RS, 0.2 mg/mL of USP Kaempferol RS, and 0.05 mg/mL of USP Isorhamnetin RS in methanol▲ (USP 1-Aug-2019)

Sample solution: Weigh and finely powder the contents of NLT 20 Capsules. Transfer an accurately weighed quantity of the powder, equivalent to about 50 mg of flavonol glycosides, to a 50-mL volumetric flask. Add 20 mL of methanol, and sonicate for 3 min. Add 20 mL of 1.5 N hydrochloric acid, and sonicate again for 10 min. Allow to cool to room temperature, and dilute with methanol to volume. Centrifuge, and transfer a portion of the clear supernatant to a rubber-capped, low-actinic glass vial. Heat in a steam bath for 25 min, and cool to room temperature in an ice bath.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 370 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1.5 mL/min

Injection volume: 20 µL

System suitability

Sample: ▲*Standard solution*▲ (USP 1-Aug-2019)

[NOTE—The relative retention times for quercetin, kaempferol, and isorhamnetin are about 1.0, 1.8, and 2.0, respectively.▲▲ (USP 1-Aug-2019)]

Suitability requirements

Relative standard deviation: NMT 2.0% for quercetin▲▲ (USP 1-Aug-2019)

Analysis

Samples: ▲*Standard solution*▲ (USP 1-Aug-2019) and *Sample solution*

Calculate the quantity, in mg, of each flavonol glycoside in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times C_S \times F \times 50$$

r_U = peak area of the relevant analyte from the *Sample solution*

r_S = peak area of the relevant analyte from ▲the *Standard solution*▲ (USP 1-Aug-2019)

C_S = concentration of the relevant analyte in ▲the *Standard solution*▲ (USP 1-Aug-2019) (mg/mL)

F = mean molecular mass factor to convert each analyte into flavonol glycoside with a mean molecular mass of 756.7 (2.504 for quercetin, 2.437 for isorhamnetin, and 2.588 for kaempferol)

Calculate the total quantity, in mg, of flavonol glycosides in the portion of Capsules taken by adding the individual quantities calculated. Calculate the total quantity, in mg/Capsule, of flavonol glycosides and the percentage of flavonol glycosides in the labeled amount of Powdered Ginkgo Extract.

Acceptance criteria: 22.0%–27.0% of flavonol glycosides

Change to read:

- **CONTENT OF TERPENE LACTONES**

▲▲ (USP 1-Aug-2019)

Buffer solution: Dissolve 1.19 g of dibasic sodium phosphate and 8.25 g of monobasic potassium phosphate in 1000 mL of water, and adjust to a pH of 5.8.

Diluent: Methanol and water (1:1)

Solution A: Water

Solution B: Methanol

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	75	25
23	52	48
28	52	48
30	25	75
35	10	90
40	75	25
50	75	25

Standard solutions: Using the labeled content of the individual terpene lactones, prepare five solutions of the USP Ginkgo Terpene Lactones RS in *Diluent* within the range of 5–500 µg/mL for each of the relevant terpene lactones. Use sonication to dissolve the analytes if necessary. Pass through a filter of 0.45-µm or finer pore size.

Sample solution: Weigh and finely powder the contents of NLT 20 Capsules. Transfer an accurately weighed quantity of the powder, equivalent to about 120 mg of Powdered Ginkgo Extract, to a ▲25-mL beaker. Add 10 mL of *Buffer solution*, and sonicate for 10 min.▲ (USP 1-Aug-2019) Quantitatively transfer the solution to a glass chromatographic tube filled with chromatographic siliceous earth capable of holding 20 mL of aqueous phase.[1] Rinse the beaker with two 5-mL portions of *Buffer solution*, and transfer the washings to the column. [NOTE—Do not exceed 20 mL of total aqueous phase or the holding capacity of the chromatographic tube.] Allow the *Buffer solution* to be absorbed into the column. After 15 min, elute the column with 100 mL of ethyl acetate,

[1] Suitable commercially available material is Extrelut® NT 20 from E Merck Science.

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collect the ethyl acetate solution, and evaporate to dryness under vacuum in a water bath maintained at 50°. Dissolve the residue in 20.0 mL of *Diluent*. ▲Pass a portion of the solution through a filter of 0.45-µm or finer pore size and discard the first few milliliters of the filtrate.▲ (USP 1-Aug-2019)

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Evaporative light-scattering. [NOTE—The parameters of the detector are adjusted to achieve the best signal-to-noise ratio, according to manufacturer recommendations.]
Column: 4.6-mm × 25-cm; packing L1
Column temperature: 25 ± 1°
Flow rate: 1 mL/min
Injection volume: 15 µL
System suitability
Samples: *Standard solutions*
Suitability requirements
Chromatogram similarity: The chromatograms of the *Standard solutions* are similar to the reference chromatogram provided with the lot of USP Ginkgo Terpene Lactones RS being used.
Relative standard deviation: NMT 2.0% for bilobalide in repeated injections
Correlation coefficient: NLT 0.995 for the regression line as determined in *Analysis*
Analysis
Samples: *Standard solutions* and *Sample solution*
Record the chromatograms, and identify the peaks of the relevant analytes in the chromatogram of the *Standard solutions* by comparison with the reference chromatogram of the USP Ginkgo Terpene Lactones RS lot being used. Measure the areas of the analyte peaks. Plot the logarithms of the relevant peak responses versus the logarithms of concentrations, in mg/mL, of each analyte of the *Standard solutions*, and determine the regression line using a least-squares analysis.
From the graphs, determine the concentration, *C*, in mg/mL, of the relevant analyte in the *Sample solution*.
Separately calculate the quantities, in mg, of bilobalide ($C_{15}H_{18}O_8$), ginkgolide A ($C_{20}H_{24}O_9$), ginkgolide B ($C_{20}H_{24}O_{10}$), and ginkgolide C ($C_{20}H_{24}O_{11}$) in the portion of Capsules taken:

$$\text{Result} = C \times 20$$

C = concentration of the relevant analyte in the *Sample solution* (mg/mL)

Calculate the total quantity of terpene lactones in the portion of Capsules taken by adding the quantities calculated for each analyte. Calculate the total quantity, in mg, of terpene lactones per Capsule and the percentage of terpene lactones in the labeled amount of Powdered Ginkgo Extract.
Acceptance criteria: 5.4%–12.0% of terpene lactones, calculated as the sum of bilobalide, ginkgolide A, ginkgolide B, and ginkgolide C

PERFORMANCE TESTS
• **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩, *Dissolution*
Medium: 0.1 N hydrochloric acid; 500 mL
Apparatus 2: 75 rpm
Time: 45 min
Standard solutions: Prepare as directed in the test for *Content of Terpene Lactones*.
Sample solution: Combine 25-mL portions of the solution under test from each of the six dissolution vessels in a separation funnel. Extract with four 50-mL portions of ethyl acetate. Combine the extracts, and evaporate under vacuum to dryness. Dissolve the residue with sonication in 5.0 mL of a mixture of water and methanol (1:1).
Analysis: Proceed as directed in the test for *Content of Terpene Lactones* to determine the concentration, *C*, in mg/mL, of ginkgolide B in the *Sample solution*.
Calculate the percentage of ginkgolide B dissolved:

$$\text{Result} = 5000C/3G$$

C = concentration of ginkgolide B in the *Sample solution* (mg/mL)
G = content of ginkgolide B as determined in the test for *Content of Terpene Lactones* (mg/Capsule)

Tolerances: NLT 75% of the content of ginkgolide B is dissolved.
• **WEIGHT VARIATION** ⟨2091⟩**:** Meet the requirements

CONTAMINANTS
• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meet the requirements

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at room temperature.
• **LABELING:** The label states the Latin binomial and, following the official name, the article used to prepare the Capsules. Label the Capsules to indicate the content, in mg/Capsule, of Powdered Ginkgo Extract.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Ginkgo Terpene Lactones RS
USP Isorhamnetin RS
USP Kaempferol RS
USP Quercetin RS

Powdered Ginkgo Extract

DEFINITION
Powdered Ginkgo Extract is prepared from dried and comminuted leaves of Ginkgo extracted with an acetone–water mixture or other suitable solvents. The ratio of the crude plant material to Powdered Extract is between 35:1 and 67:1. It contains NLT 22.0% and NMT 27.0% of flavonoids, calculated as flavonol glycosides, with a mean molecular mass of 756.7, on the dried basis. It contains NLT 5.4% and NMT 12.0% of terpene lactones, consisting of between 2.6% and 5.8% of bilobalide ($C_{15}H_{18}O_8$) and between 2.8% and 6.2% of the sum of ginkgolide A ($C_{20}H_{24}O_9$), ginkgolide B ($C_{20}H_{24}O_{10}$), and ginkgolide C ($C_{20}H_{24}O_{11}$), on the dried basis.

IDENTIFICATION
• **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩
Test for flavanoids
Standard solution: A solution of 0.6 mg/mL of USP Rutin RS and 0.2 mg/mL each of USP Chlorogenic Acid RS and USP Quercetin RS in methanol
Sample solution: 5 mg/mL of Powdered Extract in a mixture of methanol and water (4:1)

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Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)
Application volume: 5 µL
Developing solvent system: Ethyl acetate, water, anhydrous formic acid, and glacial acetic acid (100:26:11:11)
Spray reagent 1: 5 mg/mL of 2-aminoethyl diphenylborinate in methanol
Spray reagent 2: 50 mg/mL of polyethylene glycol 400 in alcohol
Analysis
Samples: *Standard solution* and *Sample solution*
Before development of the chromatograms, saturate the chamber for 20 min with *Developing solvent system*. Record temperature and humidity in the laboratory. If the relative humidity exceeds 50%, condition the plate to about 35% relative humidity using a suitable device. Apply the samples separately as bands to a suitable thin-layer chromatographic plate (see *Chromatography* ⟨621⟩), and allow the bands to dry. Develop the plate over a path of 6 cm, remove the plate from the chromatographic chamber, and dry in a circulating air oven at 105° for 5 min. Immediately spray the hot plate with *Spray reagent 1*, then with *Spray reagent 2*. Dry, and examine under long-wavelength UV light.
Acceptance criteria: The *Standard solution* shows in its lower part with increasing R_F values a yellowish-brown fluorescent zone due to rutin (R_F 0.28), a light blue fluorescent zone due to chlorogenic acid (R_F 0.36), and a yellow fluorescent zone due to quercetin (R_F 0.92). The *Sample solution* shows a yellowish-brown fluorescent zone, a light blue fluorescent zone, and a yellowish-brown fluorescent zone at R_F similar to those of rutin, chlorogenic acid, and quercetin, respectively, in the *Standard solution*. Additional yellowish to yellowish-green zones due to flavonoids detected in the *Sample solution* chromatogram include one zone below the rutin zone, two zones between the rutin and chlorogenic acid zones, and two zones above the chlorogenic acid zone. Other zones may be seen in the *Sample solution* chromatogram.

• **B. HPLC:** In the test for *Content of Flavonol Glycosides*, the retention times of the peaks for quercetin, isorhamnetin, and kaempferol of the *Sample solution* correspond to those of the *Standard solution*. In the chromatogram of the *Sample solution*, the ratio of the kaempferol peak to the quercetin peak is NLT 0.7, and the peak for isorhamnetin is NLT 0.1 times the size of the quercetin peak.

COMPOSITION

• **CONTENT OF FLAVONOL GLYCOSIDES**
Extraction solvent: Alcohol, hydrochloric acid, and water (25:4:10)
Mobile phase: Methanol, water, and phosphoric acid (100:100:1)
Standard solution A: 0.125 mg/mL of USP Quercetin RS in methanol
Standard solution B: 0.125 mg/mL of USP Kaempferol RS in methanol
Standard solution C: 0.03 mg/mL of USP Isorhamnetin RS in methanol
Sample solution: Transfer about 0.3 g of Powdered Extract, accurately weighed, to a 250-mL flask fitted with a reflux condenser. Add 78 mL of *Extraction solvent*, and reflux in a hot water bath for 135 min. [NOTE—The solution will turn deep red. The color of the solution is not a definitive indication of reaction completeness.] Allow to cool to room temperature. Transfer to a 100-mL volumetric flask, dilute with water to volume, and mix.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 370 nm
Column: 4.6-mm × 25-cm; packing L1
Flow rate: 1.5 mL/min
Injection volume: 20 µL
System suitability
Samples: *Standard solution A*, *Standard solution B*, and *Standard solution C*
[NOTE—The relative retention times for quercetin, kaempferol, and isorhamnetin are about 1.0, 1.8, and 2.0, respectively; *Standard solution A*, *Standard solution B*, and *Standard solution C*.]
Suitability requirements
Relative standard deviation: NMT 2.0% determined from the quercetin peak in repeated injections, *Standard solution A*
Analysis
Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*
Calculate the percentage of each flavonol glycoside in the portion of Powdered Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/W) \times F \times 10$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of the relevant analyte from *Standard solution A*, *Standard solution B*, or *Standard solution C*
C_S = concentration of the relevant analyte in *Standard solution A*, *Standard solution B*, or *Standard solution C* (mg/mL)
W = weight of Powdered Extract taken to prepare the *Sample solution* (g)
F = mean molecular mass factor to convert each analyte into flavonol glycoside with a mean molecular mass of 756.7: 2.504 for quercetin, 2.437 for isorhamnetin, and 2.588 for kaempferol

Calculate the total percentage of flavonol glycosides by adding the individual percentages calculated.
Acceptance criteria: 22.0%–27.0% of flavonoids, calculated as flavonol glycosides with a mean molecular mass of 756.7, on the dried basis

• **CONTENT OF TERPENE LACTONES**
Solvent: Methanol and water (9:1)
Buffer solution: Dissolve 1.19 g of dibasic sodium phosphate and 8.25 g of monobasic potassium phosphate in 1000 mL of water, and adjust to a pH of 5.8.
Diluent: Methanol and water (1:1)
Solution A: Water
Solution B: Methanol
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	75	25
23	52	48
28	52	48
30	25	75
35	10	90
40	75	25

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Table 1 *(continued)*

Time (min)	Solution A (%)	Solution B (%)
50	75	25

Standard solutions: Using the labeled content of the individual terpene lactones, prepare five solutions of the USP Ginkgo Terpene Lactones RS in *Diluent* within the range of 5–500 µg/mL for each of the relevant terpene lactones. Use sonication to dissolve the analytes if necessary. Pass through a filter of 0.45-µm or finer pore size.

Sample solution: Transfer about 120 mg of Powdered Extract, accurately weighed, to a 25-mL beaker. Add 10 mL of *Buffer solution* to the residue, and sonicate for 5 min. Quantitatively transfer the solution to a glass chromatographic tube filled with chromatographic siliceous earth capable of holding 20 mL of aqueous phase.[1] Rinse the beaker with two 5-mL portions of *Buffer solution*, and transfer the washings to the column. Do not exceed 20 mL of the total aqueous phase or the holding capacity of the chromatographic tube. Allow the *Buffer solution* to be absorbed into the column. After 15 min, elute the column with 100 mL of ethyl acetate, collect the ethyl acetate solution, and evaporate to dryness under vacuum in a water bath maintained at 50°. Dissolve the residue in 20.0 mL of *Diluent*.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Evaporative light-scattering. [NOTE—The parameters of the detector are adjusted to achieve the best signal-to-noise ratio, according to manufacturer recommendations.]

Column: 4.6-mm × 25-cm; packing L1

Column temperature: 25 ± 1°

Flow rate: 1 mL/min

Injection volume: 15 µL

System suitability

Samples: *Standard solutions*

Suitability requirements

Chromatogram similarity: The chromatograms from the *Standard solutions* are similar to the reference chromatogram provided with the lot of USP Ginkgo Terpene Lactones RS being used.

Relative standard deviation: NMT 2.0% determined from the bilobalide peak in repeated injections

Correlation coefficient: NLT 0.995 for the regression line as determined in *Analysis*

Analysis

Samples: *Standard solutions* and *Sample solution*

Record the chromatograms, and identify the peaks of the relevant analytes in the chromatograms of the *Standard solutions*, by comparing them with the reference chromatogram of the USP Ginkgo Terpene Lactones RS lot being used. Measure the areas of the analyte peaks. Plot the logarithms of the relevant peak responses versus the logarithms of concentrations, in mg/mL, of each analyte of the *Standard solutions*, and determine the regression line by using a least-squares analysis.

From the graphs, determine the concentration, *C*, in mg/mL, of the relevant analyte in the *Sample solution*.

Separately calculate the percentages of bilobalide ($C_{15}H_{18}O_8$), ginkgolide A ($C_{20}H_{24}O_9$), ginkgolide B ($C_{20}H_{24}O_{10}$), and ginkgolide C ($C_{20}H_{24}O_{11}$) in the portion of Powdered Extract taken:

$$\text{Result} = (C/W) \times 2000$$

C = concentration of the relevant analyte in the *Sample solution* (mg/mL)

W = weight of Powdered Extract taken to prepare the *Sample solution* (mg)

Calculate the total percentage of terpene lactones in the portion of Powdered Extract taken by adding the percentages calculated for each analyte.

Acceptance criteria

Total terpene lactones: 5.4%–12.0%

Bilobalide: 2.6%–5.8%

Sum of ginkgolide A, ginkgolide B, and ginkgolide C: 2.8%–6.2%

CONTAMINANTS

Change to read:

- **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020)**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

- **LIMIT OF RUTIN AND QUERCETIN**

Solution A: 0.1% of formic acid in water

Solution B: Acetonitrile

Mobile phase: See *Table 2*.

Table 2

Time (min)	Solution A (%)	Solution B (%)
0	90	10
40	64	36
45	0	100
50	0	100
51	90	10
60	90	10

Standard solution: Prepare a composite solution of 0.4 mg/mL USP Rutin RS and 0.05 mg/mL USP Quercetin RS in methanol. Sonicate to dissolve, if necessary, and mix well.

Sample solution: Transfer 100 mg of Powdered Extract into a 10-mL volumetric flask. Add about 7 mL of methanol, and sonicate to dissolve. Dilute with methanol to volume, and mix well. Pass through a filter of 0.45-µm or finer pore size.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; 5-µm base-deactivated packing L1

Column temperature: 30°

Flow rate: 1.0 mL/min

Injection volume: 10 µL

System suitability

Sample: *Standard solution*

[1] Suitable commercially available material is Extrelut® NT 20 from E. Merck Science.

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[NOTE—The relative retention times are 1.0 and 1.8 for rutin and quercetin, respectively.]

Suitability requirements

Column efficiency: NLT 15,000 theoretical plates for the rutin peak and NLT 20,000 for the quercetin peak

Tailing factor: 0.8–2.0 for the rutin peak

Relative standard deviation: NMT 2.0% for the rutin peak in repeated injections

[NOTE—If deterioration of peak shapes is observed, wash the column using a mixture of acetonitrile and water (9:1) at 1.0 mL/min for 30 min.]

Analysis

Samples: *Standard solution* and *Sample solution*

Use the chromatogram of the *Standard solution* to identify the rutin and quercetin peaks.

Calculate the percentages of rutin and quercetin in the portion of Powdered Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*

r_S = peak area of the relevant analyte from the *Standard solution*

C_S = concentration of USP Rutin RS or USP Quercetin RS in the *Standard solution* (mg/mL)

C_U = concentration of Powdered Extract in the *Sample solution* (mg/mL)

Acceptance criteria: NMT 4% of rutin and NMT 0.5% of quercetin

• **LIMIT OF GINKGOLIC ACIDS**

Solution A: 0.01% phosphoric acid in water

Solution B: 0.01% phosphoric acid in acetonitrile

Mobile phase: See *Table 3*.

Table 3

Time (min)	Solution A (%)	Solution B (%)
0	25	75
6	10	90
7	10	90
8	25	75
10	25	75

Standard solution: Dissolve USP Ginkgolic Acids RS in methanol, and dilute, if necessary, with water to obtain a concentration of 0.25 µg/mL of ginkgolic acids, calculated as the sum of the congeners ginkgolic acid C13:0, ginkgolic acid C15:1, and ginkgolic acid C17:1.

Sample solution: Transfer 0.5 g of Powdered Extract to a 10-mL volumetric flask. Add 8 mL of methanol to dissolve, and dilute with water to volume.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 210 nm

Column: 4.6-mm × 5-cm; base-deactivated packing L7

Column temperature: 35°

Flow rate: 1 mL/min

Injection volume: 100 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Chromatogram similarity: The chromatogram is similar to the reference chromatogram provided with the lot of USP Ginkgolic Acids RS being used.

Tailing factor: NMT 2.0 for the ginkgolic acid C15:1 peak

Relative standard deviation: NMT 5.0% for the ginkgolic acid C15:1 peak in repeated injections

Analysis

Samples: *Standard solution* and *Sample solution*

[NOTE—Identify the peaks of the relevant analytes by comparison with the reference chromatogram of the USP Ginkgolic Acids RS lot being used. If deterioration of peak shapes is observed, wash the column using a mixture of methanol and water (9:1) for 30 min.]

Calculate the concentration, in µg/g, of each ginkgolic acid in the portion of Powdered Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/W) \times P \times 10$$

r_U = peak area of the relevant analyte from the *Sample solution*

r_S = peak area of the relevant analyte from the *Standard solution*

C_S = concentration of USP Ginkgolic Acids RS in the *Standard solution* (mg/mL)

W = weight of Powdered Extract taken to prepare the *Sample solution* (mg)

P = content of the relevant ginkgolic acid in USP Ginkgolic Acids RS (µg/g)

Calculate the total amount of ginkgolic acids by adding the individual contents.

Acceptance criteria: NMT 5 µg/g

• **LOSS ON DRYING** ⟨731⟩

Sample: 1.0 g of Powdered Extract

Analysis: Dry the *Sample* at 105° for 2 h.

Acceptance criteria: NMT 5.0%

• **OTHER REQUIREMENTS:** Meets the requirements for *Residual Solvents* in *Botanical Extracts* ⟨565⟩

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, protect from moisture, and store at controlled room temperature.

• **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. The label also indicates the content of flavonol glycosides and of terpene lactones, the extracting solvent used for preparation, and the ratio of the starting crude plant material to the Powdered Extract.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Chlorogenic Acid RS
USP Ginkgo Terpene Lactones RS
USP Ginkgolic Acids RS
USP Isorhamnetin RS
USP Kaempferol RS
USP Quercetin RS
USP Rutin RS

Ginkgo Tablets

DEFINITION

Ginkgo Tablets are prepared from Powdered Ginkgo Extract and contain, in the labeled amount of Powdered Ginkgo Extract, NLT 22.0% and NMT 27.0% of flavonol glycosides and NLT 5.4% and NMT 12.0% of terpene lactones, consisting of bilobalide ($C_{15}H_{18}O_8$), ginkgolide A ($C_{20}H_{24}O_9$), ginkgolide B ($C_{20}H_{24}O_{10}$), and ginkgolide C ($C_{20}H_{24}O_{11}$).

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IDENTIFICATION

- **A. HPLC:** The retention times of the quercetin, isorhamnetin, and kaempferol peaks of the *Sample solution* correspond to those of the *Standard solution*, as obtained in the test for *Content of Flavonol Glycosides*. In the chromatogram of the *Sample solution*, the ratio of the kaempferol peak to the quercetin peak is NLT 0.7, and the isorhamnetin peak is NLT 0.1 times the size of the quercetin peak.
- **B. HPLC:** The retention times of the bilobalide, ginkgolide A, ginkgolide B, and ginkgolide C peaks of the *Sample solution* correspond to those of the *Standard solutions*, as obtained in the test for *Content of Terpene Lactones*.

STRENGTH

Change to read:

- **CONTENT OF FLAVONOL GLYCOSIDES**

Mobile phase: Methanol, water, and phosphoric acid (100:100:1)

▲Standard solution: 0.2 mg/mL of USP Quercetin RS, 0.2 mg/mL of USP Kaempferol RS, and 0.05 mg/mL of USP Isorhamnetin RS in methanol▲ (USP 1-Aug-2019)

Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer an accurately weighed quantity of the powder, equivalent to about 50 mg of flavonol glycosides, to a 50-mL volumetric flask. Add 20 mL of methanol, and sonicate for 3 min. Add 20 mL of 1.5 N hydrochloric acid, and sonicate again for 10 min. Allow to cool to room temperature, and dilute with methanol to volume. Centrifuge, and transfer a portion of the clear supernatant to a rubber-capped, low-actinic glass vial. Heat in a steam bath for 25 min, and cool to room temperature in an ice bath.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 370 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1.5 mL/min

Injection volume: 20 µL

System suitability

Sample: ▲*Standard solution*▲ (USP 1-Aug-2019)

[NOTE—The relative retention times for quercetin, kaempferol, and isorhamnetin are about 1.0, 1.8, and 2.0, respectively.▲▲ (USP 1-Aug-2019)]

Suitability requirements

Relative standard deviation: NMT 2.0% for quercetin▲▲ (USP 1-Aug-2019)

Analysis

Samples: ▲*Standard solution*▲ (USP 1-Aug-2019) and *Sample solution*

Calculate the quantity, in mg, of each flavonol glycoside in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times C_S \times F \times 50$$

r_U = peak area of the relevant analyte from the *Sample solution*

r_S = peak area of the relevant analyte from ▲the *Standard solution*▲ (USP 1-Aug-2019)

C_S = concentration of the relevant analyte in ▲the *Standard solution*▲ (USP 1-Aug-2019) (mg/mL)

F = mean molecular mass factor to convert each analyte into flavonol glycoside with a mean molecular mass of 756.7 (2.504 for quercetin, 2.437 for isorhamnetin, and 2.588 for kaempferol)

Calculate the total quantity, in mg, of flavonol glycosides in the portion of Tablets taken by adding the individual quantities calculated. Calculate the total quantity, in mg/Tablet, of flavonol glycosides and the percentage of flavonol glycosides in the labeled amount of Powdered Ginkgo Extract.

Acceptance criteria: 22.0%–27.0% of flavonol glycosides

Change to read:

- **CONTENT OF TERPENE LACTONES**

▲▲ (USP 1-Aug-2019)

Buffer solution: Dissolve 1.19 g of dibasic sodium phosphate and 8.25 g of monobasic potassium phosphate in 1000 mL of water, and adjust to a pH of 5.8.

Diluent: Methanol and water (1:1)

Solution A: Water

Solution B: Methanol

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	75	25
23	52	48
28	52	48
30	25	75
35	10	90
40	75	25
50	75	25

Standard solutions: Using the labeled content of the individual terpene lactones, prepare five solutions of the USP Ginkgo Terpene Lactones RS in *Diluent* within the range of 5–500 µg/mL for each of the relevant terpene lactones. Use sonication to dissolve the analytes if necessary. Pass through a filter of 0.45-µm or finer pore size.

Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer an accurately weighed quantity of the powder, equivalent to about 120 mg of Powdered Ginkgo Extract, to a ▲25-mL beaker. Add 10 mL of *Buffer solution*, and sonicate for 10 min.▲ (USP 1-Aug-2019) Quantitatively transfer the solution to a glass chromatographic tube filled with chromatographic siliceous earth capable of holding 20 mL of aqueous phase.[1] Rinse the beaker with two 5-mL portions of *Buffer solution*, and transfer the washings to the column. [NOTE—Do not exceed 20 mL of total aqueous phase or the holding capacity of the chromatographic tube.] Allow the *Buffer solution* to be absorbed into the column. After 15 min, elute the column with 100 mL of ethyl acetate, collect the ethyl acetate solution, and evaporate to dryness under vacuum in a water bath maintained at 50°. Dissolve the residue in 20.0 mL of *Diluent*. ▲Pass a portion of the solution through a filter of 0.45-µm or finer pore size and discard the first few milliliters of the filtrate.▲ (USP 1-Aug-2019)

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Evaporative light-scattering. [NOTE—The parameters of the detector are adjusted to achieve the best signal-to-noise ratio, according to manufacturer recommendations.]

[1] Suitable commercially available material is Extrelut® NT 20 from E Merck Science.

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Column: 4.6-mm × 25-cm; packing L1
Column temperature: 25 ± 1°
Flow rate: 1 mL/min
Injection volume: 15 µL
System suitability
Samples: *Standard solutions*
Suitability requirements
Chromatogram similarity: The chromatograms of the *Standard solutions* are similar to the reference chromatogram provided with the lot of USP Ginkgo Terpene Lactones RS being used.
Relative standard deviation: NMT 2.0% for bilobalide in repeated injections
Correlation coefficient: NLT 0.995 for the regression line as determined in *Analysis*
Analysis
Samples: *Standard solutions* and *Sample solution*
Record the chromatograms, and identify the peaks of the relevant analytes in the chromatogram of the *Standard solutions* by comparison with the reference chromatogram of the USP Ginkgo Terpene Lactones RS lot being used. Measure the areas of the analyte peaks. Plot the logarithms of the relevant peak responses versus the logarithms of concentrations, in mg/mL, of each analyte of the *Standard solutions*, and determine the regression line using a least-squares analysis.
From the graphs, determine the concentration, *C*, in mg/mL, of the relevant analyte in the *Sample solution*.
Separately calculate the quantities, in mg, of bilobalide ($C_{15}H_{18}O_8$), ginkgolide A ($C_{20}H_{24}O_9$), ginkgolide B ($C_{20}H_{24}O_{10}$), and ginkgolide C ($C_{20}H_{24}O_{11}$) in the portion of Tablets taken:

$$\text{Result} = C \times 20$$

C = concentration of the relevant analyte in the *Sample solution* (mg/mL)

Calculate the total quantity of terpene lactones in the portion of Tablets taken by adding the quantities calculated for each analyte. Calculate the total quantity, in mg, of terpene lactones per Tablet and the percentage of terpene lactones in the labeled amount of Powdered Ginkgo Extract.
Acceptance criteria: 5.4%–12.0% of terpene lactones, consisting of bilobalide, ginkgolide A, ginkgolide B, and ginkgolide C

PERFORMANCE TESTS

• **Disintegration and Dissolution** ⟨2040⟩, *Dissolution*
Medium: 0.1 N hydrochloric acid; 500 mL
Apparatus 2: 75 rpm
Time: 45 min
Standard solutions: Prepare as directed in the test for *Content of Terpene Lactones*.
Sample solution: Combine 25-mL portions of the solution under test from each of the six dissolution vessels in a separation funnel. Extract with four 50-mL portions of ethyl acetate. Combine the extracts, and evaporate under vacuum to dryness. Dissolve the residue with sonication in 5.0 mL of a mixture of water and methanol (1:1).
Analysis: Proceed as directed in the test for *Content of Terpene Lactones* to determine the concentration, *C*, in mg/mL, of ginkgolide B in the *Sample solution*.
Calculate the percentage of ginkgolide B dissolved:

$$\text{Result} = 5000C/3G$$

C = concentration of ginkgolide B in the *Sample solution* (mg/mL)
G = content of ginkgolide B as determined in the test for *Content of Terpene Lactones* (mg/Tablet)

Tolerances: NLT 75% of the content of ginkgolide B is dissolved.
• **Weight Variation** ⟨2091⟩**:** Meet the requirements

CONTAMINANTS

• **Microbial Enumeration Tests** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
• **Absence of Specified Microorganisms** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meet the requirements

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Preserve in tight, light-resistant containers, and store at room temperature.
• **Labeling:** The label states the Latin binomial and, following the official name, the article used to prepare the Tablets. Label the Tablets to indicate the content, in mg/Tablet, of Powdered Ginkgo Extract.
• **USP Reference Standards** ⟨11⟩
USP Ginkgo Terpene Lactones RS
USP Isorhamnetin RS
USP Kaempferol RS
USP Quercetin RS

Ginseng, American—*see American Ginseng*

Ginseng, Asian—*see Asian Ginseng*

Ginseng, Siberian—*see Eleuthero*

Ginseng, Tienchi—*see Tienchi Ginseng Root and Rhizome*

Glucosamine and Chondroitin Sulfate Sodium Tablets

DEFINITION

Glucosamine and Chondroitin Sulfate Sodium Tablets are prepared from either Glucosamine Hydrochloride, Glucosamine Sulfate Sodium Chloride, Glucosamine Sulfate Potassium Chloride, or a mixture of any of them, with Chondroitin Sulfate Sodium. Tablets contain NLT 90.0% and NMT 120.0% of the labeled amounts of chondroitin sulfate sodium and glucosamine ($C_6H_{13}NO_5$).
[Note—Chondroitin Sulfate Sodium is extremely hygroscopic once dried. Avoid exposure to atmosphere, and weigh promptly.]

IDENTIFICATION

• **A.** The retention time of the major peaks of the *Sample solution* correspond to those of the *Standard solution*, as obtained in the test for *Content of Glucosamine*.

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• **B. ELECTROPHORESIS**

Barium acetate buffer: Dissolve 25.24 g of barium acetate in 900 mL of water. Adjust with acetic acid to a pH of 5.0, and dilute with water to 1000 mL.

Staining reagent: 0.1% (w/v) toluidine blue in 0.1 M acetic acid

Standard solution: Use the *Standard solution* of middle concentration from the test for *Content of Chondroitin Sulfate Sodium*.

Sample solution: Prepare as directed in the test for *Content of Chondroitin Sulfate Sodium*.

Analysis: Fill the chambers of an electrophoresis apparatus suitable for separations on cellulose acetate membranes[1] (a small submarine gel chamber or one dedicated to membrane media) with *Barium acetate buffer*. Soak a cellulose acetate membrane 5–6 cm × 12–14 cm in *Barium acetate buffer* for 10 min, or until evenly wetted, then blot dry between two sheets of absorbent paper. Using an applicator[2] suitable for electrophoresis, apply equal volumes (0.5 µL) of the *Sample solution* and *Standard solution* to the brighter side of the membrane held in position in an appropriate applicator stand or on a separating bridge in the chamber. Ensure that both ends of the membrane are dipped at least 0.5–1.0 cm deep into the buffer chambers. Apply a constant 60 V (6 mA at the start) for 2 h. [NOTE—Perform the application of solutions and voltage within 5 min because further drying of the blotted paper reduces sensitivity.]

Place the membrane in a plastic staining tray, and with the application side down, float or gently immerse in *Staining reagent* for 5 min. Then stir the solution gently for 1 min. Remove the membrane, and destain in 5% acetic acid until the background clears.

Acceptance criteria: The principal spot of the *Sample solution* has the same migration as the principal spot of the *Standard solution*.

[NOTE—Document the results by taking a picture within 15 min of completion of destaining.]

STRENGTH

• **CONTENT OF GLUCOSAMINE**

Diluent: Transfer 29 µL of acetic acid and 5 mL of acetonitrile to a 100-mL volumetric flask containing 50 mL of water. Dilute with water to volume.

Borate buffer: 0.2 M (76.3 g/L of sodium borate in water) adjusted with hydrochloric acid TS to a pH of 9.5

Acetate buffer: 6.80 g/L of sodium acetate trihydrate in water adjusted with dilute acetic acid to a pH of 5.9

Derivatizing reagent: In a 14-mL polypropylene culture tube, dissolve 50 mg of *o*-phthalaldehyde in 1.25 mL of anhydrous methanol. Add 50 µL of 3-mercaptopropionic acid and 11.2 mL of *Borate buffer*, and mix gently. Allow to stand in the dark for 30 min before use. [NOTE—Reagent strength is maintained by adding 10 µL of 3-mercaptopropionic acid every 2 days. Storage should be in the dark at room temperature, and can be used for NMT 2 weeks.]

Mobile phase: Methanol and *Acetate buffer* (1:9)

Standard solution: 1.0 mg/mL of USP Glucosamine Hydrochloride RS in water. Allow to stand at room temperature for 1 h.

Sample solution: Transfer an equivalent to 25 mg of glucosamine, from finely powdered Tablets (NLT 20), to a 25-mL volumetric flask. Dilute with *Diluent* to volume. Mix on a vortex mixer to suspend the powder in solution. Sonicate in a 65° water bath for 20 min. Remove from the bath, stir for 5 min with the aid of a magnetic stirrer, and centrifuge.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 340 nm

Column: 3.0-mm × 5-cm; packing L1

Flow rate: 1 mL/min

Injection size: 10 µL

System suitability

Samples: Five individual aliquots of the *Standard solution* derivatized as directed in the *Analysis*. Each derivatized aliquot is injected only once.

[NOTE—The relative retention times for the β-anomer and the α-anomer are 1.0 and 1.8, respectively. The retention time for the β-anomer is NLT 4 min.]

Suitability requirements

Relative standard deviation: NMT 2.0% from five replicate injections

Analysis

Samples: *Standard solution* and *Sample solution*

Transfer 100 µL of the *Derivatizing reagent* and 100 µL of the *Standard solution* or *Sample solution* to a vial containing 400 µL of *Borate buffer*. Allow the derivatization to proceed for 1 min. Inject the derivatized solutions immediately after the derivatization reaction.

Calculate the percentage of the labeled amount of glucosamine ($C_6H_{13}NO_5$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak response of the β-anomer from the derivatized *Sample solution*

r_S = peak response of the β-anomer from the derivatized *Standard solution*

C_S = concentration of USP Glucosamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of glucosamine in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of glucosamine, 179.17

M_{r2} = molecular weight of glucosamine hydrochloride, 215.63

Acceptance criteria: 90.0%–120.0%

• **CONTENT OF CHONDROITIN SULFATE SODIUM**

Diluent: Weigh about 297 mg of monobasic potassium phosphate, 492 mg of dibasic potassium phosphate, and 250 mg of polysorbate 80, and transfer into a 1-L beaker. Dissolve in approximately 900 mL of water, and adjust with potassium hydroxide or phosphoric acid to a pH of 7.0 ± 0.2. Dilute with water to 1 L, and mix thoroughly.

Standard solutions: 1.5, 1.0, and 0.5 mg/mL of USP Chondroitin Sulfate Sodium RS in water

Sample solution: Transfer an equivalent to 100 mg of chondroitin sulfate sodium, from finely powdered Tablets (NLT 20), to 60 mL of water. Shake to suspend the powder in solution. Sonicate in a 65° water bath for 20 min. Remove from the bath, and stir or shake for 5 min. Dilute with water to 100 mL, and centrifuge or pass through a suitable filter.

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Photometric titration

Titrant: 1 mg/mL of cetylpyridinium chloride in water. Degas before use.

[1] Suitable cellulose acetate membranes for electrophoresis are available from Fluka Chemical Corp., Milwaukee, WI; and DiaSys Corp., Waterbury, CT (www.diasys.com).

[2] Suitable applicators are available from DiaSys Corp., Waterbury, CT (www.diasys.com) and Helena Laboratories, Beaumont, TX (www.helena.com).

Supplement 3

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Endpoint detection: Turbidimetric with a photoelectric probe

Analysis

Samples: *Standard solutions* and *Sample solution*

Transfer 5.0 mL of each *Standard solution* and the *Sample solution* to separate titration vessels. Add 25 mL of *Diluent* to each. Stir until a steady reading is obtained with a photoelectric probe either at 420, 550, or 660 nm. Set the instrument to zero in absorbance mode. Titrate with *Titrant* using the photoelectric probe to determine the endpoint turbidimetrically. From a linear regression equation calculated using the volumes of *Titrant* consumed versus concentrations of the *Standard solutions*, determine the concentration of chondroitin sulfate sodium in the *Sample solution*.

Calculate the percentage of the labeled amount of chondroitin sulfate sodium in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = determined concentration of chondroitin sulfate sodium in the *Sample solution* (mg/mL)

C_U = nominal concentration of chondroitin sulfate sodium in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–120.0%

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** ⟨2040⟩**:** Meet the requirements for *Dissolution*

Medium: Water; 900 mL

Apparatus 2: 75 rpm

Time: 60 min

Determine the percentage of the labeled amount of glucosamine ($C_6H_{13}NO_5$) dissolved by using the following method.

Standard solution: Prepare as directed in the test for *Content of Glucosamine*. Dilute with a suitable quantity of water, if necessary.

Sample solution: Use the solution under test.

Borate buffer, Acetate buffer, Derivatizing reagent, Mobile phase, and **Chromatographic system:** Proceed as directed in the test for *Content of Glucosamine*.

Analysis: Proceed as directed in the test for *Content of Glucosamine*.

Calculate the percentage of the labeled amount of glucosamine ($C_6H_{13}NO_5$) dissolved:

$$\text{Result} = (r_U/r_S) \times (C_S \times V/L) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area from the derivatized *Sample solution*

r_S = peak area from the derivatized *Standard solution*

C_S = concentration of USP Glucosamine Hydrochloride RS in the *Standard solution* (mg/mL)

V = volume of *Medium*, 900 mL

L = label claim of glucosamine (mg/Tablet)

M_{r1} = molecular weight of glucosamine, 179.17

M_{r2} = molecular weight of glucosamine hydrochloride, 215.63

Tolerances: NLT 75% of the labeled amount of glucosamine ($C_6H_{13}NO_5$) is dissolved.

Determine the percentage of the labeled amount of chondroitin sulfate sodium dissolved by using the following method.

Standard solutions, Titrant, and **Diluent:** Proceed as directed in the test for *Content of Chondroitin Sulfate Sodium*.

Sample solution: Use the solution under test.

Analysis: Proceed as directed in the test for *Content of Chondroitin Sulfate Sodium*.

Calculate the percentage of the labeled amount of chondroitin sulfate sodium dissolved:

$$\text{Result} = (C \times V/L) \times 100$$

C = determined concentration of chondroitin sulfate sodium in the *Sample solution* (mg/mL)

V = volume of *Medium*, 900 mL

L = label claim of chondroitin sulfate sodium (mg/Tablet)

Tolerances: NLT 75% of the labeled amount of chondroitin sulfate sodium is dissolved.

- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩**:** Meet the requirements

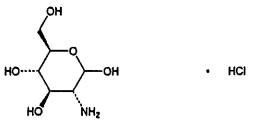
ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** The label indicates the types of glucosamine salts contained in the article and the species source from which the chondroitin was derived. Label it to state the source(s) of chondroitin sulfate sodium, whether bovine, porcine, avian, or a mixture of any of them. The label states on the front panel the content of chondroitin sulfate sodium on the dried basis.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Chondroitin Sulfate Sodium RS

USP Glucosamine Hydrochloride RS

Glucosamine Hydrochloride



$C_6H_{13}NO_5 \cdot HCl$ 215.63

D-Glucose, 2-amino-2-deoxy-, hydrochloride; 2-Amino-2-deoxy-β-D-glucopyranose hydrochloride [66-84-2].

DEFINITION

Glucosamine Hydrochloride contains NLT 98.0% and NMT 102.0% of glucosamine hydrochloride ($C_6H_{13}NO_5 \cdot HCl$), calculated on the dried basis.

IDENTIFICATION

Change to read:

- **A.** ▲**SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
- **B. IDENTIFICATION TESTS—GENERAL,** *Chloride* ⟨191⟩**:** Meets the requirements
- **C.** The retention time of the glucosamine peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

DS Monographs

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ASSAY

• **PROCEDURE**

Buffer: In a 1-L volumetric flask, dissolve 3.5 g of dibasic potassium phosphate in water. Add 0.25 mL of ammonium hydroxide, dilute with water to volume, and mix. Adjust with phosphoric acid to a pH of 7.5.

Mobile phase: Acetonitrile and *Buffer* (75:25)

Diluent: Acetonitrile and water (50:50)

Standard solution: 3.8 mg/mL of USP Glucosamine Hydrochloride RS in *Diluent*

Sample solution: 3.8 mg/mL of Glucosamine Hydrochloride in *Diluent*. [NOTE—Shake by mechanical means to aid dissolution.]

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 195 nm

Column: 4.6-mm × 15-cm; 5-µm packing L8

Column temperature: 35°

Flow rate: 1.5 mL/min

Injection size: 10 µL

System suitability

Sample: *Standard solution*

[NOTE—The peak for the glucosamine moiety elutes at about 10 min. The chromatogram shows a large additional peak near the void volume, due to the chloride ion.]

Suitability requirements

Tailing factor: NMT 2.0 for the glucosamine peak

Efficiency: NLT 1500 theoretical plates

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of glucosamine hydrochloride ($C_6H_{13}NO_5 \cdot HCl$) in the portion of Glucosamine Hydrochloride taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Glucosamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = concentration of Glucosamine Hydrochloride in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–102.0% on the dried basis

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%

• **CHLORIDE AND SULFATE,** *Sulfate* ⟨221⟩: A 0.10-g portion shows no more sulfate than corresponds to 0.25 mL of 0.020 N sulfuric acid (NMT 0.24%).

• **ARSENIC,** *Method II* ⟨211⟩: NMT 3 ppm

SPECIFIC TESTS

• **OPTICAL ROTATION,** *Specific Rotation* ⟨781S⟩: +70.0° to +73.0°

Sample solution: 25 mg/mL. Measure the specific rotation 3 h after preparation.

• **pH** ⟨791⟩

Sample solution: 20 mg/mL

Acceptance criteria: 3.0–5.0

• **LOSS ON DRYING** ⟨731⟩: Dry a sample at 105° for 2 h: it loses NMT 1.0% of its weight.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Glucosamine Hydrochloride RS

Glucosamine Tablets

DEFINITION

Glucosamine Tablets are prepared from Glucosamine Hydrochloride, Glucosamine Sulfate Sodium Chloride, Glucosamine Sulfate Potassium Chloride, or a mixture of any of them. Tablets contain NLT 90.0% and NMT 110.0% of the labeled amount of glucosamine ($C_6H_{13}NO_5$).

IDENTIFICATION

• **A.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Glucosamine*.

• **B. IDENTIFICATION TESTS—GENERAL,** *Chloride* ⟨191⟩: Meets the requirements

• **C. IDENTIFICATION TESTS—GENERAL,** *Sulfate* ⟨191⟩: Meets the requirements. [NOTE—Only for Tablets labeled as containing glucosamine sodium sulfate or glucosamine potassium sulfate]

STRENGTH

• **CONTENT OF GLUCOSAMINE**

Buffer: In a 1-L volumetric flask dissolve 3.5 g of dibasic potassium phosphate in water. Add 0.25 mL of ammonium hydroxide, dilute with water to volume, and mix. Adjust with phosphoric acid to a pH of 7.5.

Mobile phase: Acetonitrile and *Buffer* (75:25)

Diluent: Acetonitrile and water (50:50)

Standard solution: 3.75 mg/mL of USP Glucosamine Hydrochloride RS in *Diluent*

Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer an accurately weighed portion of the finely powdered material, equivalent to about 312 mg of glucosamine, to a 100-mL volumetric flask. Add 60 mL of *Diluent*, and sonicate for 10 min. Shake by mechanical means for 15 min. Dilute with *Diluent* to volume, and mix. Pass a portion of this solution through a membrane filter of 0.45-µm or finer pore size.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 195 nm

Column: 4.6-mm × 15-cm; 5-µm packing L8

Column temperature: 35°

Flow rate: 1.5 mL/min

Injection size: 10 µL

[NOTE—The peak for glucosamine moiety elutes at about 10 min. The chromatogram shows a large additional peak near the void volume, due to the chloride ion.]

System suitability

Sample: *Standard solution*

Suitability requirements

Tailing factor: NMT 2.0 for the glucosamine peak

Column efficiency: NLT 1500 theoretical plates

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of glucosamine ($C_6H_{13}NO_5$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

DS Monographs

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r_U = peak response of glucosamine from the *Sample solution*
r_S = peak response of glucosamine from the *Standard solution*
C_S = concentration of USP Glucosamine Hydrochloride RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of glucosamine in the *Sample solution* (mg/mL)
M_{r1} = molecular weight of glucosamine, 179.17
M_{r2} = molecular weight of glucosamine hydrochloride, 215.63

Acceptance criteria: 90.0%–110.0%

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** ⟨2040⟩**:** Meet the requirements for *Dissolution*

Medium: Water; 900 mL
Apparatus 2: 75 rpm
Time: 60 min
Standard solution: Dissolve a suitable amount of USP Glucosamine Hydrochloride RS in water to obtain a concentration similar to that expected in the *Sample solution*.
Sample solution: Filtered portion of the solution under test
Buffer: Mix 1.0 mL of phosphoric acid with 2 L of water, and adjust with potassium hydroxide to a pH of 3.0.
Mobile phase: Acetonitrile and *Buffer* (2:3)
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 195 nm
Column: 4.6-mm × 25-cm; packing L7
Flow rate: 0.6 mL/min
Injection size: 10 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Tailing factor: NMT 2.0 for the glucosamine peak
Relative standard deviation: NMT 2.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of glucosamine ($C_6H_{13}NO_5$) dissolved:

$$\text{Result} = (r_U/r_S) \times (C_S \times V/L) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area from the *Sample solution*
r_S = peak area from the *Standard solution*
C_S = concentration of USP Glucosamine Hydrochloride RS in the *Standard solution* (mg/mL)
V = volume of *Medium*, 900 mL
L = labeled amount of glucosamine (mg/Tablet)
M_{r1} = molecular weight of glucosamine, 179.17
M_{r2} = molecular weight of glucosamine hydrochloride, 215.63

Tolerances: NLT 75% of the labeled amount of glucosamine ($C_6H_{13}NO_5$) is dissolved.

- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩**:** Meet the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** The label indicates the type of glucosamine salt contained in the article.

- **USP REFERENCE STANDARDS** ⟨11⟩
USP Glucosamine Hydrochloride RS

Glucosamine Sulfate Potassium Chloride

$(C_6H_{14}NO_5)_2SO_4 \cdot 2KCl$ 605.52
Bis(D-glucose, 2-amino-2-deoxy-), sulfate potassium chloride complex;
Bis(2-amino-2-deoxy-β-D-glucopyranose) sulfate potassium chloride complex (–,–) [1296149-08-0].

DEFINITION
Glucosamine Sulfate Potassium Chloride contains NLT 98.0% and NMT 102.0% of glucosamine sulfate potassium chloride [$(C_6H_{14}NO_5)_2SO_4 \cdot 2KCl$], calculated on the dried basis.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

Sample: Transfer 50 mg of Glucosamine Sulfate Potassium Chloride to a centrifuge tube, and dissolve in 2 mL of water. Add 0.5 mL of barium chloride TS, and centrifuge. Collect the supernatant, and evaporate to dryness. Dry the residue at 105° for 2 h.
Acceptance criteria: The IR spectrum of the *Sample* matches that of a similar preparation of USP Glucosamine Hydrochloride RS, except that the addition of barium chloride TS is omitted.

- **B. IDENTIFICATION TESTS—GENERAL,** *Chloride* ⟨191⟩ and *Potassium* ⟨191⟩**:** Meets the requirements
- **C.** The retention time of the glucosamine peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.
- **D. SULFATE:** In the test for *Content of Sulfate*, after the addition of barium chloride TS a white precipitate is formed.

ASSAY

- **PROCEDURE**

Buffer: In a 1-L volumetric flask, dissolve 3.5 g of dibasic potassium phosphate in water, add 0.25 mL of ammonium hydroxide, dilute with water to volume, and mix. Adjust with phosphoric acid to a pH of 7.5.
Mobile phase: Acetonitrile and *Buffer* (75:25)
Diluent: Acetonitrile and water (50:50)
Standard solution: 3.8 mg/mL of USP Glucosamine Hydrochloride RS in *Diluent*. Shake for 5 min by mechanical means to completely dissolve.
Sample solution: Transfer 263 mg of Glucosamine Sulfate Potassium Chloride to a 50-mL volumetric flask. Dissolve in 30 mL of *Diluent*, and shake by mechanical means. Dilute with *Diluent* to volume.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 195 nm
Column: 4.6-mm × 15-cm; 5-µm packing L8
Column temperature: 35°
Flow rate: 1.5 mL/min
Injection size: 10 µL
System suitability
Sample: *Standard solution*

DS Monographs

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[NOTE—The peak for the glucosamine moiety elutes at about 10 min. The chromatogram shows additional peaks near the void volume, due to the counter ions.]

Suitability requirements

Tailing factor: NMT 2.0 for the glucosamine peak

Efficiency: NLT 1500 theoretical plates

Relative standard deviation: NMT 2.0%.

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of glucosamine sulfate potassium chloride [$(C_6H_{14}NO_5)_2SO_4 \cdot 2KCl$] in the portion of Glucosamine Sulfate Potassium Chloride taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Glucosamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = concentration of Glucosamine Sulfate Potassium Chloride in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of glucosamine sulfate potassium chloride, 605.52

M_{r2} = twice the molecular weight of glucosamine hydrochloride, 431.26

Acceptance criteria: 98.0%–102.0% on the dried basis

OTHER COMPONENTS

- **CONTENT OF SULFATE**

Sample: 1 g of Glucosamine Sulfate Potassium Chloride

Analysis: Transfer the *Sample* to a 250-mL beaker, and dissolve in 100 mL of water. Add 4 mL of 6 N hydrochloric acid. Heat the solution to boiling, and add, with constant stirring, sufficient boiling barium chloride TS to completely precipitate the sulfate. Add an additional 2 mL of barium chloride TS, and digest on a steam bath for 1 h. Pass the mixture through ashless filter paper. Transfer the residue quantitatively to a new filter, and wash the residue with hot water until no precipitate is obtained when 1 mL of silver nitrate TS is added to 5 mL of washing. Transfer the paper containing the residue to a tared crucible. Char the paper, without burning, and ignite the crucible and its contents to constant weight. Calculate the content of sulfate by multiplying the weight obtained by 0.4116.

Acceptance criteria: 15.5%–16.5%

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩: 26.5%–31.0%
- **SODIUM:** A solution (1 in 10), tested on a platinum wire, does not impart a pronounced yellow color to a nonluminous flame.
- **ARSENIC,** *Method II* ⟨211⟩: NMT 3 µg/g

SPECIFIC TESTS

- **OPTICAL ROTATION,** *Specific Rotation* ⟨781S⟩

Sample solution: 35 mg/mL. Measure the specific rotation 3 h after preparation.

Acceptance criteria: +47.0° to +53.0°

- **pH** ⟨791⟩

Sample solution: 20 mg/mL

Acceptance criteria: 3.0–5.0

- **LOSS ON DRYING** ⟨731⟩: Dry a sample at 105° for 2 h: it loses NMT 1.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Glucosamine Hydrochloride RS

Glucosamine Sulfate Sodium Chloride

$(C_6H_{14}NO_5)_2SO_4 \cdot 2NaCl$ 573.31

Bis(D-glucose, 2-amino-2-deoxy-), sulfate sodium chloride complex;

Bis(2-amino-2-deoxy-β-D-glucopyranose) sulfate sodium chloride complex (–,–) [1296149-13-7].

DEFINITION

Glucosamine Sulfate Sodium Chloride contains NLT 98.0% and NMT 102.0% of glucosamine sulfate sodium chloride [$(C_6H_{14}NO_5)_2SO_4 \cdot 2NaCl$], calculated on the dried basis.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

Sample: Transfer 50 mg of Glucosamine Sulfate Sodium Chloride to a centrifuge tube, and dissolve in 2 mL of water. Add 0.5 mL of barium chloride TS, and centrifuge. Collect the supernatant, and evaporate to dryness. Dry the residue at 105° for 2 h.

Acceptance criteria: The IR spectrum of the *Sample* matches that of a similar preparation of USP Glucosamine Hydrochloride RS, except that the addition of barium chloride TS is omitted.

- **B. IDENTIFICATION TESTS—GENERAL,** *Chloride* ⟨191⟩ and *Sodium* ⟨191⟩: Meets the requirements
- **C.** The retention time of the glucosamine peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.
- **D. SULFATE:** In the test for *Content of Sulfate*, after the addition of barium chloride TS a white precipitate is formed.

ASSAY

- **PROCEDURE**

Buffer: In a 1-L volumetric flask, dissolve 3.5 g of dibasic potassium phosphate in water, add 0.25 mL of ammonium hydroxide, dilute with water to volume, and mix. Adjust with phosphoric acid to a pH of 7.5.

Mobile phase: Acetonitrile and *Buffer* (75:25)

Diluent: Acetonitrile and water (50:50)

Standard solution: 3.8 mg/mL of USP Glucosamine Hydrochloride RS in *Diluent*. Shake for 5 min by mechanical means to completely dissolve.

Sample solution: Transfer 250 mg of Glucosamine Sulfate Sodium Chloride to a 50-mL volumetric flask. Dissolve in 30 mL of *Diluent*, and shake by mechanical means. Dilute with *Diluent* to volume.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 195 nm

Column: 4.6-mm × 15-cm; 5-µm packing L8

Column temperature: 35°

Flow rate: 1.5 mL/min

Injection size: 10 µL

System suitability

Sample: *Standard solution*

[NOTE—The peak for the glucosamine moiety elutes at about 10 min. The chromatogram shows additional peaks near the void volume, due to the counter ions.]

Suitability requirements

Tailing factor: NMT 2.0 for the glucosamine peak

Efficiency: NLT 1500 theoretical plates

Relative standard deviation: NMT 2.0%.

DS Monographs

www.webofpharma.com

Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of glucosamine sulfate sodium chloride $[(C_6H_{14}NO_5)_2SO_4 \cdot 2NaCl]$ in the portion of Glucosamine Sulfate Sodium Chloride taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP Glucosamine Hydrochloride RS in the *Standard solution* (mg/mL)
C_U = concentration of Glucosamine Sulfate Sodium Chloride in the *Sample solution* (mg/mL)
M_{r1} = molecular weight of glucosamine sulfate sodium chloride, 573.31
M_{r2} = twice the molecular weight of glucosamine hydrochloride, 431.26

Acceptance criteria: 98.0%–102.0% on the dried basis

OTHER COMPONENTS
- **CONTENT OF SULFATE**

Sample: 1 g of Glucosamine Sulfate Sodium Chloride
Analysis: Transfer the *Sample* to a 250-mL beaker, and dissolve in 100 mL of water. Add 4 mL of 6 N hydrochloric acid. Heat the solution to boiling, and add, with constant stirring, sufficient boiling barium chloride TS to completely precipitate the sulfate. Add an additional 2 mL of barium chloride TS, and digest on a steam bath for 1 h. Pass the mixture through ashless filter paper. Transfer the residue quantitatively to a new filter, and wash the residue with hot water until no precipitate is obtained when 1 mL of silver nitrate TS is added to 5 mL of washing. Transfer the paper containing the residue to a tared crucible. Char the paper, without burning, and ignite the crucible and its contents to constant weight. Calculate the content of sulfate by multiplying the weight obtained by 0.4116.
Acceptance criteria: 16.3%–17.3%

IMPURITIES
- **RESIDUE ON IGNITION** ⟨281⟩: 22.5%–26.0%
- **ARSENIC,** *Method II* ⟨211⟩: NMT 3 µg/g
- **POTASSIUM**

Analysis: Acidify 5 mL of a solution (1 in 20) with 6 N acetic acid, and add 5 drops of sodium cobaltinitrite TS.
Acceptance criteria: No precipitate is formed.

SPECIFIC TESTS
- **OPTICAL ROTATION,** *Specific Rotation* ⟨781S⟩

Sample solution: 35 mg/mL. Measure the specific rotation 3 h after preparation.
Acceptance criteria: +50.0° to +55.0°
- **pH** ⟨791⟩

Sample solution: 20 mg/mL
Acceptance criteria: 3.0–5.0
- **LOSS ON DRYING** ⟨731⟩: Dry a sample at 105° for 2 h: it loses NMT 1.0% of its weight.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Glucosamine Hydrochloride RS

Glucosamine and Methylsulfonylmethane Tablets

DEFINITION
Glucosamine and Methylsulfonylmethane Tablets are prepared from either Glucosamine Hydrochloride, Glucosamine Sulfate Sodium Chloride, Glucosamine Sulfate Potassium Chloride, or a mixture of any of them, with Methylsulfonylmethane. Tablets contain NLT 90.0% and NMT 120.0% of the labeled amount of glucosamine ($C_6H_{13}NO_5$) and NLT 90.0% and NMT 110.0% of the labeled amount of methylsulfonylmethane ($C_2H_6O_2S$).

IDENTIFICATION
- **A. PRESENCE OF GLUCOSAMINE:** The retention times of the major peaks of the *Sample solution* correspond to those of the *Standard solution*, as obtained in the *Content of Glucosamine*.
- **B. PRESENCE OF METHYLSULFONYLMETHANE:** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Content of Methylsulfonylmethane*.

STRENGTH
- **CONTENT OF GLUCOSAMINE**

Diluent: Transfer 29 µL of acetic acid and 5 mL of acetonitrile to a 100-mL volumetric flask containing 50 mL of water, and dilute with water to volume.
Borate buffer: 0.2 M (76.3 g/L of sodium borate in water) adjusted with hydrochloric acid TS to a pH of 9.5. [NOTE—Buffer must be stored at room temperature. It must be warmed to dissolve if crystallization occurs.]
Acetate buffer: 6.80 g/L of sodium acetate trihydrate in water adjusted with dilute acetic acid to a pH of 5.9
Derivatizing reagent: In a 14-mL polypropylene culture tube dissolve 50 mg of o-phthalaldehyde in 1.25 mL of anhydrous methanol. Add 50 µL of 3-mercaptopropionic acid and 11.2 mL of *Borate buffer*, and mix gently. Allow to stand in the dark for 30 min before use. [NOTE—Reagent strength is maintained by adding 10 µL of 3-mercaptopropionic acid every 2 days. Storage should be in the dark, at room temperature, and can be used for NMT 2 weeks.]
Mobile phase: Methanol and *Acetate buffer* (1:9)
Standard solution: 1.0 mg/mL of USP Glucosamine Hydrochloride RS in water. Allow to stand at room temperature for 1 h.
Sample solution: Transfer an equivalent to 25 mg of glucosamine from NLT 20 Tablets, finely powdered, to a 25-mL volumetric flask, and dilute with *Diluent* to volume. Mix on a vortex mixer to suspend the powder in solution. Sonicate in a 65° water bath for 20 min. Remove from the bath, stir for 5 min with the aid of a magnetic stirrer, and centrifuge.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 340 nm
Column: 3.0-mm × 5-cm; packing L1
Flow rate: 1 mL/min
Injection size: 10 µL
System suitability
Samples: Five individual aliquots of the *Standard solution* derivatized as directed for *Analysis*. Each derivatized aliquot is injected only once.
[NOTE—The relative retention times for the β-anomer and the α-anomer are 1.0 and 1.8, respectively. The retention time for the β-anomer is NLT 4 min.]

DS Monographs

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Suitability requirements
Relative standard deviation: NMT 2.0% for five replicate injections
Analysis
Samples: *Standard solution* and *Sample solution*
Transfer 100 µL of the *Derivatizing reagent* and 100 µL of the *Standard solution* or the *Sample solution* to a vial containing 400 µL of *Borate buffer*. Allow the derivatization to proceed for 1 min. Inject the derivatized solutions immediately after the derivatization reaction.
Calculate the percentage of the labeled amount of glucosamine ($C_6H_{13}NO_5$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak response of the β-anomer from the derivatized *Sample solution*
r_S = peak response of the β-anomer from the derivatized *Standard solution*
C_S = concentration of USP Glucosamine Hydrochloride RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of glucosamine in the *Sample solution* (mg/mL)
M_{r1} = molecular weight of glucosamine, 179.17
M_{r2} = molecular weight of glucosamine hydrochloride, 215.63

Acceptance criteria: 90.0%–120.0% of the labeled claim

• **CONTENT OF METHYLSULFONYLMETHANE**
Diluent: Transfer 950 mL of methanol to a 1-L volumetric flask. Add 0.60 mL of diethylene glycol methyl ether, and dilute with methanol to volume.
Standard solution: 0.4 mg/mL of USP Methylsulfonylmethane RS in *Diluent*. Sonicate at 50° for 1 min, and allow to cool to room temperature.
Sample solution: Finely powder NLT 20 Tablets. Dissolve a portion of the finely powdered material, equivalent to 1 Tablet, in *Diluent*, and sonicate for 15 min at 50°. Allow to cool to room temperature, dilute with *Diluent* to volume, and mix. Quantitatively dilute with *Diluent* to obtain a final concentration of 0.4 mg/mL of methylsulfonylmethane. Transfer 1 mL of the suspension to a 1.5-mL microcentrifuge tube, and centrifuge for 20 s. Use the supernatant.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.53-mm × 30-m capillary; 5-µm phase G2 coating
Temperature
Column: 120°
Injector: 250°
Detector: 250°
Carrier gas: Helium
Flow rate: 5 mL/min
Injection size: 1 µL
Injector type: Split ratio, 2:1
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 2.0% for the peak response ratio of methylsulfonylmethane to diethylene glycol methyl ether from replicate injections
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of methylsulfonylmethane ($C_2H_6O_2S$) in the portion of Tablets taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of methylsulfonylmethane to diethylene glycol methyl ether from the *Sample solution*
R_S = peak response ratio of methylsulfonylmethane to diethylene glycol methyl ether from the *Standard solution*
C_S = concentration of USP Methylsulfonylmethane RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of methylsulfonylmethane in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–110.0% of the label claim

PERFORMANCE TESTS

• **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩**:** Meet the requirements for *Dissolution*
Medium: Water; 900 mL
Apparatus 2: 75 rpm
Time: 60 min
Determine the percentage of glucosamine dissolved as follows.
Standard solution: Prepare as directed in the test for *Content of Glucosamine*. Dilute with a suitable quantity of water, if necessary.
Sample solution: Use the solution under test.
Borate buffer, Acetate buffer, Derivatizing reagent, Mobile phase, Chromatographic system, and **Analysis:** Proceed as directed in the test for *Content of Glucosamine*.
Calculate the percentage of the labeled amount of glucosamine ($C_6H_{13}NO_5$) dissolved:

$$\text{Result} = (r_U/r_S) \times (C_S \times V/L) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area obtained from the derivatized *Sample solution*
r_S = peak area obtained from the derivatized *Standard solution*
C_S = concentration of USP Glucosamine Hydrochloride RS in the *Standard solution* (mg/mL)
V = volume of *Medium*, 900 mL
L = label claim of glucosamine (mg/Tablet)
M_{r1} = molecular weight of glucosamine, 179.17
M_{r2} = molecular weight of glucosamine hydrochloride, 215.63

Tolerances: NLT 75% of the labeled amount of glucosamine ($C_6H_{13}NO_5$) is dissolved.

• **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩ **:** Meet the requirements

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
• **LABELING:** The label indicates the types of glucosamine salts contained in the article.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Glucosamine Hydrochloride RS
USP Methylsulfonylmethane RS
Dimethyl sulfone.
$C_2H_6O_2S$ 94.13

DS Monographs

www.webofpharma.com

Glucosamine, Chondroitin Sulfate Sodium, and Methylsulfonylmethane Tablets

DEFINITION

Glucosamine, Chondroitin Sulfate Sodium, and Methylsulfonylmethane Tablets are prepared from either Glucosamine Hydrochloride, Glucosamine Sulfate Sodium Chloride, Glucosamine Sulfate Potassium Chloride, or a mixture of any of them, with Chondroitin Sulfate Sodium and Methylsulfonylmethane. Tablets contain NLT 90.0% and NMT 120.0% of the labeled amounts of chondroitin sulfate sodium and glucosamine ($C_6H_{13}NO_5$) and NLT 90.0% and NMT 110.0% of the labeled amount of methylsulfonylmethane ($C_2H_6O_2S$).

[NOTE—Chondroitin Sulfate Sodium is extremely hygroscopic once dried. Avoid exposure to atmosphere, and weigh promptly.]

IDENTIFICATION

- **A. PRESENCE OF GLUCOSAMINE:** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Glucosamine*.
- **B. PRESENCE OF CHONDROITIN SULFATE**

Barium acetate buffer: Dissolve 25.24 g of barium acetate in 900 mL of water. Adjust with acetic acid to a pH of 5.0, and dilute with water to 1000 mL.

Staining reagent: 0.1% (w/v) toluidine blue in 0.1 M acetic acid

Standard solution: Use the *Standard solution* of middle concentration from *Content of Chondroitin Sulfate Sodium*.

Sample solution: Prepare as directed in *Content of Chondroitin Sulfate Sodium*.

Analysis: Fill the chambers of an electrophoresis apparatus suitable for separations on cellulose acetate membranes[1] (a small submarine gel chamber or one dedicated to membrane media) with *Barium acetate buffer*. Soak a cellulose acetate membrane 5–6 cm × 12–14 cm in *Barium acetate buffer* for 10 min, or until evenly wetted, then blot dry between two sheets of absorbent paper. Using an applicator[2] suitable for electrophoresis, apply equal volumes (0.5 µL) of the *Sample solution* and *Standard solution* to the brighter side of the membrane held in position in an appropriate applicator stand or on a separating bridge in the chamber. Ensure that both ends of the membrane are dipped at least 0.5–1.0 cm deep into the buffer chambers. Apply a constant 60 volts (6 mA at the start) for 2 h. [NOTE—Perform the application of solutions and voltage within 5 min because further drying of the blotted paper reduces sensitivity.]

Place the membrane in a plastic staining tray, and with the application side down, float or gently immerse in *Staining reagent* for 5 min. Then stir the solution gently for 1 min. Remove the membrane, and destain in 5% acetic acid until the background clears.

Acceptance criteria: The principal band from the *Sample solution* has the same migration as the principal band from the *Standard solution*. [NOTE—Document the results by taking a picture within 15 min of completion of destaining.]

- **C. PRESENCE OF METHYLSULFONYLMETHANE:** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Content of Methylsulfonylmethane*.

STRENGTH

- **CONTENT OF GLUCOSAMINE**

Diluent: Transfer 29 µL of acetic acid and 5 mL of acetonitrile to a 100-mL volumetric flask containing 50 mL of water, and dilute with water to volume.

Borate buffer: 0.2 M (76.3 g/L of sodium borate in water) adjusted with hydrochloric acid TS to a pH of 9.5

Acetate buffer: 6.80 g/L of sodium acetate trihydrate in water adjusted with dilute acetic acid to a pH of 5.9. [NOTE—Buffer must be stored at room temperature and can be warmed to dissolve if crystallization occurs.]

Derivatizing reagent: In a 14-mL polypropylene culture tube, dissolve 50 mg of *o*-phthalaldehyde in 1.25 mL of anhydrous methanol. Add 50 µL of 3-mercaptopropionic acid and 11.2 mL of *Borate buffer*, and mix gently. Allow to stand in the dark for 30 min before use. [NOTE—Reagent strength is maintained by adding 10 µL of 3-mercaptopropionic acid every 2 days. Storage should be in the dark, at room temperature, and can be used for NMT 2 weeks.]

Mobile phase: Methanol and *Acetate buffer* (1:9)

Standard solution: 1.0 mg/mL of USP Glucosamine Hydrochloride RS in water. Allow to stand at room temperature for 1 h.

Sample solution: Transfer an equivalent to 25 mg of glucosamine from NLT 20 Tablets, finely powdered, to a 25-mL volumetric flask, and dilute with *Diluent* to volume. Mix on a vortex mixer to suspend the powder in solution. Sonicate in a 65° water bath for 20 min. Remove from the bath, stir for 5 min with the aid of a magnetic stirrer, and centrifuge.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 340 nm

Column: 3.0-mm × 5-cm; packing L1

Flow rate: 1 mL/min

Injection size: 10 µL

System suitability

Samples: Five individual aliquots of the *Standard solution* derivatized as directed in *Analysis*. Each derivatized aliquot is injected only once.

[NOTE—The relative retention times for the β-anomer and the α-anomer are 1.0 and 1.8, respectively. The retention time for the β-anomer is NLT 4 min.]

Suitability requirements

Relative standard deviation: NMT 2.0% for five replicate injections

Analysis

Samples: *Standard solution* and *Sample solution*

Transfer 100 µL of the *Derivatizing reagent* and 100 µL of the *Standard solution* or the *Sample solution* to a vial containing 400 µL of *Borate buffer*, and allow the derivatization to proceed for 1 min. Inject the derivatized solutions immediately after the derivatization reaction.

Calculate the percentage of the labeled amount of glucosamine ($C_6H_{13}NO_5$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak response of the β-anomer from the derivatized *Sample solution*

r_S = peak response of the β-anomer from the derivatized *Standard solution*

C_S = concentration of USP Glucosamine Hydrochloride RS in the *Standard solution* (mg/mL)

[1] Suitable cellulose acetate membranes for electrophoresis are available from Fluka Chemical Corp., Milwaukee, WI; and DiaSys Corp., Waterbury, CT (www.diasys.com).

[2] Suitable applicators are available from DiaSys Corp., Waterbury, CT (www.diasys.com) and Helena Laboratories, Beaumont, TX (www.helena.com).

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Suitability requirements
Relative standard deviation: NMT 2.0% for five replicate injections
Analysis
Samples: *Standard solution* and *Sample solution*
Transfer 100 µL of the *Derivatizing reagent* and 100 µL of the *Standard solution* or the *Sample solution* to a vial containing 400 µL of *Borate buffer*. Allow the derivatization to proceed for 1 min. Inject the derivatized solutions immediately after the derivatization reaction.
Calculate the percentage of the labeled amount of glucosamine ($C_6H_{13}NO_5$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak response of the β-anomer from the derivatized *Sample solution*
r_S = peak response of the β-anomer from the derivatized *Standard solution*
C_S = concentration of USP Glucosamine Hydrochloride RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of glucosamine in the *Sample solution* (mg/mL)
M_{r1} = molecular weight of glucosamine, 179.17
M_{r2} = molecular weight of glucosamine hydrochloride, 215.63

Acceptance criteria: 90.0%–120.0% of the labeled claim

• **CONTENT OF METHYLSULFONYLMETHANE**
Diluent: Transfer 950 mL of methanol to a 1-L volumetric flask. Add 0.60 mL of diethylene glycol methyl ether, and dilute with methanol to volume.
Standard solution: 0.4 mg/mL of USP Methylsulfonylmethane RS in *Diluent*. Sonicate at 50° for 1 min, and allow to cool to room temperature.
Sample solution: Finely powder NLT 20 Tablets. Dissolve a portion of the finely powdered material, equivalent to 1 Tablet, in *Diluent*, and sonicate for 15 min at 50°. Allow to cool to room temperature, dilute with *Diluent* to volume, and mix. Quantitatively dilute with *Diluent* to obtain a final concentration of 0.4 mg/mL of methylsulfonylmethane. Transfer 1 mL of the suspension to a 1.5-mL microcentrifuge tube, and centrifuge for 20 s. Use the supernatant.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.53-mm × 30-m capillary; 5-µm phase G2 coating
Temperature
Column: 120°
Injector: 250°
Detector: 250°
Carrier gas: Helium
Flow rate: 5 mL/min
Injection size: 1 µL
Injector type: Split ratio, 2:1
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 2.0% for the peak response ratio of methylsulfonylmethane to diethylene glycol methyl ether from replicate injections
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of methylsulfonylmethane ($C_2H_6O_2S$) in the portion of Tablets taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of methylsulfonylmethane to diethylene glycol methyl ether from the *Sample solution*
R_S = peak response ratio of methylsulfonylmethane to diethylene glycol methyl ether from the *Standard solution*
C_S = concentration of USP Methylsulfonylmethane RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of methylsulfonylmethane in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–110.0% of the label claim

PERFORMANCE TESTS

• **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩**:** Meet the requirements for *Dissolution*
Medium: Water; 900 mL
Apparatus 2: 75 rpm
Time: 60 min
Determine the percentage of glucosamine dissolved as follows.
Standard solution: Prepare as directed in the test for *Content of Glucosamine*. Dilute with a suitable quantity of water, if necessary.
Sample solution: Use the solution under test.
Borate buffer, Acetate buffer, Derivatizing reagent, Mobile phase, Chromatographic system, and **Analysis:** Proceed as directed in the test for *Content of Glucosamine*.
Calculate the percentage of the labeled amount of glucosamine ($C_6H_{13}NO_5$) dissolved:

$$\text{Result} = (r_U/r_S) \times (C_S \times V/L) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area obtained from the derivatized *Sample solution*
r_S = peak area obtained from the derivatized *Standard solution*
C_S = concentration of USP Glucosamine Hydrochloride RS in the *Standard solution* (mg/mL)
V = volume of *Medium*, 900 mL
L = label claim of glucosamine (mg/Tablet)
M_{r1} = molecular weight of glucosamine, 179.17
M_{r2} = molecular weight of glucosamine hydrochloride, 215.63

Tolerances: NLT 75% of the labeled amount of glucosamine ($C_6H_{13}NO_5$) is dissolved.

• **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩ **:** Meet the requirements

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
• **LABELING:** The label indicates the types of glucosamine salts contained in the article.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Glucosamine Hydrochloride RS
USP Methylsulfonylmethane RS
Dimethyl sulfone.
$C_2H_6O_2S$ 94.13

DS Monographs

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Glucosamine, Chondroitin Sulfate Sodium, and Methylsulfonylmethane Tablets

DEFINITION

Glucosamine, Chondroitin Sulfate Sodium, and Methylsulfonylmethane Tablets are prepared from either Glucosamine Hydrochloride, Glucosamine Sulfate Sodium Chloride, Glucosamine Sulfate Potassium Chloride, or a mixture of any of them, with Chondroitin Sulfate Sodium and Methylsulfonylmethane. Tablets contain NLT 90.0% and NMT 120.0% of the labeled amounts of chondroitin sulfate sodium and glucosamine ($C_6H_{13}NO_5$) and NLT 90.0% and NMT 110.0% of the labeled amount of methylsulfonylmethane ($C_2H_6O_2S$).

[NOTE—Chondroitin Sulfate Sodium is extremely hygroscopic once dried. Avoid exposure to atmosphere, and weigh promptly.]

IDENTIFICATION

- **A. PRESENCE OF GLUCOSAMINE:** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Glucosamine*.
- **B. PRESENCE OF CHONDROITIN SULFATE**

Barium acetate buffer: Dissolve 25.24 g of barium acetate in 900 mL of water. Adjust with acetic acid to a pH of 5.0, and dilute with water to 1000 mL.

Staining reagent: 0.1% (w/v) toluidine blue in 0.1 M acetic acid

Standard solution: Use the *Standard solution* of middle concentration from *Content of Chondroitin Sulfate Sodium*.

Sample solution: Prepare as directed in *Content of Chondroitin Sulfate Sodium*.

Analysis: Fill the chambers of an electrophoresis apparatus suitable for separations on cellulose acetate membranes[1] (a small submarine gel chamber or one dedicated to membrane media) with *Barium acetate buffer*. Soak a cellulose acetate membrane 5–6 cm × 12–14 cm in *Barium acetate buffer* for 10 min, or until evenly wetted, then blot dry between two sheets of absorbent paper. Using an applicator[2] suitable for electrophoresis, apply equal volumes (0.5 µL) of the *Sample solution* and *Standard solution* to the brighter side of the membrane held in position in an appropriate applicator stand or on a separating bridge in the chamber. Ensure that both ends of the membrane are dipped at least 0.5–1.0 cm deep into the buffer chambers. Apply a constant 60 volts (6 mA at the start) for 2 h. [NOTE—Perform the application of solutions and voltage within 5 min because further drying of the blotted paper reduces sensitivity.]

Place the membrane in a plastic staining tray, and with the application side down, float or gently immerse in *Staining reagent* for 5 min. Then stir the solution gently for 1 min. Remove the membrane, and destain in 5% acetic acid until the background clears.

Acceptance criteria: The principal band from the *Sample solution* has the same migration as the principal band from the *Standard solution*. [NOTE—Document the results by taking a picture within 15 min of completion of destaining.]

- **C. PRESENCE OF METHYLSULFONYLMETHANE:** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Content of Methylsulfonylmethane*.

STRENGTH

- **CONTENT OF GLUCOSAMINE**

Diluent: Transfer 29 µL of acetic acid and 5 mL of acetonitrile to a 100-mL volumetric flask containing 50 mL of water, and dilute with water to volume.

Borate buffer: 0.2 M (76.3 g/L of sodium borate in water) adjusted with hydrochloric acid TS to a pH of 9.5

Acetate buffer: 6.80 g/L of sodium acetate trihydrate in water adjusted with dilute acetic acid to a pH of 5.9. [NOTE—Buffer must be stored at room temperature and can be warmed to dissolve if crystallization occurs.]

Derivatizing reagent: In a 14-mL polypropylene culture tube, dissolve 50 mg of *o*-phthalaldehyde in 1.25 mL of anhydrous methanol. Add 50 µL of 3-mercaptopropionic acid and 11.2 mL of *Borate buffer*, and mix gently. Allow to stand in the dark for 30 min before use. [NOTE—Reagent strength is maintained by adding 10 µL of 3-mercaptopropionic acid every 2 days. Storage should be in the dark, at room temperature, and can be used for NMT 2 weeks.]

Mobile phase: Methanol and *Acetate buffer* (1:9)

Standard solution: 1.0 mg/mL of USP Glucosamine Hydrochloride RS in water. Allow to stand at room temperature for 1 h.

Sample solution: Transfer an equivalent to 25 mg of glucosamine from NLT 20 Tablets, finely powdered, to a 25-mL volumetric flask, and dilute with *Diluent* to volume. Mix on a vortex mixer to suspend the powder in solution. Sonicate in a 65° water bath for 20 min. Remove from the bath, stir for 5 min with the aid of a magnetic stirrer, and centrifuge.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 340 nm

Column: 3.0-mm × 5-cm; packing L1

Flow rate: 1 mL/min

Injection size: 10 µL

System suitability

Samples: Five individual aliquots of the *Standard solution* derivatized as directed in *Analysis*. Each derivatized aliquot is injected only once.

[NOTE—The relative retention times for the β-anomer and the α-anomer are 1.0 and 1.8, respectively. The retention time for the β-anomer is NLT 4 min.]

Suitability requirements

Relative standard deviation: NMT 2.0% for five replicate injections

Analysis

Samples: *Standard solution* and *Sample solution*

Transfer 100 µL of the *Derivatizing reagent* and 100 µL of the *Standard solution* or the *Sample solution* to a vial containing 400 µL of *Borate buffer*, and allow the derivatization to proceed for 1 min. Inject the derivatized solutions immediately after the derivatization reaction.

Calculate the percentage of the labeled amount of glucosamine ($C_6H_{13}NO_5$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak response of the β-anomer from the derivatized *Sample solution*

r_S = peak response of the β-anomer from the derivatized *Standard solution*

C_S = concentration of USP Glucosamine Hydrochloride RS in the *Standard solution* (mg/mL)

[1] Suitable cellulose acetate membranes for electrophoresis are available from Fluka Chemical Corp., Milwaukee, WI; and DiaSys Corp., Waterbury, CT (www.diasys.com).

[2] Suitable applicators are available from DiaSys Corp., Waterbury, CT (www.diasys.com) and Helena Laboratories, Beaumont, TX (www.helena.com).

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C_U = nominal concentration of glucosamine in the *Sample solution* (mg/mL)
M_{r1} = molecular weight of glucosamine, 179.17
M_{r2} = molecular weight of glucosamine hydrochloride, 215.63

Acceptance criteria: 90.0%–120.0% of the label claim

• **CONTENT OF CHONDROITIN SULFATE SODIUM**

Standard solutions: 1.5, 1.0, and 0.5 mg/mL of USP Chondroitin Sulfate Sodium RS in water

Sample solution: Transfer an equivalent to 100 mg of chondroitin sulfate sodium from NLT 20 Tablets, finely powdered, to 60 mL of water, and shake to suspend the powder in solution. Sonicate in a 65° water bath for 20 min. Remove from the bath, stir or shake for 5 min, dilute with water to 100 mL, and centrifuge or pass through a suitable filter.

Diluent: Weigh about 297 mg of monobasic potassium phosphate, 492 mg of dibasic potassium phosphate, and 250 mg of polysorbate 80, and transfer into a 1-L beaker. Dissolve in approximately 900 mL of water, and adjust with potassium hydroxide or phosphoric acid to a pH of 7.0 ± 0.2. Dilute with water to 1 L, and mix thoroughly.

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Photometric titration

Titrant: 1 mg/mL of cetylpyridinium chloride in water

Endpoint detection: Turbidimetric with photoelectric probe

Analysis: Transfer 5.0 mL of each *Standard solution* and the *Sample solution* to separate titration vessels, and add 25 mL of *Diluent* to each. Stir until a steady reading is obtained with a photoelectric probe either at 420, 550, or 660 nm. Set the instrument to zero in absorbance mode. Titrate with *Titrant* using the photoelectric probe to determine the endpoint turbidimetrically. From a linear regression equation, calculated using the volumes of *Titrant* consumed versus concentrations of the *Standard solutions*, determine the concentration of chondroitin sulfate sodium in the *Sample solution*.

Calculate the percentage of chondroitin sulfate sodium in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = determined concentration of chondroitin sulfate sodium in the *Sample solution* (mg/mL)
C_U = nominal concentration of chondroitin sulfate sodium in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–120.0% of the label claim

• **CONTENT OF METHYLSULFONYLMETHANE**

Diluent: Transfer 950 mL of methanol to a 1-L volumetric flask. Add 0.60 mL of diethylene glycol methyl ether, and dilute with methanol to volume.

Standard solution: 0.4 mg/mL of USP Methylsulfonylmethane RS in *Diluent*. Sonicate at 50° for 1 min, and allow to cool to room temperature.

Sample solution: Finely powder NLT 20 Tablets. Dissolve a portion of the finely powdered material, equivalent to 1 Tablet, in *Diluent*, and sonicate for 15 min at 50°. Allow to cool to room temperature. Quantitatively dilute with *Diluent* to obtain a final concentration of 0.4 mg/mL of methylsulfonylmethane. Transfer 1 mL of the suspension to a 1.5-mL microcentrifuge tube, and centrifuge for 20 s. Use the supernatant.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.53-mm × 30-m capillary column coated with a 5-µm phase G2

Temperature

Column: 120°

Injector: 250°

Detector: 250°

Carrier gas: Helium

Flow rate: 5 mL/min

Injection size: 1 µL

Injection type Split ratio, 2:1

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0% for the peak response ratio of methylsulfonylmethane to diethylene glycol methyl ether from replicate injections

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of methylsulfonylmethane ($C_2H_6O_2S$) in the portion of Tablets taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak area ratio of methylsulfonylmethane to diethylene glycol methyl ether from the *Sample solution*
R_S = peak area ratio of methylsulfonylmethane to diethylene glycol methyl ether from the *Standard solution*
C_S = concentration of USP Methylsulfonylmethane RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of methylsulfonylmethane in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–110.0% of of the label claim

PERFORMANCE TESTS

• **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩**:** Meet the requirements for *Dissolution*

Medium: Water; 900 mL

Apparatus 2: 75 rpm

Time: 60 min

Determine the percentage of glucosamine dissolved as follows.

Borate buffer, Acetate buffer, Derivatizing reagent, Mobile phase, Chromatographic system, and **Analysis:** Proceed as directed in the test for *Content of Glucosamine*.

Standard solution: Prepare as directed in the test for *Content of Glucosamine*. Dilute with a suitable quantity of water, if necessary.

Sample solution: Use the solution under test.

Calculate the percentage of the labeled amount of glucosamine ($C_6H_{13}NO_5$) dissolved:

$$\text{Result} = (r_U/r_S) \times (C_S \times V/L) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area obtained from the derivatized *Sample solution*
r_S = peak area obtained from the derivatized *Standard solution*
C_S = concentration of USP Glucosamine Hydrochloride RS in the *Standard solution* (mg/mL)
V = volume of *Medium*, 900 mL
L = label claim of glucosamine (mg/Tablet)
M_{r1} = molecular weight of glucosamine, 179.17

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M_{r2} = molecular weight of glucosamine hydrochloride, 215.63

Tolerances: NLT 75% of the labeled amount of glucosamine ($C_6H_{13}NO_5$) is dissolved.
Determine the percentage of chondroitin sulfate sodium dissolved as follows.
Titrant, Diluent, Standard solutions, and Analysis: Proceed as directed in the test for *Content of Chondroitin Sulfate Sodium*.
Sample solution: Use the solution under test.
Calculate the percentage of the labeled amount of chondroitin sulfate sodium dissolved:

$$\text{Result} = (C \times V/L) \times 100$$

C = determined concentration of chondroitin sulfate sodium in the *Sample solution* (mg/mL)
V = volume of *Medium*, 900 mL
L = label claim of chondroitin sulfate sodium (mg/Tablet)

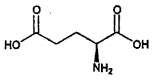
Tolerances: NLT 75% of the labeled amount of chondroitin sulfate sodium is dissolved.

- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩: Meet the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** The label indicates the types of glucosamine salts contained in the article and the species source from which chondroitin was derived. Label it to state the source(s) of chondroitin sulfate sodium, whether bovine, porcine, avian, or a mixture of any of them. The label states on the front panel the content of chondroitin sulfate sodium on the dried basis.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Chondroitin Sulfate Sodium RS
  USP Glucosamine Hydrochloride RS
  USP Methylsulfonylmethane RS
  Dimethyl sulfone.
  $C_2H_6O_2S$ 94.13

Glutamic Acid



$C_5H_9NO_4$ 147.13
L-Glutamic acid;
S-2-Aminopentanedioic acid [56-86-0].

DEFINITION
Glutamic Acid contains NLT 98.5% and NMT 101.5% of L-glutamic acid ($C_5H_9NO_4$), calculated on the dried basis.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

ASSAY

- **PROCEDURE**
  Sample: 140 mg of Glutamic Acid
  Blank: Mix 6 mL of formic acid and 50 mL of glacial acetic acid.
  Titrimetric system
  (See *Titrimetry* ⟨541⟩.)
  Mode: Direct titration
  Titrant: 0.1 N perchloric acid VS
  Endpoint detection: Potentiometric
  Analysis: Dissolve the *Sample* in 6 mL of formic acid and 50 mL of glacial acetic acid, and titrate with the *Titrant*. Perform the *Blank* determination.
  Calculate the percentage of glutamic acid ($C_5H_9NO_4$) in the *Sample* taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

V_S = volume of *Titrant* consumed by the *Sample* (mL)
V_B = volume of *Titrant* consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 147.1 mg/mEq
W = *Sample* weight (mg)

Acceptance criteria: 98.5%–101.5% on the dried basis

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%
- **CHLORIDE AND SULFATE,** *Chloride* ⟨221⟩
  Standard solution: 0.40 mL of 0.010 N hydrochloric acid
  Sample: 0.7 g of Glutamic Acid
  Acceptance criteria: NMT 0.02%
- **CHLORIDE AND SULFATE,** *Sulfate* ⟨221⟩
  Standard solution: 0.25 mL of 0.020 N sulfuric acid
  Sample: 1.2 g of Glutamic Acid
  Acceptance criteria: NMT 0.02%
- **IRON** ⟨241⟩: NMT 10 µg/g
- **RELATED COMPOUNDS**
  Standard solution: 0.05 mg/mL of USP Glutamic Acid RS in water
  Sample solution: 10 mg/mL in a solution of ammonia TS and water (1:1)
  System suitability solution: 0.4 mg/mL each of USP Aspartic Acid RS and USP Glutamic Acid RS in water
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
  Mode: TLC
  Adsorbent: 0.25-mm layer of chromatographic silica gel mixture
  Application volume: 5 µL
  Developing solvent system: Butyl alcohol, glacial acetic acid, and water (3:1:1)
  Spray reagent: 2 mg/mL of ninhydrin in a mixture of butyl alcohol and 2 N acetic acid (95:5)
  System suitability
  Suitability requirements: The chromatogram from the *System suitability solution* exhibits two clearly separated spots.
  Analysis: After air-drying the plate, repeat the development process. After air-drying a second time, spray with *Spray reagent*, and heat between 100° and 105° for about 15 min. Examine the plate under white light.
  Acceptance criteria: Any secondary spot of the *Sample solution* is not larger or more intense than the principal spot of the *Standard solution*.
  Individual impurities: NMT 0.5%
  Total impurities: NMT 2.0%

SPECIFIC TESTS

- **OPTICAL ROTATION,** *Specific Rotation* ⟨781S⟩
  Sample solution: 100 mg/mL in 2 N hydrochloric acid
  Analysis: Proceed as directed in the chapter, except measure at 20°.

DS Monographs

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Acceptance criteria: +31.5° to +32.5°

• **LOSS ON DRYING** ⟨731⟩**:** Dry a sample at 105° for 3 h: it loses NMT 0.1% of its weight.

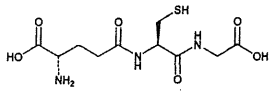
ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at controlled room temperature.

• **USP REFERENCE STANDARDS** ⟨11⟩
USP Aspartic Acid RS
USP Glutamic Acid RS

Glutamine—*see Glutamine General Monographs*

Glutathione



$C_{10}H_{17}N_3O_6S$ 307.32

Pentanoic acid, 2-amino-5-[(*R*)-1-(carboxymethylamino)-3-mercapto-1-oxopropan-2-ylamino]-5-oxo, (*S*);
N-(*N*-L-γ-Glutamyl-L-cysteinyl)glycine [70-18-8].

DEFINITION

Glutathione contains NLT 98.0% and NMT 101.0% of glutathione ($C_{10}H_{17}N_3O_6S$), calculated on the dried basis.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K▲** (CN 1-May-2020)

• **B. OPTICAL ROTATION,** *Specific Rotation* ⟨781S⟩
Sample solution: 40 mg/mL in water
Acceptance criteria: −15.5° to −17.5°, at 20°

ASSAY

• **PROCEDURE**
Sample: 500 mg of Glutathione previously dried
Blank: 50 mL of metaphosphoric acid (1 in 50)
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.1 N iodine VS
Endpoint detection: Visual
Analysis: Dissolve the *Sample* in 50 mL of metaphosphoric acid (1 in 50) and titrate with the *Titrant*.
Calculate the percentage of glutathione ($C_{10}H_{17}N_3O_6S$) in the portion of Glutathione taken:

$$\text{Result} = [(V_U - V_B) \times N \times F \times 100]/W$$

V_U = titrant volume of the *Sample* (mL)
V_B = titrant volume of the *Blank* (mL)
N = titrant normality (mEq/mL)
F = equivalency factor, 307.32 mg/mEq
W = weight of the *Sample* (mg)

Acceptance criteria: 98.0%–101.0% on the dried basis

IMPURITIES

• **AMMONIUM**
Standard solution: 10 µg of ammonium from a diluted ammonium chloride solution
Sample solution: 50 mg of Glutathione
Silver manganese paper: Immerse strips of slow filter paper (Whatman® filter paper, grade 5 or equivalent) into a solution containing 8.5 mg/mL of manganese sulfate and 8.5 mg/mL of silver nitrate. Maintain for a few min and allow to dry over phosphorus pentoxide protected from acid and alkaline vapors.
Analysis: Transfer the *Sample solution* and the *Standard solution* to separate 25-mL jars fitted with caps, and dissolve in 1 mL of water. Add 0.30 g of magnesium oxide. Close immediately after placing a 5-mm square piece of *Silver manganese paper*, wetted with a few drops of water, under the caps. Swirl, avoiding projections of liquid, and allow to stand at 40° for 30 min.
Acceptance criteria: If the *Silver manganese paper* shows a gray color, it is not more intense than that of the Standard (NMT 200 ppm).

• **ARSENIC** ⟨211⟩**:** NMT 2 ppm

• **CHLORIDE AND SULFATE,** *Chloride* ⟨221⟩**:** Dissolve 0.7 g in water to make 15 mL. The solution shows no more chloride than corresponds to 0.20 mL of 0.020 N hydrochloric acid (NMT 200 ppm).

• **CHLORIDE AND SULFATE,** *Sulfate* ⟨221⟩**:** Dissolve 0.8 g in water to make 15 mL. The solution shows no more sulfate than corresponds to 0.25 mL of 0.020 N sulfuric acid (NMT 300 ppm).

• **IRON** ⟨241⟩**:** NMT 10 ppm

• **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 0.1%

• **RELATED COMPOUNDS**
Mobile phase: 6.8 g/L of potassium dihydrogen phosphate with 2.02 g/L of sodium 1-heptane sulfonate. Adjust with phosphoric acid to a pH of 3.0. Mix 970 mL of this solution with 30 mL of methanol.
System suitability solution: 0.1 mg/mL of USP L-Phenylalanine RS, 0.5 mg/mL of USP Glutathione RS, and 0.5 mg/mL of USP Ascorbic Acid RS in *Mobile phase*
Standard solution: 0.01 mg/mL of USP Glutathione RS in *Mobile phase*. [NOTE—This solution has a concentration equivalent to 2.0% of that of the *Sample solution*.]
Sample solution: 50 mg of Glutathione in 100 mL of *Mobile phase*. [NOTE—Allow the solution to stand for 5 min before use.]
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 210 nm
Column: 4.6-mm × 15-cm; 5-µm packing L1
Column temperature: 30°
Flow rate: Adjust so that the retention time of glutathione is about 5 min.
Injection volume: 10 µL
System suitability
Sample: *System suitability solution*
Suitability requirements
Resolution: NLT 5.0 between the ascorbic acid and glutathione peaks; and NLT 5.0 between the glutathione and L-phenylalanine peaks
Relative standard deviation: NMT 1.5% for replicate injections
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of any impurity in the portion of Glutathione taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

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r_U = peak response of any peak from the *Sample solution* other than glutathione
r_S = peak response of the glutathione peak from the *Standard solution*
C_S = concentration of USP Glutathione RS in the *Standard solution* (mg/mL)
C_U = concentration of Glutathione in the *Sample solution* (mg/mL)

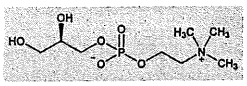
Acceptance criteria
Individual impurity: NMT 1.5% for the impurity with the relative retention time of about 4
Total impurities: NMT 2.0%

SPECIFIC TESTS
• **CLARITY AND COLOR OF SOLUTION**
Sample solution: 0.1 g/mL in water
Analysis: Using identical tubes of colorless, transparent, neutral glass with a flat base and an internal diameter of 15–25 mm, compare the liquid to be examined with water, the depth of the layer being 40 mm. Compare the colors in diffused daylight, viewing vertically against a white background.
Acceptance criteria: The solution is clear and colorless.
• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry at 105° for 3 h.
Acceptance criteria: NMT 0.5%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight containers.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Ascorbic Acid RS
USP Glutathione RS
USP L-Phenylalanine RS

Add the following:

▲L-alpha-Glycerylphosphorylcholine



$C_8H_{20}NO_6P$ 257.22
Choline hydroxide, (*R*)-2,3-dihydroxypropyl hydrogen phosphate, inner salt;
(*R*)-2,3-Dihydroxypropyl 2-(trimethylammonio)ethyl phosphate;
L-α-Glycerylphosphorylcholine [28319-77-9].

DEFINITION
L-alpha-Glycerylphosphorylcholine (L-alpha-GPC) is obtained by base catalyzed transesterification of enriched phosphatidylcholine soy lecithin extract and subsequent physicochemical purification processes.
L-alpha-Glycerylphosphorylcholine is anhydrous or an aqueous solution containing NLT 14.0% and NMT 16.0% of water. It contains NLT 98.0% and NMT 102.0% of L-alpha-glycerylphosphorylcholine ($C_8H_{20}NO_6P$), calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197A▲** (CN 1-May-2020)**:** Use USP L-alpha-Glycerylphosphorylcholine RS or USP L-alpha-Glycerylphosphorylcholine Solution RS, as appropriate.
• **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *System suitability solution*, as obtained in the test for *Organic Impurities*.

ASSAY
• **PROCEDURE**
Sample: 350 mg of L-alpha-GPC or L-alpha-GPC Solution
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.1 N perchloric acid in glacial acetic acid VS
Electrode system: A glass–calomel with lithium chloride salt bridge in acetic acid
Endpoint detection: Potentiometric
Analysis: Dissolve the *Sample* in 100 mL of glacial acetic acid and add 5 mL of acetic anhydride. Titrate with the *Titrant*. Perform a blank determination and make any necessary correction.
Calculate the percentage of L-alpha-glycerylphosphorylcholine ($C_8H_{20}NO_6P$) in the portion of *Sample* taken:

$$\text{Result} = [(V_S - V_B) \times N_A \times F \times 100]/W$$

V_S = *Titrant* volume consumed by the *Sample* (mL)
V_B = *Titrant* volume consumed by the blank (mL)
N_A = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 257.2 mg/mEq
W = *Sample* weight (mg)

Acceptance criteria: 98.0%–102.0% on the anhydrous basis

IMPURITIES
• **LIMIT OF CHLORIDE, SULFATE, PHOSPHATE, AND ACETATE**
Mobile phase: Potassium hydroxide solution, concentration gradient with the use of eluent generator cartridge.[1] See *Table 1*.

Table 1

Time (min)	Potassium Hydroxide (KOH) Concentration (mM)
0	5
3	5
4	10
5	20
8	40
10	5
15	5

Acetate standard stock solution: Transfer 230 mg of sodium acetate trihydrate to a 100-mL volumetric flask. Dissolve in and dilute with water to volume. Transfer 1.0 mL of the resultant solution to a 10-mL volumetric flask and dilute with water to volume. The acetate concentration is 0.1 mg/mL.
Chloride and sulfate standard stock solution: Transfer 210 mg of potassium chloride and 148 mg of sodium sulfate to a 100-mL volumetric flask. Dissolve in and dilute with water to volume. Transfer 1.0 mL of the resultant

[1] Dionex EGC III KOH or equivalent.

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solution to a 10-mL volumetric flask and dilute with water to volume. The concentration for chloride and sulfate is 0.1 mg/mL each.

Phosphate standard stock solution: Transfer 143 mg of potassium dihydrogen phosphate to a 100-mL volumetric flask. Dissolve in and dilute with water to volume. Transfer 1.0 mL of the resultant solution to a 100-mL volumetric flask and dilute with water to volume. The phosphate concentration is 0.01 mg/mL.

Standard solution: Transfer 1.0 mL of *Acetate standard stock solution*, 0.2 mL of *Chloride and sulfate standard stock solution*, and 0.5 mL of *Phosphate standard stock solution* to a 10-mL volumetric flask. Dilute with water to volume.

Sample solution: Transfer 100 mg of L-alpha-GPC or 120 mg of L-alpha-GPC Solution to a 10-mL volumetric flask. Dissolve in and dilute with water to volume.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: HPIC
Detector: Conductivity
Columns
Precolumn: 4.0-mm × 5-cm; 10.5-µm packing L83
Analytical: 4.0-mm × 25-cm; 10.5-µm packing L83
Flow rate: 1.0 mL/min
Injection volume: 25 µL

System suitability
Sample: *Standard solution*
[NOTE—The relative retention times of the analytes are listed in *Table 2*.]

Table 2

Name	Relative Retention Time	Acceptance Criteria, NMT (%)
Acetate	1.0	0.1
Chloride	1.7	0.02
Sulfate	4.4	0.02
Phosphate	5.0	0.005

Suitability requirements
Resolution: NLT 5 between the acetate and chloride peaks
Relative standard deviation: NMT 10% each for acetate, chloride, sulfate, and phosphate

Analysis
Samples: *Standard solution* and *Sample solution*
Separately calculate the percentage of acetate, chloride, sulfate, and phosphate in the portion of L-alpha-GPC or L-alpha-GPC Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of acetate, chloride, sulfate, or phosphate from the appropriate *Sample solution*
r_S = peak response of acetate, chloride, sulfate, or phosphate from the *Standard solution*
C_S = concentration of acetate, chloride, sulfate, or phosphate in the *Standard solution* (mg/mL)
C_U = concentration of L-alpha-GPC or L-alpha-GPC Solution in the *Sample solution* (mg/mL)

Acceptance criteria: See *Table 2*.

• **LIMIT OF GLYCEROL**

Mobile phase: Acetonitrile and water (55:45)

System suitability solution: 2 mg/mL of USP Glycerin RS and 400 mg/mL of L-alpha-GPC or 470 mg/mL of L-alpha-GPC Solution in water

Standard solution: 2 mg/mL of USP Glycerin RS in water

Sample solution: 400 mg/mL of L-alpha-GPC or 470 mg/mL of L-alpha-GPC Solution in water

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Refractive index
Column: 4.6-mm × 25-cm; 5-µm packing L14
Column temperature: 40°
Flow rate: 0.5 mL/min
Injection volume: 20 µL

System suitability
Samples: *System suitability solution* and *Standard solution*
Suitability requirements
[NOTE—The relative retention times for glycerol and L-alpha-GPC are about 0.6 and 1.0, respectively.]
Resolution: NLT 2.0 between the glycerol and L-alpha-GPC peaks, *System suitability solution*
Relative standard deviation: NMT 5.0% for the glycerol peak, *Standard solution*

Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of glycerol in the portion of *Sample solution* taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of glycerol from the appropriate *Sample solution*
r_S = peak response of glycerin from the *Standard solution*
C_S = concentration of USP Glycerin RS in the *Standard solution* (mg/mL)
C_U = concentration of L-alpha-GPC or L-alpha-GPC Solution in the *Sample solution* (mg/mL)

Acceptance criteria: NMT 0.5%

• **ORGANIC IMPURITIES**

Solution A: Mixture of acetonitrile and methanol (3:1)
Solution B: Transfer 1.98 g of ammonium acetate to a 500-mL volumetric flask. Dissolve in about 470 mL of water. Adjust with acetic acid to a pH of 4.5 and dilute with water to volume.
Solution C: Transfer 100.0 mL of *Solution B* to a 1000-mL volumetric flask and dilute with water to volume. Check the pH of the solution. If it is not 4.5, adjust with diluted ammonia solution or diluted acetic acid to a pH of 4.5.
Solution D: Mix 300 mL of *Solution B* and 1700 mL of *Solution A*.
Solution E: Mix 700 mL of *Solution C* and 1300 mL of *Solution A*.
Mobile phase: See *Table 3*.

Table 3

Time (min)	Solution D (%)	Solution E (%)
0	98	2
5	98	2
18	44	56
30	32	68
34	10	90

DS Monographs

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Table 3 *(continued)*

Time (min)	Solution D (%)	Solution E (%)
42	10	90
43	98	2
55	98	2

Diluent: Methanol and water (80:20)

Internal standard solution: Transfer 100 mg of USP L-Serine RS to a 100-mL volumetric flask. Dissolve in and dilute with *Diluent* to volume. Transfer 5.0 mL of the resultant solution to a 250-mL volumetric flask and dilute with *Diluent* to volume.

L-alpha-GPE, beta-GPC, and serine solution: Transfer 2.5 mg each of USP L-alpha-Glycerophosphorylethanolamine RS (USP L-alpha-GPE RS) and USP beta-Glycerylphosphorylcholine RS (USP beta-GPC RS) to a 10-mL volumetric flask. Dissolve in and dilute with the *Internal standard solution* to volume. Transfer 0.8 mL of the resultant solution to a 10-mL volumetric flask and dilute with the *Internal standard solution* to volume.

System suitability solution: Transfer 100 mg of USP L-alpha-Glycerylphosphorylcholine RS or 120 mg of USP L-alpha-Glycerylphosphorylcholine Solution RS to a 5-mL volumetric flask. Dissolve in and dilute with the *L-alpha-GPE, beta-GPC, and serine solution* to volume.

Sample solution: Transfer 200 mg of L-alpha-GPC or L-alpha-GPC Solution to a 10-mL volumetric flask. Dissolve in and dilute with the *Internal standard solution* to volume.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Evaporative light-scattering detector (ELSD)

Detector temperature: 90°

Column: 4.6-mm × 25-cm; 5-µm packing L111

Carrier gas: Nitrogen

Flow rate: 0.7 mL/min

Injection volume: 40 µL

System suitability

Sample: *System suitability solution*

[NOTE—Adjust the flow rate and/or isocratic portion of the gradient program to obtain the retention time of serine at 28 ± 2 min. The relative retention times of the analytes are listed in *Table 4*.]

Table 4

Name	Relative Retention Time	Relative Response Factor	Acceptance Criteria, NMT (%)
Pinitol[a]	0.76	0.48	0.1
beta-GPC[b]	0.96	1.0	0.1
L-alpha-GPC[c]	1.0	—	—
Sucrose[d]	1.38	1.0	0.1
Serine[e]	1.88	1.0	—
L-alpha-GPE[f]	1.93	1.0	0.1
Any individual unspecified impurity	—	1.0	0.1
Total impurities	—	—	2.0

[a] 3-*O*-Methyl-*chiro*-inositol.
[b] 1,3-Dihydroxypropan-2-yl 2-(trimethylammonio)ethyl phosphate.
[c] (*R*)-2,3-Dihydroxypropyl 2-(trimethylammonio)ethyl phosphate.
[d] β-D-Fructofuranosyl α-D-glucopyranoside.
[e] L-2-Amino-3-hydroxypropanoic acid.
[f] 2-Aminoethyl [(*R*)-2,3-dihydroxypropyl] hydrogen phosphate.

Suitability requirements

Resolution: NLT 1.2 between the serine and L-alpha-GPE peaks; NLT 0.4 between the beta-GPC and L-alpha-GPC peaks

Relative standard deviation: NMT 10% for the serine peak

Analysis

Samples: *Diluent*, *Internal standard solution*, and *Sample solution*

[NOTE—The response factor of serine equals that of L-alpha-GPC. Disregard peaks that are observed in both *Diluent* and *Sample solution* chromatograms. Disregard peaks below 0.05% observed in the chromatogram of the *Sample solution*.]

Calculate the percentage of each impurity in the portion of L-alpha-GPC or L-alpha-GPC Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (1/F) \times 100$$

r_U = peak response of each impurity from the *Sample solution*
r_S = peak response of serine from the *Internal standard solution*
C_S = concentration of USP L-Serine RS in the *Internal standard solution* (mg/mL)
C_U = concentration of L-alpha-GPC or L-alpha-GPC Solution in the *Sample solution* (mg/mL)
F = relative response factor (see *Table 4*)

Acceptance criteria: See *Table 4*.

SPECIFIC TESTS

- **OPTICAL ROTATION** ⟨781S⟩, *Procedures, Specific Rotation*

Sample solution: 100 mg/mL of L-alpha-GPC or L-alpha-GPC Solution in water

Acceptance criteria: –2.4° to –2.8°, determined at 20°, on the anhydrous basis

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total bacterial count does not exceed 1000 cfu/g, and the total combined molds and yeasts count does not exceed 100 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species, Test for Absence of Escherichia coli*, and *Test for Absence of Staphylococcus aureus*: It meets the requirements.
- **PH** ⟨791⟩

Sample solution: 8.5 g/100 mL of L-alpha-GPC or 10 g/100 mL of L-alpha-GPC Solution in water

Acceptance criteria: 5.0–7.0

- **WATER DETERMINATION** ⟨921⟩, *Method I, Method Ia*

L-alpha-GPC: NMT 1.0%

L-alpha-GPC Solution: 14%–16%

ADDITIONAL REQUIREMENTS

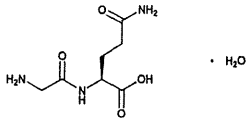
- **PACKAGING AND STORAGE:** Preserve in well-closed containers.

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- **LABELING:** Label it to indicate whether the material is the L-alpha-Glycerylphosphorylcholine or L-alpha-Glycerylphosphorylcholine Solution.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Glycerin RS
  USP L-alpha-Glycerophosphorylethanolamine RS
  USP L-alpha-Glycerylphosphorylcholine RS
  USP L-alpha-Glycerylphosphorylcholine Solution RS
  USP beta-Glycerylphosphorylcholine RS
  USP L-Serine RS▲ (USP 1-Dec-2019)

Glycine— *see Glycine General Monographs*

Glycyl-L-glutamine



$C_7H_{13}N_3O_4 \cdot H_2O$ 221.21

L-2-(Amino acetamide)-4-amino-4-oxo-butanoic acid monohydrate;
Glycyl-L-glutamine hydrate [13115-71-4].

DEFINITION

Glycyl-L-glutamine contains NLT 98.0% and NMT 101.5% of glycyl-L-glutamine ($C_7H_{13}N_3O_4$), calculated on the anhydrous and solvent-free basis, and excluding glycine and glutamine.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197A**▲ (CN 1-May-2020)
- **B.** It meets the requirements for *Optical Rotation, Specific Rotation* ⟨781S⟩ in *Specific Tests*.

ASSAY

- **PROCEDURE**

Sample: 300 mg
Blank: Mix 5 mL of formic acid with 50 mL of glacial acetic acid.
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.1 N perchloric acid VS
Endpoint detection: Potentiometric
Analysis: Dissolve the *Sample* in 5 mL of formic acid, add 50 mL of glacial acetic acid, and titrate with the *Titrant*. Perform the *Blank* determination, and make any necessary correction.
Calculate the percentage of glycyl-L-glutamine ($C_7H_{13}N_3O_4$) in the *Sample* taken:

$$\text{Result}_1 = [(V_S - V_B) \times N \times (F/W)] \times 100$$

V_S = *Titrant* volume consumed by the *Sample* (mL)
V_B = *Titrant* volume consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 203.2 mg/mEq
W = *Sample* weight (mg)

Calculate the percentage of glycyl-L-glutamine ($C_7H_{13}N_3O_4$) in the *Sample* taken, excluding glycine and glutamine:

$$\text{Result}_2 = [(\text{Result}_1 - a - b)]/[(100 - Gly - Gln)] \times 100$$

$$a = Gly \times (M_{r1}/M_{r2})$$

$$b = Gln \times (M_{r1}/M_{r3})$$

Gly = percentage of glycine from the *Related Compounds* test
Gln = percentage of glutamine from the *Related Compounds* test
M_{r1} = molecular weight of glycyl-L-glutamine, 203.2
M_{r2} = molecular weight of glycine, 75.1
M_{r3} = molecular weight of glutamine, 146.1

Acceptance criteria: 98.0%–101.5% on the anhydrous and solvent-free basis, and excluding glycine and glutamine

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 0.1%
- **CHLORIDE AND SULFATE,** *Chloride* ⟨221⟩
  Sample: 0.89 g
  Standard solution: 0.50 mL of 0.010 N hydrochloric acid
  Acceptance criteria: NMT 200 µg/g
- **CHLORIDE AND SULFATE,** *Sulfate* ⟨221⟩
  Sample: 0.96 g
  Standard solution: 0.20 mL of 0.010 N sulfuric acid
  Acceptance criteria: NMT 200 µg/g
- **IRON** ⟨241⟩**:** NMT 10 µg/g
- **RESIDUAL SOLVENTS** ⟨467⟩
  Acceptance criteria
  Ethanol: NMT 0.5%
  [NOTE—For the *Acceptance criteria* for any other residual solvents, see *Residual Solvents* ⟨467⟩.]
- **LIMIT OF AMMONIUM**
  Standard stock solution: Dissolve 1.486 g of ammonium chloride in 500.0 mL of water.
  Standard calibration solutions: Transfer 0.01, 0.1, 1.0, and 10.0 mL of *Standard stock solution* into separate 100-mL volumetric flasks, and dilute with water to volume. The final concentrations are 0.1, 1, 10, and 100 µg/mL of ammonium ions (NH_4^+), respectively.
  Sample solution: Transfer 1.0 g of Glycyl-L-glutamine to a 150-mL beaker containing a plastic-coated stirring bar, add 100.0 mL of water, and stir until dissolved.
  Electrode system: Use a gas-sensing, ammonia-specific indicating electrode with internal reference connected to a pH meter capable of measuring potentials with a minimum reproducibility of ±0.1 mV (see *pH* ⟨791⟩). Condition the electrode according to the manufacturer's instructions.
  Analysis
  Standard response line: Transfer 100 mL of water into a 150-mL beaker containing a plastic-coated stirring bar, insert the electrode into the water, stir, and measure the potential. Add 1 mL of 10 N sodium hydroxide solution, stir, and measure the potential after stabilization (about 3 min). The potential difference must be below 20 mV. Transfer 100.0 mL of each of *Standard calibration solutions* (0.1, 1, 10, 100 µg/mL of ammonium ions) into separate 150-mL beakers, and add 1 mL of 10 N sodium hydroxide. Insert the electrode into each solution, stir, and measure the potential after stabilization (about 3 min). Plot a curve (four calibration points) of the potential (mV) as function of ammonium ion concentrations (µg/mL).
  Sample: *Sample solution*

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Rinse the electrode, insert it into the *Sample solution*, add 1 mL of 10 N sodium hydroxide, and stir. Check the pH, which must be above 11; if not, adjust with 10 N sodium hydroxide. After 3 min, measure the potential, and determine the corresponding ammonium ion concentration from the calibration curve.

Calculate the content of ammonium in the portion of the *Sample* taken:

$$\text{Result} = (V \times C)/W$$

V = volume of the *Sample solution* (mL)
C = concentration of ammonium ions in the *Sample solution* determined from the *Standard response line* (µg/mL)
W = weight of Glycyl-L-glutamine taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 200 µg/g

• **RELATED COMPOUNDS**

Buffer solution: Dissolve 6.84 g of potassium phosphate in 1000 mL of water.

Mobile phase: Acetonitrile and *Buffer solution* (65:35)

System suitability solution: Transfer 25 mg of USP L-Alanyl-L-glutamine RS and 5 mg of USP L-Alanyl-alanine RS into a 25-mL volumetric flask, and dilute with water to volume. Transfer 1.0 mL of this solution into a 10-mL volumetric flask containing 20 mg of USP Glycyl-L-glutamine RS, and dilute with *Mobile phase* to volume.

Standard solution 1: 0.0125 mg/mL of USP Glutamine RS in *Mobile phase*

Standard solution 2: 0.025 mg/mL of USP Glycine RS in *Mobile phase*

Sample solution: 2.5 mg/mL of Glycyl-L-glutamine in *Mobile phase*

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 215 nm
Column: 4.6-mm × 25-cm; 5-µm packing L8
Flow rate: 0.7 mL/min
Injection volume: 20 µL

System suitability
Sample: *System suitability solution*
[NOTE—The relative retention times for L-alanyl-L-alanine, L-alanyl-L-glutamine, and glycyl-L-glutamine are 0.83, 0.96, and 1.0, respectively.]
Suitability requirements
Column efficiency: NLT 8000 theoretical plates for the L-alanyl-L-glutamine peak
Resolution: NLT 2.0 between L-alanyl-L-glutamine and L-alanyl-L-alanine

Analysis
Samples: *Standard solution 1*, *Standard solution 2*, and *Sample solution*

Calculate the percentage of glutamine and glycine in the portion of the *Sample* taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of glutamine or glycine from the *Sample solution*
r_S = peak response of glutamine from *Standard solution 1* or glycine from *Standard solution 2*
C_S = concentration of USP Glutamine RS in *Standard solution 1* (mg/mL) or concentration of USP Glycine RS in *Standard solution 2* (mg/mL)
C_U = concentration of Glycyl-L-glutamine in the *Sample solution* (mg/mL)

Calculate the percentage of any other specified and unspecified impurities in the portion of the *Sample* taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of each individual impurity
r_T = sum of the responses of all the peaks, excluding peak responses of glycine and glutamine

Acceptance criteria: See *Table 1*.

Table 1

Name	Relative Retention Time	Acceptance Criteria, NMT (%)
Cyclo(gly-gln)	0.28	0.2
Glutamine	0.54	0.5
Pyroglutamic acid	0.56	0.1
Glycine	0.59	1.0
Gly-gly-gln	1.20	0.3
Gly-glu	2.10	0.2
Any unspecified impurity	—	0.1
Total unspecified impurities	—	0.5

SPECIFIC TESTS

• **OPTICAL ROTATION,** *Specific Rotation* ⟨781S⟩
Sample solution: 100 mg/mL in 2 N hydrochloric acid. Perform the measurement at 20°.
Acceptance criteria: −5.5° to −7.5° on the anhydrous basis

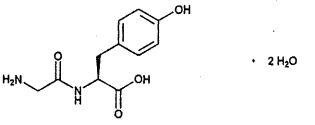
• **WATER DETERMINATION,** *Method Ia* ⟨921⟩**:** 7.0%–9.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed, tight, light-resistant containers.

• **USP REFERENCE STANDARDS** ⟨11⟩
USP L-Alanyl-L-alanine RS
USP L-Alanyl-L-glutamine RS
USP Glutamine RS
USP Glycine RS
USP Glycyl-L-glutamine RS

Glycyl-L-tyrosine



$C_{11}H_{14}N_2O_4 \cdot 2H_2O$ 274.27
L-2-Aminoacetamide-3-(parahydroxyphenyl) propionic acide dihydrate;
Glycyl-L-tyrosine dihydrate [658-79-7].

DEFINITION

Glycyl-L-tyrosine contains NLT 98.0% and NMT 101.5% of glycyl-L-tyrosine ($C_{11}H_{14}N_2O_4$), calculated on the anhydrous and solvent-free basis, and excluding glycine and tyrosine.

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IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197A**▲ (CN 1-May-2020)
- **B.** It meets the requirements for *Optical Rotation, Specific Rotation* ⟨781S⟩ in *Specific Tests*.

ASSAY

- **PROCEDURE**

Sample: 300 mg

Blank: Mix 5 mL of formic acid with 50 mL of glacial acetic acid.

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Direct titration

Titrant: 0.1 N perchloric acid VS

Endpoint detection: Potentiometric

Analysis: Dissolve the *Sample* in 5 mL of formic acid, add 50 mL of glacial acetic acid, and titrate with the *Titrant*. Perform the *Blank* determination, and make any necessary correction.

Calculate the percentage of glycyl-L-tyrosine ($C_{11}H_{14}N_2O_4$) in the *Sample* taken:

$$\text{Result}_1 = [(V_S - V_B) \times N \times (F/W)] \times 100$$

V_S = *Titrant* volume consumed by the *Sample* (mL)
V_B = *Titrant* volume consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 238.2 mg/mEq
W = *Sample* weight (mg)

Calculate the percentage of glycyl-L-tyrosine ($C_{11}H_{14}N_2O_4$) in the *Sample* taken, excluding glycine and tyrosine:

$$\text{Result}_2 = [(\text{Result}_1 - a - b)]/[(100 - Gly - Tyr)] \times 100$$

$$a = Gly \times (M_{r1}/M_{r2})$$

$$b = Tyr \times (M_{r1}/M_{r3})$$

Gly = percentage of glycine from the *Related Compounds* test
Tyr = percentage of tyrosine from the *Related Compounds* test
M_{r1} = molecular weight of glycyl-L-tyrosine, 238.2
M_{r2} = molecular weight of glycine, 75.1
M_{r3} = molecular weight of tyrosine, 181.2

Acceptance criteria: 98.0%–101.5% on the anhydrous and solvent-free basis, and excluding glycine and tyrosine

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%
- **CHLORIDE AND SULFATE,** *Chloride* ⟨221⟩

Sample: 0.89 g

Standard solution: 0.50 mL of 0.010 N hydrochloric acid

Acceptance criteria: NMT 200 µg/g

- **CHLORIDE AND SULFATE,** *Sulfate* ⟨221⟩

Sample: 0.96 g

Standard solution: 0.20 mL of 0.010 N sulfuric acid

Acceptance criteria: NMT 200 µg/g

- **IRON** ⟨241⟩: NMT 10 µg/g
- **RESIDUAL SOLVENTS** ⟨467⟩

Acceptance criteria

Ethanol: NMT 0.5%

[NOTE—For the *Acceptance criteria* for any other residual solvents, see *Residual Solvents* ⟨467⟩.]

- **LIMIT OF AMMONIUM**

Standard stock solution: Dissolve 1.486 g of ammonium chloride in 500.0 mL of water.

Standard calibration solutions: Transfer 0.01, 0.1, 1.0, and 10.0 mL of *Standard stock solution* into separate 100-mL volumetric flasks, and dilute with water to volume. The final concentrations are 0.1, 1, 10, and 100 µg/mL of ammonium ions (NH_4^+), respectively.

Sample solution: Transfer 1.0 g of Glycyl-L-tyrosine to a 150-mL beaker containing a plastic-coated stirring bar, add 100.0 mL of water, and stir until dissolved.

Electrode system: Use a gas-sensing, ammonia-specific indicating electrode with internal reference connected to a pH meter capable of measuring potentials with a minimum reproducibility of ±0.1 mV (see *pH* ⟨791⟩). Condition the electrode according to the manufacturer's instructions.

Analysis

Standard response line: Transfer 100 mL of water into a 150-mL beaker containing a plastic-coated stirring bar, insert the electrode into the water, stir, and measure the potential. Add 1 mL of 10 N sodium hydroxide solution, stir, and measure the potential after stabilization (about 3 min). The potential difference must be below 20 mV. Transfer 100.0 mL of each of *Standard calibration solutions* (0.1, 1, 10, 100 µg/mL of ammonium ions) into separate 150-mL beakers, and add 1 mL of 10 N sodium hydroxide. Insert the electrode into each solution, stir, and measure the potential after stabilization (about 3 min). Plot a curve (four calibration points) of the potential (mV) as function of ammonium ion concentrations (µg/mL).

Sample: *Sample solution*

Rinse the electrode, insert it into the *Sample solution*, add 1 mL of 10 N sodium hydroxide, and stir. Check the pH, which must be above 11; if not, adjust with 10 N sodium hydroxide. After 3 min, measure the potential, and determine the corresponding ammonium ion concentration from the calibration curve.

Calculate the content of ammonium in the portion of the *Sample* taken:

$$\text{Result} = (V \times C)/W$$

V = volume of the *Sample solution* (mL)
C = concentration of ammonium ions in the *Sample solution* determined from the *Standard response line* (µg/mL)
W = weight of Glycyl-L-tyrosine taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 200 µg/g

- **RELATED COMPOUNDS**

Buffer solution: Dissolve 6.84 g of potassium phosphate in 1000 mL of water.

Mobile phase: Acetonitrile and *Buffer solution* (65:35)

System suitability solution: Transfer 25 mg of USP L-Alanyl-L-glutamine RS and 5 mg of USP L-Alanyl-alanine RS into a 25-mL volumetric flask, and dilute with water to volume. Transfer 1.0 mL of this solution into a 10-mL volumetric flask containing 20 mg of USP Glycyl-l-tyrosine RS, and dilute with *Mobile phase* to volume.

Standard solution 1: 0.037 mg/mL of USP L-Tyrosine RS in *Mobile phase*

Standard solution 2: 0.025 mg/mL of USP Glycine RS in *Mobile phase*

Sample solution: 2.5 mg/mL of Glycyl-L-tyrosine in *Mobile phase*

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

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Mode: LC
Detector: UV 215 nm
Column: 4.6-mm × 25-cm; 5-µm packing L8
Flow rate: 0.7 mL/min
Injection volume: 20 µL
System suitability
Sample: *System suitability solution*
[NOTE—The relative retention times for glycyl-L-tyrosine, L-alanyl-L-alanine, and L-alanyl-L-glutamine are 1.0, 1.6, and 1.8, respectively.]
Suitability requirements
Column efficiency: NLT 8000 theoretical plates for the L-alanyl-L-glutamine peak
Resolution: NLT 2.0 between L-alanyl-L-glutamine and L-alanyl-L-alanine
Analysis
Samples: *Standard solution 1*, *Standard solution 2*, and *Sample solution*
Calculate the percentage of tyrosine and glycine in the portion of the *Sample* taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of tyrosine or glycine from the *Sample solution*
r_S = peak response of tyrosine from *Standard solution 1* or glycine from *Standard solution 2*
C_S = concentration of USP L-Tyrosine RS in *Standard solution 1* (mg/mL) or concentration of USP Glycine RS in *Standard solution 2* (mg/mL)
C_U = concentration of Glycyl-L-tyrosine in the *Sample solution* (mg/mL)

Calculate the percentage of any other specified and unspecified impurities in the portion of the *Sample* taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of each individual impurity
r_T = sum of the responses of all the peaks, excluding peak responses of glycine and tyrosine

Acceptance criteria: See *Table 1*.

Table 1

Name	Relative Retention Time	Acceptance Criteria, NMT (%)
Cyclo(gly-tyr)	0.41	0.2
Pivaloyl-L-tyrosine	0.48	0.1
Tyrosine	0.67	1.5
Glycine	1.15	1.0
Gly-gly-tyr	1.27	0.3
Any unspecified impurity	—	0.1
Total unspecified impurities	—	0.5

SPECIFIC TESTS
• **OPTICAL ROTATION,** *Specific Rotation* ⟨781S⟩
Sample solution: 20 mg/mL in water. Perform the measurement at 20°.
Acceptance criteria: +46.0° to +50.0° on the anhydrous basis
• **WATER DETERMINATION,** *Method Ia* ⟨921⟩**:** 10.0%–16.0%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed, tight, light-resistant containers.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Alanyl-L-alanine RS
USP L-Alanyl-L-glutamine RS
USP Glycine RS
USP Glycyl-l-tyrosine RS
USP L-Tyrosine RS

Goldenseal

DEFINITION
Goldenseal consists of the dried roots and rhizomes of *Hydrastis canadensis* L. (Fam. Ranunculaceae). It contains NLT 2.0% of hydrastine ($C_{21}H_{21}NO_6$) and NLT 2.5% of berberine ($C_{20}H_{18}NO_4$), calculated on the dried basis.

IDENTIFICATION
• **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩
Standard solution: Prepare a composite solution containing 0.5 mg/mL of USP Hydrastine RS and 0.025 mg/mL of USP Berberine Chloride RS in methanol
Sample solution: Sonicate 0.25 g of Goldenseal root and rhizome in 4 mL of 80% methanol for 30 min. Withdraw the supernatant, and wash the residue twice with 2-mL aliquots of methanol. Combine the supernatant and rinsates in a 20-mL volumetric flask, and adjust with methanol to volume. Mix well and filter before use. [NOTE—If prolonged storage is required, keep the *Sample solution* refrigerated.]
Chromatographic system
Mode: HPTLC
Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plate)[1]
Application volume: 5 µL each of *Standard solution* and *Sample solution* as 8-mm bands
Temperature: Ambient, not to exceed 30°
Developing solvent system: A mixture of ethyl acetate, methanol, formic acid, and water (50:10:6:3)
System suitability
Sample: *Standard solution*
Apply as a band and dry in air. Condition at relative humidity of about 33%. Develop in a saturated chamber until the solvent front has migrated over a path of 6 cm. Dry in a stream of cold air, and examine under the long-wave (365 nm) UV light.
Suitability requirements
Chromatographic pattern: Under long-wave UV (365 nm), the chromatogram exhibits the dark-blue band at about one-third of the migration distance due to hydrastine, and a bright yellow band at roughly halfway due to berberine.
Analysis
Samples: *Standard solution* and *Sample solution*
Treat and examine the *Samples* as described in *System suitability*.
Acceptance criteria: The chromatogram of the *Sample solution* exhibits a bright blue fluorescent band due to hydrastinine close to the application line; additional deep blue zones may be observed immediately above and below this band. In the middle third of the chromatogram, a deep blue band coincident with hydrastine, and a bright yellow fluorescent band coincident with berberine in the *Standard*

[1] Suitable commercially available plates are HPTLC Silica Gel 60 F_{254} from EMD Millipore (e.g., Part No. 1.05642.0001).

DS Monographs

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solution are observed. Proximal to the solvent front, a deep blue band is seen. The presence of a second yellow band below that of berberine, commonly ascribed to palmatine, is a likely sign of adulteration. The deep blue hydrastine band helps distinguish Goldenseal from numerous berberine-containing species.

COMPOSITION

- **CONTENT OF BERBERINE AND HYDRASTINE AND LIMIT OF PALMATINE**

Mobile phase: Dissolve 9.93 g of monobasic potassium phosphate in 730 mL of water and add 270 mL of acetonitrile. Mix, filter, and degas.

Solvent: A mixture of 0.1 M monobasic potassium phosphate and acetonitrile (60:40)

Standard solution: 0.05 mg/mL each of USP Berberine Chloride RS and USP Hydrastine RS in a mixture of methanol and water (1:1)

System suitability solution: Prepare a solution of palmatine chloride in a mixture of water and methanol (1:1) with a known concentration of about 0.05 mg/mL. Mix equal volumes of this solution and the *Standard solution*.

Sample solution: Finely powder a quantity of Goldenseal, and transfer about 0.12 g, accurately weighed, to a 50-mL volumetric flask. Add 40 mL of *Solvent*, sonicate for 5 min, and shake for 10 min. Dilute with *Solvent* to volume, mix, and filter.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 235 nm

Column: 4.6-mm × 150-mm; packing L1

Flow rate: 1.8 mL/min

Injection volume: 10 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 1.5 between the berberine and palmatine peaks; NLT 1.5 between the hydrastine and palmatine peaks, *System suitability solution*

Capacity factor: NLT 3.0 for the hydrastine peak, *Standard solution*

Column efficiency: NLT 5000 theoretical plates, determined from the hydrastine and berberine peaks, *Standard solution*

Tailing factor: NMT 2.0, determined from the hydrastine and berberine peaks, *Standard solution*

Relative standard deviation: NMT 2.5%, determined from the hydrastine and berberine peaks in repeated injections, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentages of hydrastine ($C_{21}H_{21}NO_6$) and berberine ($C_{20}H_{18}NO_4$) in the portion of Goldenseal taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of berberine or hydrastine in the *Sample solution*

r_S = peak area of berberine or hydrastine in the *Standard solution*

C_S = concentration of berberine or hydrastine in the *Standard solution* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of powdered Goldenseal used to prepare the *Sample solution* (mg)

Using the values obtained from the chromatogram of the *Sample solution*, divide the peak area of berberine by the area of any peak at the locus for palmatine (if present).

Acceptance criteria: NLT 2.0% of hydrastine ($C_{21}H_{21}NO_6$) and NLT 2.5% of berberine ($C_{20}H_{18}NO_4$) on the dried basis. The ratio of the berberine peak area to any peak area at the locus for palmatine is more than 50:1.

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩: Meets the requirements

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: The rhizome is knotty, subcylindrical, and occasionally has an aerial stem. It is 1–5 cm in length and 2–10 mm in diameter. Externally, the rhizome is brown to dusky yellowish orange, deeply furrowed, and marked by numerous stem and bud scale scars. Numerous brittle roots arise roughly from the same side of the main axis. Fractures are short and resinous, with a dark yellow to yellowish-brown bark, greenish-yellow margins, and a yellowish-orange center that is waxy in appearance. An interrupted circle of small, radially elongated fibrovascular bundles are also present. The roots are filiform, up to 35 cm in length and 1 mm in diameter, and are either curved or twisted, tangled together, or broken. Fractures are short and brittle, and show an internal color of yellowish orange to greenish yellow.

Microscopic

Transverse section of rhizome and root: The rhizome has polygonal, yellowish-brown, thin- to slightly thick-walled cork cells. Wedge-shaped vascular bundles are separated by wide medullary rays. Tracheary elements are lignified and have slit-shaped pits. A few large vessels with reticulate thickenings are also present. The parenchyma tissue is composed of polygonal cells with abundant simple or compound starch grains up to 8 µm in diameter. A few irregularly shaped resin cells are present in the cortex and the pith. Masses of granular, orange-brown matter are present in the parenchyma tissues. The roots have a single layer of irregularly elongated cork cells. The tracheary elements are associated with lignified fibers. Fragments of the epidermis are sometimes present near the base of the rhizome and are composed of cells with thick, lignified, beaded walls.

- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩: NMT 2.0%
- **LOSS ON DRYING** ⟨731⟩

Sample: 2.0 g of Goldenseal, finely powdered

Analysis: Dry the *Sample* at 100° for 5 h.

Acceptance criteria: NMT 12.0%

- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩

Sample: 1.0 g of Goldenseal, finely powdered

Analysis: NMT 9.0%

- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩: NMT 5%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and store protected from light, moisture, and heat.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Berberine Chloride RS

USP Hydrastine RS

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Powdered Goldenseal

DEFINITION

Powdered Goldenseal is Goldenseal reduced to a fine or very fine powder. It contains NLT 2.0% of hydrastine ($C_{21}H_{21}NO_6$) and NLT 2.5% of berberine ($C_{20}H_{18}NO_4$), calculated on the dried basis.

IDENTIFICATION

• **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution: Prepare a composite solution containing 0.5 mg/mL of USP Hydrastine RS and 0.025 mg/mL of USP Berberine Chloride RS in methanol

Sample solution: Sonicate 0.25 g of Powdered Goldenseal root and rhizome in 4 mL of 80% methanol for 30 min. Withdraw the supernatant, and wash the residue twice with 2-mL aliquots of methanol. Combine the supernatant and rinsates in a 20-mL volumetric flask, and adjust with methanol to volume. Mix well and filter before use. [NOTE—If prolonged storage is required, keep the *Sample solution* refrigerated.]

Chromatographic system

Mode: HPTLC

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plate)[1]

Application volume: 5 µL each of *Standard solution* and *Sample solution* as 8-mm bands

Temperature: Ambient, not to exceed 30°

Developing solvent system: A mixture of ethyl acetate, methanol, formic acid, and water (50:10:6:3)

System suitability

Sample: *Standard solution*

Apply as a band and dry in air. Condition at a relative humidity of about 33%. Develop in a saturated chamber until the solvent front has migrated over a path of 6 cm. Dry in a stream of cold air, and examine under long-wave (365 nm) UV light.

Suitability requirements

Chromatographic pattern: Under long-wave UV (365 nm), the chromatogram exhibits a dark-blue band at about one-third of the migration distance due to hydrastine and a bright yellow band at roughly halfway due to berberine.

Analysis

Samples: *Standard solution* and *Sample solution*

Treat and examine the *Samples* as described in *System suitability*.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits a bright blue fluorescent band due to hydrastinine close to the application line; additional deep blue zones may be observed immediately above and below this band. In the middle third of the chromatogram, a deep blue band coincident with hydrastine and a bright yellow fluorescent band coincident with berberine in the *Standard solution* are observed. Proximal to the solvent front, a deep blue band is seen. The presence of a second yellow band below that of berberine, commonly ascribed to palmatine, is a likely sign of adulteration. The deep blue hydrastine band helps distinguish Powdered Goldenseal from numerous berberine-containing species.

COMPOSITION

• **CONTENT OF BERBERINE AND HYDRASTINE AND LIMIT OF PALMATINE**

Mobile phase: Dissolve 9.93 g of monobasic potassium phosphate in 730 mL of water and add 270 mL of acetonitrile. Mix, filter, and degas.

Solvent: A mixture of 0.1 M monobasic potassium phosphate and acetonitrile (60:40)

Standard solution: 0.05 mg/mL each of USP Berberine Chloride RS and USP Hydrastine RS in a mixture of methanol and water (1:1)

System suitability solution: Prepare a solution of palmatine chloride in a mixture of water and methanol (1:1) with a known concentration of about 0.05 mg/mL. Mix equal volumes of this solution and the *Standard solution*.

Sample solution: Transfer about 0.12 g of Powdered Goldenseal, accurately weighed, to a 50-mL volumetric flask. Add 40 mL of *Solvent*, sonicate for 5 min, and shake for 10 min. Dilute with *Solvent* to volume, mix, and filter.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 235 nm

Column: 4.6-mm × 150-mm; packing L1

Flow rate: 1.8 mL/min

Injection volume: 10 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 1.5 between the berberine and palmatine peaks; NLT 1.5 between the hydrastine and palmatine peaks, *System suitability solution*

Capacity factor: NLT 3.0 for the hydrastine peak, *Standard solution*

Column efficiency: NLT 5000 theoretical plates, determined from the hydrastine and berberine peaks, *Standard solution*

Tailing factor: NMT 2.0, determined from the hydrastine and berberine peaks, *Standard solution*

Relative standard deviation: NMT 2.5%, determined from the hydrastine and berberine peaks in repeated injections, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentages of hydrastine ($C_{21}H_{21}NO_6$) and berberine ($C_{20}H_{18}NO_4$) in the portion of Powdered Goldenseal taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of berberine or hydrastine from the *Sample solution*

r_S = peak area of berberine or hydrastine from the *Standard solution*

C_S = concentration of berberine or hydrastine in the *Standard solution* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Powdered Goldenseal used to prepare the *Sample solution* (mg)

Using the values from the chromatogram of the *Sample solution*, divide the peak area of berberine by the area of any peak at the locus for palmatine (if present).

Acceptance criteria: NLT 2.0% of hydrastine ($C_{21}H_{21}NO_6$) and NLT 2.5% of berberine ($C_{20}H_{18}NO_4$) on the dried basis. The ratio of the berberine peak area to any peak area at the locus for palmatine is more than 50:1.

CONTAMINANTS

• **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements

• **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements

[1] Suitable commercially available plates are HPTLC Silica Gel 60 F_{254} from EMD Millipore (e.g., Part No. 1.05642.0001).

DS Monographs

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SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS:** The powder is dark yellow to moderately greenish-yellow with an aromatic odor and bitter taste. Abundant starch granules are present and are either spherical or ovoid; the granules are simple or compounded, with an occasional slit-shaped hilum. Parenchyma cells range from polygonal to round and are filled with starch or brown resin. Tracheary elements with slit-shaped pits and a few large reticulated vessels are present, as are beaded, thick-walled elongated epidermal cells. Thin fragments of cork layer are also present.
- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩: NMT 2.0%
- **LOSS ON DRYING** ⟨731⟩
  Sample: 2 g of Powdered Goldenseal, finely powdered
  Analysis: Dry the *Sample* at 100° for 5 h.
  Acceptance criteria: NMT 12.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩: NMT 9%
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩: NMT 5%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and store protected from light and moisture.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Berberine Chloride RS
  USP Hydrastine RS

Powdered Goldenseal Extract

DEFINITION

Powdered Goldenseal Extract is prepared from the pulverized dried roots and rhizomes of *Hydrastis canadensis* L. (Fam. Ranunculaceae), using suitable solvents. It contains NLT 5% of hydrastine ($C_{21}H_{21}NO_6$) and NLT 10% of the sum of berberine ($C_{20}H_{18}NO_4$) and hydrastine, calculated on the dried basis. The ratio of starting crude plant material to Powdered Goldenseal Extract is 2:1.

IDENTIFICATION

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩
  Standard solution: Prepare a composite solution containing 0.5 mg/mL of USP Hydrastine RS and 0.025 mg/mL of USP Berberine Chloride RS in methanol.
  Sample solution: 10 mg/mL of Powdered Goldenseal Extract in a mixture of methanol and water (1:1). Sonicate for 20 min, cool to room temperature, and filter. [NOTE—If prolonged storage is required, keep the *Sample solution* refrigerated.]
  Chromatographic system
  Mode: HPTLC
  Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plate)[1]
  Application volume: 5 µL each of *Standard solution* and *Sample solution* as 8-mm bands
  Temperature: Ambient, not to exceed 30°
  Developing solvent system: Ethyl acetate, methanol, formic acid, and water (50:10:6:3)
  System suitability
  Sample: *Standard solution*
  Apply as a band and dry in air. Condition at relative humidity of about 33%. Develop in a saturated chamber until the solvent front has migrated over a path of 6 cm. Dry in a current of cold air, and examine under long-wave (365 nm) UV light.
  Suitability requirements
  Chromatographic pattern: The chromatogram exhibits a dark-blue band at about one-third of the migration distance due to hydrastine and a bright-yellow band at roughly halfway due to berberine.
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Treat and examine the *Samples* as described in *System suitability*.
  Acceptance criteria: The chromatogram of the *Sample solution* exhibits a bright blue fluorescent band due to hydrastinine close to the application line; additional deep blue zones may be observed immediately above and below this band. In the middle third of the chromatogram, a deep blue band coincident with hydrastine and a bright-yellow fluorescent band coincident with berberine in the *Standard solution* are observed. Proximal to the solvent front, a deep blue band is seen. The presence of a second yellow band below that of berberine, commonly ascribed to palmatine, is a likely sign of adulteration. The deep blue hydrastine band helps distinguish Powdered Goldenseal Extract from numerous berberine-containing species.

COMPOSITION

- **CONTENT OF BERBERINE AND HYDRASTINE AND LIMIT OF PALMATINE**
  Mobile phase: Dissolve 9.93 g of monobasic potassium phosphate in 730 mL of water and add 270 mL of acetonitrile. Mix, filter, and degas.
  Standard solution: 0.05 mg/mL each of USP Berberine Chloride RS and USP Hydrastine RS in a mixture of methanol and water (1:1)
  System suitability solution: Prepare a solution of palmatine chloride in a mixture of water and methanol (1:1) with a known concentration of about 0.05 mg/mL. Mix equal volumes of this solution and the *Standard solution*.
  Sample solution: 2 mg/mL of Powdered Goldenseal Extract in a mixture of methanol and water (1:1). Sonicate for 20 min, cool to room temperature, and filter. [NOTE—The sample to be used in this test should not be subjected to the conditions specified in the test for *Loss on Drying*. A separate sample is used to determine the content on the dried basis.]
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: LC
  Detector: UV 235 nm
  Column: 4.6-mm × 150-mm; packing L1
  Flow rate: 1.8 mL/min
  Injection volume: 10 µL
  System suitability
  Samples: *Standard solution* and *System suitability solution*
  Suitability requirements
  Resolution: NLT 1.5 between the berberine and palmatine peaks; NLT 1.5 between the hydrastine and palmatine peaks, *System suitability solution*
  Capacity factor: NLT 3.0 for the hydrastine peak, *Standard solution*
  Column efficiency: NLT 5000 theoretical plates, determined from the hydrastine and berberine peaks, *Standard solution*
  Tailing factor: NMT 2.0, determined from the hydrastine and berberine peaks, *Standard solution*

[1] Suitable commercially available plates are HPTLC Silica Gel 60 F_{254} from EMD Millipore (e.g., Part No. 1.05642.0001).

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Relative standard deviation: NMT 2.5%, determined from the hydrastine and berberine peaks in replicate injections, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentages of hydrastine ($C_{21}H_{21}NO_6$) and berberine ($C_{20}H_{18}NO_4$) in the portion of Powdered Goldenseal Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of berberine or hydrastine from the *Sample solution*

r_S = peak area of berberine or hydrastine from the *Standard solution*

C_S = concentration of berberine or hydrastine in the *Standard solution* (mg/mL)

C_U = concentration of Powdered Goldenseal Extract in the *Sample solution* (mg/mL)

Using the values from the chromatogram of the *Sample solution*, divide the peak area of berberine by the area of any peak at the locus for palmatine (if present).

Acceptance criteria: NLT 5% of hydrastine and NLT 10% of the sum of hydrastine and berberine on the dried basis. The ratio of the berberine peak area to any peak area at the locus for palmatine is more than 50:1.

CONTAMINANTS

Change to read:

- **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020) : Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

- **LOSS ON DRYING** ⟨731⟩

Sample: 1.0 g of Powdered Goldenseal Extract

Analysis: Dry the *Sample* at 105° for 2 h.

Acceptance criteria: NMT 5.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and store protected from light and moisture.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. Label to indicate the content of hydrastine and berberine, the extracting solvent used for preparation, and the ratio of the starting crude plant material to the Powdered Goldenseal Extract.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Berberine Chloride RS

USP Hydrastine RS

Grape Seeds Oligomeric Proanthocyanidins

DEFINITION

Grape Seeds Oligomeric Proanthocyanidins is a fraction of an extract of the ripe seeds of *Vitis vinifera* L. (Fam. Vitaceae). It contains NLT 75.0% of oligomeric proanthocyanidins, on the anhydrous basis. The extract is prepared using suitable solvents such as alcohol, methanol, acetone, ethyl acetate, water, or mixtures of these solvents, in a ratio of starting plant material to extract between 70:1 and 10:1. The extract is further enriched in oligomeric proanthocyanidins by fractionation with ethyl acetate or by other means.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm and a layer thickness of about 0.2 mm (HPTLC plates)

Standard solution A: Dissolve a quantity of USP Purified Grape Seeds Oligomeric Proanthocyanidins RS in methanol, using sonication, to obtain a solution having a concentration of about 5 mg/mL. Centrifuge if necessary, and use the clear supernatant. [NOTE—Prepare fresh.]

Standard solution B: Dissolve a quantity of USP (+)-Catechin RS in methanol, using sonication, to obtain a solution having a concentration of about 1 mg/mL.

Sample solution: Proceed as directed for *Standard solution A*, except use the Grape Seeds Oligomeric Proanthocyanidins.

Developing solvent system: A mixture of acetone, toluene, and formic acid (15:15:5)

Spray reagent: Dissolve about 100 mg of vanillin in 3-mL of methanol using sonication. Add about 3 mL of hydrochloric acid, dilute with methanol to 10 mL, and carefully mix under cold water. [NOTE—Prepare fresh.]

Application volume: 15 µL, as 5–10-mm bands

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands to a suitable thin-layer chromatographic plate (see *Chromatography* ⟨621⟩). Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about 90% of the plate. Remove the plate from the chamber, dry, spray with the *Spray reagent*, dry, and examine under visible light.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits pink-violet bands, corresponding in color and R_F to those in the chromatogram of *Standard solution A*, at the following approximate R_F values: a pair of bands between 0.20 and 0.23 (trimeric proanthocyanidins), a band at 0.28 (proanthocyanidin-B_2-3′-*O*-gallate), a band at 0.31 (B-type dimeric proanthocyanidins), and a band at 0.43 ((−)-epicatechin-3-*O*-gallate). The chromatogram of the *Sample solution* may exhibit a pink-violet band at an approximate R_F of 0.49 (residual flavan 3-ol monomers and/or gallic acid) corresponding to the band in the chromatogram of *Standard solution B*. Other pink-violet bands may also be observed.

- **B.** The chromatogram of the *Sample solution* obtained in the test for *Limit of Catechin and Epicatechin* exhibits peaks due to proanthocyanidin dimer B_1, proanthocyanidin dimer B_2, (−)-epicatechin-3-*O*-gallate, and a broad peak due to other oligomeric proanthocyanidins at retention times corresponding to those in the chromatogram of *Standard solution B*.

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COMPOSITION

• **CONTENT OF OLIGOMERIC PROANTHOCYANIDINS**

Internal standard solution: Prepare a solution of butylated hydroxytoluene in *Mobile phase* containing about 0.3 mg/mL.

Standard solution A: Dissolve a weighed quantity of USP Purified Grape Seeds Oligomeric Proanthocyanidins RS in *Internal standard solution* to obtain a solution having a known concentration of about 1.0 mg/mL.

Standard solution B: Dissolve a portion of USP (+)-Catechin RS in *Internal standard solution* to obtain a solution having a known concentration of about 0.2 mg/mL.

Sample solution: Dissolve a weighed quantity of Grape Seeds Oligomeric Proanthocyanidins in *Internal standard solution* to obtain a solution having a known concentration of about 1.0 mg/mL. Centrifuge, and use the clear supernatant.

Mobile phase: Prepare a filtered and degassed mixture of tetrahydrofuran and an aqueous solution of lithium bromide (about 1 mg/mL) (95:5).

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 7.5-mm × 30-cm; 5-µm, 500-Å, packing L21

Column temperature: 25° ± 1

Flow rate: 1.0 mL/min

Injection size: 10 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements: Measure the responses as determined under *Analysis*.

Relative standard deviation: NMT 2.0% determined from the the peak area ratio of the oligomeric proanthocyanidins to the internal standard in repeated injections, *Standard solution A*

Resolution: NLT 3.0 between the peaks of monomers and the internal standard, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Chromatograph *Standard solution A* and determine the beginning and end of the retention time window for the integration of oligomeric proanthocyanidins, at the points where the response of the main peak is about 0.5% of its maximum. Record the peak area ratio of the oligomeric proanthocyanidins to the internal standard. Chromatograph *Standard solution B* and the *Sample solution* and identify the locus for the monomers. Integrate the areas of the main peaks within the retention time window as determined for *Standard solution A*, excluding the area above the main peak, at the locus identified for the monomers, using a proper integration method.

Calculate the percentage of the oligomeric proanthocyanidins in the portion of the Grape Seeds Oligomeric Proanthocyanidins taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of the oligomeric proanthocyanidins to the internal standard from the *Sample solution*

R_S = peak response ratio of the oligomeric proanthocyanidins to the internal standard from *Standard solution A*

C_S = concentration of USP Purified Grape Seeds Oligomeric Proanthocyanidins RS in *Standard solution A* (mg/mL)

C_U = concentration of Grape Seeds Oligomeric Proanthocyanidins in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 75.0% on the anhydrous basis

IMPURITIES

Change to read:

ORGANIC IMPURITIES

• **▲Botanical Extracts** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues:* ▲ (CN 1-May-2020) Meets the requirements

SPECIFIC TESTS

• **LIMIT OF CATECHIN AND EPICATECHIN**

Solution A: Use acetonitrile.

Solution B: Use a 0.3% aqueous solution of 85% phosphoric acid.

Solvent: Prepare a mixture of *Solution A* and *Solution B* (1:9).

Standard solution A: Dissolve, using sonication, a weighed quantity of USP (+)-Catechin RS in *Solvent* to obtain a solution having a known concentration of about 0.5 mg/mL.

Standard solution B: Dissolve, using sonication, a weighed quantity of USP Grape Seeds Oligomeric Proanthocyanidins RS in *Solvent* to obtain a solution having a known concentration of about 5 mg/mL. Centrifuge, and use the clear supernatant.

Sample solution: Proceed as directed for *Standard solution B*, except use the Grape Seeds Oligomeric Proanthocyanidins.

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	10	90
45	20	80
65	60	40
66	10	90
85	10	90

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 278 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Flow rate: 0.7 mL/min

Injection size: 10 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

The chromatogram obtained from *Standard solution B* is similar to the Reference Chromatogram provided with the lot of the USP Grape Seeds Oligomeric Proanthocyanidins RS being used.

Tailing factor: NMT 2.0 for the catechin peak, *Standard solution A*

Relative standard deviation: NMT 2% determined from the catechin peak in repeated injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatogram of *Standard solution A*, *Standard solution B*, and the Reference Chromatogram provided

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with the lot of USP Grape Seeds Oligomeric Proanthocyanidins RS being used, identify the retention times of the peaks corresponding to (+)-catechin and (–)-epicatechin. The approximate relative retention times of the peaks are 1.0 and 1.43 for (+)-catechin and (–)-epicatechin, respectively.

Calculate the sum of the percentages of (+)-catechin and (–)-epicatechin in the portion of the Grape Seeds Oligomeric Proanthocyanidins taken:

$$(r_U/r_S) \times (C \times V/W) \times 100$$

r_U = sum of the peak responses for (+)-catechin and (–)-epicatechin from the *Sample solution*
r_S = peak response for (+)-catechin in *Standard solution A*
C_S = concentration of USP (+)-Catechin RS in *Standard solution A* (mg/mL)
V = final volume of the *Sample solution* (mL)
W = weight of Grape Seeds Oligomeric Proanthocyanidins taken to prepare the *Sample solution* (mg)

Acceptance criteria: NMT 19.0% on the anhydrous basis

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^4 cfu/g. The total combined yeast and mold count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.
- **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 0.5%, determined on 5.0 g
- **WATER DETERMINATION,** *Method Ia* ⟨921⟩ **:** NMT 8.0%
- **WATER-INSOLUBLE FRACTION**

Analysis: Transfer about 1 g, weighed, to a suitable flask. Add 100 mL of water, and shake vigorously for 15 min. Pass the solution through a previously tared sintered-glass filter, wash the flask with 30 mL of water, and transfer the washings to the filter. Wash the filter with 30 mL of water in 5-mL portions. Dry the filter for 2 h at 105°, cool in a desiccator, and weigh. Calculate the percentage of the water-insoluble fraction.

Acceptance criteria: NMT 2%

- **OTHER REQUIREMENTS:** It meets the requirements of the test for *Residual Solvents* under *Botanical Extracts* ⟨565⟩.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, it states "Grape Seeds Oligomeric Proanthocyanidins". It meets other labeling requirements under *Botanical Extracts* ⟨565⟩.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP (+)-Catechin RS
USP Grape Seeds Oligomeric Proanthocyanidins RS
USP Purified Grape Seeds Oligomeric Proanthocyanidins RS

Powdered Decaffeinated Green Tea Extract

DEFINITION

Powdered Decaffeinated Green Tea Extract is prepared from young unfermented leaves and leaf buds of *Camellia sinensis* (L.) Kuntze (Family Theaceae) [syn. *Thea sinensis* L.] using suitable solvents such as alcohol, methanol, acetone, water, or mixtures of these solvents; the caffeine has been removed. The ratio of the starting crude plant material to Powdered Decaffeinated Green Tea Extract is 6:1–10:1. It contains NLT 60.0% of polyphenols, calculated as (–)-epigallocatechin-3-*O*-gallate, NLT 40.0% of (–)-epigallocatechin-3-*O*-gallate, and NMT 0.1% of caffeine, all calculated on the anhydrous basis.

IDENTIFICATION

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution: 4 mg/mL of USP Powdered Decaffeinated Green Tea Extract RS in alcohol and water (4:1), sonicated for 10 min, and centrifuged. Use the clear supernatant. [NOTE—Prepare fresh. Store below –20°, if storage is needed.]

Sample solution: 4 mg/mL of Powdered Decaffeinated Green Tea Extract in alcohol and water (4:1), sonicated for 10 min, and centrifuged. Use the clear supernatant.

Chromatographic system

Application volume: 1 µL

Developing solvent system: Toluene, acetone, and formic acid (9:9:2)

Immersion reagent: Dissolve 140 mg of fast blue B salt in 10 mL of water, and add 140 mL of methanol and 50 mL of dichloromethane. [NOTE—Prepare weekly, and store at 4° in the dark.]

Analysis

Samples: *Standard solution* and *Sample solution*

Use an unsaturated chamber. Develop the chromatograms, dry the plate at 100°, treat with the *Immersion reagent*, dry in a current of cold air, and immediately examine under white light. [NOTE—The chromatogram is stable up to 30 min; afterward, the plate's background darkens significantly.]

System suitability: Under white light, the derivatized chromatogram of the *Standard solution* exhibits, in the middle third of the plate, four prominent brownish-orange bands corresponding, in the order of increasing R_F, to (–)-epigallocatechin-3-*O*-gallate, (–)-epigallocatechin, (–)-epicatechin-3-*O*-gallate, and (–)-epicatechin, respectively. The most intense band is that of (–)-epigallocatechin-3-*O*-gallate.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits major bands similar in position and color to the major bands of the *Standard solution*.

- **B. HPLC**

Analysis: Proceed as directed in the test for *Content of Polyphenols*.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits the peaks for (–)-epigallocatechin, (+)-catechin, (–)-epicatechin, (–)-epigallocatechin-3-*O*-gallate, (–)-gallocatechin-3-*O*-gallate, (–)-epigallocatechin-3-*O*-(3′-*O*-methyl)-gallate, and (–)-epicatechin-3-*O*-gallate at retention times corresponding to those of *Standard solution B*.

COMPOSITION

- **CONTENT OF POLYPHENOLS**

Solution A: Methanol, 85% phosphoric acid, and water (50: 3.5: 946.5)

Solution B: Acetonitrile and methanol (95:5)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	94	6
20	94	6

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Table 1 *(continued)*

Time (min)	Solution A (%)	Solution B (%)
50	78	22
70	38	62
75	94	6
90	94	6

Standard solution A: 0.1 mg/mL of USP (–)-Epigallocatechin-3-*O*-gallate RS in *Solution A*
Standard solution B: 0.4 mg/mL of USP Powdered Decaffeinated Green Tea Extract RS in *Solution A*. Mix and centrifuge.
Sample solution: 0.4 mg/mL of Powdered Decaffeinated Green Tea Extract in *Solution A*. Mix and centrifuge.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 278 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 25°
Flow rate: 0.8 mL/min
Injection volume: 15 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Resolution: NLT 1 between the (–)-epigallocatechin-3-*O*-gallate peak and the preceding peak, *Standard solution B*
Tailing factor: 0.8–2.0 for the (–)-epigallocatechin-3-*O*-gallate peak, *Standard solution A*
Relative standard deviation: NMT 2.0% for the (–)-epigallocatechin-3-*O*-gallate peak in replicate injections, *Standard solution A*
Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Decaffeinated Green Tea Extract RS being used.
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Record the chromatograms, and measure the areas of the analyte peaks. Using the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Decaffeinated Green Tea Extract RS, identify the retention times of the peaks corresponding to the different polyphenols. The approximate relative retention times of the polyphenols are provided in *Table 2*.

Table 2

Analyte	Relative Retention Time
(–)-Epigallocatechin	0.56
(+)-Catechin	0.68
(–)-Epicatechin	0.98
(–)-Epigallocatechin-3-*O*-gallate	1.00
(–)-Gallocatechin-3-*O*-gallate	1.09
(–)-Epigallocatechin-3-*O*-(3′-*O*-methyl)-gallate	1.19
(–)-Epicatechin-3-*O*-gallate	1.27

Separately calculate the percentages of (–)-epigallocatechin, (+)-catechin, (–)-epicatechin, (–)-epigallocatechin-3-*O*-gallate, (–)-gallocatechin-3-*O*-gallate, (–)-epigallocatechin-3-*O*-(3′-*O*-methyl)-gallate, and (–)-epicatechin-3-*O*-gallate as (–)-epigallocatechin-3-*O*-gallate in the portion of Powdered Decaffeinated Green Tea Extract taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of each of the polyphenols in the *Sample solution*
r_S = peak area of (–)-epigallocatechin-3-*O*-gallate in *Standard solution A*
C_S = concentration of USP (–)-Epigallocatechin-3-*O*-gallate RS in *Standard solution A* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Powdered Decaffeinated Green Tea Extract taken to prepare the *Sample solution* (mg)

Add the percentages calculated for the individual polyphenols.
Acceptance criteria: NLT 40.0% of (–)-epigallocatechin-3-*O*-gallate and NLT 60.0% of polyphenols, calculated as (–)-epigallocatechin-3-*O*-gallate, both on the anhydrous basis

CONTAMINANTS

Change to read:

- **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020) **:** Meets the requirements
- **BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Residual Solvents***:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^4 cfu/g, and the total combined yeasts and molds count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

- **LIMIT OF GALLIC ACID**

Solution A, Solution B, Mobile phase, and **Chromatographic system:** Proceed as directed in the test for *Content of Polyphenols*.
Standard solution: 0.2 mg/mL of gallic acid in *Solution A*
Sample solution: 20 mg/mL of Powdered Decaffeinated Green Tea Extract in *Solution A*. Mix and centrifuge.
Analysis
Samples: *Standard solution* and *Sample solution*
Record the chromatograms, and measure the areas of the gallic acid peaks.
Calculate the percentage of gallic acid in the portion of Powdered Decaffeinated Green Tea Extract taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of gallic acid from the *Sample solution*
r_S = peak area of gallic acid from the *Standard solution*
C_S = concentration of gallic acid in the *Standard solution* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Powdered Decaffeinated Green Tea Extract taken to prepare the *Sample solution* (mg)

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Acceptance criteria: NMT 1.0% on the anhydrous basis

- **LIMIT OF CAFFEINE**

Solution A: Methanol, tetrahydrofuran, 85% phosphoric acid, and water (50: 10: 3.5: 936.5)
Solution B: Acetonitrile, methanol, and 85% phosphoric acid (946.5: 50: 3.5)
Mobile phase: See *Table 3*.

Table 3

Time (min)	Solution A (%)	Solution B (%)
0	100	0
30	100	0
35	0	100
40	0	100
45	100	0
55	100	0

Standard solution: 1 µg/mL of USP Caffeine RS in methanol
Sample solution: 1 mg/mL of Powdered Decaffeinated Green Tea Extract in methanol
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 272 nm
Column: 4.6-mm × 25-cm; 5-µm packing L60[1]
Column temperature: 25°
Flow rate: 1 mL/min
Injection volume: 15 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 2.0% determined from the caffeine peak in replicate injections
Analysis
Samples: *Standard solution* and *Sample solution*
Record the chromatograms, and measure the areas of the caffeine peaks.
Calculate the percentage of caffeine in the portion of Powdered Decaffeinated Green Tea Extract taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of caffeine from the *Sample solution*
r_S = peak area of caffeine from the *Standard solution*
C_S = concentration of USP Caffeine RS in the *Standard solution* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Powdered Decaffeinated Green Tea Extract taken to prepare the *Sample solution* (mg)

Acceptance criteria: NMT 0.1% on the anhydrous basis

- **WATER DETERMINATION** ⟨921⟩, *Method I, Method Ia*

Sample: 0.5 g of Powdered Decaffeinated Green Tea Extract
Acceptance criteria: NMT 6.0%

- **RESIDUE ON IGNITION** ⟨281⟩

Sample: 1.0 g of Powdered Decaffeinated Green Tea Extract
Acceptance criteria: NMT 0.5%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived. It meets other labeling requirements in *Botanical Extracts* ⟨565⟩. Dosage forms prepared with this article should bear the following statement: Do not take on an empty stomach. Take with food. Do not use if you have a liver problem. Discontinue use and consult a healthcare practitioner if you develop symptoms of liver trouble, such as abdominal pain, dark urine, or jaundice (yellowing of the skin or eyes).
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Caffeine RS
USP (–)-Epigallocatechin-3-*O*-gallate RS
USP Powdered Decaffeinated Green Tea Extract RS

[1] Endcapped packing L1 columns can also be used in this test.

Guggul

DEFINITION

Guggul is the oleo-gum-resin obtained by incision or produced by spontaneous exudation from the stem and branches of *Commiphora wightii* (Arn.) Bhandari, also known as *Commiphora mukul* (Hook. ex. Stocks) Engl. or *Balsamodendrum mukul* (Hook.) (Fam. Burseraceae). It contains NLT 1.0% of guggulsterones *E* and *Z*, calculated on the dried basis as guggulsterone *Z*.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHY**

Standard solution: 10 mg/mL of USP Purified Guggul Extract RS with heating, in acetonitrile
Sample solution: Transfer about 0.5 g of crushed Guggul to a centrifuge tube. Add 25 mL of acetonitrile, and shake for 1 min. Heat in a water bath for 10–15 min with occasional shaking, cool, centrifuge, and use the supernatant.
Chromatographic system
(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
Developing solvent system: A mixture of hexane and ethyl acetate (6:4)
Adsorbent: 0.25-mm layer of chromatographic silica gel mixture, typically 20 cm in length (TLC plates)
Application volume: 10 µL
Analysis
Samples: *Standard solution* and *Sample solution*
Apply the *Samples* as bands. Use a saturated chamber. Develop until the solvent front has moved about three-fourths the length of the plate, dry the plate, and examine under short-wave UV light (254 nm).
Acceptance criteria: The chromatogram of the *Sample solution* exhibits bands at R_F values of about 0.38 and 0.47, due to guggulsterones *E* and *Z*, respectively. Both bands correspond in R_F values to bands from the *Standard solution*.

- **B. HPLC**

Analysis: Proceed as directed in the test for *Content of Guggulsterones E and Z*.
Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks for guggulsterones *E* and *Z* at retention times that correspond to those of *Standard solution A*.

COMPOSITION

- **CONTENT OF GUGGULSTERONES *E* AND *Z***

Mobile phase: A mixture of acetonitrile and water (45:55)
Standard solution A: 10 mg/mL of USP Purified Guggul Extract RS with heating, in acetonitrile. Pass the solution through a filter of 0.45-µm pore size before injection.

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Standard solution B: 0.1 mg/mL of USP Guggulsterone Z RS in acetonitrile. Pass the solution through a filter of 0.45-µm pore size before injection.

Sample solution: Transfer about 2.0 g of crushed Guggul to a conical flask, and extract four times with 50-mL portions of acetonitrile. Shake for 1 min, and reflux in a water bath for 30 min, stirring magnetically. Evaporate the combined extracts to about 50 mL, and transfer to a 100-mL volumetric flask. Dilute with acetonitrile to volume, and pass through a filter of 0.45-µm pore size before injection.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 242 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Flow rate: 2.0 mL/min
Injection volume: 20 µL
Column temperature: 27 ± 1°

System suitability
Samples: *Standard solution A* and *Standard solution B*
[NOTE—The relative retention times for guggulsterones *E* and *Z* are about 0.69 and 1.0, respectively.]
Suitability requirements
Chromatogram similarity: The chromatogram of *Standard solution A* is similar to the reference chromatogram provided with the lot of USP Purified Guggul Extract RS being used.
Resolution: NLT 2.0 between the guggulsterone *Z* peak and the peak before, *Standard solution A*
Tailing factor: NMT 1.5 for the guggulsterone *Z* peak, *Standard solution B*
Relative standard deviation: NMT 2.0% for replicate injections for the guggulsterone *Z* peak, *Standard solution B*

Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Allow *Standard solution A* to elute for NLT 2 times the retention time of guggulsterone *Z*, as determined from *Standard solution B*. Using the chromatogram of *Standard solution A* and the reference chromatogram provided with the lot of USP Purified Guggul Extract RS being used, identify the retention times of the peaks corresponding to guggulsterone *E* and guggulsterone *Z*.
Calculate the percentage of guggulsterones *E* and *Z* as guggulsterone *Z* in the portion of Guggul taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = sum of the peak responses of guggulsterones *E* and *Z* from the *Sample solution*
r_S = peak response of guggulsterone *Z* from *Standard solution B*
C_S = concentration of USP Guggulsterone Z RS in *Standard solution B* (mg/mL)
V = final volume of the *Sample solution*, 100 mL
W = weight of Guggul taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 1.0% on the dried basis

CONTAMINANTS
- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements

SPECIFIC TESTS
- **BOTANICAL CHARACTERISTICS**
  Macroscopic: Guggul occurs in rounded or irregular conglomerates of tears, of variable sizes, light to dark brown, slightly sticky to touch, with characteristic and aromatic odor and an aromatic, astringent taste.
- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩**:** NMT 1%
- **ARTICLES OF BOTANICAL ORIGIN,** *Alcohol-Soluble Extractives, Method 2* ⟨561⟩**:** NLT 33%
- **ETHYL ACETATE SOLUBLE EXTRACTIVES**
  Sample: About 5.0 g of coarsely powdered Guggul
  Analysis: Transfer the *Sample* to a glass-stoppered, conical flask. Add 25 mL of solvent hexane, insert a stopper into the flask, shake for 1 h, filter, and discard the filtrate. Repeat twice, and dry the residue in a vacuum over phosphorus pentoxide at room temperature for 8 h. Crush the dried material, and extract with four 25-mL quantities of ethyl acetate, by shaking each time for 1 h at room temperature, followed by filtration through a sintered glass funnel (porosity No. 3). Evaporate the combined filtrates under reduced pressure in a tared flask, dry the residue in a vacuum over phosphorus pentoxide at room temperature for 12 h, and weigh. Determine the percentage of the ethyl acetate soluble extractives calculated from the weight of Guggul taken.
  Acceptance criteria: 22%–30%
- **LOSS ON DRYING** ⟨731⟩
  Sample: 1.0 g of Guggul
  Analysis: Dry the *Sample* at 105° for 2 h.
  Acceptance criteria: NMT 8.0%
- **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 10.0%, ignited at 800 ± 25°
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 2.0%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store in a cool place.
- **LABELING:** The label states the Latin binomial of the species of *Commiphora* from which the oleo-gum-resin was obtained and the official name.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Guggulsterone Z RS
  USP Purified Guggul Extract RS

Native Guggul Extract

DEFINITION
Native Guggul Extract is prepared from Guggul, using ethyl acetate, alcohol, or methanol. The ratio of starting crude plant material to Native Extract is approximately 9:1. It contains NLT 5.0% of guggulsterones *E* and *Z*, calculated on the anhydrous basis as guggulsterone *Z*. It does not contain added substances.

IDENTIFICATION
- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**
  Standard solution: 10 mg/mL of USP Purified Guggul Extract RS, with heating, in acetonitrile
  Sample solution: Homogenize Native Guggul Extract by heating in a water bath at 60°–80°. Prepare a 5-mg/mL solution in acetonitrile with heating.
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

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Developing solvent system: A mixture of hexane and ethyl acetate (6:4)
Adsorbent: 0.25-mm layer of chromatographic silica gel mixture, typically 20 cm in length
Application volume: 10 µL
Analysis
Samples: *Standard solution* and *Sample solution*
Apply the *Samples* as bands to a suitable plate. Use a saturated chamber. Develop until the solvent front has moved about about three-fourths the length of the plate, dry the plate, and examine under UV light at 254 nm.
Acceptance criteria: The chromatogram of the *Sample solution* exhibits bands at R_F values of about 0.38 and 0.47, due to guggulsterone *E* and *Z*, respectively. Both bands correspond in R_F to bands in the chromatogram from the *Standard solution*.

- **B. HPLC IDENTIFICATION TEST**

Analysis: Proceed as directed in the test for *Content of Guggulsterones E and Z*.
Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks for guggulsterones *E* and *Z* at retention times that correspond to those of *Standard solution A*.

COMPOSITION

- **CONTENT OF GUGGULSTERONES *E* AND *Z***

Mobile phase: A mixture of acetonitrile and water (45:55)
Standard solution A: 10 mg/mL of USP Purified Guggul Extract RS, with heating, in acetonitrile. Pass the solution through a filter of 0.45-µm pore size before injection.
Standard solution B: 0.1 mg/mL of USP Guggulsterone Z RS in acetonitrile. Pass the solution through a filter of 0.45-µm pore size before injection.
Sample solution: Homogenize the Native Guggul Extract by heating in a water bath at 60°–80°. Dissolve a weighed quantity of homogenized Native Extract, with heating, in acetonitrile to obtain a solution having a known concentration of about 0.2 mg/mL of guggulsterones *E* and *Z*. Pass through a filter of 0.45-µm pore size before injection.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 242 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Flow rate: 2.0 mL/min
Injection size: 20 µL
Column temperature: 27 ± 1°
System suitability
Samples: *Standard solution A* and *Standard solution B*
[NOTE—The relative retention times for guggulsterones *E* and *Z* are about 0.69 and 1.0, respectively.]
Suitability requirements
Chromatogram similarity: The chromatogram from *Standard solution A* is similar to the reference chromatogram provided with the lot of USP Purified Guggul Extract RS being used.
Resolution: NLT 2.0 between the guggulsterone *Z* peak and the peak before, *Standard solution A*
Tailing factor: NMT 1.5 for the guggulsterone *Z* peak, *Standard solution B*
Relative standard deviation: NMT 2.0% for the guggulsterone *Z* peak (replicate injections), *Standard solution B*
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Allow *Standard solution A* to elute for NLT two times the retention time of guggulsterone *Z*, as determined in *Standard solution B*. Using the chromatogram of *Standard solution A* and the reference chromatogram provided with the lot of USP Purified Guggul Extract RS being used, identify the retention times of the peaks corresponding to guggulsterone *E* and guggulsterone *Z*.
Calculate the percentage of guggulsterones *E* and *Z* as guggulsterone *Z* in the portion of Native Guggul Extract taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = sum of the peak responses of guggulsterones *E* and *Z* from the *Sample solution*
r_S = peak response of guggulsterone *Z* from *Standard solution B*
C_S = concentration of USP Guggulsterone Z RS in *Standard solution B* (mg/mL)
V = final volume of *Sample solution* (mL)
W = weight of Native Extract taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 5.0% of guggulsterones *E* and *Z*, calculated as guggulsterone *Z*, on the anhydrous basis

CONTAMINANTS

Change to read:

- **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020) **:** Meets the requirements
- **BOTANICAL EXTRACTS,** *Residual Solvents* ⟨565⟩**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^4 cfu/g, and the total combined yeasts and molds count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

- **WATER DETERMINATION,** *Method 1a* ⟨921⟩**:** NMT 5.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store in a cool place.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived. It meets other labeling requirements under *Botanical Extracts* ⟨565⟩.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Guggulsterone Z RS
USP Purified Guggul Extract RS

Purified Guggul Extract

DEFINITION

Purified Guggul Extract is prepared from Native Guggul Extract by fractionation using aqueous methanol. It contains NLT 90.0% and NMT 110.0% of the labeled amount of guggulsterones *E* and *Z*, calculated as guggulsterones *Z*. It may be a semisolid extract with no added substances or powder extract containing suitable added substances.

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IDENTIFICATION

• **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution: 10 mg/mL of USP Purified Guggul Extract RS, with heating, in acetonitrile.

Sample solution: 10 mg/mL in acetonitrile from Purified Guggul Extract, dissolved with heating, cooled, and centrifuged.

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Developing solvent system: A mixture of hexane and ethyl acetate (6:4)

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture, typically 20 cm in length

Application volume: 10 µL

Analysis

Samples: *Standard solution* and *Sample solution*

Apply the *Samples* as bands to a suitable plate. Use a saturated chamber. Develop until the solvent front has moved about about three-fourths the length of the plate, dry the plate, and examine under UV light at 254 nm.

Acceptance criteria: The chromatograph of the *Sample solution* exhibits bands at R_F values of about 0.38 and 0.47, due to guggulsterone *E* and *Z*, respectively. Both bands correspond in R_F to bands in the chromatogram from the *Standard solution*.

• **B. HPLC IDENTIFICATION TEST**

Analysis: Proceed as directed in the test for *Content of Guggulsterones E and Z*.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks for guggulsterones *E* and *Z* at retention times that correspond to those of *Standard solution A*.

COMPOSITION

• **CONTENT OF GUGGULSTERONES *E* AND *Z***

Mobile phase: A mixture of acetonitrile and water (45:55)

Standard solution A: 10 mg/mL of USP Purified Guggul Extract RS, with heating, in acetonitrile. Pass the solution through a filter of 0.45-µm pore size before injection.

Standard solution B: 0.1 mg/mL of USP Guggulsterone Z RS in acetonitrile. Pass the solution through a filter of 0.45-µm pore size before injection.

Sample solution: Dissolve a weighed quantity of Purified Guggul Extract, with heating, in acetonitrile to obtain a solution having a known concentration of about 0.2 mg/mL of guggulsterones *E* and *Z*. Pass through a filter of 0.45-µm pore size before injection.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 242 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Flow rate: 2.0 mL/min

Injection size: 20 µL

Column temperature: 27 ± 1°

System suitability

Samples: *Standard solution A* and *Standard solution B*

[NOTE—The relative retention times for guggulsterones *E* and *Z* are about 0.69 and 1.0, respectively.]

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution A* is similar to the reference chromatogram provided with the lot of USP Purified Guggul Extract RS being used.

Resolution: NLT 2.0 between the guggulsterone *Z* peak and the peak before, *Standard solution A*

Tailing factor: NMT 1.5 for guggulsterone *Z* peak, *Standard solution B*

Relative standard deviation: NMT 2.0% for replicate injections for the guggulsterone *Z* peak, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Allow *Standard solution A* to elute for NLT two times the retention time of guggulsterone *Z*, as determined in *Standard solution B*. Using the chromatogram of *Standard solution A* and the reference chromatogram provided with the lot of USP Purified Guggul Extract RS being used, identify the retention times of the peaks corresponding to guggulsterone *E* and guggulsterone *Z*.

Calculate the percentage of guggulsterones *E* and *Z* as guggulsterone *Z* in the portion of Purified Guggul Extract taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = sum of the peak responses of guggulsterones *E* and *Z* from the *Sample solution*

r_S = peak response of guggulsterone *Z* from *Standard solution B*

C_S = concentration of USP Guggulsterone Z RS in *Standard solution B* (mg/mL)

V = final volume of the *Sample solution* (mL)

W = weight of Purified Guggul Extract taken to prepare the *Sample solution* (mg)

Acceptance criteria: 90.0%–110.0% of the labeled amount of guggulsterones *E* and *Z*, calculated as guggulsterones *Z*

CONTAMINANTS

Change to read:

• **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020)**:** Meets the requirements

• **BOTANICAL EXTRACTS,** *Residual Solvents* ⟨565⟩**:** Meets the requirements

• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^4 cfu/g, and the total combined yeasts and molds count does not exceed 10^3 cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store in a cool place.

• **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived. It meets other labeling requirements in *Botanical Extracts* ⟨565⟩.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Guggulsterone Z RS

USP Purified Guggul Extract RS

Guggul Tablets

DEFINITION

Guggul Tablets are prepared from Native Guggul Extract or Purified Guggul Extract. Tablets contain NLT 90.0% and NMT 110.0% of the labeled amount of Extract, calculated as the sum of guggulsterones *E* and *Z*.

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IDENTIFICATION

• **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution: 10 mg/mL of USP Purified Guggul Extract RS, with heating, in acetonitrile

Sample solution: Powder and transfer a portion of the Tablets equivalent to 100 mg of Extract to a conical flask. Extract three times, each with 25 mL of acetonitrile, in a 55° water bath for 15 min, stirring with a magnetic stirrer, and filter. Evaporate the combined extracts to dryness in a vacuum between 45° and 50°, dissolve the residue in 10 mL of acetonitrile, centrifuge, and use the clear supernatant.

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Developing solvent system: A mixture of hexane and ethyl acetate (6:4)

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture, typically 20 cm in length

Application volume: 10 µL

Analysis

Samples: *Standard solution* and *Sample solution*

Apply the *Samples* as bands to a suitable plate. Use a saturated chamber. Develop until the solvent front has moved about about three-fourths the length of the plate, dry the plate, and examine under UV light at 254 nm.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits bands at R_F values of about 0.38 and 0.47, due to guggulsterone *E* and *Z*, respectively. Both bands correspond in R_F to bands in the chromatogram from the *Standard solution*.

• **B. HPLC IDENTIFICATION TEST**

Analysis: Proceed as directed in the test for *Content of Guggulsterones E and Z*.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks for guggulsterones *E* and *Z* at retention times that correspond to those of *Standard solution A*.

STRENGTH

• **CONTENT OF GUGGULSTERONES *E* AND *Z***

Mobile phase: A mixture of acetonitrile and water (45:55)

Standard solution A: 10 mg/mL of USP Purified Guggul Extract RS, with heating, in acetonitrile. Pass the solution through a filter of 0.45-µm pore size before injection.

Standard solution B: 0.1 mg/mL of USP Guggulsterone Z RS in acetonitrile. Pass the solution through a filter of 0.45-µm pore size before injection.

Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer a weighed amount of the powder, equivalent to about 10 mg of guggulsterones *E* and *Z*, to a conical flask, and extract five times, each with a 20-mL portion of acetonitrile, shake for 1 min, and reflux in a water bath for 30 min, stirring with a magnetic stirrer. Evaporate the combined extracts to dryness in a vacuum at 45°–50°. Dissolve the residue in 50.0 mL of acetonitrile, and pass through a filter of 0.45-µm pore size before injection.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 242 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Flow rate: 2.0 mL/min

Injection size: 20 µL

Column temperature: 27 ± 1°

System suitability

Samples: *Standard solution A* and *Standard solution B*

[NOTE—The relative retention times for guggulsterones *E* and *Z* are about 0.69 and 1.0, respectively.]

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution A* is similar to the reference chromatogram provided with the lot of USP Purified Guggul Extract RS being used.

Resolution: NLT 2.0 between the guggulsterone *Z* peak and the peak before, *Standard solution A*

Tailing factor: NMT 1.5 for the guggulsterone *Z* peak, *Standard solution B*

Relative standard deviation: NMT 2.0% for the guggulsterone *Z* peak (replicate injections), *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Allow *Standard solution A* to elute for NLT two times the retention time of guggulsterone *Z*, as determined in *Standard solution B*. Using the chromatogram of *Standard solution A* and the reference chromatogram provided with the lot of USP Purified Guggul Extract RS being used, identify the retention times of the peaks corresponding to guggulsterone *E* and guggulsterone *Z*.

Calculate the content of guggulsterones *E* and *Z* as guggulsterone *Z* in the portion of Tablets taken:

$$C_I = (r_U/r_S) \times C_S \times V$$

r_U = sum of the peak responses of guggulsterones *E* and *Z* from the *Sample solution*

r_S = peak response of guggulsterone *Z* from *Standard solution B*

C_S = concentration of USP Guggulsterone Z RS in *Standard solution B* (mg/mL)

V = final volume of the *Sample solution* (mL)

Calculate the percentage of the labeled amount of Guggul Extract taken:

$$\text{Result} = C_I \times (A_{WT}/W) \times (100/L_E) \times (100/L)$$

C_I = content of guggulsterones *E* and *Z* in the portion of Tablets taken (mg)

A_{WT} = average weight of Tablets (mg)

W = weight of the powdered Tablets taken (mg)

L_E = labeled percentage of the sum of guggulsterones *E* and *Z* in the Extract used to prepare the Tablets

L = label claim of Extract (mg/Tablet)

Acceptance criteria: 90.0%–110.0% of the labeled amount of Extract, calculated as the sum of guggulsterones *E* and *Z*

PERFORMANCE TESTS

• **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩**:** It meets the requirement for *Disintegration* only; 30 min, the use of the disk being omitted.

• **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩**:** Meet the requirements

CONTAMINANTS

• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^4 cfu/g, and the total combined yeasts and molds count does not exceed 10^3 cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

• **RESIDUAL SOLVENTS** ⟨467⟩**:** Meets the requirements

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ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the article from which the Tablets were prepared. The label also indicates the amount of Extract, in mg/Tablet, and the content, in mg, of guggulsterones *E* and *Z* per 100 mg of Extract.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Guggulsterone Z RS
  USP Purified Guggul Extract RS

Gymnema

DEFINITION

Gymnema consists of the dried leaves of *Gymnema sylvestre* (Retz.) R. Br. ex Schult. (Fam. Asclepiadaceae). It contains NLT 1.0% of gymnemic acids, calculated as gymnemagenin on the dried basis.

IDENTIFICATION

- **A.** Meets the requirements for *Specific Tests, Botanical Characteristics*
- **B. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 0.5 mg/mL of USP Gymnemagenin RS in methanol

Standard solution B: 20 mg/mL of USP Native Gymnema Extract RS in methanol. Sonicate for 10 min, centrifuge, and use the supernatant.

Sample solution: About 0.5 g of Gymnema, finely powdered, in 5 mL of methanol. Sonicate for 10 min, centrifuge, and use the supernatant.

Chromatographic system

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)

Application volume: 5 µL as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

Developing solvent system: A mixture of dichloromethane, methanol, and formic acid (75:25:10)

Developing distance: 6 cm

Derivatization reagent: A mixture of methanol and sulfuric acid (9:1)

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the samples as bands. Develop in a saturated chamber, remove the plate from the chamber, dry in air, treat with *Derivatization reagent*, heat at 110° for 3 min, and examine under white light and UV at 365 nm.

System suitability: The chromatogram of *Standard solution B* shows two bands clearly separated at an R_F of about 0.6–0.7 below the band due to gymnemagenin in *Standard solution A*. The most prominent band is located at about one-third of the chromatogram, visible as brown in color under white light and blue under UV.

Acceptance criteria: The chromatogram of the *Sample solution* shows the following bands corresponding in color and position to bands in the chromatogram of *Standard solution B*: two bands at an R_F of about 0.6–0.7, below the band due to gymnemagenin in *Standard solution A*. The most prominent band is at about one-third of the chromatogram, visible as brown in color under white light and blue under UV; in the lower third of the chromatogram, under UV, are one light blue-greenish band and a dark band underneath.

- **C. HPLC**

Analysis: Proceed as directed in the test for *Content of Gymnemic Acids*.

Acceptance criteria: The chromatogram of the *Sample solution* shows a major peak at a retention time corresponding to that of the gymnemagenin peak in the chromatogram of *Standard solution A* and an additional peak corresponding to deacylgymnemic acid.

COMPOSITION

- **CONTENT OF GYMNEMIC ACIDS**

Solution A: Dissolve 0.14 g of anhydrous potassium dihydrogen phosphate in 900 mL of water, and add 0.5 mL of phosphoric acid. Dilute with water to 1000 mL, mix, filter, and degas.

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	75	25
20	45	55
25	40	60
30	40	60
35	75	25
40	75	25

Solvent: 50% Ethanol in water

Potassium hydroxide solution: 12% Potassium hydroxide in water

Hydrochloric acid solution: 4 N hydrochloric acid

Standard solution A: 0.3 mg/mL of USP Gymnemagenin RS in methanol

Standard solution B: Transfer about 0.25 g of USP Native Gymnema Extract RS to a 100-mL round-bottom flask fitted with a reflux condenser. Add 25 mL of *Solvent* and 2 mL of *Potassium hydroxide solution*, reflux for 1 h, and cool to room temperature. Add 5.5 mL of *Hydrochloric acid solution*, reflux for 2 h, and cool to room temperature. Adjust the solution with *Potassium hydroxide solution* to a pH of 7.5–8.5, transfer to a 50-mL volumetric flask, dilute with *Solvent* to volume, and mix. Before injection, pass through a membrane filter having a 0.45-µm or finer pore size, discarding the first few mL of the filtrate.

Sample solution: Transfer about 0.75 g of Gymnema, finely powdered and accurately weighed, to a 100-mL round-bottom flask fitted with a reflux condenser. Add 25 mL of *Solvent* and 2 mL of *Potassium hydroxide solution*, reflux for 1 h, and cool to room temperature. Add 5.5 mL of *Hydrochloric acid solution*, reflux for 2 h, and cool to room temperature. Adjust the solution with *Potassium hydroxide solution* to a pH of 7.5–8.5, filter into a 100-mL volumetric flask, dilute with *Solvent* to volume, and mix. Before injection, pass through a membrane filter having a 0.45-µm or finer pore size, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 210 nm

Column: 4.6-mm × 25-cm; 5-µm, 100 Å, packing L1

Column temperature: 25°

Flow rate: 1.6 mL/min

Injection volume: 20 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

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Suitability requirements
Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Native Gymnema Extract RS being used.
Tailing factor: NMT 1.5 for the gymnemagenin peak, *Standard solution A*
Relative standard deviation: NMT 2.0% for the gymnemagenin peak, *Standard solution A*
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Native Gymnema Extract RS being used, identify the retention times of the peaks corresponding to deacylgymnemic acid and gymnemagenin from the *Sample solution*.
Calculate the percentage of gymnemic acids, calculated as gymnemagenin, in the portion of Gymnema taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of gymnemagenin from the *Sample solution*
r_S = peak area of gymnemagenin from *Standard solution A*
C_S = concentration of gymnemagenin in *Standard solution A* (mg/mL)
V = final volume of the *Sample solution* (mL)
W = weight of Gymnema used to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 1.0% on the dried basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria do not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: Leaves opposite; petiolate; simple, elliptic or ovate, acute apex, margin entire, acute to acuminate at base; reticulate venation; pubescent on both surfaces, the dorsal surface is more pubescent; about 2–6 cm long, 1–4 cm broad; upper surface is yellowish-brown, lower surface is dark green; bitter taste, when chewed has the property of paralyzing the sense of taste for a few hours for both sweet and bitter substances and in particular sweet substances. Pharmacopeial article consists of dry, brittle, and green to yellowish-green leaves.
Microscopic
Transverse section of petiole: Horseshoe shaped. Trichomes present profusely all over the periphery, and are uniseriate, multicellular and thick-walled. A layer of epidermis, thick-walled cells; 4–5 layers of collenchymatous cortex; and inner parenchyma. The vascular bundles occur as an arc in the middle, three in number—two small lateral, and one large median. The median bundle consists of xylem elements arranged in radial rows, surrounded by phloem. Xylem consists of vessel elements, tracheids, and fibers. Rosette crystals are present in the collenchyma and parenchyma cells, with more in the cells toward the center. The starch grains are polygonal, simple or compound, in two to many in groups, hilum indistinct.
Transverse section of lamina: Upper and lower epidermal cells, thin wall, covered with thin striated cuticle. Trichomes are present on both surfaces, uniseriate, uni- to tricellular, and thick-walled. A single layer of palisade cells below the upper epidermis occupies one-third of the thickness of the lamina, followed by 3–5 layers of parenchyma cells with large intercellular spaces.
Transverse section of midrib: Upper and lower epidermal cells, thin wall, covered with thin striated cuticle. Trichomes are present, uniseriate, uni- to tricellular, and thick-walled, followed by 4–5 layers of compact parenchyma cells on both sides of the midrib; collateral vascular bundles are present, forming an arc shape.

- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩**:** NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Alcohol-Soluble Extractives, Method 1* ⟨561⟩**:** NLT 20%
- **ARTICLES OF BOTANICAL ORIGIN,** *Water-Soluble Extractives, Method 1* ⟨561⟩**:** NLT 20%
- **LOSS ON DRYING** ⟨731⟩
  Sample: 1.0 g of finely powdered Gymnema
  Analysis: Dry the *Sample* at 105° for 3 h.
  Acceptance criteria: NMT 7.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩
  Sample: 1.0 g of finely powdered Gymnema
  Acceptance criteria: NMT 15%
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩
  Sample: 1.0 g of finely powdered Gymnema
  Acceptance criteria: NMT 2.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture. Store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Gymnemagenin RS
  USP Native Gymnema Extract RS

Powdered Gymnema

DEFINITION

Powdered Gymnema is Gymnema reduced to powder or very fine powder. It contains NLT 1.0% of gymnemic acids, calculated as gymnemagenin on the dried basis.

IDENTIFICATION

- **A.** Meets the requirements for *Specific Tests, Botanical Characteristics*
- **B. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩
  Standard solution A: 0.5 mg/mL of USP Gymnemagenin RS in methanol
  Standard solution B: 20 mg/mL of USP Native Gymnema Extract RS in methanol. Sonicate for 10 min, centrifuge, and use the supernatant.
  Sample solution: About 0.5 g of Powdered Gymnema in 5 mL of methanol. Sonicate for 10 min, centrifuge, and use the supernatant.

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Chromatographic system
Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)
Application volume: 5 µL as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33%, using a suitable device.
Developing solvent system: A mixture of dichloromethane, methanol, and formic acid (75:25:10)
Developing distance: 6 cm
Derivatization reagent: A mixture of methanol and sulfuric acid (9:1)
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*.
Apply the *Samples* as bands, and dry in air. Develop the chromatograms in a saturated chamber, remove the plate from the chamber, dry in air, derivatize the plate with *Derivatization reagent*, heat at 110° for 3 min, and examine under white light and under UV at 365 nm.
System suitability: The chromatogram of *Standard solution B* shows two bands clearly separated at an R_F of about 0.6–0.7 below the band due to gymnemagenin in *Standard solution A*. The most prominent band is located at about one-third of the chromatogram, visible as brown in color under white light and blue under UV.
Acceptance criteria: The chromatogram of the *Sample solution* shows the following bands corresponding in color and position to bands in the chromatogram of *Standard solution B*: two bands at an R_F of about 0.6–0.7, below the band due to gymnemagenin in *Standard solution A*. The most prominent band is at about one-third of the chromatogram, visible as brown in color under white light and blue under UV; in the lower third of the chromatogram, under UV, are one light blue-greenish band and a dark band underneath.

• **C. HPLC**
Analysis: Proceed as directed in the test for *Content of Gymnemic Acids*.
Acceptance criteria: The chromatogram of the *Sample solution* shows a major peak at a retention time corresponding to that of the gymnemagenin peak in the chromatogram of *Standard solution A* and an additional peak corresponding to deacylgymnemic acid.

COMPOSITION

• **CONTENT OF GYMNEMIC ACIDS**
Solution A: Dissolve 0.14 g of anhydrous potassium dihydrogen phosphate in 900 mL of water, and add 0.5 mL of phosphoric acid. Dilute with water to 1000 mL, mix, filter, and degas.
Solution B: Acetonitrile
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	75	25
20	45	55
25	40	60
30	40	60
35	75	25
40	75	25

Solvent: 50% Ethanol in water
Potassium hydroxide solution: 12% Potassium hydroxide in water
Hydrochloric acid solution: 4 N hydrochloric acid
Standard solution A: 0.3 mg/mL of USP Gymnemagenin RS in methanol
Standard solution B: Transfer about 0.25 g of USP Native Gymnema Extract RS to a 100-mL round-bottom flask fitted with a reflux condenser. Add 25 mL of *Solvent* and 2 mL of *Potassium hydroxide solution*, reflux on a water bath for 1 h, and cool to room temperature. Add 5.5 mL of *Hydrochloric acid solution*, reflux on a water bath for 2 h, and cool to room temperature. Adjust the solution with *Potassium hydroxide solution* to a pH of 7.5–8.5, transfer to a 50-mL volumetric flask, dilute with *Solvent* to volume, and mix. Before injection, pass through a membrane filter having a 0.45-µm or finer pore size, discarding the first few mL of the filtrate.
Sample solution: Transfer about 0.75 g of Powdered Gymnema, accurately weighed, to a 100-mL round-bottom flask fitted with a reflux condenser. Add 25 mL of *Solvent* and 2 mL of *Potassium hydroxide solution*, reflux on a water bath for 1 h, and cool to room temperature. Add 5.5 mL of *Hydrochloric acid solution*, reflux on a water bath for 2 h, and cool to room temperature. Adjust the solution with *Potassium hydroxide solution* to a pH of 7.5–8.5, filter into a 100-mL volumetric flask, dilute with *Solvent* to volume, and mix. Before injection, pass through a membrane filter having a 0.45-µm or finer pore size, discarding the first few mL of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 210 nm
Column: 4.6-mm × 25-cm; 5-µm, 100 Å, packing L1
Column temperature: 25°
Flow rate: 1.6 mL/min
Injection volume: 20 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Native Gymnema Extract RS being used.
Tailing factor: NMT 1.5 for the gymnemagenin peak, *Standard solution A*
Relative standard deviation: NMT 2.0% for the gymnemagenin peak, *Standard solution A*
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Native Gymnema Extract RS being used, identify the retention times of the peaks corresponding to deacylgymnemic acid and gymnemagenin from the *Sample solution*.
Calculate the percentage of gymnemic acids, calculated as gymnemagenin, in the portion of Powdered Gymnema taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of gymnemagenin from the *Sample solution*
r_S = peak area of gymnemagenin from *Standard solution A*
C_S = concentration of gymnemagenin in *Standard solution A* (mg/mL)
V = final volume of the *Sample solution* (mL)

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W = weight of Powdered Gymnema used to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 1.0% on the dried basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria do not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Microscopic: Under a microscope, it shows fragments of upper and lower epidermal cells, polygonal, thin and straight walls, covered with striated cuticle, anomocytic stomata; trichomes are present on both surfaces, uniseriate, uni- to tricellular, and thick walled; fragments of collenchyma and parenchyma cells, some containing rosettes of calcium oxalate; starch grains, polygonal, simple or compound, hilum indistinct; fragments of reticulate and spiral vessels, and tracheids.

- **ARTICLES OF BOTANICAL ORIGIN,** *Alcohol-Soluble Extractives, Method 1* ⟨561⟩**:** NLT 20%
- **ARTICLES OF BOTANICAL ORIGIN,** *Water-Soluble Extractives, Method 1* ⟨561⟩**:** NLT 20%
- **LOSS ON DRYING** ⟨731⟩

Sample: 1.0 g of Powdered Gymnema
Analysis: Dry the *Sample* at 105° for 3 h.
Acceptance criteria: NMT 7.0%

- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩

Sample: 1.0 g of Powdered Gymnema
Acceptance criteria: NMT 15%

- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩

Sample: 1.0 g of Powdered Gymnema
Acceptance criteria: NMT 2.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture. Store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant used to prepare the article.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Gymnemagenin RS
USP Native Gymnema Extract RS

Native Gymnema Extract

DEFINITION

Native Gymnema Extract is prepared from Gymnema using hydroalcoholic mixtures. The ratio of plant material to extract is about 8:1. It contains NLT 5.0% of gymnemic acids, calculated as gymnemagenin on the dried basis.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHY**

Standard solution A: 0.5 mg/mL of USP Gymnemagenin RS in methanol
Standard solution B: 20 mg/mL of USP Native Gymnema Extract RS in methanol. Sonicate for 10 min, centrifuge, and use the supernatant.
Sample solution: 20 mg/mL of Native Gymnema Extract in methanol. Sonicate for 10 min, centrifuge, and use the supernatant.
Chromatographic system
Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)
Application volume: 5 µL as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33%, using a suitable device.
Developing solvent system: A mixture of dichloromethane, methanol, and formic acid (75:25:10)
Developing distance: 6 cm
Derivatization reagent: A mixture of methanol and sulfuric acid (9:1)
Analysis
Samples: *Standard solution A, Standard solution B,* and *Sample solution.* Apply the samples as bands to a suitable high-performance thin-layer chromatographic plate, and dry in air (see *Chromatography* ⟨621⟩). Develop the chromatograms in a saturated chamber, remove the plate from the chamber, dry in air, derivatize the plate with *Derivatization reagent,* heat at 110° for 3 min, and examine under visible light and UV at 366 nm.
System suitability: The chromatogram of *Standard solution B* shows two bands clearly separated at an R_F of about 0.6–0.7 below the band due to gymnemagenin in *Standard solution A,* and the most prominent band is located at about one-third of the chromatogram, visible as brown in color under white light and blue under UV.
Acceptance criteria: The chromatogram of the *Sample solution* shows the following bands corresponding in color and position to bands in the chromatogram of *Standard solution B*: two bands at an R_F of about 0.6–0.7, below the band due to gymnemagenin in *Standard solution A*; the most prominent band at about one-third of the chromatogram, visible as brown in color under white light and blue under UV; and in the lower third of the chromatogram, under UV, one light blue-greenish band and a dark band underneath.

- **B. HPLC**

Analysis: Proceed as directed in the test for *Content of Gymnemic Acids.*
Acceptance criteria: The chromatogram of the *Sample solution* shows a major peak at a retention time corresponding to that of the gymnemagenin peak in the chromatogram of *Standard solution A* and an additional peak corresponding to deacylgymnemic acid.

COMPOSITION

- **CONTENT OF GYMNEMIC ACIDS**

Solution A: Dissolve 0.14 g of anhydrous potassium dihydrogen phosphate in 900 mL of water, and add 0.5 mL of phosphoric acid. Dilute with water to 1000 mL, mix, filter, and degas.
Solution B: Acetonitrile
Mobile phase: See *Table 1.*

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	75	25
20	45	55
25	40	60
30	40	60

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Table 1 *(continued)*

Time (min)	Solution A (%)	Solution B (%)
35	75	25
40	75	25

Solvent: 50% ethanol in water
Potassium hydroxide solution: 12% potassium hydroxide in water
Hydrochloric acid solution: 4 N hydrochloric acid
Standard solution A: 0.3 mg/mL of USP Gymnemagenin RS in methanol
Standard solution B: Transfer about 0.25 g of USP Native Gymnema Extract RS to a 100-mL round-bottom flask fitted with a reflux condenser. Add 25 mL of *Solvent* and 2 mL of *Potassium hydroxide solution*, reflux on a water bath for 1 h, and cool to room temperature. Add 5.5 mL of *Hydrochloric acid solution*, reflux on a water bath for 2 h, and cool to room temperature. Adjust the solution with *Potassium hydroxide solution* to a pH of 7.5–8.5, transfer to a 50-mL volumetric flask, dilute with *Solvent* to volume, and mix. Before injection, pass through a membrane filter having a 0.45-µm or finer pore size, discarding the first few mL of the filtrate.
Sample solution: Transfer an amount of Native Gymnema Extract, equivalent to about 30 mg of gymnemic acids, to a 100-mL round-bottom flask fitted with a reflux condenser. Add 25 mL of *Solvent* and 2 mL of *Potassium hydroxide solution*, reflux on a water bath for 1 h, and cool to room temperature. Add 5.5 mL of *Hydrochloric acid solution*, reflux on a water bath for 2 h, and cool to room temperature. Adjust the solution with *Potassium hydroxide solution* to a pH of 7.5–8.5, transfer to a 100-mL volumetric flask, dilute with *Solvent* to volume, and mix. Before injection, pass through a membrane filter having a 0.45-µm or finer pore size, discarding the first few mL of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 210 nm
Column: 4.6-mm × 25-cm; 5-µm, 100 Å, packing L1
Column temperature: 25 ± 1°
Flow rate: 1.6 mL/min
Injection volume: 20 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Native Gymnema Extract RS being used.
Tailing factor: NMT 1.5 for the gymnemagenin peak, *Standard solution A*
Relative standard deviation: NMT 2.0% determined from the gymnemagenin peak in repeated injections, *Standard solution A*
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Native Gymnema Extract RS being used, identify the retention times of the peaks corresponding to deacylgymnemic acid and gymnemagenin from the *Sample solution*.
Calculate the percentage of gymnemic acids, calculated as gymnemagenin, in the portion of Native Gymnema Extract taken:

$$Result = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of gymnemagenin from the *Sample solution*
r_S = peak area of gymnemagenin from *Standard solution A*
C_S = concentration of gymnemagenin in *Standard solution A* (mg/mL)
C_U = concentration of Native Gymnema Extract in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 5.0% on the dried basis

CONTAMINANTS

Change to read:

- **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020) **:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS
- **LOSS ON DRYING** ⟨731⟩
Sample: 1.0 g of Native Gymnema Extract
Analysis: Dry at 105° for 3 h.
Acceptance criteria: NMT 5.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩
Sample: 1.0 g of Native Gymnema Extract
Acceptance criteria: NMT 8%
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩
Sample: 1.0 g of Native Gymnema Extract
Acceptance criteria: NMT 2.0%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture. Store at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived. It meets other labeling requirements under *Botanical Extracts* ⟨565⟩.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Gymnemagenin RS
USP Native Gymnema Extract RS

Purified Gymnema Extract

DEFINITION
Purified Gymnema Extract is prepared from Native Gymnema Extract by precipitation using dilute hydrochloric acid solution. The ratio of plant material to extract is about 25:1. It contains NLT 90.0% and NMT 110.0% of the labeled amount of gymnemic acids, calculated as gymnemagenin on the dried basis. It may contain suitable added substances as carriers.

IDENTIFICATION
- **A. THIN-LAYER CHROMATOGRAPHY**
Standard solution A: 0.5 mg/mL of USP Gymnemagenin RS in methanol

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Standard solution B: 20 mg/mL of USP Native Gymnema Extract RS in methanol. Sonicate for 10 min, centrifuge, and use the supernatant.
Sample solution: 20 mg/mL of Purified Gymnema Extract in methanol. Sonicate for 10 min, centrifuge, and use the supernatant.
Chromatographic system
Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)
Application volume: 5 µL as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33%, using a suitable device.
Developing solvent system: A mixture of dichloromethane, methanol, and formic acid (75:25:10)
Developing distance: 6 cm
Derivatization reagent: A mixture of methanol and sulfuric acid (9:1)
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*. Apply the samples as bands to a suitable high-performance thin-layer chromatographic plate, and dry in air (see *Chromatography* ⟨621⟩). Develop the chromatograms in a saturated chamber, remove the plate from the chamber, dry in air, derivatize the plate with *Derivatization reagent*, heat at 110° for 3 min, and examine under visible light and UV at 366 nm.
System suitability: The chromatogram of *Standard solution B* shows two bands clearly separated at an R_F of about 0.6–0.7 below the band due to gymnemagenin in *Standard solution A*, and the most prominent band is located at about one-third of the chromatogram, visible as brown in color under white light and blue under UV.
Acceptance criteria: The chromatogram of the *Sample solution* shows the following bands corresponding in color and position to bands in the chromatogram of *Standard solution B*: two bands at an R_F of about 0.6–0.7, below the band due to gymnemagenin in *Standard solution A*; the most prominent band at about one-third of the chromatogram, visible as brown in color under white light and blue under UV; and in the lower third of the chromatogram, under UV, one light blue-greenish band and a dark band underneath.

• **B. HPLC**

Analysis: Proceed as directed in the test for *Content of Gymnemic Acids*.
Acceptance criteria: The chromatogram of the *Sample solution* shows a major peak at a retention time corresponding to that of the gymnemagenin peak in the chromatogram of *Standard solution A* and an additional peak corresponding to deacylgymnemic acid.

COMPOSITION

• **CONTENT OF GYMNEMIC ACIDS**

Solution A: Dissolve 0.14 g of anhydrous potassium dihydrogen phosphate in 900 mL of water, and add 0.5 mL of phosphoric acid. Dilute with water to 1000 mL, mix, filter, and degas.
Solution B: Acetonitrile
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	75	25
20	45	55
25	40	60
30	40	60
35	75	25
40	75	25

Solvent: 50% ethanol in water
Potassium hydroxide solution: 12% potassium hydroxide in water
Hydrochloric acid solution: 4 N hydrochloric acid
Standard solution A: 0.3 mg/mL of USP Gymnemagenin RS in methanol
Standard solution B: Transfer about 0.25 g of USP Native Gymnema Extract RS to a 100-mL round-bottom flask fitted with a reflux condenser. Add 25 mL of *Solvent* and 2 mL of *Potassium hydroxide solution*, reflux on a water bath for 1 h, and cool to room temperature. Add 5.5 mL of *Hydrochloric acid solution*, reflux on a water bath for 2 h, and cool to room temperature. Adjust the solution with *Potassium hydroxide solution* to a pH of 7.5–8.5, transfer to a 50-mL volumetric flask, dilute with *Solvent* to volume, and mix. Before injection, pass through a membrane filter having a 0.45-µm or finer pore size, discarding the first few mL of the filtrate.
Sample solution: Transfer an amount of Purified Gymnema Extract, equivalent to about 30 mg of gymnemic acids, to a 100-mL round-bottom flask fitted with a reflux condenser. Add 25 mL of *Solvent* and 2 mL of *Potassium hydroxide solution*, reflux on a water bath for 1 h, and cool to room temperature. Add 5.5 mL of *Hydrochloric acid solution*, reflux on a water bath for 2 h, and cool to room temperature. Adjust the solution with *Potassium hydroxide solution* to a pH of 7.5–8.5, transfer to a 100-mL volumetric flask, dilute with *Solvent* to volume, and mix. Before injection, pass through a membrane filter having a 0.45-µm or finer pore size, discarding the first few mL of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 210 nm
Column: 4.6-mm × 25-cm; 5-µm, 100 Å, packing L1
Column temperature: 25 ± 1°
Flow rate: 1.6 mL/min
Injection volume: 20 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Native Gymnema Extract RS being used.
Tailing factor: NMT 1.5 for the gymnemagenin peak, *Standard solution A*
Relative standard deviation: NMT 2.0% for the gymnemagenin peak, *Standard solution A*
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Native Gymnema Extract RS being used, identify the retention times of the peaks corresponding to deacylgymnemic acid and gymnemagenin in the *Sample solution* chromatogram.
Calculate the percentage (*P*) of gymnemic acids, calculated as gymnemagenin, in the portion of Purified Gymnema Extract taken:

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$$P = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of gymnemagenin from the *Sample solution*
r_S = peak area of gymnemagenin from *Standard solution A*
C_S = concentration of gymnemagenin in *Standard solution A* (mg/mL)
C_U = concentration of Purified Gymnema Extract in the *Sample solution* (mg/mL)

Calculate the percentage of the labeled amount of gymnemic acids in the portion of Purified Gymnema Extract taken:

$$\text{Result} = (P/L) \times 100$$

P = content of gymnemic acids as determined above (%)
L = labeled amount of gymnemic acids (%)

Acceptance criteria: 90.0%–110.0% of the labeled amount on the dried basis

CONTAMINANTS

Change to read:

- **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020) **:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

- **LOSS ON DRYING** ⟨731⟩
  Sample: 1.0 g of Purified Gymnema Extract
  Analysis: Dry at 105° for 3 h.
  Acceptance criteria: NMT 5.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩
  Sample: 1.0 g of Purified Gymnema Extract
  Acceptance criteria: NMT 8%
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩
  Sample: 1.0 g of Purified Gymnema Extract
  Acceptance criteria: NMT 2.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture. Store at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived. Label it to indicate the content of gymnemic acids, in percentage. It meets other labeling requirements under *Botanical Extracts* ⟨565⟩.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Gymnemagenin RS
  USP Native Gymnema Extract RS

Hawthorn Leaf with Flower

DEFINITION

Hawthorn Leaf with Flower consists of the dried tips of the flower-bearing branches up to 7 cm in length of *Crataegus monogyna* Jacq. emend Lindman. or *Crataegus laevigata* (Poir.) DC., also known as *Crataegus oxycantha* Linné (Fam. Rosaceae). It contains NLT 0.6% of *C*-glycosylated flavones, expressed as vitexin ($C_{21}H_{20}O_{10}$), and NLT 0.45% of *O*-glycosylated flavones, expressed as hyperoside ($C_{21}H_{20}O_{12}$), calculated on the dried basis.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩
  Standard solution: 0.1 mg/mL each of chlorogenic acid, rutin, USP Hyperoside RS, and USP Vitexin RS in methanol. [NOTE—Reserve a portion of this solution for use in *Identification* test *B.*]
  Sample solution: Finely powder 10 g of Hawthorn Leaf with Flower. Transfer 1 g of the powder to a flask, and add 10 mL of methanol. Heat the flask on a water bath maintained at 65° for 5 min, cool, filter, and use the filtrate.
  Adsorbent: 0.50-mm layer of chromatographic silica gel mixture (TLC plates)
  Application volume: 10 µL
  Developing solvent system: Ethyl acetate, glacial acetic acid, formic acid, and water (10: 1.1: 1.1: 2.6)
  Derivatization reagent A: 2-Aminoethyl diphenylborinate in methanol (1 in 100)
  Derivatization reagent B: Polyethylene glycol 4000 in methanol (5 in 100)
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Proceed as directed in the chapter, except to dry the plate at 105°, and spray the plate while still hot with 10 mL of *Derivatization reagent A* and then with 10 mL of *Derivatization reagent B.* Allow the plate to air-dry for 30 min, and examine under long-wave UV light (365 nm).
  Acceptance criteria: The chromatogram of the *Standard solution* exhibits an intense orange zone (at R_F value of 0.3) due to rutin; a light blue fluorescent zone (at R_F value of 0.4) due to chlorogenic acid; a yellowish-orange zone (at R_F value of 0.55) due to hyperoside; and a yellowish-green zone (at R_F value of 0.65) due to vitexin. The chromatogram of the *Sample solution*, in addition to the zones due to rutin, chlorogenic acid, hyperoside, and vitexin, exhibits a yellowish-green zone (at R_F value of 0.35) due to vitexin-2-rhamnoside; a light blue fluorescent zone (at R_F value of 0.6) due to spiraeoside; and a light blue fluorescent zone near the solvent front (at R_F value of 0.9) due to caffeic acid. The chromatogram of the *Sample solution* also exhibits additional zones of weaker intensity.
- **B. HPLC**
  Solution A: Tetrahydrofuran, acetonitrile, and methanol (92.4: 3.4: 4.2)
  Solution B: 0.5% Phosphoric acid in water
  Mobile phase: See *Table 1.*

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	12	88
12	12	88
25	18	82
30	18	82

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Standard solution: Use the *Standard solution* reserved from *Identification* test *A*.
Sample solution: Transfer 3 g of finely powdered Hawthorn Leaf with Flower to a 100-mL round-bottom flask, add 60 mL of a mixture of methanol and water (4:1), and maintain under reflux for 1 h. Cool, filter, and collect the filtrate in a separate flask. Transfer the residue from the filter back to the flask, add 40 mL of a mixture of methanol and water (4:1), and maintain under reflux for 10 min. Cool, filter, and combine the filtrate with the filtrate obtained from the first extraction. Evaporate the solvent from the combined filtrates under vacuum to a volume of 20 mL. Dilute the resulting solution with a mixture of methanol and water (4:1) to 25.0 mL. Pass 5.0 mL of this solution through a freshly conditioned solid-phase extraction column containing 360 mg of packing L1, collect the eluate in a 10-mL volumetric flask, and dilute with a mixture of methanol and water (4:1) to volume.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 336 nm
Column: 4.0-mm × 10-cm; 5-µm packing L1
Column temperature: 25°
Flow rate: 1 mL/min
Injection volume: 5 µL
System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for chlorogenic acid, vitexin, rutin, and hyperoside are 0.26, 1.0, 1.16, and 1.4, respectively.]
Suitability requirements
Relative standard deviation: NMT 2.0%
Analysis
Samples: *Standard solution* and *Sample solution*
[NOTE—The relative retention times for acetyl vitexin-2″-*O*-rhamnoside, vitexin, isovitexin, and vitexin-2″-*O*-rhamnoside are 1.53, 1.0, 0.73, and 0.67, respectively.]
Measure the retention times for the major peaks.
Acceptance criteria: The retention times of the peaks for chlorogenic acid, vitexin, rutin, and hyperoside from the *Sample solution* correspond to those from the *Standard solution*.

COMPOSITION

• **CONTENT OF C-GLYCOSYLATED FLAVONES**
Solution A: 0.5% solution of phosphoric acid in water
Solution B: Tetrahydrofuran, isopropyl alcohol, and acetonitrile (10:8:3)
Mobile phase: *Solution A* and *Solution B* (22:3)
Standard solution: 0.3 mg/mL of USP Vitexin RS in *Solution B*, with heating if necessary
Sample solution: Finely powder 100 g of Hawthorn Leaf with Flower. Transfer about 4 g of the powder, accurately weighed, to a continuous-extraction apparatus fitted with a flask containing 80 mL of methanol, and extract for 5 h. Cool, remove the flask, and evaporate the solvent from the extract under vacuum to 40 mL. Transfer this solution to a 50-mL volumetric flask, and dilute with methanol to volume. Transfer 10.0 mL of the solution to a suitable flask fitted with a reflux condenser, add 4 mL of 25% hydrochloric acid, and maintain under reflux for 90 min. Cool, transfer the contents of the flask to a 50-mL volumetric flask, and dilute with methanol to volume.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 336 nm
Column: 4-mm × 10-cm; packing L1
Flow rate: 1 mL/min
Injection volume: 5 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Column efficiency: NLT 3000 theoretical plates
Tailing factor: 0.8–2
Relative standard deviation: NMT 2.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the areas of the major peaks. Calculate the percentage of C-glycosylated flavones, expressed as vitexin ($C_{21}H_{20}O_{10}$), in the portion of Hawthorn Leaf with Flower taken:

$$\text{Result} = (C_S/C_U) \times (\Sigma r_U/r_S) \times 100$$

C_S = concentration of USP Vitexin RS in the *Standard solution* (mg/mL)
C_U = concentration of Hawthorn Leaf with Flower in the *Sample solution* (mg/mL)
Σr_U = sum of the peak areas of vitexin and isovitexin, with a relative retention time of about 1.0 and 0.85, respectively, in the chromatogram of the *Sample solution*
r_S = vitexin peak area in the *Standard solution*

Acceptance criteria: NLT 0.6% of C-glycosylated flavones, expressed as vitexin ($C_{21}H_{20}O_{10}$) on the dried basis

• **CONTENT OF O-GLYCOSYLATED FLAVONES**
Mobile phase: Methanol, phosphoric acid, and water (100:1:100)
Standard solution: 0.05 mg/mL of USP Quercetin RS in methanol
Sample solution: Proceed as directed for the *Sample solution* in *Content of C-Glycosylated Flavones*, except to use 1 mL of 25% hydrochloric acid for 60 min instead of 4 mL of 25% hydrochloric acid for 90 min.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 370 nm
Column: 4.6-mm × 25-cm; packing L1
Flow rate: 1.5 mL/min
Injection volume: 10 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Column efficiency: NLT 3000 theoretical plates
Tailing factor: 0.8–2
Relative standard deviation: NMT 2.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the areas of the major peaks. Calculate the percentage of *O*-glycosylated flavones, expressed as hyperoside ($C_{21}H_{20}O_{12}$), in the portion of Hawthorn Leaf with Flower taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = quercetin peak area from the *Sample solution*
r_S = quercetin peak area from the *Standard solution*
C_S = concentration of USP Quercetin RS in the *Standard solution* (mg/mL)
C_U = concentration of Hawthorn Leaf with Flower in the *Sample solution* (mg/mL)
M_{r1} = molecular weight of hyperoside, 464.38
M_{r2} = molecular weight of quercetin, 302.24

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Acceptance criteria: NLT 0.45% of *O*-glycosylated flavones, expressed as hyperoside ($C_{21}H_{20}O_{12}$) on the dried basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩: It meets the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: It shows fragments of dark brown, lignified branches, usually from 1 mm to NMT 2.5 mm in diameter, bearing alternate petiolate leaves, with small, often deciduous styles, and bearing numerous white flowers in a corymbose arrangement. The leaves are more or less strongly lobate, and their margins are slightly or very slightly serrate. *C. laevigata* has pinnatilobate to pinnatifid leaves, divided into three, five, or seven obtuse lobes; the leaves of *C. monogyna* are almost pinnatisect with three to five acute lobes. The adaxial surface of the leaf is dark green to brownish-green; the abaxial surface is lighter, greyish-green, and shows a dense network of clearly visible veinlets and slightly prominent principal veins. The leaves of *C. laevigata* and *C. monogyna* are glabrous or bear isolated trichomes. The flowers consist of a brownish-green tubulous calyx, ending in its upper part in five triangular segments, and of five yellowish-white to brownish free petals, rounded to widely oval, shortly unguiculate, and with numerous stamens. The ovary, fused to the tubulous calyx, bears one to three long styles and consists of the same number of carpels, each containing one fertile ovule. *C. monogyna* has one style and one carpel, and *C. laevigata* has two or three styles and carpels.

Microscopic: When reduced to a fine powder, it shows the following characteristics: unicellular covering trichomes, usually with thick walls and wide lumens, almost straight to somewhat curved, pitted at the base; fragments of leaf epidermis with cells that have sinuous to polygonal walls and large anomocytic stomata surrounded by four to seven subsidiary cells; clusters of parenchymatous cells containing calcium oxalate crystals, usually from 10–20 µm in length; fragments of petals showing rounded polygonal epidermal cells, strongly papillose, with thick walls, the cuticle of which clearly shows wavy striations; fragments of anthers whose endothecium has an arched and regularly thickened margin; fragments of stems containing collenchymatous cells, vessels and fibers of lignified sclerenchyma, with narrow lumens; numerous rounded to elliptical triangular pollen grains up to 45 µm in diameter, with free exines and three germinal pores.

- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩: NMT 8.0% of lignified matter
- **LOSS ON DRYING** ⟨731⟩

Sample: 1.0 g of Hawthorn Leaf with Flower, finely powdered
Analysis: Dry at 105° for 2 h.
Acceptance criteria: NMT 10.0%

- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩: NMT 9.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Store in a well-closed container, protected from light.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Hyperoside RS
  USP Quercetin RS
  USP Vitexin RS

Powdered Hawthorn Leaf with Flower

DEFINITION

Powdered Hawthorn Leaf with Flower is Hawthorn Leaf with Flower reduced to a fine or very fine powder.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩

Standard solution: 0.1 mg/mL each of chlorogenic acid, rutin, USP Hyperoside RS, and USP Vitexin RS in methanol. [NOTE—Reserve a portion of this solution for use in *Identification* test *B*.]
Sample solution: Transfer about 1 g of Powdered Hawthorn Leaf with Flower to a flask, and add 10 mL of methanol. Heat the flask on a water bath maintained at 65° for 5 min, cool, filter, and use the filtrate.
Adsorbent: 0.50-mm layer of chromatographic silica gel mixture (TLC plates)
Application volume: 10 µL
Developing solvent system: Ethyl acetate, glacial acetic acid, formic acid, and water (10: 1.1: 1.1: 2.6)
Derivatization reagent A: 2-Aminoethyl diphenylborinate in methanol (1 in 100)
Derivatization reagent B: Polyethylene glycol 4000 in methanol (5 in 100)
Analysis
Samples: *Standard solution* and *Sample solution*
Proceed as directed in the chapter, except to dry the plate at 105°, and spray the plate while still hot with 10 mL of *Derivatization reagent A* and then with 10 mL of *Derivatization reagent B*. Allow the plate to air-dry for 30 min, and examine under long-wave UV light (365 nm).
Acceptance criteria: The chromatogram of the *Standard solution* exhibits an intense orange zone (at R_F value of 0.3) due to rutin; a light blue fluorescent zone (at R_F value of 0.4) due to chlorogenic acid; a yellowish-orange zone (at R_F value of 0.55) due to hyperoside; and a yellowish-green zone (at R_F value of 0.65) due to vitexin. The chromatogram of the *Sample solution*, in addition to the zones due to rutin, chlorogenic acid, hyperoside, and vitexin, exhibits a yellowish-green zone (at R_F value of 0.35) due to vitexin-2-rhamnoside; a light blue fluorescent zone (at R_F value of 0.6) due to spiraeoside; and a light blue fluorescent zone near the solvent front (at R_F value of 0.9) due to caffeic acid. The chromatogram of the *Sample solution* also exhibits additional zones of weaker intensity.

- **B. HPLC**

Solution A: Tetrahydrofuran, acetonitrile, and methanol (92.4: 3.4: 4.2)
Solution B: 0.5% Phosphoric acid in water
Mobile phase: See *Table 1*.

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Table 1

Time (min)	Solution A (%)	Solution B (%)
0	12	88
12	12	88
25	18	82
30	18	82

Standard solution: Use the *Standard solution* reserved from *Identification* test *A*.
Sample solution: Transfer about 3 g of Powdered Hawthorn Leaf with Flower to a 100-mL round-bottom flask, add 60 mL of a mixture of methanol and water (4:1), and maintain under reflux for 1 h. Cool, filter, and collect the filtrate in a separate flask. Transfer the residue from the filter back to the flask, add 40 mL of a mixture of methanol and water (4:1), and maintain under reflux for 10 min. Cool, filter, and combine the filtrate with the filtrate obtained from the first extraction. Evaporate the solvent from the combined filtrates under vacuum to a volume of 20 mL. Dilute the resulting solution with a mixture of methanol and water (4:1) to 25.0 mL. Pass 5.0 mL of this solution through a freshly conditioned solid-phase extraction column containing 360 mg of packing L1, collect the eluate in a 10-mL volumetric flask, and dilute with a mixture of methanol and water (4:1) to volume.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 336 nm
Column: 4.0-mm × 10-cm; 5-µm packing L1
Column temperature: 25°
Flow rate: 1 mL/min
Injection volume: 5 µL
System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for chlorogenic acid, vitexin, rutin, and hyperoside are 0.26, 1.0, 1.16, and 1.4, respectively.]
Suitability requirements
Relative standard deviation: NMT 2.0%
Analysis
Samples: *Standard solution* and *Sample solution*
[NOTE—The relative retention times for acetyl vitexin-2″-*O*-rhamnoside, vitexin, isovitexin, and vitexin-2″-*O*-rhamnoside are 1.53, 1.0, 0.73, and 0.67, respectively.]
Measure the retention times for the major peaks.
Acceptance criteria: The retention times of the peaks for chlorogenic acid, vitexin, rutin, and hyperoside from the *Sample solution* correspond to those of the *Standard solution*.

COMPOSITION

• **CONTENT OF C-GLYCOSYLATED FLAVONES**
Solution A: 0.5% Solution of phosphoric acid in water
Solution B: Tetrahydrofuran, isopropyl alcohol, and acetonitrile (10:8:3)
Mobile phase: *Solution A* and *Solution B* (22:3)
Standard solution: 0.3 mg/mL of USP Vitexin RS in *Solution B*, with heating if necessary
Sample solution: Transfer about 4 g of Powdered Hawthorn Leaf with Flower, accurately weighed, to a continuous-extraction apparatus fitted with a flask containing 80 mL of methanol, and extract for 5 h. Cool, remove the flask, and evaporate the solvent from the extract under vacuum to 40 mL. Transfer this solution to a 50-mL volumetric flask, and dilute with methanol to volume. Transfer 10.0 mL of the solution to a suitable flask fitted with a reflux condenser, add 4 mL of 25% hydrochloric acid, and maintain under reflux for 90 min. Cool, transfer the contents of the flask to a 50-mL volumetric flask, and dilute with methanol to volume.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 336 nm
Column: 4-mm × 10-cm; packing L1
Flow rate: 1 mL/min
Injection volume: 5 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Column efficiency: NLT 3000 theoretical plates
Tailing factor: 0.8–2
Relative standard deviation: NMT 2.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the areas of the major peaks.
Calculate the percentage of *C*-glycosylated flavones, expressed as vitexin ($C_{21}H_{20}O_{10}$), in the portion of Powdered Hawthorn Leaf with Flower taken:

$$\text{Result} = (\Sigma r_U / r_S) \times (C_S / C_U) \times 100$$

Σr_U = sum of the peak areas of vitexin and isovitexin from the *Sample solution*, with relative retention times of about 1.0 and 0.85, respectively
r_S = vitexin peak area from the *Standard solution*
C_S = concentration of USP Vitexin RS in the *Standard solution* (mg/mL)
C_U = concentration of Powdered Hawthorn Leaf with Flower in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 0.6% of *C*-glycosylated flavones, expressed as vitexin ($C_{21}H_{20}O_{10}$) on the dried basis

• **CONTENT OF *O*-GLYCOSYLATED FLAVONES**
Mobile phase: Methanol, phosphoric acid, and water (100:1:100)
Standard solution: 0.05 mg/mL of USP Quercetin RS in methanol
Sample solution: Proceed as directed for the *Sample solution* in *Content of C-Glycosylated Flavones*, except use 1 mL of 25% hydrochloric acid for 60 min instead of 4 mL of 25% hydrochloric acid for 90 min.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 370 nm
Column: 4.6-mm × 25-cm; packing L1
Flow rate: 1.5 mL/min
Injection volume: 10 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Column efficiency: NLT 3000 theoretical plates
Tailing factor: 0.8–2
Relative standard deviation: NMT 2.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the areas of the major peaks.
Calculate the percentage of *O*-glycosylated flavones, expressed as hyperoside ($C_{21}H_{20}O_{12}$), in the portion of Powdered Hawthorn Leaf with Flower taken:

$$\text{Result} = (r_U / r_S) \times (C_S / C_U) \times (M_{r1} / M_{r2}) \times 100$$

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r_U = quercetin peak area from the *Sample solution*
r_S = quercetin peak area from the *Standard solution*
C_S = concentration of USP Quercetin RS in the *Standard solution* (mg/mL)
C_U = concentration of Powdered Hawthorn Leaf with Flower in the *Sample solution* (mg/mL)
M_{r1} = molecular weight of hyperoside, 464.38
M_{r2} = molecular weight of quercetin, 302.24

Acceptance criteria: NLT 0.45% of *O*-glycosylated flavones, expressed as hyperoside ($C_{21}H_{20}O_{12}$) on the dried basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩: It meets the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Microscopic: The yellowish-green powder shows the following characteristics: unicellular covering trichomes, usually with thick walls and wide lumens, almost straight to somewhat curved, pitted at the base; fragments of leaf epidermis with cells that have sinuous to polygonal walls and large anomocytic stomata surrounded by four to seven subsidiary cells; clusters of parenchymatous cells containing calcium oxalate crystals, usually from 10–20 µm in length; fragments of petals showing rounded polygonal epidermal cells, strongly papillose, with thick walls, the cuticle of which clearly shows wavy striations; fragments of anthers whose endothecium has an arched and regularly thickened margin; fragments of stems containing collenchymatous cells, vessels, and fibers of lignified sclerenchyma, with narrow lumens; and numerous rounded to elliptical triangular pollen grains up to 45 µm in diameter, with free exines and three germinal pores.

- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩: NMT 8.0% of lignified matter
- **LOSS ON DRYING** ⟨731⟩

Sample: 1.0 g of Powdered Hawthorn Leaf with Flower
Analysis: Dry at 105° for 2 h.
Acceptance criteria: NMT 10.0%

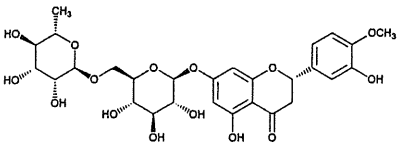
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩: NMT 9.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Store in a well-closed container, protected from light.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant source from which the article was derived.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Hyperoside RS
USP Quercetin RS
USP Vitexin RS

Hesperidin



$C_{28}H_{34}O_{15}$ 610.57

4*H*-1-Benzopyran-4-one, 7-[[6-*O*-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-2,3-dihydro-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-, (2*S*)-; (2*S*)-5-Hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-oxo-3,4-dihydro-2*H*-chromen-7-yl 6-*O*-(α-L-rhamnopyranosyl)-β-D-glucopyranoside [520-26-3].

DEFINITION

Hesperidin contains NLT 90.0% and NMT 102.0% of hesperidin ($C_{28}H_{34}O_{15}$), calculated on the dried basis.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

ASSAY

- **PROCEDURE**

Solution A: Dilute 5 mL of glacial acetic acid with water to 1000 mL.
Mobile phase: Methanol and *Solution A* (30:70)
System suitability solution: 0.2 mg/mL each of USP Isonaringin RS, USP Hesperidin RS, USP Neohesperidin RS, USP Diosmin RS, and USP Didymin RS in dimethyl sulfoxide
Standard solution: 1.0 mg/mL of USP Hesperidin RS in dimethyl sulfoxide
Sample solution: 1.0 mg/mL of Hesperidin in dimethyl sulfoxide
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 284 nm
Column: 3.9-mm × 15-cm; 4-µm packing L1
Column temperature: 40°
Flow rate: 1.0 mL/min
Injection volume: 10 µL
Run time: At least 5 times the retention time of hesperidin
System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The relative retention times for hesperidin and its related compounds are shown in *Table 1*.]
Suitability requirements
Resolution: NLT 1.8 between hesperidin and neohesperidin, *System suitability solution*
Relative standard deviation: NMT 2.0%, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of hesperidin ($C_{28}H_{34}O_{15}$) in the portion of Hesperidin taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*

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C_S = concentration of USP Hesperidin RS in the *Standard solution* (mg/mL)
C_U = concentration of Hesperidin in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–102.0% on the dried basis

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩
Sample: 1.0 g
Acceptance criteria: NMT 0.2%

• **RELATED COMPOUNDS**
Solution A, Mobile phase, System suitability solution, Chromatographic system, and System suitability: Proceed as directed in the *Assay*.
Standard solution: 0.05 mg/mL of USP Hesperidin RS in dimethyl sulfoxide
Sample solution: 1.0 mg/mL of Hesperidin in dimethyl sulfoxide
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of each impurity in the portion of Hesperidin taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak response of each impurity from the *Sample solution*
r_S = peak response of hesperidin from the *Standard solution*
C_S = concentration of USP Hesperidin RS in the *Standard solution* (mg/mL)
C_U = concentration of Hesperidin in the *Sample solution* (mg/mL)
F = correction factor for each individual impurity (see *Table 1*)

Acceptance criteria: See *Table 1*. [NOTE—Disregard any impurity less than 0.1%.]

Table 1

Name	Relative Retention Time	Correction Factor (*F*)	Acceptance Criteria, NMT (%)
Eriocitrin[a]	0.4	1.00	1.0
Isonaringin	0.7	1.07	4.0
Hesperidin	1.0	—	—
Neohesperidin	1.2	0.91	1.0
Diosmin	1.5	1.67	1.0
Naringenin[b]	2.6	0.51	1.0
Didymin	3.0	1.02	3.0
Hesperetin[c]	3.8	0.45	1.0
Any unspecified impurity	—	1.00	1.0
Total unspecified impurities	—	—	3.0
Total impurities	—	—	10.0

[a] (*S*)-5-Hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-oxo-3,4-dihydro-2*H*-chromen-7-yl 6-*O*-(α-L-rhamnopyranosyl)-β-D-glucopyranoside.
[b] (2*S*)-5,7-Dihydroxy-2-(4-hydroxyphenyl)chroman-4-one.
[c] (*S*)-5,7-Dihydroxy-2-(3-hydroxy-4-methoxyphenyl)chroman-4-one.

SPECIFIC TESTS

• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry at 105° for 4 h.
Acceptance criteria: NMT 5.0%

• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total bacterial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed, tight containers.

• **USP REFERENCE STANDARDS** ⟨11⟩
USP Didymin RS
(*RS*)-5-Hydroxy-2-(4-methoxyphenyl)-4-oxo-3,4-dihydro-2*H*-chromen-7-yl 6-*O*-(α-L-rhamnopyranosyl)-β-D-glucopyranoside.
$C_{28}H_{34}O_{14}$ 594.57
USP Diosmin RS
USP Hesperidin RS
USP Isonaringin RS
(*RS*)-5-Hydroxy-2-(4-hydroxyphenyl)-4-oxo-3,4-dihydro-2*H*-chromen-7-yl 6-*O*-(α-L-rhamnopyranosyl)-β-D-glucopyranoside.
$C_{27}H_{32}O_{14}$ 580.54
USP Neohesperidin RS
(*S*)-5-Hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-oxo-3,4-dihydro-2*H*-chromen-7-yl 2-*O*-(α-L-rhamnopyranosyl)-β-D-glucopyranoside.
$C_{28}H_{34}O_{15}$ 610.57

Histidine—*see Histidine General Monographs*

Holy Basil Leaf

DEFINITION

Holy Basil Leaf consists of the dried leaf of *Ocimum tenuiflorum* L. (Fam. Lamiaceae). It contains NLT 0.5% of triterpenes, calculated as the sum of oleanolic acid and ursolic acid, on the dried basis.

IDENTIFICATION

• **A.** Holy Basil Leaf meets the requirements for *Specific Tests, Botanic Characteristics*.

• **B. THIN-LAYER CHROMATOGRAPHY**
Standard solution A: 0.5 mg/mL of USP Rosmarinic Acid RS in methanol
Standard solution B: 10 mg/mL of USP Powdered Holy Basil Extract RS in methanol
Sample solution: Sonicate about 1 g of Holy Basil Leaf, finely powdered, in 10 mL of methanol for 10 min, centrifuge, and use the supernatant. [NOTE—Reserve a portion of the supernatant for *Identification* test C.]
Chromatographic system
Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)
Application volume: 2 µL of *Standard solution A*, 4 µL of *Standard solution B*, and 8 µL of *Sample solution* as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
Developing solvent system: A mixture of ethyl acetate, formic acid, and water (15:1:1)
Developing distance: 6 cm

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Derivatization reagent A: 5-mg/mL solution of 2-aminoethyl diphenylborinate in ethyl acetate
Derivatization reagent B: 50-mg/mL solution of polyethylene glycol 400 in dichloromethane
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Apply the *Samples* as bands to a suitable high performance thin-layer chromatographic plate, and dry in air (see *Chromatography* ⟨621⟩). Develop the chromatograms in a saturated chamber, remove the plate from the chamber, and dry in air. Heat the plate at 100° for 3 min, derivatize the plate while still warm with *Derivatization reagent A*, and dry in air. Then derivatize with *Derivatization reagent B*, dry in air, and examine under UV light at 366 nm.
System suitability: The chromatogram of *Standard solution B* exhibits, in the upper third section, a blue fluorescent band corresponding to the band due to rosmarinic acid in the chromatogram of *Standard solution A*, and a less intense blue fluorescent band directly above the band due to rosmarinic acid, corresponding to caffeic acid, and a red band above the caffeic acid band. The bands due to rosmarinic acid and caffeic acid are clearly separated. The chromatogram of *Standard solution B* also shows the most intense band, an orange band, in the lower third section of the chromatogram; and two greenish bands in the middle third section of the chromatogram.
Acceptance criteria: The chromatogram of the *Sample solution* exhibits the following bands corresponding to similar bands in the chromatogram of *Standard solution B*: a blue fluorescent band at an R_F corresponding to the rosmarinic acid band; a less intense blue fluorescent band at an R_F above that of the rosmarinic acid band, corresponding to caffeic acid; a red band above the caffeic acid band; a red band at the solvent front due to chlorophyll; two greenish bands in the middle third section of the chromatogram; and the most intense band, an orange band, with a blue band right above in the lower third section of the chromatogram (distinction from sage leaf, thyme leaf, basil leaf, and oregano leaf, all of which show two orange bands in the lower third section of the chromatogram; and lemon balm leaf which lacks this band). The *Sample solution* does not exhibit yellowish-orange bands in the middle section of the chromatogram (distinction from rosemary leaf and thyme leaf).

• **C. Thin-Layer Chromatography**
Standard solution A: 0.2 mg/mL of USP Ursolic Acid RS in methanol
Standard solution B: 1.0 mg/mL of eugenol in methanol
Standard solution C: 1.0 mg/mL of methyleugenol in methanol
Standard solution D: 10 mg/mL of USP Powdered Holy Basil Extract RS in methanol
Sample solution: Use the solution prepared in *Identification* test *B*.
Chromatographic system
Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)
Application volume: 2 µL each of *Standard solution A*, *Standard solution B*, and *Standard solution C*, 4 µL of *Standard solution D*, and 8 µL of the *Sample solution* as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
Developing solvent system: A mixture of toluene and ethyl acetate (85:15)
Developing distance: 6 cm
Derivatization reagent: Place 85 mL of methanol in a 100-mL glass bottle, and cool in a water–ice cubes–salt bath or in a freezer. To the ice-cold methanol add 10 mL of acetic acid and 5 mL of sulfuric acid, slowly and carefully, and mix well. Allow the mixture to cool to room temperature, and then add 0.5 mL of *p*-anisaldehyde.
Analysis
Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, *Standard solution D*, and *Sample solution*
Apply the *Samples* as bands to a suitable high performance thin-layer chromatographic plate, and dry in air (see *Chromatography* ⟨621⟩). Develop the chromatograms in a saturated chamber, remove the plate from the chamber, dry in air, derivatize with *Derivatization reagent*, heat at 100° for 3–5 min, set aside to cool, and examine under visible light.
System suitability: *Standard solution A* exhibits a violet band. *Standard solution B* and *Standard solution C* each exhibit a greenish band. *Standard solution D* exhibits a purple band above the green band corresponding to eugenol or methyleugenol in *Standard solution B* or *Standard solution C*.
Acceptance criteria: The chromatogram of the *Sample solution* exhibits the most intense band, a violet band at an R_F corresponding to the ursolic acid band in the chromatogram of *Standard solution A* and *Standard solution D*, and a greenish band at an R_F corresponding to the eugenol band or methyleugenol band in the chromatograms of *Standard solution B* or *Standard solution C*. [Note—Holy basil leaf may occur in two chemotypes, one characterized by the presence of eugenol and the other by the presence of methyleugenol.]

• **D. HPLC**
Analysis: Proceed as directed in the test for *Content of Triterpenes*.
Acceptance criteria: The chromatogram of the *Sample solution* exhibits two peaks at the retention times corresponding to the ursolic acid and oleanolic acid peaks from the *Standard solutions*.

COMPOSITION

• **Content of Triterpenes**
Mobile phase: A mixture of acetonitrile and a 2.5-mg/mL solution of ammonium acetate in water (67:33)
Standard solution A: 0.1 mg/mL of USP Ursolic Acid RS in methanol. Sonicate to dissolve, if necessary.
Standard solution B: 4.0 mg/mL of USP Powdered Holy Basil Extract RS in methanol. Sonicate to dissolve, if necessary. Before injection, pass through a membrane filter of 0.45-µm or finer pore size.
Sample solution: Transfer about 3.0 g of Holy Basil Leaf, finely powdered and accurately weighed, to a 100-mL round-bottom flask fitted with a reflux condenser. Add 50 mL of methanol, reflux on a water bath for 20 min, cool to room temperature, and decant the supernatant. Repeat until the last extract is colorless. Combine the extracts, filter, concentrate under vacuum, and adjust with methanol to a volume of 100 mL. Before injection, pass through a membrane filter having a 0.45-µm or finer pore size, discarding the first few mL of filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV, 205 nm
Column: 3.0-mm × 10-cm; 2.5-µm packing L1
Flow rate: 0.3 mL/min
Injection volume: 5 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*

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Suitability requirements
Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Holy Basil Extract RS being used.
Resolution: NLT 1.3 between the oleanolic acid and ursolic acid peaks, *Standard solution B*
Relative standard deviation: NMT 2% for the ursolic acid peak, *Standard solution A*

Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Holy Basil Extract RS being used, identify the retention time of the peaks corresponding to oleanolic acid and ursolic acid in the *Sample solution* chromatogram.
Calculate the percentage of triterpenes, oleanolic acid, and ursolic acid in the portion of Holy Basil Leaf taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = sum of the peak areas of oleanolic acid and ursolic acid from the *Sample solution*
r_S = peak area of ursolic acid from *Standard solution A*
C_S = concentration of ursolic acid in *Standard solution A* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Holy Basil Leaf taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 0.5% on the dried basis

CONTAMINANTS

• **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩
Acceptance criteria
Arsenic: NMT 1.0 µg/g
Cadmium: NMT 0.5 µg/g
Lead: NMT 5.0 µg/g
Mercury: NMT 1.0 µg/g

Change to read:

• **▲ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Pesticide Residue Analysis*:▲ (CN 1-May-2019) Meets the requirements
• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria does not exceed 10^3 cfu/g.
• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

• **BOTANIC CHARACTERISTICS**
Macroscopic: The leaves are petiolate; simple; elliptic oblong to elliptic; apex acute or obtuse; margin serrate or occasionally entire; base acute or obtuse; pubescent, more trichomes on the lower surface around its midrib; venation reticulate, veins more prominent on lower surface; petiole cylindrical, slightly grooved on the upper surface, with trichomes. The upper surface is green; the lower surface is pale green; characteristic aromatic odor. Pharmacopeial article consists of dry, yellowish-green, brittle, leaves.
Microscopic
Transverse section of leaf: Lamina is dorsiventral; upper epidermis and lower epidermis, each a single layer of rectangular cells covered with thin cuticle, showing plenty of covering and glandular trichomes, more on the lower epidermis; covering trichomes are uniseriate, unicellular to multicellular, often bent, with bulging basal cells; glandular trichomes are of various sizes and shapes, majority are sessil with 4-celled head, few with a single-celled stalk, some with 1- to 2-celled stalk and 1- to 2-celled head; a layer of palisade cells under the upper epidermis in the lamina region; 2–4 rows of collenchyma cells under each epidermis in the midrib region; an arc of conjoint, collateral vascular bundles in the center, and 2–4 patches of phloem bundles or rudimentary small vascular bundles above the arc; and spongy parenchyma cells rich in oleoresin contents.
Transverse section of petiole: Similar to the transverse section through the midrib of the leaf; upper epidermis and lower epidermis, each a single layer of rectangular cells covered with thin cuticle, showing plenty of covering and glandular trichomes; 2–4 rows of collenchyma cells under each epidermis; an arc of conjoint, collateral vascular bundle in the center, a row of small rudimentary vascular bundles above the arc facing the upper side, and an isolated vascular bundle at each end of the arc; and spongy parenchyma cells
• **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩**:** NMT 5% of stems; NMT 2.0% of other foreign matter
• **LOSS ON DRYING** ⟨731⟩
Sample: 1.0 g of finely powdered Holy Basil Leaf
Analysis: Dry at 105° for 2 h.
Acceptance criteria: NMT 10%
• **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩
Sample: 2 g of finely powdered Holy Basil Leaf
Acceptance criteria: NMT 18%
• **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩
Sample: 2–4 g of finely powdered Holy Basil Leaf
Acceptance criteria: NMT 3%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
• **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Powdered Holy Basil Extract RS
USP Rosmarinic Acid RS
USP Ursolic Acid RS

Powdered Holy Basil Leaf

DEFINITION

Powdered Holy Basil Leaf is the pulverized dried leaf of *Ocimum tenuiflorum* L. (Fam. Lamiaceae). It contains NLT 0.5% of triterpenes, calculated as the sum of oleanolic acid and ursolic acid, on the dried basis.

IDENTIFICATION

• **A.** Powdered Holy Basil Leaf meets the requirements for *Specific Tests, Botanic Characteristics*.
• **B. THIN-LAYER CHROMATOGRAPHY**
Standard solution A: 0.5 mg/mL of USP Rosmarinic Acid RS in methanol
Standard solution B: 10 mg/mL of USP Powdered Holy Basil Extract RS in methanol
Sample solution: Sonicate about 1 g of Powdered Holy Basil Leaf in 10 mL of methanol for 10 min, centrifuge, and use

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the supernatant. [NOTE—Reserve a portion of the supernatant for *Identification* test *C*.]

Chromatographic system

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)

Application volume: 2 µL of *Standard solution A*, 4 µL of *Standard solution B*, and 8 µL of the *Sample solution* as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

Developing solvent system: A mixture of ethyl acetate, formic acid, and water (15:1:1)

Developing distance: 6 cm

Derivatization reagent A: 5-mg/mL solution of 2-aminoethyl diphenylborinate in ethyl acetate

Derivatization reagent B: 50-mg/mL solution of polyethylene glycol 400 in dichloromethane

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands to a suitable high performance thin-layer chromatographic plate, and dry in air (see *Chromatography* ⟨621⟩). Develop the chromatograms in a saturated chamber, remove the plate from the chamber, dry in air, heat at 100° for 3 min, derivatize the plate while still warm with *Derivatization reagent A*, dry in air, then derivatize with *Derivatization reagent B*, dry in air, and examine under UV light at 366 nm.

System suitability: The chromatogram of *Standard solution B* exhibits, in the upper third section, a blue fluorescent band corresponding to the band due to rosmarinic acid in the chromatogram of *Standard solution A*, and a less intense blue fluorescent band, right above the band due to rosmarinic acid, corresponding to caffeic acid, and a red band above the caffeic band. The bands due to rosmarinic acid and caffeic acid are clearly separated. The chromatogram of *Standard solution B* also shows the most intense band, an orange band, in the lower third section of the chromatogram, and two greenish bands in the middle third section of the chromatogram.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits the following bands corresponding to similar bands in *Standard solution B*: a blue fluorescent band at an R_F corresponding to the rosmarinic acid band; a less intense blue fluorescent band at an R_F above that of the rosmarinic acid band, corresponding to caffeic acid; a red band above the caffeic acid band; and a red band at the solvent front due to chlorophyll; two greenish bands in the middle third section of the chromatogram; and the most intense band, an orange band, with a blue band right above in the lower third section of the chromatogram (distinction from sage leaf, thyme leaf, basil leaf, and oregano leaf, all show two orange bands in the lower third section of the chromatogram; and lemon balm leaf which lacks the band). The *Sample solution* does not exhibit yellowish-orange bands at the middle of the chromatogram (distinction from rosemary leaf and thyme leaf).

• **C. THIN-LAYER CHROMATOGRAPHY**

Standard solution A: 0.2 mg/mL of USP Ursolic Acid RS in methanol

Standard solution B: 1.0 mg/mL of eugenol in methanol

Standard solution C: 1.0 mg/mL of methyleugenol in methanol

Standard solution D: 10 mg/mL of USP Powdered Holy Basil Extract RS in methanol

Sample solution: Use the solution prepared in *Identification* test *B*.

Chromatographic system

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)

Application volume: 2 µL of *Standard solution A*, *Standard solution B*, and *Standard solution C*; 4 µL of *Standard solution D*, and 8 µL of the *Sample solution* as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

Developing solvent system: A mixture of toluene and ethyl acetate (85:15)

Developing distance: 6 cm

Derivatization reagent: Place 85 mL of methanol in a 100-mL glass bottle, and cool it down in a water–ice cubes–salt bath or in a freezer. To the ice-cold methanol, add 10 mL of acetic acid and 5 mL of sulfuric acid, slowly and carefully, and mix well. Allow the mixture to cool to room temperature, and then add 0.5 mL of *p*-anisaldehyde.

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, *Standard solution D*, and *Sample solution*

Apply the *Samples* as bands to a suitable high performance thin-layer chromatographic plate, and dry in air (see *Chromatography* ⟨621⟩). Develop the chromatograms in a saturated chamber, remove the plate from the chamber, dry in air, derivatize with *Derivatization reagent*, heat at 100° for 3–5 min, set aside to cool, and examine under visible light.

System suitability: *Standard solution A* exhibits a violet band. *Standard solution B* and *Standard solution C* each exhibit a greenish band. *Standard solution D* exhibits a purple band above the green band corresponding to eugenol or methyleugenol in *Standard solution B* or *Standard solution C*.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits the most intense band, a violet band at an R_F corresponding to the ursolic acid band in the chromatogram of *Standard solution A* and *Standard solution D*, and a greenish band at an R_F corresponding to the eugenol band or methyleugenol band in the chromatograms of *Standard solution B* or *Standard solution C*. [NOTE—Holy basil leaf may occur in two chemotypes, one characterized by the presence of eugenol and the other by the presence of methyleugenol.]

• **D. HPLC**

Analysis: Proceed as directed in the test for *Content of Triterpenes*.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits two peaks at the retention times corresponding to the ursolic acid and oleanolic acid in the *Standard solutions*.

COMPOSITION

• **CONTENT OF TRITERPENES**

Mobile phase: A mixture of acetonitrile and a solution of 2.5 mg/mL ammonium acetate in water (67:33)

Standard solution A: 0.1 mg/mL of USP Ursolic Acid RS in methanol. Sonicate to dissolve if necessary.

Standard solution B: 4.0 mg/mL of USP Powdered Holy Basil Extract RS in methanol. Sonicate to dissolve if necessary. Before injection, pass through a membrane filter of 0.45-µm or finer pore size.

Sample solution: Transfer about 3.0 g of Powdered Holy Basil Leaf, accurately weighed, to a 100-mL round-bottom flask fitted with a reflux condenser. Add 50 mL of methanol, reflux on a water bath for 20 min, cool to room temperature, and decant the supernatant. Repeat until the last extract is colorless. Combine the extracts, filter, concentrate under vacuum and adjust the volume to

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100 mL using methanol. Before injection, pass through a membrane filter having a 0.45-µm or finer pore size, discarding the first few mL of the filtrate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV, 205 nm
Column: 3.0-mm × 10-cm; 2.5-µm packing L1
Flow rate: 0.3 mL/min
Injection volume: 5 µL

System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Holy Basil Extract RS being used.
Resolution: NLT 1.3 between the oleanolic acid and ursolic acid peaks, *Standard solution B*
Relative standard deviation: NMT 2% determined from the ursolic acid peak in repeated injections, *Standard solution A*

Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Holy Basil Extract RS being used, identify the retention time of the peaks corresponding to oleanolic acid and ursolic acid in the *Sample solution* chromatogram.
Calculate the percentage of triterpenes, oleanolic acid, and ursolic acid in the portion of Powdered Holy Basil Leaf taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = sum of peak areas of oleanolic acid and ursolic acid in the *Sample solution* chromatogram
r_S = peak area for ursolic acid in the *Standard solution A* chromatogram
C_S = concentration of ursolic acid in *Standard solution A* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Powdered Holy Basil Leaf taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 0.5% on the dried basis

CONTAMINANTS

- **Elemental Impurities—Procedures** ⟨233⟩

Acceptance criteria
Arsenic: NMT 1.0 µg/g
Cadmium: NMT 0.5 µg/g
Lead: NMT 5.0 µg/g
Mercury: NMT 1.0 µg/g

Change to read:

- **▲Articles of Botanical Origin** ⟨561⟩, *Pesticide Residue Analysis*:▲ (CN 1-May-2019) Meets the requirements
- **Microbial Enumeration Tests** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria does not exceed 10^3 cfu/g.
- **Absence of Specified Microorganisms** ⟨2022⟩: Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

- **Botanic Characteristics**

Macroscopic: Yellowish-green powder; characteristic aromatic odor
Microscopic: It shows fragments of epidermal cell, straight anticlinal walls on the upper surface and slightly wavy on the lower surface, with diacytic stomata, with covering and glandular trichomes; covering trichomes, uniseriate, unicellular to multicellular, often bent, with bulging basal cells; 4-celled head and 1- to 2-celled head glandular trichomes; fibres; and vascular tissue.

- **Loss on Drying** ⟨731⟩

Sample: 1.0 g of Powdered Holy Basil Leaf
Analysis: Dry at 105° for 2 h.
Acceptance criteria: NMT 10%

- **Articles of Botanical Origin,** *Total Ash* ⟨561⟩

Sample: 2 g of Powdered Holy Basil Leaf
Acceptance criteria: NMT 18%

- **Articles of Botanical Origin,** *Acid-insoluble Ash* ⟨561⟩

Sample: 2–4 g of Powdered Holy Basil Leaf
Acceptance criteria: NMT 3%

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **Labeling:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived.
- **USP Reference Standards** ⟨11⟩
USP Powdered Holy Basil Extract RS
USP Rosmarinic Acid RS
USP Ursolic Acid RS

Powdered Holy Basil Leaf Extract

DEFINITION

Powdered Holy Basil Leaf Extract is prepared from Holy Basil Leaf by extraction with mixtures of alcohol–water or methanol–water. It contains NLT 90.0% and NMT 110.0% of the labeled amount of triterpenes, calculated as the sum of oleanolic acid and ursolic acid, on the dried basis. It may contain suitable added substances as carriers.

IDENTIFICATION

- **A. Thin-Layer Chromatography**

Standard solution A: 0.5 mg/mL of USP Rosmarinic Acid RS in methanol
Standard solution B: 10 mg/mL of USP Powdered Holy Basil Extract RS in methanol
Sample solution: 10 mg/mL of Powdered Holy Basil Leaf Extract in methanol. [Note—Reserve a portion for use in *Identification* test *B*.]
Chromatographic system
Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)
Application volume: 2 µL of *Standard solution A*, 4 µL of *Standard solution B*, and 8 µL of the *Sample solution* as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
Developing solvent system: A mixture of ethyl acetate, formic acid, and water (15:1:1)
Developing distance: 6 cm
Derivatization reagent A: 5-mg/mL solution of 2-aminoethyl diphenylborinate in ethyl acetate

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Derivatization reagent B: 50-mg/mL solution of polyethylene glycol 400 in dichloromethane

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands to a suitable high performance thin-layer chromatographic plate, and dry in air (see *Chromatography* ⟨621⟩). Develop the chromatograms in a saturated chamber, remove the plate from the chamber, dry in air, heat at 100° for 3 min, derivatize the plate while still warm with *Derivatization reagent A*, dry in air, then derivatize with *Derivatization reagent B*, dry in air, and examine under UV light at 366 nm.

System suitability: The chromatogram of *Standard solution B* exhibits, in the upper third section, a blue fluorescent band corresponding to the band due to rosmarinic acid in the chromatogram of *Standard solution A*, and a less intense blue fluorescent band, right above the band due to rosmarinic acid, corresponding to caffeic acid, and a red band above the caffeic acid band. The bands due to rosmarinic acid and caffeic acid are clearly separated. The chromatogram of *Standard solution B* also shows the most intense band, an orange band, in the lower third section of the chromatogram, and two greenish bands in the middle third section of the chromatogram.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits the following bands corresponding to similar bands in the chromatogram of *Standard solution B*: a blue fluorescent band at an R_F corresponding to the rosmarinic acid band; a less intense blue fluorescent band at an R_F above that of the rosmarinic acid band, corresponding to caffeic acid; a red band above the caffeic acid band; and a red band at the solvent front due to chlorophyll; two greenish bands in the middle third section of the chromatogram; and the most intense band, an orange band, with a blue band above in the lower third section of the chromatogram (distinction from sage leaf, thyme leaf, basil leaf, and oregano leaf, all show two orange bands in the lower third section of the chromatogram; and lemon balm leaf which lacks this band). The *Sample solution* does not exhibit yellowish-orange bands in the middle section of the chromatogram (distinction from rosemary leaf and thyme leaf).

• **B. Thin-Layer Chromatography**

Standard solution A: 0.2 mg/mL of USP Ursolic Acid RS in methanol

Standard solution B: 1.0 mg/mL of eugenol in methanol

Standard solution C: 1.0 mg/mL of methyleugenol in methanol

Standard solution D: 10 mg/mL of USP Powdered Holy Basil Extract RS in methanol

Sample solution: Use the solution prepared in *Identification* test *A*.

Chromatographic system

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)

Application volume: 2 µL of *Standard solution A*, *Standard solution B*, and *Standard solution C*, 4 µL of *Standard solution D*, and 8 µL of the *Sample solution* as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

Developing solvent system: A mixture of toluene and ethyl acetate (85:15)

Developing distance: 6 cm

Derivatization reagent: Place 85 mL of methanol in a 100-mL glass bottle, and cool it down in a water–ice cubes–salt bath or in a freezer. To the ice-cold methanol, add 10 mL of acetic acid and 5 mL of sulfuric acid, slowly and carefully, and mix well. Allow the mixture to cool to room temperature, and then add 0.5 mL of *p*-anisaldehyde.

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, *Standard solution D*, and *Sample solution*

Apply the *Samples* as bands to a suitable high performance thin-layer chromatographic plate, and dry in air (see *Chromatography* ⟨621⟩). Develop the chromatograms in a saturated chamber, remove the plate from the chamber, dry in air, derivatize with *Derivatization reagent*, heat at 100° for 3–5 min, set aside to cool, and examine under visible light.

System suitability: *Standard solution A* exhibits a violet band. *Standard solution B* and *Standard solution C* each exhibit a greenish band. *Standard solution D* exhibits a purple band above the green band corresponding to eugenol or methyleugenol in *Standard solution B* or *Standard solution C*.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits the most intense band, a violet band at an R_F corresponding to the ursolic acid band in the chromatogram of *Standard solution A* and *Standard solution D*, and a greenish band at an R_F corresponding to the eugenol band or methyleugenol band in the chromatograms of *Standard solution B* or *Standard solution C*. [Note—Holy basil leaf may occur in two chemotypes, one characterized by the presence of eugenol and the other by the presence of methyleugenol.]

• **C. HPLC**

Analysis: Proceed as directed in the test for *Content of Triterpenes*.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits two peaks at the retention times corresponding to the ursolic acid and oleanolic acid in the *Standard solutions*.

COMPOSITION

• **Content of Triterpenes**

Mobile phase: A mixture of acetonitrile and a solution of 2.5 mg/mL ammonium acetate in water (67:33)

Standard solution A: 0.1 mg/mL of USP Ursolic Acid RS in methanol. Sonicate to dissolve if necessary.

Standard solution B: 4.0 mg/mL of USP Powdered Holy Basil Extract RS in methanol. Sonicate to dissolve if necessary. Before injection, pass through a membrane filter of 0.45-µm or finer pore size.

Sample solution: An amount of Powdered Holy Basil Leaf Extract equivalent to 5 mg of triterpenes in 50 mL of methanol. Sonicate to dissolve if necessary. Before injection, pass through a membrane filter of 0.45-µL or finer pore size.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV, 205 nm

Column: 3.0-mm × 10-cm; 2.5-µm packing L1

Flow rate: 0.3 mL/min

Injection volume: 5 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Holy Basil Extract RS being used.

Resolution: NLT 1.3 between the oleanolic acid and ursolic acid peaks, *Standard solution B*

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Relative standard deviation: NMT 2% determined from the ursolic acid peak in repeated injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Holy Basil Extract RS being used, identify the retention time of the peaks corresponding to oleanolic acid and ursolic acid in the *Sample solution* chromatogram.

Calculate the percentage of triterpenes, oleanolic acid, and ursolic acid in the portion of Powdered Holy Basil Leaf Extract taken:

$$P = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = sum of peak areas of oleanolic acid and ursolic acid in the *Sample solution* chromatogram

r_S = peak area for ursolic acid in the *Standard solution A* chromatogram

C_S = concentration of ursolic acid in *Standard solution A* (mg/mL)

C_U = concentration of Powdered Holy Basil Leaf Extract in the *Sample solution* (mg/mL)

Calculate the percentage of the labeled amount of triterpenes, oleanolic acid, and ursolic acid in the portion of Powdered Holy Basil Leaf Extract taken:

$$\text{Result} = (P/L) \times 100$$

P = content of triterpenes, oleanolic acid, and ursolic acid, as determined above (%)

L = labeled amount of triterpenes, oleanolic acid, and ursolic acid (%)

Acceptance criteria: 90%–110% of the labeled amount on the dried basis

CONTAMINANTS

- **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩

Acceptance criteria

Arsenic: NMT 1.0 µg/g

Cadmium: NMT 0.5 µg/g

Lead: NMT 5.0 µg/g

Mercury: NMT 1.0 µg/g

Change to read:

- **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020)**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

- **LOSS ON DRYING** ⟨731⟩

Sample: 2.0 g of Powdered Holy Basil Leaf Extract

Analysis: Dry at 105° for 2 h.

Acceptance criteria: NMT 5.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived. It meets other labeling requirements for *Botanical Extracts* ⟨565⟩.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Powdered Holy Basil Extract RS

USP Rosmarinic Acid RS

USP Ursolic Acid RS

Hydroxocobalamin—*see Hydroxocobalamin General Monographs*

Isoleucine—*see Isoleucine General Monographs*

Japanese Honeysuckle Flower

DEFINITION

Japanese Honeysuckle Flower consists of the dried flower buds or dried flowers in the early-opening stage of *Lonicera japonica* Thunb. (Fam. Caprifoliaceae) collected in early summer. It contains NLT 3.8% of caffeoylquinic acids, calculated as the sum of chlorogenic acid, 3,5-di-*O*-caffeoylquinic acid, and 4,5-di-*O*-caffeoylquinic acid on the dried basis; and NLT 0.80% of iridoids, calculated as the sum of sweroside, secoxyloganin, and centauroside on the dried basis.

IDENTIFICATION

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 0.2 mg/mL of USP Chlorogenic Acid RS, 0.25 mg/mL of USP Rutin RS, and 0.1 mg/mL of USP Luteolin-7-*O*-Glucoside RS in methanol

Standard solution B: 10 mg/mL of USP *Lonicera japonica* Flower Dry Extract RS in methanol. Sonicate for 10 min, and filter. Evaporate the filtrate at about 50° under reduced pressure to dryness. Add 5 mL of water to dissolve the residue, and then add 10 mL of ethyl acetate and mix. After the solution separates into two layers, take the ethyl acetate layer. Repeat the extraction one more time. Combine the ethyl acetate extracts, evaporate the solvent at about 50° under reduced pressure to dryness, and dissolve the residue in methanol equivalent to one-fifth of the initial volume of USP *Lonicera japonica* Flower Dry Extract RS solution.

Sample solution: 500 mg of Japanese Honeysuckle Flower, finely powdered, in 10 mL of methanol. Sonicate for 10 min, and filter. Evaporate the filtrate at about 50° under reduced pressure to dryness. Add 5 mL of water to dissolve the residue, and then add 10 mL of ethyl acetate and mix. After the solution separates into two layers, take the ethyl acetate layer. Repeat the extraction one more time. Combine the ethyl acetate extracts, evaporate the solvent at about 50° under reduced pressure to dryness, and add 2 mL of methanol to dissolve the residue.

Chromatographic system

Adsorbent: Chromatographic silica gel mixture with an average particle size of about 5 µm

Application volume: 5 µL, as 10-mm bands

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Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
Temperature: About 25°
Developing solvent system: The upper layer solution of a mixture of *n*-butyl acetate, formic acid, and water (7:5:5)
Developing distance: 8 cm
Derivatization reagent A: 10 mg/mL of 2-aminoethyl diphenylborinate in methanol
Derivatization reagent B: 50 mg/mL of polyethylene glycol 4000 in alcohol

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands and dry in air. Develop in a saturated chamber, remove the plate from the chamber, and dry in air. Treat the plate with *Derivatization reagent A* and allow to air-dry. Immediately, treat the plate with *Derivatization reagent B*, allow to air-dry, and examine under UV light at 366 nm.

System suitability: Under UV light at 366 nm, in the lower-third section, *Standard solution B* exhibits one blue fluorescent band corresponding in R_F to the band due to chlorogenic acid and one yellow band above chlorogenic acid corresponding in R_F to the band due to luteolin-7-*O*-glucoside in *Standard solution A*. One faint blue fluorescent band is below the chlorogenic acid band and another yellow band, due to rutin, is below the faint blue fluorescent band. In the middle-third section, *Standard solution B* exhibits three blue fluorescent bands: the band with the highest R_F is due to 3,5-di-*O*-caffeoylquinic acid; the band with the middle R_F is due to 4,5-di-*O*-caffeoylquinic acid; the band with the lowest R_F is due to 3,4-di-*O*-caffeoylquinic acid.

Acceptance criteria: Under UV light at 366 nm, the *Sample solution* exhibits the most intense band corresponding in R_F and color to the band of chlorogenic acid and a yellow band above chlorogenic acid corresponding in R_F and color to the band due to luteolin-7-*O*-glucoside in *Standard solution A*. The *Sample solution* exhibits another yellow band in the lower-third section corresponding to rutin and two blue bands in the middle-third section due to 3,5-di-*O*-caffeoylquinic acid and 4,5-di-*O*-caffeoylquinic acid corresponding in R_F and color to similar bands in *Standard solution B*.

• **B. Caffeoylquinic Acids HPLC Profile**

Analysis: Proceed as directed in the test for *Content of Caffeoylquinic Acids*.

Acceptance criteria: The *Sample solution* exhibits the most intense peak with a retention time corresponding to chlorogenic acid in *Standard solution A* and peaks at the retention times corresponding to 3,5-di-*O*-caffeoylquinic acid and 4,5-di-*O*-caffeoylquinic acid in *Standard solution B*. It meets the content ratios in *Table 2*.

• **C. Iridoids HPLC Profile**

Analysis: Proceed as directed in the test for *Content of Iridoids*.

Acceptance criteria: The *Sample solution* exhibits a peak with a retention time corresponding to secoxyloganin in *Standard solution A* and two additional iridoid peaks of sweroside and centauroside at retention times corresponding to the same iridoids in *Standard solution B*.

COMPOSITION

• **Content of Caffeoylquinic Acids**

Solution A: 0.1% phosphoric acid in water
Solution B: Acetonitrile
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	86	14
8	81	19
14	81	19
34	69	31
35	10	90
39.5	10	90
40	86	14
48	86	14

[NOTE—Protect from light and proceed under low actinic light. The *Standard solutions* and the *Sample solution* are stable for 24 h at room temperature.]

Solvent: Methanol and water (7.5: 2.5)
Standard solution A: 0.30 mg/mL of USP Chlorogenic Acid RS in methanol
Standard solution B: 2.5 mg/mL of USP *Lonicera japonica* Flower Dry Extract RS in *Solvent*. Sonicate for 15 min, and pass through a membrane filter of 0.45-µm or finer pore size.
Sample solution: Accurately transfer about 100 mg of Japanese Honeysuckle Flower, finely powdered, into a suitable stoppered conical flask, and accurately add 10.0 mL of *Solvent*. Weigh the filled flask with a precision of ±0.1 mg, stopper, and sonicate for 30 min. After cooling to room temperature, adjust to the initial weight by adding *Solvent*. Pass through a membrane filter of 0.45-µm or finer pore size, and discard the first portion of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 327 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 15°
Flow rate: 0.7 mL/min
Injection volume: 2 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Resolution: NLT 1.5 between the chlorogenic acid and cryptochlorogenic acid peaks, and between the 3,5-di-*O*-caffeoylquinic acid and 4,5-di-*O*-caffeoylquinic acid peaks, *Standard solution B*
Tailing factor: NMT 2.0 for the chlorogenic acid peak, *Standard solution A*
Relative standard deviation: NMT 2.0% for the chlorogenic acid peak in repeated injections, *Standard solution A*
Chromatographic similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram for caffeoylquinic acids provided with the lot of USP *Lonicera japonica* Flower Dry Extract RS being used.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatograms of *Standard solution A* and *Standard solution B*, and the reference chromatogram for caffeoylquinic acids provided with the lot of USP *Lonicera japonica* Flower Dry Extract RS being used, identify the retention times of the peaks of neochlorogenic acid, chlorogenic acid, cryptochlorogenic acid, 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid,

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and 4,5-di-*O*-caffeoylquinic acid in the *Sample solution*. [NOTE—See *Table 2* for relative retention times. These values are not monograph requirements. They may vary due to differences in the chromatographic conditions allowed by the system suitability requirements.]

Table 2

Analyte	Approximate Relative Retention Time	Conversion Factor	Content Ratio
Neochlorogenic acid[a]	0.75	1.00	Minor peak[b]
Chlorogenic acid[c]	1.00	1.00	1.0
Cryptochlorogenic acid[d]	1.05	1.00	Minor peak[b]
3,4-Di-*O*-caffeoylquinic acid[e]	2.65	0.81	Minor peak[b]
3,5-Di-*O*-caffeoylquinic acid[f]	2.88	0.77	0.3–0.8
4,5-Di-*O*-caffeoylquinic acid[g]	3.01	0.77	0.04–0.2

[a] (1*R*,3*R*,4*S*,5*R*)-3-{[(*E*)-3-(3,4-Dihydroxyphenyl)acryloyl]oxy}-1,4,5-trihydroxycyclohexane-1-carboxylic acid.
[b] The peak area is lower than the peak area of 4,5-di-*O*-caffeoylquinic acid.
[c] (1*S*,3*R*,4*R*,5*R*)-3-{[(*E*)-3-(3,4-Dihydroxyphenyl)acryloyl]oxy}-1,4,5-trihydroxycyclohexane-1-carboxylic acid.
[d] (1*S*,3*R*,4*S*,5*R*)-4-{[(*E*)-3-(3,4-Dihydroxyphenyl)acryloyl]oxy}-1,4,5-trihydroxycyclohexane-1-carboxylic acid.
[e] (1*S*,3*R*,4*R*,5*R*)-3,4-Bis{[(*E*)-3-(3,4-dihydroxyphenyl)acryloyl]oxy}-1,5-dihydroxycyclohexane-1-carboxylic acid.
[f] (1*S*,3*R*,4*S*,5*R*)-3,5-Bis{[(*E*)-3-(3,4-dihydroxyphenyl)acryloyl]oxy}-1,5-dihydroxycyclohexane-1-carboxylic acid.
[g] (1*R*,3*R*,4*S*,5*R*)-3,4-Bis{[(*E*)-3-(3,4-dihydroxyphenyl)acryloyl]oxy}-1,5-dihydroxycyclohexane-1-carboxylic acid.

Separately calculate the percentages of chlorogenic acid, 3,5-di-*O*-caffeoylquinic acid, and 4,5-di-*O*-caffeoylquinic acid in the portion of Japanese Honeysuckle Flower taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of chlorogenic acid from *Standard solution A*
C_S = concentration of USP Chlorogenic Acid RS in *Standard solution A* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Japanese Honeysuckle Flower taken to prepare the *Sample solution* (mg)
F = conversion factor for the analyte (see *Table 2*)

Calculate the content of caffeoylquinic acids as the sum of the percentages of chlorogenic acid, 3,5-di-*O*-caffeoylquinic acid, and 4,5-di-*O*-caffeoylquinic acid.

Acceptance criteria: NLT 3.8% on the dried basis

- **CONTENT OF IRIDOIDS**

Solution A, Solution B, Mobile phase, Standard solution B, and Sample solution: Prepare as directed in the test for *Content of Caffeoylquinic Acids*.

Standard solution A: 0.05 mg/mL of USP Secoxyloganin RS in methanol

Chromatographic system: Proceed as directed in the test for *Content of Caffeoylquinic Acids* except for the *Detector*.

Detector: UV 240 nm

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Resolution: NLT 1.5 between the peak of secoxyloganin and the peak before it, *Standard solution B*

Tailing factor: NMT 2.0 for the secoxyloganin peak, *Standard solution A*

Relative standard deviation: NMT 2.0% for the secoxyloganin peak in repeated injections, *Standard solution A*

Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram for iridoids provided with the lot of USP *Lonicera japonica* Flower Dry Extract RS being used.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatograms of *Standard solution A* and *Standard solution B*, and the reference chromatogram for iridoids provided with the lot of USP *Lonicera japonica* Flower Dry Extract RS being used, identify the peaks of sweroside, secoxyloganin, and centauroside in the *Sample solution*. [NOTE—The approximate relative retention times for the peaks of sweroside, secoxyloganin, and centauroside are 0.85, 1.00, and 1.80, respectively.]

Separately calculate the percentages of secoxyloganin, sweroside, and centauroside in the portion of Japanese Honeysuckle Flower taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of secoxyloganin from *Standard solution A*
C_S = concentration of USP Secoxyloganin RS in *Standard solution A* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Japanese Honeysuckle Flower taken to prepare the *Sample solution* (mg)
F = conversion factor for the analyte (1.00 for secoxyloganin, 1.03 for sweroside, and 0.89 for centauroside)

Calculate the content of iridoids as the sum of the percentages of secoxyloganin, sweroside, and centauroside.

Acceptance criteria: NLT 0.80% on the dried basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Limits of Elemental Impurities*: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Pesticide Residue Analysis*: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures*, *Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: Flower buds are clavate, tapered downwards, slightly curved; 2–3 cm long, about 3 mm in diameter in the upper part, and 1.5 mm in diameter in the lower part with densely pubescent surface; externally yellowish white, greenish white to yellowish green, gradually darkening over

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time to a golden color. The foliaceous bracts are occasionally visible. The calyx is green, pubescent, five-lobed at the apex, about 2 mm long. The corollas of opening flowers are tubular and two-lipped at the apex. The stamens are epipetalous, yellow, in groups of five, and the one ovary is glabrous.

Microscopic: The powder contains numerous glandular hairs. The heads of glandular hairs are multicellular, subround or slightly oblate, usually 30–70 µm in diameter, exceptionally up to 110 µm. The stalks of glandular hairs are unicellular or multicellular with up to five cells, usually 20–70 µm long, exceptionally up to 700 µm. Non-glandular hairs occur in two types: one with thick walls, unicellular, 45–900 µm long, 15–40 µm in diameter, with fine verrucae on the surface, some having corneous spirals; another type with thin walls, slender, curved or shrunken, with fine verrucae on the surface. Clusters of calcium oxalate are usually 6–45 µm in diameter. Pollen grains are spherical, with three germinal pores, 60–90 µm in diameter.

- **LIMIT OF TRITERPENOID SAPONINS**

Standard solution: 5.0 mg/mL of USP *Lonicera macranthoides* Flower Dry Extract RS in methanol. Sonicate for 10 min, and filter.

Sample solution: 500 mg of Japanese Honeysuckle Flower, finely powdered, in 10 mL of methanol. Sonicate for 10 min, and filter.

Chromatographic system

(See *HPTLC for Articles of Botanical Origin* ⟨203⟩.)

Adsorbent: Chromatographic silica gel mixture with an average particle size of about 5 µm

Application volume: 3 µL, as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

Developing solvent system: The upper layer solution of a mixture of *n*-butanol, formic acid, and water (4:1:5)

Developing distance: 6 cm

Derivatization reagent: 10% sulfuric acid in ethanol

Analysis

Samples: *Standard solution* and *Sample solution*

Apply the *Samples* as bands and dry in air. Develop in a saturated chamber, remove the plate from the chamber, and dry in air. Treat the plate with *Derivatization reagent*, heat at 105° for 5 min, and examine immediately under UV light at 366 nm.

System suitability: Under UV light at 366 nm, in the lower-third section, the *Standard solution* exhibits three clearly separated brown bands: the band with the highest R_F is due to dipsacoside B; the middle band is due to macranthoidin A; the band with the lowest R_F is due to macranthoidin B.

Acceptance criteria: Under UV light at 366 nm, the *Sample solution* does not exhibit any bands corresponding in R_F and color to the bands due to dipsacoside B, macranthoidin A, and macranthoidin B in the *Standard solution*.

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Foreign Organic Matter*: NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Alcohol-Soluble Extractives, Method 1*: NLT 30.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Water-Soluble Extractives, Method 2*: NLT 35.0%
- **LOSS ON DRYING** ⟨731⟩

Sample: 2 g of Japanese Honeysuckle Flower, finely powdered

Analysis: Dry the *Sample* at 105° for 2 h.

Acceptance criteria: NMT 12.0%

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Total Ash*

Sample: 4 g of Japanese Honeysuckle Flower, finely powdered

Acceptance criteria: NMT 10.0%

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Acid-Insoluble Ash*: NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store in a cool place.
- **LABELING:** The label states the Latin binomial following the official name of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Chlorogenic Acid RS
USP *Lonicera japonica* Flower Dry Extract RS
USP *Lonicera macranthoides* Flower Dry Extract RS
USP Luteolin-7-*O*-Glucoside RS
USP Rutin RS
USP Secoxyloganin RS

Japanese Honeysuckle Flower Dry Extract

DEFINITION

Japanese Honeysuckle Flower Dry Extract is prepared from the dried flower buds or dried flowers in the early opening stage of *Lonicera japonica* Thunb. (Fam. Caprifoliaceae) collected in early summer and extracted with water or a hydroalcoholic mixture containing NMT 15% alcohol. Extracts may be further processed by adding alcohol, filtering, evaporating, and then adding water, filtering, and evaporating. It contains NLT 90.0% and NMT 110.0% of the labeled amount of caffeoylquinic acids, calculated as the sum of neochlorogenic acid, chlorogenic acid, cryptochlorogenic acid, 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, and 4,5-di-*O*-caffeoylquinic acid on the dried basis; and NLT 90.0% and NMT 110.0% of the labeled amount of iridoids, calculated as the sum of sweroside, secoxyloganin, and centauroside on the dried basis. It may contain suitable added substances as carriers.

IDENTIFICATION

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 0.2 mg/mL of USP Chlorogenic Acid RS, 0.25 mg/mL of USP Rutin RS, and 0.1 mg/mL of USP Luteolin-7-*O*-Glucoside RS in methanol

Standard solution B: 10 mg/mL of USP *Lonicera japonica* Flower Dry Extract RS in methanol. Sonicate for 10 min, and filter. Evaporate the filtrate at about 50° under reduced pressure to dryness. Add 5 mL of water to dissolve the residue, and then add 10 mL of ethyl acetate and mix. After the solution separates into two layers, take the ethyl acetate layer. Repeat the extraction one more time. Combine the ethyl acetate extracts, evaporate the solvent at about 50° under reduced pressure to dryness, and dissolve the residue in methanol equivalent to one-fifth of the initial volume of the USP *Lonicera japonica* Flower Dry Extract RS solution.

Sample solution: 100 mg of Japanese Honeysuckle Flower Dry Extract in 10 mL of methanol. Sonicate for 10 min, and filter. Evaporate the filtrate at about 50° under reduced pressure to dryness. Add 5 mL of water to dissolve the residue, and then add 10 mL of ethyl acetate and mix. After the solution separates into two layers, take the ethyl acetate layer. Repeat the extraction one more time. Combine the ethyl acetate extracts, evaporate the solvent at about 50°

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under reduced pressure to dryness, and add 2 mL of methanol to dissolve the residue.

Chromatographic system

Adsorbent: Chromatographic silica gel mixture with an average particle size of about 5 µm

Application volume: 5 µL, as 10-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

Temperature: About 25°

Developing solvent system: The upper layer solution of a mixture of *n*-butyl acetate, formic acid, and water (7:5:5)

Developing distance: 8 cm

Derivatization reagent A: 10 mg/mL of 2-aminoethyl diphenylborinate in methanol

Derivatization reagent B: 50 mg/mL of polyethylene glycol 4000 in alcohol

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands and dry in air. Develop in a saturated chamber, remove the plate from the chamber, and dry in air. Treat the plate with *Derivatization reagent A* and allow to air-dry. Immediately, treat the plate with *Derivatization reagent B*, allow to air-dry, and examine under UV light at 366 nm.

System suitability: Under UV light at 366 nm, in the lower-third section, *Standard solution B* exhibits one blue fluorescent band corresponding in R_F to the band due to chlorogenic acid and one yellow band above chlorogenic acid corresponding in R_F to the band due to luteolin-7-*O*-glucoside in *Standard solution A*. One faint blue fluorescent band is below the chlorogenic acid band and another yellow band, due to rutin, is below the faint blue fluorescent band. In the middle-third section, *Standard solution B* exhibits three blue fluorescent bands: the band with the highest R_F is due to 3,5-di-*O*-caffeoylquinic acid; the band with the middle R_F is due to 4,5-di-*O*-caffeoylquinic acid; the band with the lowest R_F is due to 3,4-di-*O*-caffeoylquinic acid.

Acceptance criteria: Under UV light at 366 nm, in the lower-third section, the *Sample solution* exhibits one blue fluorescent band corresponding in R_F and color to the band of chlorogenic acid and one yellow band above chlorogenic acid corresponding in R_F and color to the band of luteolin-7-*O*-glucoside in *Standard solution A*. One faint blue fluorescent band is below the chlorogenic acid band and another yellow band, due to rutin, is below the faint blue fluorescent band corresponding in R_F and color to similar bands in *Standard solution B*. In the middle-third section, the *Sample solution* exhibits three blue fluorescent bands due to 3,5-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid, and 3,4-di-*O*-caffeoylquinic acid corresponding in R_F and color to similar bands in *Standard solution B*.

• **B. Caffeoylquinic Acids HPLC Profile**

Analysis: Proceed as directed in the test for *Content of Caffeoylquinic Acids*.

Acceptance criteria: The *Sample solution* exhibits the most intense peak with a retention time corresponding to chlorogenic acid in *Standard solution A* and peaks at the retention times corresponding to neochlorogenic acid, cryptochlorogenic acid, 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, and 4,5-di-*O*-caffeoylquinic acid in *Standard solution B*. The chlorogenic acid content is NMT 65% of the total caffeoylquinic acids.

• **C. Iridoids HPLC Profile**

Analysis: Proceed as directed in the test for *Content of Iridoids*.

Acceptance criteria: The *Sample solution* exhibits a peak with a retention time corresponding to secoxyloganin in *Standard solution A* and two additional iridoid peaks of sweroside and centauroside at retention times corresponding to the same iridoids in *Standard solution B*.

COMPOSITION

• **Content of Caffeoylquinic Acids**

Solution A: 0.1% phosphoric acid in water

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	86	14
8	81	19
14	81	19
34	69	31
35	10	90
39.5	10	90
40	86	14
48	86	14

[NOTE—Protect from light and proceed under low actinic light. The *Standard solutions* and the *Sample solution* are stable for 24 h at room temperature.]

Solvent: Methanol and water (7.5: 2.5)

Standard solution A: 0.30 mg/mL of USP Chlorogenic Acid RS in methanol

Standard solution B: 2.5 mg/mL of USP *Lonicera japonica* Flower Dry Extract RS in *Solvent*. Sonicate for 15 min, and pass through a membrane filter of 0.45-µm or finer pore size.

Sample solution: Accurately transfer about 25 mg of Japanese Honeysuckle Flower Dry Extract into a suitable stoppered conical flask, and accurately add 10.0 mL of *Solvent*. Weigh the filled flask with a precision of ±0.1 mg, stopper, and then sonicate for 30 min. After cooling to room temperature, adjust to the initial weight by adding *Solvent*. Pass through a membrane filter of 0.45-µm or finer pore size, and discard the first portion of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 327 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 15°

Flow rate: 0.7 mL/min

Injection volume: 2 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Resolution: NLT 1.5 between the chlorogenic acid and cryptochlorogenic acid peaks, and between the 3,5-di-*O*-caffeoylquinic acid and 4,5-di-*O*-caffeoylquinic acid peaks, *Standard solution B*

Tailing factor: NMT 2.0 for the chlorogenic acid peak, *Standard solution A*

Relative standard deviation: NMT 2.0% for the chlorogenic acid peak in repeated injections, *Standard solution A*

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Chromatographic similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram for caffeoylquinic acids provided with the lot of USP *Lonicera japonica* Flower Dry Extract RS being used.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatograms of *Standard solution A* and *Standard solution B*, and the reference chromatogram for caffeoylquinic acids provided with the lot of USP *Lonicera japonica* Flower Dry Extract RS being used, identify the retention times of the peaks of neochlorogenic acid, chlorogenic acid, cryptochlorogenic acid, 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, and 4,5-di-*O*-caffeoylquinic acid in the *Sample solution*. [NOTE—See *Table 2* for relative retention times. These values are not monograph requirements. They may vary due to differences in the chromatographic conditions allowed by the system suitability requirements.]

Table 2

Analyte	Approximate Relative Retention Time	Conversion Factor
Neochlorogenic acid[a]	0.75	1.00
Chlorogenic acid[b]	1.00	1.00
Cryptochlorogenic acid[c]	1.05	1.00
3,4-Di-*O*-caffeoylquinic acid[d]	2.65	0.81
3,5-Di-*O*-caffeoylquinic acid[e]	2.88	0.77
4,5-Di-*O*-caffeoylquinic acid[f]	3.01	0.77

[a] (1*R*,3*R*,4*S*,5*R*)-3-{[(*E*)-3-(3,4-Dihydroxyphenyl)acryloyl]oxy}-1,4,5-trihydroxycyclohexane-1-carboxylic acid.
[b] (1*S*,3*R*,4*R*,5*R*)-3-{[(*E*)-3-(3,4-Dihydroxyphenyl)acryloyl]oxy}-1,4,5-trihydroxycyclohexane-1-carboxylic acid.
[c] (1*S*,3*R*,4*S*,5*R*)-4-{[(*E*)-3-(3,4-Dihydroxyphenyl)acryloyl]oxy}-1,4,5-trihydroxycyclohexane-1-carboxylic acid.
[d] (1*S*,3*R*,4*R*,5*R*)-3,4-Bis{[(*E*)-3-(3,4-dihydroxyphenyl)acryloyl]oxy}-1,5-dihydroxycyclohexane-1-carboxylic acid.
[e] (1*S*,3*R*,4*S*,5*R*)-3,5-Bis{[(*E*)-3-(3,4-dihydroxyphenyl)acryloyl]oxy}-1,5-dihydroxycyclohexane-1-carboxylic acid.
[f] (1*R*,3*R*,4*S*,5*R*)-3,4-Bis{[(*E*)-3-(3,4-dihydroxyphenyl)acryloyl]oxy}-1,5-dihydroxycyclohexane-1-carboxylic acid.

Separately calculate the percentages of neochlorogenic acid, chlorogenic acid, cryptochlorogenic acid, 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, and 4,5-di-*O*-caffeoylquinic acid in the portion of Japanese Honeysuckle Flower Dry Extract taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of chlorogenic acid from *Standard solution A*
C_S = concentration of USP Chlorogenic Acid RS in *Standard solution A* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Japanese Honeysuckle Flower Dry Extract taken to prepare the *Sample solution* (mg)
F = conversion factor for the analyte (see *Table 2*)

Calculate the content of caffeoylquinic acids as the sum of the percentages of neochlorogenic acid, chlorogenic acid, cryptochlorogenic acid, 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, and 4,5-di-*O*-caffeoylquinic acid.

Calculate the percentage of the labeled amount of caffeoylquinic acids in the portion of Dry Extract taken:

$$\text{Result} = (P/L) \times 100$$

P = content of caffeoylquinic acids as determined above (%)
L = labeled amount of caffeoylquinic acids (%)

Acceptance criteria: 90.0%–110.0% on the dried basis

• **CONTENT OF IRIDOIDS**

Solution A, Solution B, Mobile phase, Standard solution B, and **Sample solution:** Prepare as directed in the test for *Content of Caffeoylquinic Acids*.

Standard solution A: 0.05 mg/mL of USP Secoxyloganin RS in methanol

Chromatographic system: Proceed as directed in the test for *Content of Caffeoylquinic Acids* except for the *Detector*.

Detector: UV 240 nm

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Resolution: NLT 1.5 between the peak of secoxyloganin and the peak before it, *Standard solution B*

Tailing factor: NMT 2.0 for the secoxyloganin peak, *Standard solution A*

Relative standard deviation: NMT 2.0% for the secoxyloganin peak in repeated injections, *Standard solution A*

Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram for iridoids provided with the lot of USP *Lonicera japonica* Flower Dry Extract RS being used.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatograms of *Standard solution A* and *Standard solution B*, and the reference chromatogram for iridoids provided with the lot of USP *Lonicera japonica* Flower Dry Extract RS being used, identify the peaks of sweroside, secoxyloganin, and centauroside in the *Sample solution*. [NOTE—The approximate relative retention times for the peaks of sweroside, secoxyloganin, and centauroside are 0.85, 1.00, and 1.80, respectively.]

Separately calculate the percentages of secoxyloganin, sweroside, and centauroside in the portion of Japanese Honeysuckle Flower Dry Extract taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of secoxyloganin from *Standard solution A*
C_S = concentration of USP Secoxyloganin RS in *Standard solution A* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Japanese Honeysuckle Flower Dry Extract taken to prepare the *Sample solution* (mg)
F = conversion factor for the analyte (1.00 for secoxyloganin, 1.03 for sweroside, and 0.89 for centauroside)

Calculate the content of iridoids as the sum of the percentages of secoxyloganin, sweroside, and centauroside.

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Calculate the percentage of the labeled amount of iridoids in the portion of Dry Extract taken:

$$\text{Result} = (P/L) \times 100$$

P = content of iridoids as determined above (%)
L = labeled amount of iridoids (%)

Acceptance criteria: 90.0%–110.0% on the dried basis

CONTAMINANTS

- **BOTANICAL EXTRACTS** ⟨565⟩, *Preparations,General Pharmacopeial Requirements,Pesticide Residues*: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS

- **LIMIT OF TRITERPENOID SAPONINS**

Standard solution: 5.0 mg/mL of USP *Lonicera macranthoides* Flower Dry Extract RS in methanol. Sonicate for 10 min, and filter.

Sample solution: 50 mg of Japanese Honeysuckle Flower Dry Extract in 10 mL of methanol. Sonicate for 10 min, and filter.

Chromatographic system

(See *HPTLC for Articles of Botanical Origin* ⟨203⟩.)

Adsorbent: Chromatographic silica gel mixture with an average particle size of about 5 µm

Application volume: 3 µL, as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

Developing solvent system: The upper layer solution of a mixture of *n*-butanol, formic acid, and water (4:1:5)

Developing distance: 6 cm

Derivatization reagent: 10% sulfuric acid in ethanol

Analysis

Samples: *Standard solution* and *Sample solution*

Apply the *Samples* as bands and dry in air. Develop in a saturated chamber, remove the plate from the chamber, and dry in air. Treat the plate with *Derivatization reagent*, heat at 105° for 5 min, and examine immediately under UV light at 366 nm.

System suitability: Under UV light at 366 nm, in the lower-third section, the *Standard solution* exhibits three clearly separated brown bands: the band with the highest R_F is due to dipsacoside B; the middle band is due to macranthoidin A; the band with the lowest R_F is due to macranthoidin B.

Acceptance criteria: Under UV light at 366 nm, the *Sample solution* does not exhibit any bands corresponding in R_F and color to the bands due to dipsacoside B, macranthoidin A, and macranthoidin B in the *Standard solution*.

- **LOSS ON DRYING** ⟨731⟩

Sample: 1 g of Japanese Honeysuckle Flower Dry Extract

Analysis: Dry the *Sample* at 105° for 2 h.

Acceptance criteria: NMT 5.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store in a cool place.
- **LABELING:** The label states the Latin binomial following the official name of the plant from which the article was prepared. It meets other labeling requirements under *Botanical Extracts* ⟨565⟩.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Chlorogenic Acid RS
USP *Lonicera japonica* Flower Dry Extract RS
USP *Lonicera macranthoides* Flower Dry Extract RS
USP Luteolin-7-*O*-Glucoside RS
USP Rutin RS
USP Secoxyloganin RS

Japanese Honeysuckle Flower Powder

DEFINITION

Japanese Honeysuckle Flower Powder consists of the dried flower buds or dried flowers in the early opening stage of *Lonicera japonica* Thunb. (Fam. Caprifoliaceae) collected in early summer and reduced to a fine or very fine powder. It contains NLT 3.8% of caffeoylquinic acids, calculated as the sum of chlorogenic acid, 3,5-di-*O*-caffeoylquinic acid, and 4,5-di-*O*-caffeoylquinic acid on the dried basis; and NLT 0.80% of iridoids, calculated as the sum of sweroside, secoxyloganin, and centauroside on the dried basis.

IDENTIFICATION

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 0.2 mg/mL of USP Chlorogenic Acid RS, 0.25 mg/mL of USP Rutin RS, and 0.1 mg/mL of USP Luteolin-7-*O*-Glucoside RS in methanol

Standard solution B: 10 mg/mL of USP *Lonicera japonica* Flower Dry Extract RS in methanol. Sonicate for 10 min, and filter. Evaporate the filtrate at about 50° under reduced pressure to dryness. Add 5 mL of water to dissolve the residue, and then add 10 mL of ethyl acetate and mix. After the solution separates into two layers, take the ethyl acetate layer. Repeat the extraction one more time. Combine the ethyl acetate extracts, evaporate the solvent at about 50° under reduced pressure to dryness, and dissolve the residue in methanol equivalent to one-fifth of the initial volume of the USP *Lonicera japonica* Flower Dry Extract RS solution.

Sample solution: 500 mg of Japanese Honeysuckle Flower Powder in 10 mL of methanol. Sonicate for 10 min, and filter. Evaporate the filtrate at about 50° under reduced pressure to dryness. Add 5 mL of water to dissolve the residue, and then add 10 mL of ethyl acetate and mix. After the solution separates into two layers, take the ethyl acetate layer. Repeat the extraction one more time. Combine the ethyl acetate extracts, evaporate the solvent at about 50° under reduced pressure to dryness, and add 2 mL of methanol to dissolve the residue.

Chromatographic system

Adsorbent: Chromatographic silica gel mixture with an average particle size of about 5 µm

Application volume: 5 µL, as 10-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

Temperature: About 25°

Developing solvent system: The upper layer solution of a mixture of *n*-butyl acetate, formic acid, and water (7:5:5)

Developing distance: 8 cm

Derivatization reagent A: 10 mg/mL of 2-aminoethyl diphenylborinate in methanol

Derivatization reagent B: 50 mg/mL of polyethylene glycol 4000 in alcohol

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands and dry in air. Develop in a saturated chamber, remove the plate from the chamber,

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and dry in air. Treat the plate with *Derivatization reagent A* and allow to air-dry. Immediately, treat the plate with *Derivatization reagent B*, allow to air-dry, and examine under UV light at 366 nm.

System suitability: Under UV light at 366 nm, in the lower-third section, *Standard solution B* exhibits one blue fluorescent band corresponding in R_F to the band due to chlorogenic acid and one yellow band above chlorogenic acid corresponding in R_F to the band due to luteolin-7-*O*-glucoside in *Standard solution A*. One faint blue fluorescent band is below the chlorogenic acid band and another yellow band, due to rutin, is below the faint blue fluorescent band. In the middle-third section, *Standard solution B* exhibits three blue fluorescent bands: the band with the highest R_F is due to 3,5-di-*O*-caffeoylquinic acid; the band with the middle R_F is due to 4,5-di-*O*-caffeoylquinic acid; the band with the lowest R_F is due to 3,4-di-*O*-caffeoylquinic acid.

Acceptance criteria: Under UV light at 366 nm, the *Sample solution* exhibits the most intense band corresponding in R_F and color to the band of chlorogenic acid and a yellow band above chlorogenic acid corresponding in R_F and color to the band due to luteolin-7-*O*-glucoside in *Standard solution A*. The *Sample solution* exhibits another yellow band in the lower-third section corresponding to rutin and two blue bands in the middle-third section due to 3,5-di-*O*-caffeoylquinic acid and 4,5-di-*O*-caffeoylquinic acid corresponding in R_F and color to similar bands in *Standard solution B*.

• **B. Caffeoylquinic Acids HPLC Profile**

Analysis: Proceed as directed in the test for *Content of Caffeoylquinic Acids*.

Acceptance criteria: The *Sample solution* exhibits the most intense peak with a retention time corresponding to chlorogenic acid in *Standard solution A* and peaks at the retention times corresponding to 3,5-di-*O*-caffeoylquinic acid and 4,5-di-*O*-caffeoylquinic acid in *Standard solution B*. It meets the content ratios in *Table 2*.

• **C. Iridoids HPLC Profile**

Analysis: Proceed as directed in the test for *Content of Iridoids*.

Acceptance criteria: The *Sample solution* exhibits a peak with a retention time corresponding to secoxyloganin in *Standard solution A* and two additional iridoid peaks of sweroside and centauroside at retention times corresponding to the same iridoids in *Standard solution B*.

COMPOSITION

• **Content of Caffeoylquinic Acids**

Solution A: 0.1% phosphoric acid in water

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	86	14
8	81	19
14	81	19
34	69	31
35	10	90
39.5	10	90
40	86	14
48	86	14

[Note—Protect from light and proceed under low actinic light. The *Standard solutions* and the *Sample solution* are stable for 24 h at room temperature.]

Solvent: Methanol and water (7.5: 2.5)

Standard solution A: 0.30 mg/mL of USP Chlorogenic Acid RS in methanol

Standard solution B: 2.5 mg/mL of USP *Lonicera japonica* Flower Dry Extract RS in *Solvent*. Sonicate for 15 min, and pass through a membrane filter of 0.45-µm or finer pore size.

Sample solution: Accurately transfer about 100 mg of Japanese Honeysuckle Flower Powder into a suitable stoppered conical flask, and accurately add 10.0 mL of *Solvent*. Weigh the filled flask with a precision of ±0.1 mg, stopper, and then sonicate for 30 min. After cooling to room temperature, adjust to the initial weight by adding *Solvent*. Pass through a membrane filter of 0.45-µm or finer pore size, and discard the first portion of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 327 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 15°

Flow rate: 0.7 mL/min

Injection volume: 2 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Resolution: NLT 1.5 between the chlorogenic acid and cryptochlorogenic acid peaks, and between the 3,5-di-*O*-caffeoylquinic acid and 4,5-di-*O*-caffeoylquinic acid peaks, *Standard solution B*

Tailing factor: NMT 2.0 for the chlorogenic acid peak, *Standard solution A*

Relative standard deviation: NMT 2.0% for the chlorogenic acid peak in repeated injections, *Standard solution A*

Chromatographic similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram for caffeoylquinic acids provided with the lot of USP *Lonicera japonica* Flower Dry Extract RS being used.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatograms of *Standard solution A* and *Standard solution B*, and the reference chromatogram for caffeoylquinic acids provided with the lot of USP *Lonicera japonica* Flower Dry Extract RS being used, identify the retention times of the peaks of neochlorogenic acid, chlorogenic acid, cryptochlorogenic acid, 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, and 4,5-di-*O*-caffeoylquinic acid in the *Sample solution*. [Note—See *Table 2* for relative retention times. These values are not monograph requirements. They may vary due to differences in the chromatographic conditions allowed by the system suitability requirements.]

Table 2

Analyte	Approximate Relative Retention Time	Conversion Factor	Content Ratio
Neochlorogenic acid[a]	0.75	1.00	Minor peak[b]
Chlorogenic acid[c]	1.00	1.00	1.0

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Table 2 *(continued)*

Analyte	Approximate Relative Retention Time	Conversion Factor	Content Ratio
Cryptochlorogenic acid[d]	1.05	1.00	Minor peak[b]
3,4-Di-*O*-caffeoylquinic acid[e]	2.65	0.81	Minor peak[b]
3,5-Di-*O*-caffeoylquinic acid[f]	2.88	0.77	0.3–0.8
4,5-Di-*O*-caffeoylquinic acid[g]	3.01	0.77	0.04–0.2

[a] (1*R*,3*R*,4*S*,5*R*)-3-{[(*E*)-3-(3,4-Dihydroxyphenyl)acryloyl]oxy}-1,4,5-trihydroxycyclohexane-1-carboxylic acid.
[b] The peak area is lower than the peak area of 4,5-di-*O*-caffeoylquinic acid.
[c] (1*S*,3*R*,4*R*,5*R*)-3-{[(*E*)-3-(3,4-Dihydroxyphenyl)acryloyl]oxy}-1,4,5-trihydroxycyclohexane-1-carboxylic acid.
[d] (1*S*,3*R*,4*S*,5*R*)-4-{[(*E*)-3-(3,4-Dihydroxyphenyl)acryloyl]oxy}-1,4,5-trihydroxycyclohexane-1-carboxylic acid.
[e] (1*S*,3*R*,4*R*,5*R*)-3,4-Bis{[(*E*)-3-(3,4-dihydroxyphenyl)acryloyl]oxy}-1,5-dihydroxycyclohexane-1-carboxylic acid.
[f] (1*S*,3*R*,4*S*,5*R*)-3,5-Bis{[(*E*)-3-(3,4-dihydroxyphenyl)acryloyl]oxy}-1,5-dihydroxycyclohexane-1-carboxylic acid.
[g] (1*R*,3*R*,4*S*,5*R*)-3,4-Bis{[(*E*)-3-(3,4-dihydroxyphenyl)acryloyl]oxy}-1,5-dihydroxycyclohexane-1-carboxylic acid.

Separately calculate the percentages of chlorogenic acid, 3,5-di-O-caffeoylquinic acid, and 4,5-di-O-caffeoylquinic acid in the portion of Japanese Honeysuckle Flower Powder taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of chlorogenic acid from *Standard solution A*
C_S = concentration of USP Chlorogenic Acid RS in *Standard solution A* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Japanese Honeysuckle Flower Powder taken to prepare the *Sample solution* (mg)
F = conversion factor for the analyte (see *Table 2*)

Calculate the content of caffeoylquinic acids as the sum of the percentages of chlorogenic acid, 3,5-di-*O*-caffeoylquinic acid, and 4,5-di-*O*-caffeoylquinic acid.

Acceptance criteria: NLT 3.8% on the dried basis

• **CONTENT OF IRIDOIDS**

Solution A, Solution B, Mobile phase, Standard solution B, and Sample solution: Prepare as directed in the test for *Content of Caffeoylquinic Acids*.

Standard solution A: 0.05 mg/mL of USP Secoxyloganin RS in methanol

Chromatographic system: Proceed as directed in the test for *Content of Caffeoylquinic Acids* except for the *Detector*.

Detector: UV 240 nm

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Resolution: NLT 1.5 between the peak of secoxyloganin and the peak before it, *Standard solution B*

Tailing factor: NMT 2.0 for the secoxyloganin peak, *Standard solution A*

Relative standard deviation: NMT 2.0% for the secoxyloganin peak in repeated injections, *Standard solution A*

Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram for iridoids provided with the lot of USP *Lonicera japonica* Flower Dry Extract RS being used.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatograms of *Standard solution A* and *Standard solution B*, and the reference chromatogram for iridoids provided with the lot of USP *Lonicera japonica* Flower Dry Extract RS being used, identify the peaks of sweroside, secoxyloganin, and centauroside in the *Sample solution*. [NOTE—The approximate relative retention times for the peaks of sweroside, secoxyloganin, and centauroside are 0.85, 1.00, and 1.80, respectively.]

Separately calculate the percentages of secoxyloganin, sweroside, and centauroside in the portion of Japanese Honeysuckle Flower Powder taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of secoxyloganin from *Standard solution A*
C_S = concentration of USP Secoxyloganin RS in *Standard solution A* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Japanese Honeysuckle Flower Powder taken to prepare the *Sample solution* (mg)
F = conversion factor for the analyte (1.00 for secoxyloganin, 1.03 for sweroside, and 0.89 for centauroside)

Calculate the content of iridoids as the sum of the percentages of secoxyloganin, sweroside, and centauroside.

Acceptance criteria: NLT 0.80% on the dried basis

CONTAMINANTS

• **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Limits of Elemental Impurities*: Meets the requirements

• **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Pesticide Residue Analysis*: Meets the requirements

• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria count does not exceed 10^3 cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS

• **BOTANICAL CHARACTERISTICS**

Macroscopic: Yellowish-white to yellowish-green powder, darkening over time to a golden color

Microscopic: Glandular hairs are numerous. The heads of glandular hairs are multicellular, subround or slightly oblate, usually 30–70 µm in diameter, exceptionally up to 110 µm. The stalks of glandular hairs are unicellular or multicellular with up to five cells, usually 20–70 µm long, exceptionally up to 700 µm. Non-glandular hairs occur in two types: one with thick walls, unicellular, 45–900 µm long, 15–40 µm in diameter, with fine verrucae on the surface, some having corneous spirals; another type with thin walls, slender, curved, or shrunken, with fine verrucae on the surface. Clusters of calcium oxalate are usually 6–45 µm in diameter. Pollen grains are spherical, with three germinal pores, 60–90 µm in diameter.

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• **Limit of Triterpenoid Saponins**
Standard solution: 5.0 mg/mL of USP *Lonicera macranthoides* Flower Dry Extract RS in methanol. Sonicate for 10 min, and filter.
Sample solution: 500 mg of Japanese Honeysuckle Flower Powder in 10 mL of methanol. Sonicate for 10 min, and filter.
Chromatographic system
(See *HPTLC for Articles of Botanical Origin* ⟨203⟩.)
Adsorbent: Chromatographic silica gel mixture with an average particle size of about 5 µm
Application volume: 3 µL, as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
Developing solvent system: The upper layer solution of a mixture of *n*-butanol, formic acid, and water (4:1:5)
Developing distance: 6 cm
Derivatization reagent: 10% sulfuric acid in ethanol
Analysis
Samples: *Standard solution* and *Sample solution*
Apply the *Samples* as bands and dry in air. Develop in a saturated chamber, remove the plate from the chamber, and dry in air. Treat the plate with *Derivatization reagent*, heat at 105° for 5 min, and examine immediately under UV light at 366 nm.
System suitability: Under UV light at 366 nm, in the lower-third section, the *Standard solution* exhibits three clearly separated brown bands: the band with the highest R_F is due to dipsacoside B; the middle band is due to macranthoidin A; the band with the lowest R_F is due to macranthoidin B.
Acceptance criteria: Under UV light at 366 nm, the *Sample solution* does not exhibit any bands corresponding in R_F and color to the bands due to dipsacoside B, macranthoidin A, and macranthoidin B in the *Standard solution*.
• **Articles of Botanical Origin** ⟨561⟩, *Methods of Analysis, Alcohol-Soluble Extractives, Method 1*: NLT 30.0%
• **Articles of Botanical Origin** ⟨561⟩, *Methods of Analysis, Water-Soluble Extractives, Method 2*: NLT 35.0%
• **Loss on Drying** ⟨731⟩
Sample: 2 g of Japanese Honeysuckle Flower Powder
Analysis: Dry the *Sample* at 105° for 2 h.
Acceptance criteria: NMT 12.0%
• **Articles of Botanical Origin** ⟨561⟩, *Methods of Analysis, Total Ash*
Sample: 4 g of Japanese Honeysuckle Flower Powder
Acceptance criteria: NMT 10.0%
• **Articles of Botanical Origin** ⟨561⟩, *Methods of Analysis, Acid-Insoluble Ash*: NMT 1.0%

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Preserve in well-closed containers, protected from light and moisture, and store in a cool place.
• **Labeling:** The label states the Latin binomial following the official name of the plant from which the article was derived.
• **USP Reference Standards** ⟨11⟩
USP Chlorogenic Acid RS
USP *Lonicera japonica* Flower Dry Extract RS
USP *Lonicera macranthoides* Flower Dry Extract RS
USP Luteolin-7-*O*-Glucoside RS
USP Rutin RS
USP Secoxyloganin RS

Krill Oil

Change to read:

DEFINITION

Krill Oil is the fixed oil extracted from Antarctic krill (*Euphausia superba* Dana) biomass using appropriate food-grade organic solvents. Krill Oil contains NLT 30% (w/w) and NMT 59% (w/w) of total phospholipids, of which 60%–96% are phosphatidylcholine, ▲1-lysophosphatidylcholine, and 2-lysophosphatidylcholine.▲ 2S (USP41) It contains NLT 10% (w/w) of eicosapentaenoic acid (EPA) and NLT 5.0% (w/w) of docosahexaenoic acid (DHA) mostly in the form of phospholipids. It also contains NLT ▲0.005%▲ 2S (USP41) of astaxanthin.

IDENTIFICATION

Change to read:

• **A. Fatty Acid Profile**
Antioxidant solution, System suitability solution 1, and **Chromatographic system:** Proceed as directed in *Fats and Fixed Oils* ⟨401⟩, *Procedures, Omega-3 Fatty Acids Determination and Profile.*
Standard solution: Prepare as directed in *Fats and Fixed Oils* ⟨401⟩, *Procedures, Omega-3 Fatty Acids Determination and Profile, Content of EPA and DHA, Test Solution 1*, except use 250 mg of USP Krill Oil RS.
Sample solution: Prepare as directed in the *Standard solution*, except replace USP Krill Oil RS with Krill Oil.
System suitability
Samples: *System suitability solution 1* and *Standard solution*
Suitability requirements
Resolution: NLT 1.0 between the methyl oleate and methyl *cis*-vaccinate peaks, *Standard solution*
Theoretical area percentages: Meets the requirements for *System suitability solution 1*
Chromatogram similarity: The chromatogram obtained from the *Standard solution* is similar to the reference chromatogram provided with the lot of USP Krill Oil RS being used.
Analysis
Sample: *Sample solution*
Identify the retention times of the relevant fatty acid methyl esters in the *Sample solution* by comparing the chromatogram of the *Sample solution* with that of the *Standard solution* and the USP reference chromatogram.
Calculate the area percentage for each fatty acid as methyl esters in the portion of Krill Oil taken:

$$\text{Result} = (r_A/r_B) \times 100$$

r_A = peak area of each individual fatty acid from the *Sample solution*
r_B = total area of all peaks, except the solvent and butylated hydroxytoluene peaks, from the *Sample solution*

Acceptance criteria: The fatty acids obtained from the *Sample solution* meet the limit requirements in *Table 1*.

Table 1

Fatty Acid	Shorthand Notation	Lower Limit (Area %)	Upper Limit (Area %)
Saturated fatty acids			

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Table 1 *(continued)*

Fatty Acid	Shorthand Notation	Lower Limit (Area %)	Upper Limit (Area %)
Myristic acid	14:0	▲5.0▲ 2S (USP41)	13.0
Palmitic acid	16:0	17.0	24.6
Palmitic acid:myristic acid ratio	16:0/14:0	1.6	▲3.6▲ 2S (USP41)
Monounsaturated fatty acids			
Palmitoleic acid	16:1 *n*–7	2.5	▲12.0▲ 2S (USP41)
cis-Vaccenic acid	18:1 *n*–7	4.7	8.0
Oleic acid	18:1 *n*–9	▲6.0▲ 2S (USP41)	14.5
Eicosenoic acid	20:1 *n*–9	0.0	2.0
Erucic acid	22:1 *n*–9	0.0	1.5
Polyunsaturated fatty acids			
Linoleic acid	18:2 *n*–6	0.0	3.0
Eicosapentaenoic acid	20:5 *n*–3	14.0	▲28.0▲ 2S (USP41)
Docosapentaenoic acid	22:5 *n*–3	0.0	0.7
Docosahexaenoic acid	22:6 *n*–3	7.1	15.7

Change to read:

- **B. PHOSPHOLIPID PROFILE**

Solution A, Line shape standard (^{1}H), Sensitivity standard (^{1}H), Sensitivity standard (^{31}P), Internal standard, Sample solution, Standard solution, Instrumental conditions, System suitability, and **Analysis:** Proceed as directed in the test for *Content of Total Phospholipids*.

Acceptance criteria: The *Sample solution* contains all of the following phospholipids: phosphatidylcholine, ▲lysophosphatidylcholine, and phosphatidylethanolamine. The sum of phosphatidylcholine, 1-lysophosphatidylcholine, and 2-lysophosphatidylcholine is 60%–96%.▲ 2S (USP41)

COMPOSITION

- **CONTENT OF EPA AND DHA**

Standard solution 1a, Standard solution 1b, Standard solution 2a, Standard solution 2b, System suitability solution 1, and **Chromatographic system:** Proceed as directed in *Fats and Fixed Oils* ⟨401⟩, *Procedures, Omega-3 Fatty Acids Determination and Profile.*

Test solution 1: Prepare as directed in *Fats and Fixed Oils* ⟨401⟩, *Procedures, Omega-3 Fatty Acids Determination and Profile, Content of EPA and DHA, Test Solution 1*, except use 250 mg of Krill Oil.

Test solution 2: Prepare as directed in *Fats and Fixed Oils* ⟨401⟩, *Procedures, Omega-3 Fatty Acids Determination and Profile, Content of EPA and DHA, Test Solution 2*, except use 250 mg of Krill Oil.

Analysis: Proceed as directed in *Fats and Fixed Oils* ⟨401⟩, *Procedures, Omega-3 Fatty Acids Determination and Profile, Content of EPA and DHA, Analysis (for triglycerides).*

Acceptance criteria: NLT 10.0% (w/w) of EPA and NLT 5.0% (w/w) of DHA

Change to read:

- **CONTENT OF TOTAL PHOSPHOLIPIDS**

(See *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩, *Qualitative and Quantitative NMR Analysis.*)

[NOTE—All deuterated solvents used in this method should be NLT 99.8 atom % D. Whenever water is used in this method, it should be of sufficient quality to ensure that no trace metals or other contaminants that may affect the analysis are present.]

Solution A: 0.2 M EDTA, adjusted with a 1 M cesium carbonate solution to a pH of 7.2–7.5. Document the final pH and the amount of 1 M cesium carbonate solution necessary to attain the desired pH. [NOTE—Use cesium carbonate of a sufficient grade for trace metals analysis.]

Line shape standard (^{1}H): 1% chloroform in acetone-d_6

Sensitivity standard (^{1}H): 0.1% ethylbenzene in chloroform-d

Sensitivity standard (^{31}P): 0.0485 M triphenyl phosphate in acetone-d_6

Internal standard: Use a suitable triphenyl phosphate NMR analytical standard with purity of NLT 99.0%.

Sample solution: [NOTE—NMR solvents containing tetramethylsilane (TMS) are readily available. If the solvents used do not contain TMS, it must be added to the *Sample solution* at an approximate concentration of 0.05% (v/v) for use as a chemical shift scale reference.] Transfer 300–350 mg of Krill Oil to a 5-mL sealable glass vial. Add 25.0 mg of the *Internal standard* to the vial. Add 1 mL each of deuterated chloroform (chloroform-d) and deuterated methanol (methanol-d_4) of a grade suitable for NMR analysis to the vial to dissolve the sample. Once dissolution is complete, add 1 mL of *Solution A*, seal the vial, and shake the solution for 10–20 min, then centrifuge the contents of the vial. Transfer the lower organic phase to an appropriate NMR tube. It is critical to collect the entire organic phase and transfer it to the NMR tube. It may be unavoidable to also transfer small amounts of the aqueous phase when collecting the organic phase in the NMR tube. This is acceptable practice, so long as the aqueous phase remains completely separated and atop the organic phase in the NMR tube. The entire amount of aqueous phase must be above the probe's radio frequency (RF) coil (outside the analysis area of the tube). Should the organic phase contain undissolved materials, they must remain suspended at the aqueous-organic interface and be outside the analysis area of the tube as well. The organic phase must be free of bubbles and suspended materials that may interfere with NMR data acquisition.

Standard solution: Prepare as directed in the *Sample solution*, using 300–350 mg of USP Krill Oil RS in place of the sample.

Instrumental conditions

(See *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩.)

Magnetic field strength: NLT 300 MHz for ^{1}H frequency

Probe: Direct observe probe capable of tuning to the resonance frequency of ^{31}P (dependent on the specific magnetic field strength used)

Instrument performance qualification

[NOTE—Testing for sensitivity and line shape should be performed on the interval specified by the manufacturer of the instrument used. Performing these tests on a minimum of a monthly basis is required for this method, but may be done more often, as required. Resolution testing is to be performed during each analysis and documented as a part of the analytical results.]

^{1}H Line shape test: Using the *Line shape standard (^{1}H)* and the protocol recommended by the instrument

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manufacturer, the instrument must achieve the line shape specifications for the probe in use, as required by the instrument manufacturer. [NOTE—A different standard solution may be required or recommended by the manufacturer of the instrument; 1% chloroform in acetone-d_6 is most commonly used.]

^{1}H Sensitivity test: Using the *Sensitivity standard (^{1}H)* and the protocol recommended by the instrument manufacturer, the instrument must achieve the sensitivity specifications required by the instrument manufacturer. [NOTE—A different standard solution may be required or recommended by the manufacturer of the instrument; 0.1% ethylbenzene in chloroform-d is most commonly used.]

^{31}P Sensitivity test: Using the *Sensitivity standard (^{31}P)* and the protocol recommended by the instrument manufacturer, the instrument must achieve the sensitivity specifications required by the instrument manufacturer. [NOTE—A different standard solution may be required or recommended by the manufacturer of the instrument; 0.0485 M triphenyl phosphate in acetone-d is most commonly used.]

^{1}H Resolution test: The resolution is demonstrated by the ability to detect both of the ^{29}Si satellite signals of TMS. The satellites must be resolved from the TMS signal in the spectrum with a line broadening factor of NMT 0.5 ppm.

^{31}P Resolution test: The resolution is demonstrated using the phosphatidylcholine ether peak and the phosphatidylcholine peak. The separation of these peaks (with an applied line broadening factor of 1.0) must be demonstrated, as follows. Using the baseline as a reference, determine the total peak height of the phosphatidylcholine ether peak , and draw a line at 30% of that total peak height (intensity). [NOTE—The phosphatidylcholine ether signal appears just downfield from the phosphatidylcholine signal.] The phosphatidylcholine ether peak and the neighboring phosphatidylcholine peak must be fully resolved at a point that is NMT 30% of the peak height of the phosphatidylcholine ether peak.

Data collection: Use the parameters specified in *Table 2*. Use 90° pulses, and calibrate pulses before use according to the recommendations supplied by the instrument manufacturer.

Table 2

Parameter	^{31}P NMR Quantitative Measurement	^{1}H NMR Qualitative Measurement
Pulse program	^{1}H-decoupled ^{31}P (inverse gated)	Single pulse ^{1}H
Spectral width	50 ppm (25 ppm to −25 ppm)	20 ppm (−3 ppm to 17 ppm)
Transmitter offset	Center of spectral width, 0 ppm	Center of spectral width, 7 ppm
Relaxation delay	5–15 s	2–5 s
Acquisition time	1–6 s	1–6 s
Size of data set	NLT 64k (32k with zero-filling)	NLT 64k (32k with zero-filling)

[NOTE—The acquisition time is dependent upon the dwell time and the number of data points collected. The number of scans acquired using a 300 MHz instrument must be NLT 512.]

System suitability: Under the conditions outlined in *Data collection*, the ^{31}P NMR signal of triphenyl phosphate should be observed at −17.80 ppm, and the ^{1}H NMR spectrum should be referenced to the ^{1}H signal of TMS (0 ppm) for all spectra acquired in the *Analysis*. For quantitative analysis, a sufficient number of scans should be acquired such that the signal-to-noise ratio for the phosphatidylcholine signal in the ^{31}P spectrum of the *Sample solution* acquired in the *Analysis* is NLT 2000.

Analysis: Acquire the data outlined in *Data collection*. Minimally acquire the ^{1}H spectrum (fingerprint) of the *Sample solution* and the *Standard solution* as well as the quantitative ^{31}P spectrum of the *Sample solution* and the *Standard solution*. Record the resulting spectra, and perform integration by hand or automated means on the quantitative ^{31}P NMR spectrum of the *Sample solution*. Integration of the peaks contained in the spectrum of the *Sample solution* must be performed such that the complete set of phospholipid peaks (as identified by comparison to the spectrum of the *Standard solution* and its reference spectrum) is included in the integration. The integration region for each signal must extend ±0.05 ppm on either side of the ^{31}P signal. Quantify the total phospholipids present, the phosphatidylcholine ether content, and the phosphatidylcholine content in the *Sample solution* using comparison to the concentration of the *Internal standard*. Compare the ^{1}H spectrum of the *Sample solution* to that of the *Standard solution* to determine the similarity of fingerprints according to which phospholipids identified in the reference spectrum of the *Standard solution* are present in the spectrum of the *Sample solution*.

Calculations: Use the following equations and molecular weights listed in *Table 3* to determine the phospholipids content in the sample taken:

$$\text{mmol}_{IS} = (W_{IS} \times C_{IS})/(MW_{IS} \times 100)$$

mmol_{IS} = millimoles of the *Internal standard* in the *Sample solution* (mmol)
W_{IS} = weight of the *Internal standard* added to the *Sample solution* (mg)
C_{IS} = purity value of the *Internal standard*, based on quantitative ^{31}P NMR analysis (% by weight)
MW_{IS} = molecular weight of the *Internal standard*, 326.28 g/mol (for triphenyl phosphate)

$$\text{mmol}_{PL} = (I_{PL} \times A_{IS} \times \text{mmol}_{IS})/(I_{IS} \times A_{PL})$$

mmol_{PL} = millimoles of the phospholipid of interest in the *Sample solution* (mmol)
I_{PL} = integrated area under the phospholipid signal of interest obtained from the spectrum of the *Sample solution*
A_{IS} = number of phosphorus atoms per molecule expected from the *Internal standard*, 1 (for triphenyl phosphate)
mmol_{IS} = millimoles of the *Internal standard* in the *Sample solution* (mmol)
I_{IS} = integrated area under the *Internal standard* obtained from the spectrum of the *Sample solution*
A_{PL} = number of phosphorus atoms per molecule expected from the phospholipid of interest, 1 (for any phospholipid listed in *Table 3*)

$$C_{PL} = (MW_{PL} \times \text{mmol}_{PL} \times 100)/W_S$$

C_{PL} = concentration of the phospholipid of interest in the *Sample solution* (%, w/w)

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MW_{PL} = molecular weight of the phospholipid of interest (g/mol, from *Table 3*) in the *Sample solution* (mg)
$mmol_{PL}$ = millimoles of the phospholipid of interest in the *Sample solution* (mmol)
W_S = weight of the sample present in the *Sample solution* (mg)

[NOTE—Use the molecular weight specified in *Table 3* for the calculations.]

Table 3

Component	Approximate Chemical Shift (ppm) in Reference to Triphenyl Phosphate	Molecular Weight (g/mol)
Triphenyl phosphate (*Internal standard*)	−17.8	—
Phosphatidylcholine (PC)	−0.89	791
1-Lysophosphatidylcholine (1-LPC)[a]	−0.48	534.5
2-Lysophosphatidylcholine (2-LPC)[a]	−0.4	534.5
Phosphatidylethanolamine (PE)	−0.24	770
N-Acylphosphatidylethanolamine (NAPE)	0	1032
Lysophosphatidylethanolamine (LPE)	0.25	492.5
Other	—	800

[a] Ability to resolve the signals of 1-lysophosphatidylcholine and 2-lysophosphatidylcholine will depend upon the applied magnetic field strength of the NMR spectrometer used for the test procedure.

Acceptance criteria
Total phospholipids: 30%–59% (w/w)
▲Sum of phosphatidylcholine, 1-lysophosphatidylcholine, and 2-lysophosphatidylcholine:▲ 2S (USP41) 60%–96% (w/w) of the *Total phospholipids*

Change to read:

• **CONTENT OF ASTAXANTHIN**
[NOTE—Perform this analysis in subdued light, using low-actinic glassware.]
Sample solution: 0.005 g/mL of Krill Oil in chloroform. [NOTE—If the solution is not clear, centrifuge it with an appropriate centrifuge to obtain a clear supernatant.]
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Analytical wavelength: 486 nm
Cell: 1 cm
Blank: Chloroform
Analysis
Sample: *Sample solution*
Calculate the percentage of astaxanthin in the portion of Krill Oil taken:

$$\text{Result} = A/(F \times C)$$

A = absorbance of the *Sample solution*
F = coefficient of extinction ($E^{1\%}$) of pure astaxanthin in chloroform (100 mL · g^{-1} · cm^{-1}), 1692
C = concentration of the *Sample solution* (g/mL)

Acceptance criteria: NLT **▲0.005%▲ 2S (USP41)**

CONTAMINANTS
• **LIMIT OF DIOXINS, FURANS, AND POLYCHLORINATED BIPHENYLS**
Analysis: Determine the content of polychlorinated dibenzo-para-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) by Method No. 1613 Revision B of the Environmental Protection Agency. Determine the content of polychlorinated biphenyls (PCBs) by Method No. 1668 Revision A of the Environmental Protection Agency.
Acceptance criteria: The sum of PCDDs and PCDFs is NMT 2.0 pg/g of World Health Organization (WHO) toxic equivalents. The sum of PCDDs, PCDFs, and dioxin-like PCBs (polychlorinated biphenyls, non-ortho IUPAC congeners PCB-77, PCB-81, PCB-126, and PCB-169, and mono-ortho IUPAC congeners PCB-105, PCB-114, PCB-118, PCB-123, PCB-156, PCB-157, PCB-167, and PCB-189) is NMT 3.0 pg/g of WHO toxic equivalents.

SPECIFIC TESTS
• **ASTAXANTHIN ESTERIFICATION**
Standard solution A: 10 mg/mL of USP Astaxanthin Esters from *Haematococcus pluvialis* RS in acetone
Standard solution B: 10 mg/mL of USP Astaxanthin (Synthetic) RS in acetone
Sample solution: 250 mg/mL of Krill Oil in acetone
Chromatographic system
(See *Chromatography* ⟨621⟩, *General Procedures, Thin-Layer Chromatography.*)
Mode: TLC
Adsorbent: 0.25-mm layer of chromatographic silica gel. [NOTE—Dry silica gel at 110° for 1 h before use.]
Application volume: 5 µL
Developing solvent system: Hexane and acetone (70:30)
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Develop the chromatogram in the *Developing solvent system* until the solvent front has moved about 15 cm of the length of the plate. Remove the plate from the chamber, and allow to dry.
Acceptance criteria: The principal spot from *Standard solution B*, located in the bottom half of the plate, is free astaxanthin. The *Sample solution* may exhibit a light, minor spot, in the same location. The principal spots from *Standard solution A* are from monoesters (primary spot, located slightly above the middle of the plate) and diesters (secondary spot, located in the top third of the plate). The principal spot from the *Sample solution* should correspond in color and R_F value to the diester spot from *Standard solution A*. The secondary spot from the *Sample solution* should correspond in color and approximately the same R_F value to the monoester spot from *Standard solution A*. [NOTE—Slight differences in R_F values within monoester spots and within diester spots may exist because of different intensities.]
• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Peroxide Value*: NMT 5.0 mEq peroxide/kg

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at controlled room temperature. It may be bottled or otherwise packaged in containers from which air has been expelled by production of a vacuum or by an inert gas.
• **LABELING:** The label states the average content of DHA and EPA in mg/g. It also states the name and concentration of any added antioxidant.

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Change to read:

• **USP REFERENCE STANDARDS** ⟨11⟩
USP Astaxanthin Esters from *Haematococcus pluvialis* RS
USP Astaxanthin (Synthetic) RS
▲ ▲ (CN 1-May-2018)
USP Krill Oil RS
▲ ▲ (CN 1-May-2018)

Krill Oil Capsules

DEFINITION

Change to read:

▲Krill Oil Capsules contain NLT 95.0% of the labeled amount of Krill Oil calculated through the content of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and total phospholipids. ▲ (USP 1-May-2019)

IDENTIFICATION

Change to read:

• **A. FATTY ACID PROFILE**
Antioxidant solution, System suitability solution 1, and **Chromatographic system:** Proceed as directed in *Fats and Fixed Oils* ⟨401⟩, *Procedures, Omega-3 Fatty Acids Determination and Profile.*
Standard solution: Prepare as directed in *Fats and Fixed Oils* ⟨401⟩, *Procedures, Omega-3 Fatty Acids Determination and Profile, Test solution 1*, except use 250 mg of USP Krill Oil RS.
Sample solution: Using the portion of oil from NLT 10 Capsules, prepare as directed in *Fats and Fixed Oils* ⟨401⟩, *Procedures, Omega-3 Fatty Acids Determination and Profile, Test solution 1.*
System suitability
Samples: *System suitability solution 1* and *Standard solution*
Suitability requirements
Chromatogram similarity: The chromatogram from the *Standard solution* is similar to the reference chromatogram provided with the lot of USP Krill Oil RS being used.
Resolution: NLT 1.0 between the methyl oleate and methyl *cis*-vaccinate peaks, *Standard solution*
Theoretical area percentages: Meet the requirements for *System suitability solution 1*
Analysis
Sample: *Standard solution* and *Sample solution*
Identify the retention times of the peaks corresponding to the relevant fatty acid methyl esters by comparing the chromatogram of the *Standard solution* to the reference chromatogram provided with the lot of USP Krill Oil RS used.
Calculate the area percentage for each fatty acid as methyl esters in the portion of oil taken from the Capsules:

$$\text{Result} = (r_A/r_B) \times 100$$

r_A = peak area of each individual fatty acid from the *Sample solution*
r_B = total area of all peaks, except the solvent and butylated hydroxytoluene peaks, from the *Sample solution*

Acceptance criteria: See *Table 1.*

Table 1

Fatty Acid	Short-Hand Notation	Lower Limit (Area %)	Upper Limit (Area %)
Saturated fatty acids			
Myristic acid	14:0	▲5.0▲ (USP 1-May-2019)	13.0
Palmitic acid	16:0	17.0	24.6
Palmitic acid:myristic acid ratio	16:0/14:0	1.6	▲3.6▲ (USP 1-May-2019)
Monounsaturated fatty acids			
Palmitoleic acid	16:1 n-7	2.5	▲12.0▲ (USP 1-May-2019)
cis-Vaccenic acid	18:1 n-7	4.7	8.0
Oleic acid	18:1 n-9	▲6.0▲ (USP 1-May-2019)	14.5
Eicosenic acid	20:1 n-9	0.0	2.0
Erucic acid	22:1 n-9	0.0	1.5
Polyunsaturated fatty acids			
Linoleic acid	18:2 n-6	0.0	3.0
Eicosapentaenoic acid	20:5 n-3	14.0	▲28.0▲ (USP 1-May-2019)
Docosapentaenoic acid	22:5 n-3	0.0	0.7
Docosahexaenoic acid	22:6 n-3	7.1	15.7

Change to read:

• **B. PHOSPHOLIPID PROFILE**
Solution A, ▲ ▲ (USP 1-May-2019) **Internal standard,** ▲**Sample stock solution,** ▲ (USP 1-May-2019) **Sample solution, Standard solution, Instrumental conditions, System suitability,** and **Analysis:** *Content of Total PhospholipidsStrength*
Acceptance criteria: ▲The *Sample solution* shows ^{31}P nuclear magnetic resonance (NMR) spectra similar to those obtained with USP Krill Oil RS. The main signal in the ^{31}P NMR is due to phosphatidylcholine (PC); a signal due to phosphatidylcholine ether has an intensity of about 10% of that due to PC; and the second signal in intensity is due to 2-lysophosphatidylcholine (2-LPC). Minor signals due to phosphatidylethanolamine (PE), *N*-acylphosphatidylethanolamine (NAPE), lysophosphatidylethanolamine (LPE), and 1-lysophosphatidylcholine (1-LPC) among others are also observed. ▲ (USP 1-May-2019)

STRENGTH

Delete the following:

▲• **CONTENT OF KRILL OIL**
Analysis: Weigh NLT 10 Capsules in a tared weighing bottle, and carefully open the Capsules, without loss of shell material. Transfer the combined Capsule contents to a 100-mL beaker. Remove any adhering substance from the emptied Capsules by washing with several small portions of 2,2,4-trimethylpentane. Discard the washings, and allow the empty Capsules to dry in a current of dry air until the 2,2,4-trimethylpentane is completely evaporated. Weigh the empty Capsules in the original tared weighing bottle, and calculate the average net weight per Capsule.
Acceptance criteria: 95.0%–105.0%▲ (USP 1-May-2019)

DS Monographs

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Change to read:

- **CONTENT OF EPA AND DHA**

(See *Fats and Fixed Oils* ⟨401⟩, *Procedures, Omega-3 Fatty Acids Determination and Profile*).

Standard solution 1a, Standard solution 1b, Standard solution 2a, Standard solution 2b, System suitability solution 1, and **Chromatographic system:** Proceed as directed in the chapter.

Test solution 1: ▲Weigh NLT 10 Capsules in a tared weighing bottle, and carefully open the Capsules without loss of shell material. Transfer the combined contents to a 100-mL beaker. Remove any adhering substance from the emptied Capsules by washing with several small portions of acetone. Discard the washings, and allow the empty Capsules to dry in a current of air until the acetone is completely evaporated. Weigh the empty Capsules in the original tared weighing bottle, and calculate the average fill weight per Capsule. Take 250 mg of the combined Capsule contents and▲ (USP 1-May-2019) proceed as directed in *Fats and Fixed Oils* ⟨401⟩, *Procedures, Omega-3 Fatty Acids Determination and Profile, Test solution 1*.

Test solution 2: ▲▲ (USP 1-May-2019) Prepare as directed in *Fats and Fixed Oils* ⟨401⟩, *Procedures, Omega-3 Fatty Acids Determination and Profile, Test solution 2* ▲using 250 mg of the combined Capsule contents.▲ (USP 1-May-2019)

Analysis: Proceed as directed in *Fats and Fixed Oils* ⟨401⟩, *Procedures, Omega-3 Fatty Acids Determination and Profile, Analysis (for triglycerides)*.

▲Calculate the percentage of the labeled amounts of EPA and DHA in the portion of Capsules taken:

$$\text{Result} = A \times W_{Av} \times 100/L$$

A = content of EPA or DHA in the portion of Capsules taken (mg/mg)
W_{Av} = average fill weight (mg/Capsule)
L = label claim of EPA or DHA (mg/Capsule)▲ (USP 1-May-2019)

Acceptance criteria: ▲NLT 95.0% of the labeled amounts of EPA and DHA▲ (USP 1-May-2019)

Change to read:

- **CONTENT OF TOTAL PHOSPHOLIPIDS**

(See *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩, *Qualitative and Quantitative NMR Analysis*.)

[NOTE—All deuterated solvents used in this method should be NLT 99.8 atom % D.▲▲ (USP 1-May-2019)]

Solution A: 0.2 M ethylenediaminetetraacetic acid (EDTA) adjusted with a 1 M cesium carbonate solution to a pH of 7.2–7.5.▲▲ (USP 1-May-2019) [NOTE—Use cesium carbonate of a sufficient grade for trace metals analysis.]

▲▲ (USP 1-May-2019)

Internal standard: Use a triphenyl phosphate nuclear magnetic resonance (NMR) reference standard with NLT 99% purity.

▲Sample stock solution: Transfer a number of Capsules (NLT 5) to a conical flask with stopper. Add 2 mL/Capsule of water and melt the gelatin shells at 50°–60°. Add 10 mL/Capsule of chloroform and methanol (2:1), insert the stopper and shake intensively for 30 min. Transfer to a separation funnel fitted with glass wool. Collect the organic phase. Wash the conical flask with chloroform and methanol (2:1) and transfer to a separation funnel. Collect the organic phase and combine with previous extract in a suitable volumetric flask. Dilute with chloroform and methanol (2:1) to volume.

Sample solution: Transfer an aliquot of the *Sample stock solution* equivalent to 300 mg of Krill Oil to a suitable sealable glass vial, and add about 25 mg of the *Internal standard* accurately weighed. Evaporate to dryness. Add 2 mL of deuterated chloroform (chloroform-d) containing 0.05% tetramethylsilane (TMS), 2 mL of deuterated methanol (methanol-d4), and 2 mL of *Solution A*. Seal the vial and vortex intensively for 30–60 s, shake for an additional 30 min on a shaking device, and centrifuge the contents of the vial. Pass the entire amount of the lower organic phase through a glass fiber filter, and collect the filtrate in the appropriate NMR tube.▲ (USP 1-May-2019)

Standard solution: ▲Transfer 300 mg of USP Krill Oil RS to a suitable sealable glass vial, and add about 25 mg of the *Internal standard* accurately weighed. Add 2 mL of methanol-d4, 2 mL of chloroform-d containing 0.05% TMS, and 2 mL of *Solution A*. Seal the vial and vortex intensively for 30–60 s, shake for an additional 30 min on a shaking device, and centrifuge the contents of the vial. Pass the entire amount of the lower organic phase through a glass fiber filter, and collect the filtrate in the appropriate NMR tube.▲ (USP 1-May-2019)

Instrumental conditions

Magnetic field strength: ▲NLT 7.05 Tesla (resonance frequencies of 121 MHz for ^{31}P or 300 MHz for ^{1}H)▲ (USP 1-May-2019)

Probe: Direct observe probe capable of tuning to the resonance frequency of ^{31}P (dependent on the specific magnetic field strength used) ▲at a temperature of 35°▲ (USP 1-May-2019)

Data collection: Use the parameters specified in *Table 2*. Use 90° pulses, and calibrate pulses before use according to the recommendations supplied by the instrument manufacturer.

Table 2

Parameter	^{31}P NMR Quantitative Measurement	▲▲ (USP 1-May-2019)
Pulse program	^{1}H-decoupled ^{31}P (inverse gated)	▲▲ (USP 1-May-2019)
Spectral width	50 ppm (25 to –25 ppm)	▲▲ (USP 1-May-2019)
Transmitter offset	Center of spectral width, 0 ppm	▲▲ (USP 1-May-2019)
Relaxation delay	5–15 s	▲▲ (USP 1-May-2019)
Acquisition time	1–6 s	▲▲ (USP 1-May-2019)
Size of data set	NLT 64k (32k with zero-filling)	▲▲ (USP 1-May-2019)

[NOTE—The acquisition time is dependent upon the dwell time and the number of data points collected. The number of scans acquired using a ▲7.05 Tesla magnet▲ (USP 1-May-2019) must be NLT 512.]

System suitability: ▲The *Standard solution* shows the ^{31}P NMR signal for triphenyl phosphate at –17.80 ppm, and the signal for phosphatidylcholine at –0.89 ppm. The signal-to-noise ratio for the phosphatidylcholine signal in the ^{31}P spectrum of the *Sample solution* obtained in the *Analysis* is NLT 2000. Using the baseline as a reference, determine the height of the phosphatidylcholine ether peak and draw a line parallel to the baseline at 30% of that total peak height (intensity). The valley between the peaks of

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phosphatidylcholine ether and phosphatidylcholine is below the line drawn.▲ (USP 1-May-2019)

Analysis: ▲Obtain the quantitative ^{31}P spectrum of the *Sample solution* and the *Standard solution* as directed in *Data collection*. Record the resulting spectra, and integrate the complete set of phospholipid peaks as identified by a comparison with the reference spectrum provided with USP Krill Oil RS. The integration region for each signal must extend ±0.05 ppm on either side of the ^{31}P signal.▲ (USP 1-May-2019)

Calculations: Use the following equations and molecular weights listed in *Table 3* to ▲calculate the content of the phospholipid of interest in the Capsules taken:▲ (USP 1-May-2019)

$$mmol_{IS} = (W_{IS} \times C_{IS})/(MW_{IS} \times 100)$$

$mmol_{IS}$ = millimoles of the *Internal standard* in the *Sample solution* (mmol)
W_{IS} = weight of the *Internal standard* added to the *Sample solution* (mg)
C_{IS} = purity value of the *Internal standard*, based on quantitative ^{31}P NMR analysis (% by weight)
MW_{IS} = molecular weight of the *Internal standard*, 326.28 g/mol (for triphenyl phosphate)

$$mmol_{PL} = (I_{PL} \times A_{IS} \times mmol_{IS})/(I_{IS} \times A_{PL})$$

$mmol_{PL}$ = millimoles of the phospholipid of interest in the *Sample solution* (mmol)
I_{PL} = integrated area under the phospholipid signal of interest obtained from the spectrum of the *Sample solution*
A_{IS} = number of phosphorus atoms per molecule expected from the *Internal standard*, 1 (for triphenyl phosphate)
$mmol_{IS}$ = millimoles of the *Internal standard* in the *Sample solution*
I_{IS} = integrated area under the *Internal standard* obtained from the spectrum of the *Sample solution*
A_{PL} = number of phosphorus atoms per molecule expected from the phospholipid of interest, 1 (for any phospholipid listed in *Table 3*)

$$▲C_{PL} = 1/n \times MW_{PL} \times mmol_{PL} \times V/a$$

C_{PL} = content of the phospholipid of interest in the Capsules taken (mg/Capsule)
n = number of Capsules used to prepare the *Sample stock solution*
MW_{PL} = molecular weight of the phospholipid of interest (mg/mmol, from *Table 3*)
$mmol_{PL}$ = millimoles of the phospholipid of interest in the *Sample solution* (mmol)
V = volume of volumetric flask used to prepare the *Sample stock solution* (mL)
a = volume of the aliquot of *Sample stock solution* used to prepare the *Sample solution* (mL)▲ (USP 1-May-2019)

Table 3

Component	Approximate Chemical Shift (ppm) in Reference to Triphenyl Phosphate	Molecular Weight (g/mol)
Triphenyl phosphate (*Internal standard*)	–17.8	—
Phosphatidylcholine, including ether (PC)	–0.89	791
1-Lysophosphatidylcholine (1-LPC)[a]	–0.48	534.5
2-Lysophosphatidylcholine (2-LPC)[a]	–0.4	534.5
Phosphatidylethanolamine (PE)	–0.24	770
N-Acylphosphatidylethanolamine (NAPE)	0	1032
Lysophosphatidylethanolamine (LPE)	0.25	492.5
Other	—	800

[a] Ability to resolve the signals of 1-LPC and 2-LPC will depend upon the applied magnetic field strength of the NMR spectrometer used for the test procedure.

▲Calculate the percentage of the labeled amount of total phospholipids per Capsule taken:

$$Result = \Sigma C_{PL} \times 100/L$$

C_{PL} = sum of the individual amounts of phospholipids of interest in the Capsules taken (mg/Capsule)
L = label claim of total phospholipids (mg/Capsule)▲ (USP 1-May-2019)

Acceptance criteria: ▲NLT 95.0% of the labeled amount of total phospholipids▲ (USP 1-May-2019)

Change to read:

- **CONTENT OF ASTAXANTHIN**

[NOTE—Perform this analysis in subdued light using low-actinic glassware.]

Sample solution: 0.005 g/mL of Krill Oil in chloroform using the portion of oil from NLT 10 Capsules. [NOTE—If the solution is not clear, centrifuge it with an appropriate centrifuge to obtain a clear supernatant.]

Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Analytical wavelength: 486 nm
Cell: 1 cm
Blank: Chloroform

Analysis
Sample: *Sample solution*
Calculate the percentage of astaxanthin in the portion of Krill Oil taken from the Capsules:

$$Result = A/(F \times C)$$

A = absorbance of the *Sample solution*
F = coefficient of extinction ($E^{1\%}$) of pure astaxanthin in chloroform (100 mL · g^{-1} · cm^{-1}), 1692
C = concentration of the *Sample solution* (g/mL)

Acceptance criteria: NLT ▲0.005%▲ (USP 1-May-2019)

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩, *Rupture Test for Soft Shell Capsules*: Meet the requirements

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• **Weight Variation** ⟨2091⟩: Meet the requirements

CONTAMINANTS

• **Limit of Dioxins, Furans, and Polychlorinated Biphenyls**

Analysis: Determine the content of polychlorinated dibenzo-para-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) by Method 1613, Revision B of the Environmental Protection Agency (EPA). Determine the content of polychlorinated biphenyls (PCBs) by Method 1668, Revision A of the EPA.

Acceptance criteria: The sum of PCDDs and PCDFs is NMT 2.0 pg/g of World Health Organization (WHO) toxic equivalents. The sum of PCDDs, PCDFs, and dioxin-like PCBs [non-ortho International Union of Pure and Applied Chemistry (IUPAC) congeners PCB-77, PCB-81, PCB-126, and PCB-169; and mono-ortho IUPAC congeners PCB-105, PCB-114, PCB-118, PCB-123, PCB-156, PCB-157, PCB-167, and PCB-189] is NMT 10.0 pg/g of WHO toxic equivalents.

• **Microbial Enumeration Tests** ⟨2021⟩: The total aerobic microbial count does not exceed 10^3 cfu/g, and the combined molds and yeasts count does not exceed 10^2 cfu/g.

• **Absence of Specified Microorganisms** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meet the requirements

SPECIFIC TESTS

• **Astaxanthin Esterification**

Standard solution A: 10 mg/mL of USP Astaxanthin Esters from *Haematococcus pluvialis* RS in acetone

Standard solution B: 10 mg/mL of USP Astaxanthin (Synthetic) RS in acetone

Sample solution: Using the portion of oil from NLT 10 Capsules, prepare a solution of 250 mg/mL in acetone.

Chromatographic system

(See *Chromatography* ⟨621⟩, *General Procedures, Thin-Layer Chromatography*.)

Mode: TLC

Adsorbent: 0.25-mm layer of chromatographic silica gel. [Note—Dry silica gel at 110° for 1 h before use.]

Application volume: 5 µL

Developing solvent system: Hexane and acetone (70:30)

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatogram in the *Developing solvent system* until the solvent front has moved about 15 cm of the length of the plate. Remove the plate from the chamber, and allow to dry.

Acceptance criteria: The principal spot from *Standard solution B*, located in the bottom half of the plate, is free astaxanthin. The *Sample solution* may exhibit a light, minor spot in the same location. The principal spots from *Standard solution A* are from monoesters (primary spot, located slightly above the middle of the plate) and diesters (secondary spot, located in the top third of the plate). The principal spot from the *Sample solution* should correspond in color and R_F value to the diester spot from *Standard solution A*. The secondary spot from the *Sample solution* should correspond in color and approximately the same R_F value to the monoester spot from *Standard solution A*. [Note—Slight differences in R_F values within monoester spots and within diester spots may exist because of different intensities.]

• **Fats and Fixed Oils** ⟨401⟩, *Procedures, Peroxide Value*: NMT 5.0 mEq peroxide/kg

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Preserve in tight containers, and store at room temperature. Protect from light.

Change to read:

• **Labeling:** The label states the amount of docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and total phospholipids ▲in mg/Capsule.▲ (USP 1-May-2019)

Change to read:

• **USP Reference Standards** ⟨11⟩

USP Astaxanthin (Synthetic) RS

USP Astaxanthin Esters from *Haematococcus pluvialis* RS ▲▲ (USP 1-May-2019)

USP Krill Oil RS ▲▲ (USP 1-May-2019)

Krill Oil Delayed-Release Capsules

DEFINITION

Change to read:

▲Krill Oil Delayed-Release Capsules contain NLT 95.0% of the labeled amount of Krill Oil calculated through the content of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and total phospholipids.▲ (USP 1-May-2019)

IDENTIFICATION

Change to read:

• **A. Fatty Acid Profile**

Antioxidant solution, System suitability solution 1, and **Chromatographic system:** Proceed as directed in *Fats and Fixed Oils* ⟨401⟩, *Procedures, Omega-3 Fatty Acids Determination and Profile*.

Standard solution: Prepare as directed in *Fats and Fixed Oils* ⟨401⟩, *Procedures, Omega-3 Fatty Acids Determination and Profile, Test solution 1*, except use 250 mg of USP Krill Oil RS.

Sample solution: Using the portion of oil from NLT 10 Capsules, prepare as directed in *Fats and Fixed Oils* ⟨401⟩, *Procedures, Omega-3 Fatty Acids Determination and Profile, Test solution 1*.

System suitability

Samples: *System suitability solution 1* and *Standard solution*

Suitability requirements

Chromatogram similarity: The chromatogram from the *Standard solution* is similar to the reference chromatogram provided with the lot of USP Krill Oil RS being used.

Resolution: NLT 1.0 between the methyl oleate and methyl *cis*-vaccinate peaks, *Standard solution*

Theoretical area percentages: Meet the requirements, *System suitability solution 1*

Analysis

Sample: *Standard solution* and *Sample solution*

Identify the retention times of the peaks corresponding to the relevant fatty acid methyl esters by comparing the chromatogram of the *Standard solution* to the reference chromatogram provided with the lot of USP Krill Oil RS used.

Calculate the area percentage for each fatty acid as methyl esters in the portion of oil taken from the Capsules:

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$$\text{Result} = (r_A/r_B) \times 100$$

r_A = peak area of each individual fatty acid from the *Sample solution*

r_B = total area of all peaks, except the solvent and butylated hydroxytoluene peaks, from the *Sample solution*

Acceptance criteria: See *Table 1*.

Table 1

Fatty Acid	Short-Hand Notation	Lower Limit (Area %)	Upper Limit (Area %)
Saturated fatty acids			
Myristic acid	14:0	▲5.0▲ (USP 1-May-2019)	13.0
Palmitic acid	16:0	17.0	24.6
Palmitic acid:myristic acid ratio	16:0/14:0	1.6	▲3.6▲ (USP 1-May-2019)
Monounsaturated fatty acids			
Palmitoleic acid	16:1 n-7	2.5	▲12.0▲ (USP 1-May-2019)
cis-Vaccenic acid	18:1 n-7	4.7	8.0
Oleic acid	18:1 n-9	▲6.0▲ (USP 1-May-2019)	14.5
Eicosenic acid	20:1 n-9	0.0	2.0
Erucic acid	22:1 n-9	0.0	1.5
Polyunsaturated fatty acids			
Linoleic acid	18:2 n-6	0.0	3.0
Eicosapentaenoic acid	20:5 n-3	14.0	▲28.0▲ (USP 1-May-2019)
Docosapentaenoic acid	22:5 n-3	0.0	0.7
Docosahexaenoic acid	22:6 n-3	7.1	15.7

Change to read:

- **B. PHOSPHOLIPID PROFILE**

Solution A, ▲▲ (USP 1-May-2019) **Internal standard,** ▲**Sample stock solution,**▲ (USP 1-May-2019) **Sample solution, Standard solution, Instrumental conditions, System suitability,** and **Analysis:** *Content of Total PhospholipidsStrength*

Acceptance criteria: ▲The *Sample solution* shows ^{31}P nuclear magnetic resonance (NMR) spectra similar to those obtained from USP Krill Oil RS. The main signal in the ^{31}P NMR is due to phosphatidylcholine (PC); a signal due to phosphatidylcholine ether has an intensity of about 10% of that due to PC; and the second signal in intensity is due to 2-lysophosphatidylcholine (2-LPC). Minor signals due to phosphatidylethanolamine (PE), *N*-acylphosphatidylethanolamine (NAPE), lysophosphatidylethanolamine (LPE), and 1-lysophosphatidylcholine (1-LPC) among others are also observed.▲ (USP 1-May-2019)

STRENGTH

Delete the following:

- **▲• CONTENT OF KRILL OIL**

Analysis: Weigh NLT 10 Capsules in a tared weighing bottle, and carefully open the Capsules, without loss of shell material. Transfer the combined Capsule contents to a 100-mL beaker. Remove any adhering substance from the emptied Capsules by washing with several small portions of 2,2,4-trimethylpentane. Discard the washings, and allow the empty Capsules to dry in a current of dry air until the 2,2,4-trimethylpentane is completely evaporated. Weigh the empty Capsules in the original tared weighing bottle, and calculate the average net weight per Capsule.

Acceptance criteria: 95.0%–105.0%▲ (USP 1-May-2019)

Change to read:

- **CONTENT OF EPA AND DHA**

(See *Fats and Fixed Oils* ⟨401⟩, *Procedures, Omega-3 Fatty Acids Determination and Profile*).

Standard solution 1a, Standard solution 1b, Standard solution 2a, Standard solution 2b, System suitability solution 1, and **Chromatographic system:** Proceed as directed in the chapter.

Test solution 1: ▲Weigh NLT 10 Capsules in a tared weighing bottle, and carefully open the Capsules without loss of shell material. Transfer the combined contents to a 100-mL beaker. Remove any adhering substance from the emptied Capsules by washing with several small portions of acetone. Discard the washings, and allow the empty Capsules to dry in a current of air until the acetone is completely evaporated. Weigh the empty Capsules in the original tared weighing bottle, and calculate the average net weight per Capsule. Take 250 mg of the combined Capsule contents and▲ (USP 1-May-2019) prepare as directed in *Fats and Fixed Oils* ⟨401⟩, *Procedures, Omega-3 Fatty Acids Determination and Profile, Test solution 1*.

Test solution 2: ▲▲ (USP 1-May-2019)Prepare as directed in *Fats and Fixed Oils* ⟨401⟩, *Procedures, Omega-3 Fatty Acids Determination and Profile, Test solution 2* ▲using 250 mg of the combined Capsule contents.▲ (USP 1-May-2019)

Analysis: Proceed as directed in *Fats and Fixed Oils* ⟨401⟩, *Procedures, Omega-3 Fatty Acids Determination and Profile, Analysis (for triglycerides)*.

▲Calculate the percentage of the labeled amounts of EPA and DHA in the portion of Capsules taken:

$$\text{Result} = A \times W_{AV} \times 100/L$$

A = content of EPA or DHA in the portion of Capsules taken (mg/mg)

W_{AV} = average fill weight (mg/Capsule)

L = label claim of EPA or DHA (mg/Capsule)▲ (USP 1-May-2019)

Acceptance criteria: ▲NLT 95.0% of the labeled amounts of EPA and DHA▲ (USP 1-May-2019)

Change to read:

- **CONTENT OF TOTAL PHOSPHOLIPIDS**

(See *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩, *Qualitative and Quantitative NMR Analysis*.)

[NOTE—All deuterated solvents used in this method should be NLT 99.8 atom % D.▲▲ (USP 1-May-2019)]

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Solution A: 0.2 M ethylenediaminetetraacetic acid (EDTA) adjusted with a 1 M cesium carbonate solution to a pH of 7.2–7.5.▲ ▲ (USP 1-May-2019) [NOTE—Use cesium carbonate of a sufficient grade for trace metals analysis.]
▲ ▲ (USP 1-May-2019)

Internal standard: Use a triphenyl phosphate nuclear magnetic resonance (NMR) reference standard with NLT 99% purity.

▲Sample stock solution: Transfer a number of Capsules (NLT 5) to a conical flask with stopper. Add 2 mL/Capsule of water and melt the gelatin shells at 50°–60°. Add 10 mL/Capsule of chloroform and methanol (2:1), insert the stopper and shake intensively for 30 min. Transfer to a separation funnel fitted with glass wool. Collect the organic phase. Wash the conical flask with chloroform and methanol (2:1) and transfer to a separation funnel. Collect the organic phase and combine with previous extract in a suitable volumetric flask. Dilute with chloroform and methanol (2:1) to volume.

Sample solution: Transfer an aliquot of the *Sample stock solution* equivalent to 300 mg of Krill Oil to a suitable sealable glass vial, and add about 25 mg of the *Internal standard* accurately weighed. Evaporate to dryness. Add 2 mL of deuterated chloroform (chloroform-d) containing 0.05% tetramethylsilane (TMS), 2 mL of deuterated methanol (methanol-d4), and 2 mL of *Solution A*. Seal the vial and vortex intensively for 30–60 s, shake for an additional 30 min on a shaking device, and centrifuge the contents of the vial. Pass the entire amount of the lower organic phase through a glass fiber filter, and collect the filtrate in the appropriate NMR tube.▲ (USP 1-May-2019)

Standard solution: ▲Transfer 300 mg of USP Krill Oil RS to a suitable sealable glass vial, and add about 25 mg of the *Internal standard* accurately weighed. Add 2 mL of methanol-d4, 2 mL of chloroform-d containing 0.05% TMS, and 2 mL of *Solution A*. Seal the vial and vortex intensively for 30–60 s, shake for an additional 30 min on a shaking device, and centrifuge the contents of the vial. Pass the entire amount of the lower organic phase through a glass fiber filter, and collect the filtrate in the appropriate NMR tube.▲ (USP 1-May-2019)

Instrumental conditions

Magnetic field strength: ▲NLT 7.05 Tesla (resonance frequencies of 121 MHz for ^{31}P or 300 MHz for ^{1}H)▲ (USP 1-May-2019)

Probe: Direct observe probe capable of tuning to the resonance frequency of ^{31}P (dependent on the specific magnetic field strength used) ▲at a temperature of 35°▲ (USP 1-May-2019)

Data collection: Use the parameters specified in *Table 2*. Use 90° pulses, and calibrate pulses before use according to the recommendations supplied by the instrument manufacturer.

Table 2

Parameter	^{31}P NMR Quantitative Measurement	▲ ▲ (USP 1-May-2019)
Pulse program	^{1}H-decoupled ^{31}P (inverse gated)	▲ ▲ (USP 1-May-2019)
Spectral width	50 ppm (25 to –25 ppm)	▲ ▲ (USP 1-May-2019)
Transmitter offset	Center of spectral width, 0 ppm	▲ ▲ (USP 1-May-2019)
Relaxation delay	5–15 s	▲ ▲ (USP 1-May-2019)
Acquisition time	1–6 s	▲ ▲ (USP 1-May-2019)
Size of data set	NLT 64k (32k with zero-filling)	▲ ▲ (USP 1-May-2019)

[NOTE—The acquisition time is dependent upon the dwell time and the number of data points collected. The number of scans acquired using a ▲7.05 Tesla magnet▲ (USP 1-May-2019) must be NLT 512.]

System suitability: ▲The *Standard solution* shows the ^{31}P NMR signal for triphenyl phosphate at –17.80 ppm, and the signal for phosphatidylcholine at –0.89 ppm. The signal-to-noise ratio for the phosphatidylcholine signal in the ^{31}P spectrum from the *Sample solution* obtained in the *Analysis* is NLT 2000. Using the baseline as a reference, determine the height of the phosphatidylcholine ether peak and draw a line parallel to the baseline at 30% of that total peak height (intensity). The valley between the peaks of phosphatidylcholine ether and phosphatidylcholine is below the line drawn.▲ (USP 1-May-2019)

Analysis: ▲Obtain the quantitative ^{31}P spectrum of the *Sample solution* and of the *Standard solution* as directed in *Data collection*. Record the resulting spectra, and integrate the complete set of phospholipid peaks as identified by a comparison with the reference spectrum provided with USP Krill Oil RS. The integration region for each signal must extend ±0.05 ppm on either side of the ^{31}P signal.▲ (USP 1-May-2019)

Calculations: Use the following equations and molecular weights listed in *Table 3* to ▲calculate the content of the phospholipid of interest in the Capsules taken:▲ (USP 1-May-2019)

$$mmol_{IS} = (W_{IS} \times C_{IS})/(MW_{IS} \times 100)$$

$mmol_{IS}$ = millimoles of the *Internal standard* in the *Sample solution* (mmol)
W_{IS} = weight of the *Internal standard* added to the *Sample solution* (mg)
C_{IS} = purity value of the *Internal standard*, based on quantitative ^{31}P NMR analysis (% by weight)
MW_{IS} = molecular weight of the *Internal standard*, 326.28 g/mol (for triphenyl phosphate)

$$mmol_{PL} = (I_{PL} \times A_{IS} \times mmol_{IS})/(I_{IS} \times A_{PL})$$

$mmol_{PL}$ = millimoles of the phospholipid of interest in the *Sample solution* (mmol)
I_{PL} = integrated area under the phospholipid signal of interest obtained from the spectrum of the *Sample solution*
A_{IS} = number of phosphorus atoms per molecule expected from the *Internal standard*, 1 (for triphenyl phosphate)
$mmol_{IS}$ = millimoles of the *Internal standard* in the *Sample solution*
I_{IS} = integrated area under the *Internal standard* obtained from the spectrum of the *Sample solution*
A_{PL} = number of phosphorus atoms per molecule expected from the phospholipid of interest, 1 (for any phospholipid listed in *Table 3*)

$$▲C_{PL} = 1/n \times MW_{PL} \times mmol_{PL} \times V/a$$

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C_{PL} = content of the phospholipid of interest in the Capsules taken (mg/Capsule)
n = number of Capsules used to prepare the *Sample stock solution*
MW_{PL} = molecular weight of the phospholipid of interest (mg/mmol, from *Table 3*)
$mmol_{PL}$ = millimoles of the phospholipid of interest in the *Sample solution* (mmol)
V = volume of volumetric flask used to prepare the *Sample stock solution* (mL)
a = volume of the aliquot of *Sample stock solution* used to prepare the *Sample solution* (mL) ▲(USP 1-May-2019)

Table 3

Component	Approximate Chemical Shift (ppm) in Reference to Triphenyl Phosphate	Molecular Weight (g/mol)
Triphenyl phosphate (*Internal standard*)	–17.8	—
Phosphatidylcholine, including ether (PC)	–0.89	791
1-Lysophosphatidylcholine (1-LPC)[a]	–0.48	534.5
2-Lysophosphatidylcholine (2-LPC)[a]	–0.4	534.5
Phosphatidylethanolamine (PE)	–0.24	770
N-Acylphosphatidylethanolamine (NAPE)	0	1032
Lysophosphatidylethanolamine (LPE)	0.25	492.5
Other	—	800

[a] Ability to resolve the signals of 1-LPC and 2-LPC will depend upon the applied magnetic field strength of the NMR spectrometer used for the test procedure.

▲Calculate the percentage of the labeled amount of total phospholipids per Capsule taken:

$$\text{Result} = \Sigma C_{PL} \times 100/L$$

C_{PL} = sum of the individual amounts of phospholipids of interest in the Capsules taken (mg/Capsule)
L = label claim of total phospholipids (mg/Capsule) ▲(USP 1-May-2019)

Acceptance criteria: NLT 95.0% of the labeled amount of total phospholipids

Change to read:

- **CONTENT OF ASTAXANTHIN**

[NOTE—Perform this analysis in subdued light using low-actinic glassware.]

Sample solution: 0.005 g/mL of Krill Oil in chloroform using the portion of oil from NLT 10 Capsules. [NOTE—If the solution is not clear, centrifuge it with an appropriate centrifuge to obtain a clear supernatant.]

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Analytical wavelength: 486 nm

Cell: 1 cm

Blank: Chloroform

Analysis

Sample: *Sample solution*

Calculate the percentage of astaxanthin in the portion of Krill Oil taken from the Capsules:

$$\text{Result} = A/(F \times C)$$

A = absorbance of the *Sample solution*
F = coefficient of extinction ($E^{1\%}$) of pure astaxanthin in chloroform (100 mL · g^{-1} · cm^{-1}), 1692
C = concentration of the *Sample solution* (g/mL)

Acceptance criteria: NLT ▲0.005%▲ (USP 1-May-2019)

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩, *Disintegration, Procedure, Delayed-release (enteric-coated) soft shell capsules*: Meet the requirements
- **WEIGHT VARIATION** ⟨2091⟩: Meet the requirements

CONTAMINANTS

- **LIMIT OF DIOXINS, FURANS, AND POLYCHLORINATED BIPHENYLS**

Analysis: Determine the content of polychlorinated dibenzo-para-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) by Method 1613, Revision B of the Environmental Protection Agency (EPA). Determine the content of polychlorinated biphenyls (PCBs) by Method 1668, Revision A of the EPA.

Acceptance criteria: The sum of PCDDs and PCDFs is NMT 2.0 pg/g of World Health Organization (WHO) toxic equivalents. The sum of PCDDs, PCDFs, and dioxin-like PCBs [non-ortho International Union of Pure and Applied Chemistry (IUPAC) congeners PCB-77, PCB-81, PCB-126, and PCB-169; and mono-ortho IUPAC congeners PCB-105, PCB-114, PCB-118, PCB-123, PCB-156, PCB-157, PCB-167, and PCB-189] is NMT 10.0 pg/g of WHO toxic equivalents.

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic microbial count does not exceed 10^3 cfu/g, and the combined molds and yeasts count does not exceed 10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meet the requirements

SPECIFIC TESTS

- **ASTAXANTHIN ESTERIFICATION**

Standard solution A: 10 mg/mL of USP Astaxanthin Esters from *Haematococcus pluvialis* RS in acetone

Standard solution B: 10 mg/mL of USP Astaxanthin (Synthetic) RS in acetone

Sample solution: Using the portion of oil from NLT 10 Capsules, prepare a solution of 250 mg/mL in acetone.

Chromatographic system

(See *Chromatography* ⟨621⟩, *General Procedures, Thin-Layer Chromatography*.)

Mode: TLC

Adsorbent: 0.25-mm layer of chromatographic silica gel. [NOTE—Dry silica gel at 110° for 1 h before use.]

Application volume: 5 µL

Developing solvent system: Hexane and acetone (70:30)

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatogram in the *Developing solvent system* until the solvent front has moved about 15 cm of the length of the plate. Remove the plate from the chamber, and allow to dry.

Acceptance criteria: The principal spot from *Standard solution B*, located in the bottom half of the plate, is free astaxanthin. The *Sample solution* may exhibit a light, minor spot in the same location. The principal spots from *Standard*

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solution A are from monoesters (primary spot, located slightly above the middle of the plate) and diesters (secondary spot, located in the top third of the plate). The principal spot from the *Sample solution* should correspond in color and R_F value to the diester spot from *Standard solution A*. The secondary spot from the *Sample solution* should correspond in color and approximately the same R_F value to the monoester spot from *Standard solution A*. [NOTE—Slight differences in R_F values within monoester spots and within diester spots may exist because of different intensities.]

- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Peroxide Value*: NMT 5.0 mEq peroxide/kg

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and store at room temperature. Protect from light.

Change to read:

- **LABELING:** The label states the amount of docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and total phospholipids ▲in mg/Capsule.▲ (USP 1-May-2019)

Change to read:

- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Astaxanthin (Synthetic) RS
  USP Astaxanthin Esters from *Haematococcus pluvialis* RS
  ▲▲ (USP 1-May-2019)
  USP Krill Oil RS
  ▲▲ (USP 1-May-2019)

Lactobacillus acidophilus La-14

DEFINITION

Lactobacillus acidophilus La-14 (ATCC strain designation SD5212) is a lactic acid-producing, Gram-positive, rod-shaped, non-motile, non-spore-forming bacterium that is homofermentative. *Lactobacillus acidophilus* La-14 occurs as a white- to cream-colored powder that is produced via fermentation of a pure, specific strain of *Lactobacillus acidophilus*. Suitable cryoprotectants may be added to the concentrated bacteria following fermentation, after which the product is frozen and then freeze-dried. The formulated product may be blended with suitable diluents and/or bulking agents. It contains NLT 100% of the labeled viable cell count of *Lactobacillus acidophilus* La-14.

IDENTIFICATION

- **A. NUCLEIC ACID-BASED IDENTIFICATION**
  [NOTE—In all cases for the *Identification* test, "sterile water" refers to sterile, nuclease-free water acceptable for use in molecular biology.[1]]
  Buffer: Use a molecular biology-grade 10 mM tris hydrochloride, 1 mM EDTA sodium buffer.[2]
  Sample solution: 100 mg/mL of the freeze-dried probiotic powder in *Buffer*
  Primer set: Use a primer set comprised of forward primer sequence (5′-3′) AAACTGCAATTTAAGATTATGAGTTTC and reverse primer sequence (5′-3′) GGTACCGTCTTGATTATTAGTGTA.[3] Primers should be diluted in *Buffer* to a stock concentration of 100 µM, then further diluted to 25 µM in *Buffer*, and stored at −20°. A positive test for this *Primer set* is expected to give an amplification product of 184 base pairs.
  Polymerase chain reaction (PCR) sample preparations: Prepare a solution containing 1 µL of the *Sample solution*, 10 µL of mastermix polymerase,[4] 1 µL of diluted forward primer (25 µM), 1 µL of diluted reverse primer (25 µM), and 12 µL of sterile water.
  PCR negative control: Prepare as directed for the *PCR sample preparations*, replacing the 1 µL of *Sample solution* with 1 µL of sterile water.
  PCR amplification: Perform PCR on each *PCR sample preparation* and the *PCR negative control* using an appropriate thermal cycler.[5] Incubate at 95° for 7 min (step 1); 95° for 30 s (step 2); 51.0° for 30 s (step 3); and at 72° for 60 s (step 4). Repeat steps 2–4 for 34 cycles, then incubate at 72° for 5 min and hold at 4°.
  Analysis: Analyze the products of the *PCR amplification* for each *PCR sample preparation* and for the *PCR negative control* using an automated on-chip electrophoresis system with a DNA kit.[6] Follow the manufacturer's instructions for analysis. Alternatively, analysis and visualization may be accomplished using gel electrophoresis. Prepare or use a commercially available 1% (w/v) agarose gel in a 1X tris-acetic acid–EDTA buffer (40 mM tris hydrochloride, 1% glacial acetic acid, and 1 mM EDTA). Stain the gel with 0.5 mg/mL of ethidium bromide in water and de-stain with deionized water. [**CAUTION**—Ethidium bromide is considered a toxic substance and a potential mutagen. Use appropriate personal protective equipment (including nitrile gloves) when handling this reagent.] Use a DNA ladder standard (1 KB plus)[7] suitable for determining the size of linear double-stranded DNA fragments between 100 and 12,000 base pairs. The ladder standard should be used in the first and last lanes on the gel to allow for proper comparison of amplicons.
  Analysis of the *PCR negative control* must result in the absence of any amplification products, or the preparation of the *PCR sample preparations* and the *PCR negative control* must be repeated, followed by *PCR amplification* and *Analysis*.
  Acceptance criteria: The *PCR sample preparations* prepared with the *Primer set* gives an amplification product of 184 base pairs. There should be no amplification product of 600 base pairs.

ASSAY

- **ENUMERATION**
  Agar medium: Prepare as follows or use a suitable commercially available agar (see *Table 1*).[8]

[1] Suitable PCR-Certified Waters, RNase and DNase Free, are available from www.teknova.com.
[2] Suitable buffers (e.g. TE Buffer 1X, Molecular Biology Grade) are available from www.promega.com.
[3] DNA primers are commercially available (custom manufacture) from Integrated DNA Technologies (www.idtdna.com) and other commercial sources.
[4] 5 Prime MasterMix polymerase from 5 Prime.
[5] Suitable thermal cyclers are available from Eppendorf® (www.eppendorf.com).
[6] Suitable automated on-chip electrophoresis systems with a DNA kit are available from Agilent (Agilent 2100 Bioanalyzer with Agilent DNA 1000 Kit www.genomics.agilent.com).
[7] Suitable 1 KB plus DNA ladders are available from www.lifetechnologies.com.
[8] Difco™ Lactobacilli MRS Agar, or equivalent. Suitable Lactobacilli MRS Agars are available from www.vwr.com or other chemical/microbiological suppliers.

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Table 1. Lactobacilli MRS Agar

Reagent	Quantity (g)
Proteose peptone no. 3	10.0
Beef extract	10.0
Yeast extract	5.0
Dextrose	20.0
Polysorbate 80	1.0
Ammonium citrate	2.0
Sodium acetate	5.0
Magnesium sulfate	0.1
Manganese sulfate	0.05
Dipotassium phosphate	2.0
Agar	15.0

Suspend Lactobacilli MRS Agar in 1 L of purified water in an appropriately sized conical flask or beaker (sufficiently large to not boil over). Cover the flask or beaker with aluminum foil and heat with stirring to boiling on a hot plate. Allow to boil for 1 min to completely dissolve the medium, then autoclave the solution at 121° for 15 min. Cool to 45° and use immediately. Boiled *Agar medium* may also be aseptically transferred into individual media bottles in 100- or 200-mL aliquots before sterilizing, and then autoclaved and stored for later use. [NOTE—Can be stored at 4° (heat gently to 45° to melt the agar before use).] Immediately before use, aseptically add 1.0 mL of a sterile 5% (w/v) cysteine hydrochloride solution for each 100 mL of *Agar medium* prepared, such that the final concentration of cysteine hydrochloride in the *Agar medium* is 0.05%.

Sample broth: Prepare as follows or use a suitable commercially available broth (see *Table 2*).[9]

Table 2. Lactobacilli MRS Broth

Reagent	Quantity (g)
Proteose peptone no. 3	10.0
Beef extract	10.0
Yeast extract	5.0
Dextrose	20.0
Polysorbate 80	1.0
Ammonium citrate	2.0
Sodium acetate	5.0
Magnesium sulfate	0.1
Manganese sulfate	0.05
Dipotassium phosphate	2.0

Suspend Lactobacilli MRS Broth in 1 L of purified water in an appropriately sized conical flask or beaker (sufficiently large to not boil over). Cover the flask or beaker with aluminum foil and heat with stirring to boiling on a hot plate. Allow to boil for 1 min to completely dissolve the broth ingredients, then autoclave the solution at 121° for 15 min. Broth may also be aseptically transferred into individual media bottles in 100- or 200-mL aliquots before sterilizing, and then autoclaved and stored for later use. [NOTE—Can be stored at 4° (allow broth to come to room temperature before use).]

Peptone diluent: Prepare a solution of 0.1% peptone[10] in water (w/v) and adjust with a solution of lactic acid to a pH of 7.0. Using an autoclave, steam sterilize the solution at 121° for NLT 15 min, then allow to cool in the unopened autoclave. Dispense into sterile containers as needed for preparing samples.

Sample preparation: Aseptically transfer 11.0 g of freeze-dried probiotic powder into a sterile stomacher bag. Add 99 mL of previously sterilized (room temperature) *Sample broth* to the bag and blend at 230 rpm for 2 min in a stomacher. Hold the mixture at room temperature for 30 min to allow rehydration of the freeze-dried sample, then blend in the stomacher for an additional 2 min at 230 rpm. This is the primary 10^{-1} dilution.

Using sterilized, filtered pipet tips, make serial dilutions by aseptically transferring 1.0 mL of the primary 10^{-1} dilution to sterile media bottles, each containing 99.0 mL of *Peptone diluent* (10^{-3} dilution). Repeat this operation until the desired dilution series is obtained. [NOTE—The dilutions used in the *Analysis* should be expected to contain 25–250 cfu/mL.] Shake the media bottles for complete mixing before proceeding with the *Analysis*.

Analysis: For each *Sample preparation* to be plated, prepare the Petri plates as follows. Using three sterile, filtered 1-mL pipet tips, aseptically transfer 1.0 mL of the *Sample preparation* separately into three appropriately labeled sterile 15-mm × 100-mm Petri plates, then pour about 15 mL of the 45° *Agar medium* into each plate, flaming the lip of the bottle between pours. Place the lid on each plate after adding the *Agar medium*, then gently swirl the plates to mix the *Sample preparation* and the *Agar medium*. [NOTE—Be careful to avoid spillage onto the lid of the dish when swirling the plates.] Repeat this procedure for additional dilutions of the *Sample preparation*. Prepare one blank plate that contains only the *Agar medium* and a second blank plate in which 1.0 mL of *Peptone diluent* has been mixed with the *Agar medium*. Allow the plates to sit at room temperature on a level surface until the *Agar medium* solidifies, then incubate the plates at 38° for 72 h under anaerobic conditions.[11]

After 72 h of incubation, count the colonies and record the results as viable cfu/g, taking into account the appropriate dilution factor of the *Sample preparation*. Only count plates containing 25–250 colonies. Determine the average plate count, in cfu/g.

Acceptance criteria: NLT 100% of the labeled viable cell count, in cfu/g

CONTAMINANTS

[NOTE—The methods of microbial analysis included in this section as examples represent currently accepted methods commonly used in industry. Users may substitute other validated test methods for the methods in this section.]

- **MICROBIAL ENUMERATION TESTS ⟨2021⟩:** The total combined molds and yeasts count does not exceed 10^2 cfu/g.
- **NON-LACTIC ACID BACTERIA:** ISO international standard number 13559 (IDF 153), available from the International Organization for Standardization (www.iso.org). The total non-lactic acid bacteria count is less than 5×10^3 cfu/g.

[9] Difco™ Lactobacilli MRS Broth, or equivalent are available from www.vwr.com or other chemical/microbiological suppliers.

[10] Suitable peptone for microbiological analysis is available from BD Bacto™ (www.bd.com).

[11] Suitable anaerobic systems are available from BD GasPak™ EZ Container System, www.bd.com.

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- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures*, ***Test for Absence of Escherichia coli*** and *Test for Absence of Salmonella Species*: It meets the requirements of the tests for absence of *Escherichia coli*. It meets the requirements of the tests for absence of *Salmonella* species in 40 g.
- ***Listeria***: (See *Food Chemicals Codex, Appendix XV*.) It meets the requirements of the tests for absence of *Listeria* in 25 g.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in high barrier foil laminate bags and store at or below 4°.
- **LABELING:** This ingredient should be labeled with the genus, species, and strain names and with the formulated enumeration in cfu/g (or similar units). This monograph applies only to *Lactobacillus acidophilus* La-14, and no other strains of *Lactobacillus acidophilus* cultures.

Lactobacillus acidophilus NCFM

DEFINITION

Lactobacillus acidophilus NCFM (ATCC strain designation SD5221) is a lactic acid-producing, Gram-positive, rod-shaped, non-spore-forming bacterium that is homofermentative. Rods occurring in short chains are commonly observed. *Lactobacillus acidophilus* NCFM occurs as a white- to cream-colored powder that is produced via fermentation of a pure, specific strain of *Lactobacillus acidophilus*. Suitable cryoprotectants may be added to the concentrated bacteria following fermentation, after which the product is frozen and then freeze-dried. The formulated product may be blended with suitable diluents and/or bulking agents. It contains NLT 100% of the labeled viable cell count of *Lactobacillus acidophilus* NCFM.

IDENTIFICATION

- **A. NUCLEIC ACID-BASED IDENTIFICATION**

[NOTE—In all cases for the *Identification* test, "sterile water" refers to sterile, nuclease-free water acceptable for use in molecular biology.[1]]

Buffer: Use a molecular biology-grade 10 mM tris hydrochloride, 1 mM EDTA sodium buffer.[2]

Sample solution: 100 mg/mL of the freeze-dried probiotic powder in *Buffer*

Primer set 1: Use a primer set comprised of forward primer sequence (5′-3′) TCGGCAAACTTAGTTGCAGAAGG and reverse primer sequence (5′-3′) GGTGCTAAGCTAGGTATGGAACG.[3] Primers should be diluted in *Buffer* to a stock concentration of 100 µM, then further diluted to 25 µM in *Buffer*, and stored at –20°. The annealing temperature for *Primer set 1* is 57.0°. A positive test for this *Primer set* is expected to give an amplification product of 365 base pairs with two single nucleotide polymorphisms (SNPs).

Primer set 2: Use a primer set comprised of forward primer sequence (5′-3′) GCTGACGGAGATTATTCAAGCCA and reverse primer sequence (5′-3′) GCAGAACACATACGCTACCAGAA.[3] Primers should be diluted in *Buffer* to a stock concentration of 100 µM, then further diluted to 25 µM in *Buffer*, and stored at –20°. The annealing temperature for *Primer set 2* is 57.0°. A positive test for this *Primer set* is expected to give an amplification product of 433 base pairs with two single nucleotide polymorphisms (SNPs).

Primer set 3: Use a primer set comprised of forward primer sequence (5′-3′) AAACTGCAATTTAAGATTATGAGTTTC and reverse primer sequence (5′-3′) GGTACCGTCTTGATTATTAGTGTA.[3] Primers should be diluted in *Buffer* to a stock concentration of 100 µM, then further diluted to 25 µM in *Buffer*, and stored at –20°. The annealing temperature for *Primer set 3* is 51.0°. A positive test for this *Primer set* is expected to give an amplification product of 600 base pairs.

Polymerase chain reaction (PCR) sample preparations: For each *Primer set*, prepare a solution containing 1 µL of the *Sample solution*, 10 µL of mastermix polymerase,[4] 1 µL of diluted forward primer (25 µM), 1 µL of diluted reverse primer (25 µM), and 12 µL of sterile water.

PCR negative control: Prepare as directed for the *PCR sample preparations*, replacing the 1 µL of *Sample solution* with 1 µL of sterile water.

PCR amplification: Perform PCR on each *PCR sample preparation* and the *PCR negative control* using an appropriate thermal cycler.[5] Incubate at 95° for 7 min (step 1); 95° for 30 s (step 2); the *Primer set* annealing temperature for 30 s (step 3); and at 72° for 30 s (for *Primer set 1*), for 30 s (for *Primer set 2*), or for 1 min (for *Primer set 3*; step 4). Repeat steps 2–4 for 34 cycles, then incubate at 72° for 5 min and hold at 4°.

Analysis: Analyze the products of the *PCR amplification* for each *PCR sample preparation* and for the *PCR negative control* using an automated on-chip electrophoresis system with a DNA kit.[6] Follow the manufacturer's instructions for analysis. Alternatively, analysis and visualization may be accomplished using gel electrophoresis. Prepare or use a commercially available 1% (w/v) agarose gel in a 1X tris-acetic acid–EDTA buffer (40 mM tris hydrochloride, 1% glacial acetic acid, and 1 mM EDTA). Stain the gel with 0.5 mg/mL of ethidium bromide in water and de-stain with deionized water. [**CAUTION**—Ethidium bromide is considered a toxic substance and a potential mutagen. Use appropriate personal protective equipment (including nitrile gloves) when handling this reagent.] Use a DNA ladder standard (1 KB plus)[7] suitable for determining the size of linear double-stranded DNA fragments between 100 and 12,000 base pairs. The ladder standard should be used in the first and last lanes on the gel to allow for proper comparison of amplicons.

Analysis of the *PCR negative control* must result in the absence of any amplification products or the preparation of the *PCR sample preparations* and the *PCR negative control* must be repeated, followed by *PCR amplification* and *Analysis*.

Acceptance criteria

Primer set 1: The *PCR sample preparation* prepared with *Primer set 1* gives an amplification product of 365 base pairs with two SNPs. The first SNP is identified as (underlined in the following 15 base pair sequence): GAGTAAATTCCATCA and the location is 49 base pairs from the 5′ end of the forward primer described in *Primer set 1*. The second SNP is identified as (underlined in the

[1] Suitable PCR-Certified Waters, RNase and DNase Free, are available from www.teknova.com.

[2] Suitable buffers (e.g., TE Buffer 1X, Molecular Biology Grade) are available from www.promega.com.

[3] DNA primers are commercially available (custom manufacture) from Integrated DNA Technologies (www.idtdna.com) and other commercial sources.

[4] 5 Prime MasterMix polymerase from 5 Prime.

[5] Suitable thermal cyclers are available from Eppendorf® (www.eppendorf.com).

[6] Suitable automated on-chip electrophoresis systems with a DNA kit are available from Agilent (Agilent 2100 Bioanalyzer with Agilent DNA 1000 Kit www.genomics.agilent.com).

[7] Suitable 1 KB plus DNA ladders are available from www.lifetechnologies.com.

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following 15 base pair sequence): TTCAATTGAAGTTTG and the location is 241 base pairs from the 5′ end of the forward primer described in *Primer set 1*. The sequence of the amplicon should be determined by validated, standard sequencing technologies.

Primer set 2: The *PCR sample preparation* prepared with *Primer set 2* gives an amplification product of 433 base pairs with two SNPs. The first SNP is identified as (underlined in the following 15 base pair sequence): AAGCATTCAATACAC and the location is 72 base pairs from the 5′ end of the forward primer described in *Primer set 2*. The second SNP is identified as (underlined in the following 15 base pair sequence): ATATGGTTCTTACCT and the location is 215 base pairs from the 5′ end of the forward primer described in *Primer set 2*. The sequence of the amplicon should be determined by validated, standard sequencing technologies.

Primer set 3: The *PCR sample preparation* prepared with *Primer set 3* gives an amplification product of 600 base pairs. There should be no amplification product of 184 base pairs.

ASSAY

• ENUMERATION

Agar medium: Prepare as follows or use a suitable commercially available agar (see *Table 1*).[8]

Table 1. Lactobacilli MRS Agar

Reagent	Quantity (g)
Proteose peptone no. 3	10.0
Beef extract	10.0
Yeast extract	5.0
Dextrose	20.0
Polysorbate 80	1.0
Ammonium citrate	2.0
Sodium acetate	5.0
Magnesium sulfate	0.1
Manganese sulfate	0.05
Dipotassium phosphate	2.0
Agar	15.0

Suspend Lactobacilli MRS Agar in 1 L of purified water in an appropriately sized conical flask or beaker (sufficiently large to not boil over). Cover the flask or beaker with aluminum foil and heat with stirring to boiling on a hot plate. Allow to boil for 1 min to completely dissolve the medium, then autoclave the solution at 121° for 15 min. Cool to 45° and use immediately. Boiled *Agar medium* may also be aseptically transferred into individual media bottles in 100- or 200-mL aliquots before sterilizing, and then autoclaved and stored for later use. [NOTE—Can be stored at 4° (heat gently to 45° to melt the agar before use).] Immediately before use, aseptically add 1.0 mL of a sterile 5% (w/v) cysteine hydrochloride solution for each 100 mL of *Agar medium* prepared, such that the final concentration of cysteine hydrochloride in the *Agar medium* is 0.05%.

Sample broth: Prepare as follows or use a suitable commercially available broth (see *Table 2*).[9]

Table 2. Lactobacilli MRS Broth

Reagent	Quantity (g)
Proteose peptone no. 3	10.0
Beef extract	10.0
Yeast extract	5.0
Dextrose	20.0
Polysorbate 80	1.0
Ammonium citrate	2.0
Sodium acetate	5.0
Magnesium sulfate	0.1
Manganese sulfate	0.05
Dipotassium phosphate	2.0

Suspend Lactobacilli MRS Broth in 1 L of purified water in an appropriately sized conical flask or beaker (sufficiently large to not boil over). Cover the flask or beaker with aluminum foil and heat with stirring to boiling on a hot plate. Allow to boil for 1 min to completely dissolve the broth ingredients, then autoclave the solution at 121° for 15 min. Broth may also be aseptically transferred into individual media bottles in 100- or 200-mL aliquots before sterilizing, and then autoclaved and stored for later use. [NOTE—Can be stored at 4° (allow broth to come to room temperature before use).]

Peptone diluent: Prepare a solution of 0.1% peptone[10] in water (w/v) and adjust with a solution of lactic acid to a pH of 7.0. Using an autoclave, steam sterilize the solution at 121° for NLT 15 min, then allow to cool in the unopened autoclave. Dispense into sterile containers as needed for preparing samples.

Sample preparation: Aseptically transfer 11.0 g of freeze-dried probiotic powder into a sterile stomacher bag. Add 99 mL of previously sterilized (room temperature) *Sample broth* to the bag and blend at 230 rpm for 2 min in a stomacher. Hold the mixture at room temperature for 30 min to allow rehydration of the freeze-dried sample, then blend in the stomacher for an additional 2 min at 230 rpm. This is the primary 10^{-1} dilution.

Using sterilized, filtered pipet tips, make serial dilutions by aseptically transferring 1.0 mL of the primary 10^{-1} dilution to sterile media bottles, each containing 99.0 mL of *Peptone diluent* (10^{-3} dilution). Repeat this operation until the desired dilution series is obtained. [NOTE—The dilutions used in the *Analysis* should be expected to contain 25–250 cfu/mL.] Shake the media bottles for complete mixing before proceeding with the *Analysis*.

Analysis: For each *Sample preparation* to be plated, prepare the Petri plates as follows. Using three sterile, filtered 1-mL pipet tips, aseptically transfer 1.0 mL of the *Sample preparation* separately into three appropriately labeled sterile 15-mm × 100-mm Petri plates, then pour about 15 mL of the 45° *Agar medium* into each plate, flaming the lip of the bottle between pours. Place the lid on each plate after adding the *Agar medium*, then gently swirl the plates to mix the *Sample preparation* and the *Agar medium*.

[8] Difco™ Lactobacilli MRS Agar, or equivalent. Suitable Lactobacilli MRS Agars are available from www.vwr.com or other chemical/microbiological suppliers.

[9] Difco™ Lactobacilli MRS Broth, or equivalent are available from www.vwr.com or other chemical/microbiological suppliers.

[10] Suitable peptone for microbiological analysis is available from BD Bacto™ (www.bd.com).

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[NOTE—Be careful to avoid spillage onto the lid of the dish when swirling the plates.] Repeat this procedure for additional dilutions of the *Sample preparation*. Prepare one blank plate that contains only the *Agar medium* and a second blank plate in which 1.0 mL of *Peptone diluent* has been mixed with the *Agar medium*. Allow the plates to sit at room temperature on a level surface until the *Agar medium* solidifies, then incubate the plates at 38° for 72 h under anaerobic conditions.[11]

After 72 h of incubation, count the colonies and record the results as viable cfu/g, taking into account the appropriate dilution factor of the *Sample preparation*. Only count plates containing 25–250 colonies. Determine the average plate count, in cfu/g.

Acceptance criteria: NLT 100% of the labeled viable cell count, in cfu/g

CONTAMINANTS

[NOTE—The methods of microbial analysis included in this section as examples represent currently accepted methods commonly used in industry. Users may substitute other validated test methods for the methods in this section.]

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total combined molds and yeasts count does not exceed 10^2 cfu/g.
- **NON-LACTIC ACID BACTERIA:** ISO international standard number 13559 (IDF 153), available from the International Organization for Standardization (www.iso.org). The total non-lactic acid bacteria count is less than 5×10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures*, *Test for Absence of Escherichia coli* and *Test for Absence of Salmonella Species***:** It meets the requirements of the tests for absence of *Escherichia coli*. It meets the requirements of the tests for absence of *Salmonella* species in 40 g.
- ***Listeria*:** (See *Food Chemicals Codex, Appendix XV*.) It meets the requirements of the tests for absence of *Listeria* in 25 g.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in high barrier foil laminate bags and store at or below 4°.
- **LABELING:** This ingredient should be labeled with the genus, species, and strain names and with the formulated enumeration in cfu/g (or similar units). This monograph applies only to *Lactobacillus acidophilus* NCFM, and no other strains of *Lactobacillus acidophilus* cultures.

Lactobacillus paracasei LPC-37

DEFINITION

Lactobacillus paracasei LPC-37 (ATCC strain designation SD5275) is a lactic acid-producing, Gram-positive, rod-shaped, non-motile, non-spore-forming bacterium that is homofermentative. *Lactobacillus paracasei* LPC-37 occurs as a white- to cream-colored powder that is produced via fermentation of a pure, specific strain of *Lactobacillus paracasei*. Suitable cryoprotectants may be added to the concentrated bacteria following fermentation, after which the product is frozen and then freeze-dried. The formulated product may be blended with suitable diluents and/or bulking agents. It contains NLT 100% of the labeled viable cell count of *Lactobacillus paracasei* LPC-37.

[11] Suitable anaerobic systems are available from BD GasPak™ EZ Container System, www.bd.com.

IDENTIFICATION

- **A. NUCLEIC ACID-BASED IDENTIFICATION**

[NOTE—In all cases for the *Identification* test, "sterile water" refers to sterile, nuclease-free water acceptable for use in molecular biology.[1]]

Buffer: Use a molecular biology-grade 10 mM tris-hydrochloride, 1 mM EDTA sodium buffer.[2]

Sample solution: 100 mg/mL of the freeze-dried probiotic powder in *Buffer*

Primer set: Use a primer set comprised of forward primer sequence (5′-3′) GTTTGTGGCGGCGTAACTTC and reverse primer sequence (5′-3′) GGTGATCCTGAACGCGGTT.[3] Primers should be diluted in *Buffer* to a stock concentration of 100 µM, then further diluted to 25 µM in *Buffer*, and stored at −20°. A positive test for this *Primer set* is expected to give an amplification product of 273 base pairs.

Polymerase chain reaction (PCR) sample preparations: Prepare a solution containing 1 µL of the *Sample solution*, 10 µL of mastermix polymerase,[4] 1 µL of diluted forward primer (25 µM), 1 µL of diluted reverse primer (25 µM), and 12 µL of sterile water.

PCR negative control: Prepare as directed for the *PCR sample preparations*, replacing the 1 µL of *Sample solution* with 1 µL of sterile water.

PCR amplification: Perform PCR on each *PCR sample preparation* and the *PCR negative control* using an appropriate thermal cycler.[5] Incubate at 95° for 7 min (step 1); 95° for 30 s (step 2); 57.0° for 30 s (step 3); and at 72° for 30 s (step 4). Repeat steps 2–4 for 34 cycles, then incubate at 72° for 5 min, and hold at 4°.

Analysis: Analyze the products of the *PCR amplification* for each *PCR sample preparation* and for the *PCR negative control* using an automated on-chip electrophoresis system with a DNA kit.[6] Follow the manufacturer's instructions for analysis. Alternatively, analysis and visualization may be accomplished using gel electrophoresis. Prepare or use a commercially available 1% (w/v) agarose gel in a 1X tris-acetic acid–EDTA buffer (40 mM tris-hydrochloride, 1% glacial acetic acid, and 1 mM EDTA). Stain the gel with 0.5 mg/mL of ethidium bromide in water and de-stain with deionized water. [**CAUTION**—Ethidium bromide is considered a toxic substance and a potential mutagen. Use appropriate personal protective equipment (including nitrile gloves) when handling this reagent.] Use a DNA ladder standard (1 KB plus)[7] suitable for determining the size of linear double-stranded DNA fragments between 100 and 12,000 base pairs. The ladder standard should be used in the first and last lanes on the gel to allow for proper comparison of amplicons.

Analysis of the *PCR negative control* must result in the absence of any amplification products, or the preparation of the *PCR sample preparations* and the *PCR negative control* must be repeated, followed by *PCR amplification* and *Analysis*.

[1] Suitable PCR-Certified Waters, RNase and DNase Free, are available from www.teknova.com.

[2] Suitable buffers (e.g. TE Buffer 1X, Molecular Biology Grade) are available from www.promega.com.

[3] DNA primers are commercially available (custom manufacture) from Integrated DNA Technologies (www.idtdna.com) and other commercial sources.

[4] 5 Prime MasterMix polymerase from 5 Prime.

[5] Suitable thermal cyclers are available from Eppendorf® (www.eppendorf.com).

[6] Suitable automated on-chip electrophoresis systems with a DNA kit are available from Agilent (Agilent 2100 Bioanalyzer with Agilent DNA 1000 Kit www.genomics.agilent.com).

[7] Suitable 1 KB plus DNA ladders are available from www.lifetechnologies.com.

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Acceptance criteria: The *PCR sample preparations* prepared with the *Primer set* gives an amplification product of 273 base pairs.

ASSAY

• **ENUMERATION**

Agar medium: Prepare as follows or use a suitable commercially available agar (see *Table 1*).[8]

Table 1. Lactobacilli MRS Agar

Reagent	Quantity (g)
Proteose peptone no. 3	10.0
Beef extract	10.0
Yeast extract	5.0
Dextrose	20.0
Polysorbate 80	1.0
Ammonium citrate	2.0
Sodium acetate	5.0
Magnesium sulfate	0.1
Manganese sulfate	0.05
Dipotassium phosphate	2.0
Agar	15.0

Suspend Lactobacilli MRS Agar in 1 L of purified water in an appropriately sized conical flask or beaker (sufficiently large to not boil over). Cover the flask or beaker with aluminum foil and heat with stirring to boiling on a hot plate. Allow to boil for 1 min to completely dissolve the medium, then autoclave the solution at 121° for 15 min. Cool to 45° and use immediately. Boiled *Agar medium* may also be aseptically transferred into individual media bottles in 100- or 200-mL aliquots before sterilizing, and then autoclaved and stored for later use. [NOTE—Can be stored at 4° (heat gently to 45° to melt the agar before use).]

Sample broth: Prepare as follows or use a suitable commercially available broth (see *Table 2*).[9]

Table 2. Lactobacilli MRS Broth

Reagent	Quantity (g)
Proteose peptone no. 3	10.0
Beef extract	10.0
Yeast extract	5.0
Dextrose	20.0
Polysorbate 80	1.0
Ammonium citrate	2.0
Sodium acetate	5.0
Magnesium sulfate	0.1
Manganese sulfate	0.05
Dipotassium phosphate	2.0

Suspend Lactobacilli MRS Broth in 1 L of purified water in an appropriately sized conical flask or beaker (sufficiently large to not boil over). Cover the flask or beaker with aluminum foil and heat with stirring to boiling on a hot plate. Allow to boil for 1 min to completely dissolve the broth ingredients, then autoclave the solution at 121° for 15 min. Broth may also be aseptically transferred into individual media bottles in 100- or 200-mL aliquots before sterilizing, and then autoclaved and stored for later use. [NOTE—Can be stored at 4° (allow broth to come to room temperature before use).]

Peptone diluent: Prepare a solution of 0.1% peptone[10] in water (w/v) and adjust with a solution of lactic acid to a pH of 7.0. Using an autoclave, steam sterilize the solution at 121° for NLT 15 min, then allow to cool in the unopened autoclave. Dispense into sterile containers as needed for preparing samples.

Sample preparation: Aseptically transfer 11.0 g of freeze-dried probiotic powder into a sterile stomacher bag. Add 99 mL of previously sterilized (room temperature) *Sample broth* to the bag and blend at 230 rpm for 2 min in a stomacher. Hold the mixture at room temperature for 30 min to allow rehydration of the freeze-dried sample, then blend in the stomacher for an additional 2 min at 230 rpm. This is the primary 10^{-1} dilution.

Using sterilized, filtered pipet tips, make serial dilutions by aseptically transferring 1.0 mL of the primary 10^{-1} dilution to sterile media bottles, each containing 99.0 mL of *Peptone diluent* (10^{-3} dilution). Repeat this operation until the desired dilution series is obtained. [NOTE—The dilutions used in the *Analysis* should be expected to contain 25–250 cfu/mL.] Shake the media bottles for complete mixing before proceeding with the *Analysis*.

Analysis: For each *Sample preparation* to be plated, prepare the Petri plates as follows. Using three sterile, filtered 1-mL pipet tips, aseptically transfer 1.0 mL of the *Sample preparation* separately into three appropriately labeled sterile 15-mm × 100-mm Petri plates, then pour about 15 mL of the 45° *Agar medium* into each plate, flaming the lip of the bottle between pours. Place the lid on each plate after adding the *Agar medium*, then gently swirl the plates to mix the *Sample preparation* and the *Agar medium*. [NOTE—Be careful to avoid spillage onto the lid of the dish when swirling the plates.] Repeat this procedure for additional dilutions of the *Sample preparation*. Prepare one blank plate that contains only the *Agar medium* and a second blank plate in which 1.0 mL of *Peptone diluent* has been mixed with the *Agar medium*. Allow the plates to sit at room temperature on a level surface until the *Agar medium* solidifies, then incubate the plates at 38° for 72 h under anaerobic conditions.[11]

After 72 h of incubation, count the colonies and record the results as viable cfu/g, taking into account the appropriate dilution factor of the *Sample preparation*. Only count plates containing 25–250 colonies. Determine the average plate count, in cfu/g.

Acceptance criteria: NLT 100% of the labeled viable cell count, in cfu/g

CONTAMINANTS

[NOTE—The methods of microbial analysis included in this section as examples represent currently accepted methods commonly used in industry. Users may substitute other validated test methods for the methods in this section.]

[8] Difco™ Lactobacilli MRS Agar, or equivalent. Suitable Lactobacilli MRS Agars are available from www.vwr.com or other chemical/microbiological suppliers.

[9] Difco™ Lactobacilli MRS Broth, or equivalent are available from www.vwr.com or other chemical/microbiological suppliers.

[10] Suitable peptone for microbiological analysis is available from BD Bacto™ (www.bd.com).

[11] Suitable anaerobic systems are available from BD GasPak™ EZ Container System, www.bd.com.

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- **Microbial Enumeration Tests** ⟨2021⟩**:** The total combined molds and yeasts count does not exceed 10^2 cfu/g.
- **Non-Lactic Acid Bacteria:** ISO international standard number 13559 (IDF 153), available from the International Organization for Standardization (www.iso.org). The total non-lactic acid bacteria count is less than 5×10^3 cfu/g.
- **Enterococci:** (See *Food Chemicals Codex, Appendix XV.*) The total enterococci count is less than 10^2 cfu/g.
- **Coliforms:** (See *FCC, Appendix XV.*) The total coliforms count is less than 10 cfu/g.
- **Absence of Specified Microorganisms** ⟨2022⟩, *Test Procedures, Test for Absence of Staphylococcus aureus, Test for Absence of Escherichia coli,* and *Test for Absence of Salmonella Species*: It meets the requirements of the tests for absence of *Staphylococcus aureus* and *Escherichia coli.* It meets the requirements of the tests for absence of *Salmonella* species in 40 g.
- ***Listeria*:** (See *FCC, Appendix XV.*) It meets the requirements of the tests for absence of *Listeria* in 25 g, if the production lot of the strain has been in contact with any dairy-derived ingredients.

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in high barrier foil laminate bags and store at or below 4°.
- **Labeling:** This ingredient should be labeled with the genus, species, and strain names and with the formulated enumeration in cfu/g (or similar units). This monograph applies only to *Lactobacillus paracasei* LPC-37, and no other strains of *Lactobacillus paracasei* cultures.

Lactobacillus rhamnosus HN001

DEFINITION

Lactobacillus rhamnosus HN001 (ATCC strain designation SD5675) is a lactic acid-producing, Gram-positive, rod-shaped, non-spore-forming bacterium that is a facultative hexose heterofermenter. Rods of varying length occurring in short chains are commonly observed. *Lactobacillus rhamnosus* HN001 occurs as a white- to cream-colored powder that is produced via fermentation of a pure, specific strain of *Lactobacillus rhamnosus.* Suitable cryoprotectants may be added to the concentrated bacteria following fermentation, after which the product is frozen and then freeze-dried. The formulated product may be blended with suitable diluents and or bulking agents. It contains NLT 100% of the labeled viable cell count of *Lactobacillus rhamnosus* HN001.

IDENTIFICATION

- **A. Nucleic Acid-Based Identification**

[Note—In all cases for the *Identification* test, "sterile water" refers to sterile, nuclease-free water acceptable for use in molecular biology.[1]]

Buffer: Use a molecular biology-grade 10 mM tris hydrochloride, 1 mM EDTA sodium buffer.[2]

Sample solution: 100 mg/mL of the freeze-dried probiotic powder in *Buffer*

Primer set: Use a primer set comprised of forward primer sequence (5′-3′) CACCTGCGATCAAAGCGAAAC and reverse primer sequence (5′-3′) GCTCCCACCGGCACATTA.[3] Primers should be diluted in *Buffer* to a stock concentration of 100 µM, then further diluted to 25 µM in *Buffer*, and stored at –20°. A positive test for this *Primer set* is expected to give an amplification product of 340 base pairs.

Polymerase chain reaction (PCR) sample preparations: Prepare a solution containing 1 µL of the *Sample solution*, 10 µL of mastermix polymerase,[4] 1 µL of diluted forward primer (25 µM), 1 µL of diluted reverse primer (25 µM), and 12 µL of sterile water.

PCR negative control: Prepare as directed for the *PCR sample preparations*, replacing the 1 µL of *Sample solution* with 1 µL of sterile water.

PCR amplification: Perform PCR on each *PCR sample preparation* and the *PCR negative control* using an appropriate thermal cycler.[5] Incubate at 95° for 7 min (step 1); 95° for 30 s (step 2); 57.0° for 30 s (step 3); and at 72° for 30 s (step 4). Repeat steps 2–4 for 34 cycles, then incubate at 72° for 5 min, and hold at 4°.

Analysis: Analyze the products of the *PCR amplification* for each *PCR sample preparation* and for the *PCR negative control* using an automated on-chip electrophoresis system with a DNA kit.[6] Follow the manufacturer's instructions for analysis. Alternatively, analysis and visualization may be accomplished using gel electrophoresis. Prepare or use a commercially available 1% (w/v) agarose gel in a 1X tris-acetic acid–EDTA buffer (40 mM tris hydrochloride, 1% glacial acetic acid, and 1 mM EDTA). Stain the gel with 0.5 mg/mL of ethidium bromide in water and de-stain with deionized water. [**Caution**—Ethidium bromide is considered a toxic substance and a potential mutagen. Use appropriate personal protective equipment (including nitrile gloves) when handling this reagent.] Use a DNA ladder standard (1 KB plus)[7] suitable for determining the size of linear double-stranded DNA fragments between 100 and 12,000 base pairs. The ladder standard should be used in the first and last lanes on the gel to allow for proper comparison of amplicons.

Analysis of the *PCR negative control* must result in the absence of any amplification products or the preparation of the *PCR sample preparations* and the *PCR negative control* must be repeated, followed by *PCR amplification* and *Analysis.*

Acceptance criteria: The *PCR sample preparations* prepared with the *Primer set* gives an amplification product of 340 base pairs.

ASSAY

- **Enumeration**

Agar medium: Prepare as follows or use a suitable commercially available agar (see *Table 1*).[8]

[1] Suitable PCR-Certified Waters, RNase and DNase Free, are available from www.teknova.com.

[2] Suitable buffers (e.g. TE Buffer 1X, Molecular Biology Grade) are available from www.promega.com.

[3] DNA primers are commercially available (custom manufacture) from Integrated DNA Technologies (www.idtdna.com) and other commercial sources.

[4] 5 Prime MasterMix polymerase from 5 Prime.

[5] Suitable thermal cyclers are available from Eppendorf® (www.eppendorf.com).

[6] Suitable automated on-chip electrophoresis systems with a DNA kit are available from Agilent (Agilent 2100 Bioanalyzer with Agilent DNA 1000 Kit www.genomics.agilent.com).

[7] Suitable 1 KB plus DNA ladders are available from www.lifetechnologies.com.

[8] Difco™ Lactobacilli MRS Agar, or equivalent. Suitable Lactobacilli MRS Agars are available from www.vwr.com or other chemical/microbiological suppliers.

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Table 1. Lactobacilli MRS Agar

Reagent	Quantity (g)
Proteose peptone no. 3	10.0
Beef extract	10.0
Yeast extract	5.0
Dextrose	20.0
Polysorbate 80	1.0
Ammonium citrate	2.0
Sodium acetate	5.0
Magnesium sulfate	0.1
Manganese sulfate	0.05
Dipotassium phosphate	2.0
Agar	15.0

Suspend Lactobacilli MRS Agar in 1 L of purified water in an appropriately sized conical flask or beaker (sufficiently large to not boil over). Cover the flask or beaker with aluminum foil and heat with stirring to boiling on a hot plate. Allow to boil for 1 min to completely dissolve the medium, then autoclave the solution at 121° for 15 min. Cool to 45° and use immediately. Boiled *Agar medium* may also be aseptically transferred into individual media bottles in 100- or 200-mL aliquots before sterilizing, and then autoclaved and stored for later use. [NOTE—Can be stored at 4° (heat gently to 45° to melt the agar before use).]

Sample broth: Prepare as follows or use a suitable commercially available broth (see *Table 2*).[9]

Table 2. Lactobacilli MRS Broth

Reagent	Quantity (g)
Proteose peptone no. 3	10.0
Beef extract	10.0
Yeast extract	5.0
Dextrose	20.0
Polysorbate 80	1.0
Ammonium citrate	2.0
Sodium acetate	5.0
Magnesium sulfate	0.1
Manganese sulfate	0.05
Dipotassium phosphate	2.0

Suspend Lactobacilli MRS Broth in 1 L of purified water in an appropriately sized conical flask or beaker (sufficiently large to not boil over). Cover the flask or beaker with aluminum foil and heat with stirring to boiling on a hot plate. Allow to boil for 1 min to completely dissolve the broth ingredients, then autoclave the solution at 121° for 15 min. Broth may also be aseptically transferred into individual media bottles in 100- or 200-mL aliquots before sterilizing, and then autoclaved and stored for later use. [NOTE—Can be stored at 4° (allow broth to come to room temperature before use).]

[9] Difco™ Lactobacilli MRS Broth, or equivalent are available from www.vwr.com or other chemical/microbiological suppliers.

Peptone diluent: Prepare a solution of 0.1% peptone[10] in water (w/v) and adjust with a solution of lactic acid to a pH of 7.0. Using an autoclave, steam sterilize the solution at 121° for NLT 15 min, then allow to cool in the unopened autoclave. Dispense into sterile containers as needed for preparing samples.

Sample preparation: Aseptically transfer 11.0 g of freeze-dried probiotic powder into a sterile stomacher bag. Add 99 mL of previously sterilized (room temperature) *Sample broth* to the bag and blend at 230 rpm for 2 min in a stomacher. Hold the mixture at room temperature for 30 min to allow rehydration of the freeze-dried sample, then blend in the stomacher for an additional 2 min at 230 rpm. This is the primary 10^{-1} dilution.

Using sterilized, filtered pipet tips, make serial dilutions by aseptically transferring 1.0 mL of the primary 10^{-1} dilution to sterile media bottles, each containing 99.0 mL of *Peptone diluent* (10^{-3} dilution). Repeat this operation until the desired dilution series is obtained. [NOTE—The dilutions used in the *Analysis* should be expected to contain 25–250 cfu/mL.] Shake the media bottles for complete mixing before proceeding with the *Analysis*.

Analysis: For each *Sample preparation* to be plated, prepare the Petri plates as follows. Using three sterile, filtered 1-mL pipet tips, aseptically transfer 1.0 mL of the *Sample preparation* separately into three appropriately labeled sterile 15-mm × 100-mm Petri plates, then pour about 15 mL of the 45° *Agar medium* into each plate, flaming the lip of the bottle between pours. Place the lid on each plate after adding the *Agar medium*, then gently swirl the plates to mix the *Sample preparation* and the *Agar medium*. [NOTE—Be careful to avoid spillage onto the lid of the dish when swirling the plates.] Repeat this procedure for additional dilutions of the *Sample preparation*. Prepare one blank plate that contains only the *Agar medium* and a second blank plate in which 1.0 mL of *Peptone diluent* has been mixed with the *Agar medium*. Allow the plates to sit at room temperature on a level surface until the *Agar medium* solidifies, then incubate the plates at 38° for 72 h under anaerobic conditions.[11]

After 72 h of incubation, count the colonies and record the results as viable cfu/g, taking into account the appropriate dilution factor of the *Sample preparation*. Only count plates containing 25–250 colonies. Determine the average plate count, in cfu/g.

Acceptance criteria: NLT 100% of the labeled viable cell count, in cfu/g

CONTAMINANTS

[NOTE—The methods of microbial analysis included in this section as examples represent currently accepted methods commonly used in industry. Users may substitute other validated test methods for the methods in this section.]

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total combined molds and yeasts count does not exceed 10^2 cfu/g.
- **NON-LACTIC ACID BACTERIA:** ISO international standard number 13559 (IDF 153), available from the International Organization for Standardization (www.iso.org). The total non-lactic acid bacteria count is less than 5×10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures*, ***Test for Absence of Escherichia coli*** and *Test for Absence of Salmonella Species*: It meets the requirements of the tests for absence of *Escherichia coli*. It meets the

[10] Suitable peptone for microbiological analysis is available from BD Bacto™ (www.bd.com).

[11] Suitable anaerobic systems are available from BD GasPak™ EZ Container System, www.bd.com.

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requirements of the tests for absence of *Salmonella* species in 40 g.
- ***Listeria*:** (See *Food Chemicals Codex, Appendix XV.*) It meets the requirements of the tests for absence of *Listeria* in 25 g.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in high barrier foil laminate bags and store at or below 4°.
- **LABELING:** This ingredient should be labeled with the genus, species, and strain names and with the formulated enumeration in cfu/g (or similar units). This monograph applies only to *Lactobacillus rhamnosus* HN001, and no other strains of *Lactobacillus rhamnosus* cultures.

Leucine—*see Leucine General Monographs*

Levocarnitine—*see Levocarnitine General Monographs*

Levocarnitine Oral Solution—*see Levocarnitine Oral Solution General Monographs*

Levocarnitine Tablets—*see Levocarnitine Tablets General Monographs*

Licorice

DEFINITION
Licorice consists of the roots, rhizomes, and stolons of *Glycyrrhiza glabra* L. or *Glycyrrhiza uralensis* Fish. ex DC. (Fam. Fabaceae). It contains NLT 2.5% of glycyrrhizic acid ($C_{42}H_{62}O_{16}$), calculated on the dried basis.

IDENTIFICATION
- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution: 5 mg/mL of USP Glycyrrhizic Acid RS in a mixture of alcohol and water (7:3)
Sample solution: 2 g of pulverized Licorice in 10 mL of a mixture of alcohol and water (7:3). Heat by shaking on a water bath for 5 min, cool, and filter.
Chromatographic system
(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography.*)
Adsorbent: 0.25-mm layer of chromatographic silica gel mixture (TLC plates)
Application volume: 2 µL
Developing solvent system: Butyl alcohol, glacial acetic acid, and water (7:1:2)
Analysis
Samples: *Standard solution* and *Sample solution*
Develop the chromatogram in an unsaturated chamber to a length of 10 cm. Examine the plate under 254-nm light.
Acceptance criteria: The chromatograms show a dark purple zone, among other spots, due to glycyrrhizic acid at an R_F value of 0.4.

COMPOSITION
- **CONTENT OF GLYCYRRHIZIC ACID**

Diluent: Alcohol and water (1:1)
Solution A: Dilute acetic acid (1:15)
Mobile phase: Acetonitrile and *Solution A* (2:3)
Standard solution: 0.25 mg/mL of USP Glycyrrhizic Acid RS in *Diluent*
Sample solution: Transfer 500 mg of Licorice, reduced to a powder, to a suitable flask. Add 70 mL of *Diluent*, shake for 15 min, centrifuge, and decant the supernatant into a 100-mL volumetric flask. Mix the residue with 25 mL of *Diluent*, shake for 15 min, centrifuge, and add the supernatant to the volumetric flask. Dilute with *Diluent* to volume, and filter.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability.*)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 15-cm; packing L1
Flow rate: 0.6 mL/min
Injection volume: 20 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Column efficiency: NLT 5000 theoretical plates determined from glycyrrhizic acid
Tailing factor: NMT 2.0 for the glycyrrhizic acid peak
Relative standard deviation: NMT 2.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of glycyrrhizic acid ($C_{42}H_{62}O_{16}$) in the portion of Licorice taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of glycyrrhizic acid from the *Sample solution*
r_S = peak area of glycyrrhizic acid from the *Standard solution*
C_S = concentration of USP Glycyrrhizic Acid RS in the *Standard solution* (mg/mL)
V = final volume of the *Sample solution* (mL)
W = weight of Licorice taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 2.5% on the dried basis

CONTAMINANTS
- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements

SPECIFIC TESTS
- **BOTANICAL CHARACTERISTICS**

Macroscopic: The terrestrial stem is nearly cylindrical, 0.5–3.0 cm in diameter, and over 1 m in length; it is externally dark brown to red-brown and longitudinally wrinkled. It often has lenticels, small buds, and scaly leaves. The transverse section reveals a rather clear border between the phloem and the xylem, and a radial structure that often has radiating splits.
Microscopic: The transverse section reveals several yellow-brown cork layers, and a layer of phelloderm that is 1–3 cells thick. The cortex exhibits medullary rays, and obliterated sieve portions radiate alternately. The phloem exhibits groups of phloem fibers, which are surrounded by crystal cells, with thick but incompletely lignified walls. The vessels are accompanied by xylem fibers, which are surrounded by crystal cells, and by xylem parenchyma cells. The parenchyma cells contain starch grains, and often contain single crystals of calcium oxalate.

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- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩: NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Alcohol-Soluble Extractives, Method 2* ⟨561⟩: NLT 25.0%
- **LOSS ON DRYING** ⟨731⟩: Dry a sample at 105° for 6 h; it loses NMT 12.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩: NMT 7.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩: NMT 2.0%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers. Store in a cool, dry place.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Glycyrrhizic Acid RS

Powdered Licorice

DEFINITION
Powdered Licorice is Licorice reduced to a fine or very fine powder. It contains NLT 2.5% of glycyrrhizic acid ($C_{42}H_{62}O_{16}$), calculated on the dried basis.

IDENTIFICATION
- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution: 5 mg/mL of USP Glycyrrhizic Acid RS in a mixture of alcohol and water (7:3)
Sample solution: 2 g of Powdered Licorice in 10 mL of a mixture of alcohol and water (7:3). Heat by shaking on a water bath for 5 min, cool, and filter.
Chromatographic system
(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
Adsorbent: 0.25-mm layer of chromatographic silica gel mixture (TLC plates)
Application volume: 2 µL
Developing solvent system: Butyl alcohol, glacial acetic acid, and water (7:1:2)
Analysis
Samples: *Standard solution* and *Sample solution*
Develop the chromatogram in an unsaturated chamber to a length of 10 cm. Examine the plate under 254-nm light.
Acceptance criteria: The chromatograms show a dark purple zone, among other spots, due to glycyrrhizic acid at an R_F value of 0.4.

COMPOSITION
- **CONTENT OF GLYCYRRHIZIC ACID**

Diluent: Alcohol and water (1:1)
Solution A: Dilute acetic acid (1:15)
Mobile phase: Acetonitrile and *Solution A* (2:3)
Standard solution: 0.25 mg/mL of USP Glycyrrhizic Acid RS in *Diluent*
Sample solution: Transfer 500 mg of Powdered Licorice to a suitable flask. Add 70 mL of *Diluent*, shake for 15 min, centrifuge, and decant the supernatant into a 100-mL volumetric flask. Mix the residue with 25 mL of *Diluent*, shake for 15 min, centrifuge, and add the supernatant to the volumetric flask. Dilute with *Diluent* to volume, and filter.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 15-cm; packing L1
Flow rate: 0.6 mL/min
Injection volume: 20 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Column efficiency: NLT 5000 theoretical plates determined from glycyrrhizic acid
Tailing factor: NMT 2.0 for the glycyrrhizic acid peak
Relative standard deviation: NMT 2.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of glycyrrhizic acid ($C_{42}H_{62}O_{16}$) in the portion of Powdered Licorice taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of glycyrrhizic acid from the *Sample solution*
r_S = peak area of glycyrrhizic acid from the *Standard solution*
C_S = concentration of USP Glycyrrhizic Acid RS in the *Standard solution* (mg/mL)
V = final volume of the *Sample solution* (mL)
W = weight of Powdered Licorice taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 2.5% on the dried basis

CONTAMINANTS
- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩: Meets the requirements

SPECIFIC TESTS
- **BOTANICAL CHARACTERISTICS**

Macroscopic: Light yellow-brown powder with slight odor and sweet taste
Microscopic: Powdered Licorice reveals parenchyma cells containing starch grains and solitary crystals of calcium oxalate, parenchyma cell fragments, cork tissue, yellow sclerenchymatous fiber bundles that are accompanied by crystal cell rows, and vessels with reticulate and scalariform pits.
- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩: NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Alcohol-Soluble Extractives, Method 2* ⟨561⟩: NLT 25.0%
- **LOSS ON DRYING** ⟨731⟩: Dry a sample at 105° for 6 h; it loses NMT 12.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩: NMT 7.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩: NMT 2.0%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Store in well-closed containers in a cool, dry place.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Glycyrrhizic Acid RS

Supplement 2

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Powdered Licorice Extract

DEFINITION

Powdered Licorice Extract is prepared from comminuted Licorice extracted with water or suitable solvents such as alcohol, water, or mixtures of these solvents. The ratio of the crude plant material to Powdered Extract is between 5:1 and 7:1. It contains NLT 6.0% of glycyrrhizic acid ($C_{42}H_{62}O_{16}$), calculated on the dried basis.

IDENTIFICATION

• **A. Thin-Layer Chromatographic Identification Test**

Standard solution: 5 mg/mL of USP Glycyrrhizic Acid RS in a mixture of alcohol and water (7:3)

Sample solution: 60 mg/mL of Powdered Licorice Extract, in a mixture of alcohol and water (1:1)

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture

Application volume: 2 µL

Developing solvent system: Butyl alcohol, glacial acetic acid, and water (7:1:2)

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the chromatogram in an unsaturated chamber to a length of 10 cm. Examine the plate under 254-nm light.

Acceptance criteria: The chromatograms show a dark purple zone, among other spots, due to glycyrrhizic acid at an R_F value of 0.4.

COMPOSITION

• **Content of Glycyrrhizic Acid**

Diluent: Alcohol and water (1:1)

Solution A: Diluted acetic acid (1 in 15)

Mobile phase: Acetonitrile and *Solution A* (2:3)

Standard solution: 0.25 mg/mL of USP Glycyrrhizic Acid RS in *Diluent*.

Sample solution: Transfer 150 mg of Powdered Licorice Extract to a flask. Add 25 mL of *Diluent*, heat at 50° for 30 min, cool, centrifuge, and decant the supernatant into a 100-mL volumetric flask. Mix the residue with 20 mL of *Diluent*, repeat the above procedure, and dilute with *Diluent* to volume.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 15-cm; packing L1

Flow rate: 0.6 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Column efficiency: NLT 5000 theoretical plates

Tailing factor: NMT 2.0

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of glycyrrhizic acid ($C_{42}H_{62}O_{16}$) in the portion of Powdered Licorice Extract taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area for glycyrrhizic acid from the *Sample solution*

r_S = peak area for glycyrrhizic acid from the *Standard solution*

C_S = concentration of USP Glycyrrhizic Acid RS in the *Standard solution* (mg/mL)

V = volume of *Sample solution*, 100 mL

W = weight of Powdered Licorice Extract taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 6.0% on the dried basis

CONTAMINANTS

Change to read:

• **▲Botanical Extracts** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020)

: Meets the requirements

SPECIFIC TESTS

• **Loss on Drying** ⟨731⟩: Dry a sample at 105° for 6 h: it loses NMT 10.0% of its weight.

• **Articles of Botanical Origin,** *Total Ash* ⟨561⟩: NMT 12.0%

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Preserve in tight containers, protected from light.

• **Labeling:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived. The label also indicates the content of glycyrrhizic acid, the extracting solvent or solvent mixture used for preparation, and the ratio of the starting material to final product. The label bears a statement indicating that "Excessive amounts or long-term use of Licorice may cause high blood pressure or low potassium, which have been associated with irregular heartbeat and/or muscle weakness. Licorice may worsen the effects of congestive heart failure, cirrhosis, or kidney failure. Diuretic use may increase the risk. If you are pregnant or nursing a baby, seek the advice of a health professional before using this product." It meets the requirements in *Botanical Extracts* ⟨565⟩, *Labeling*.

• **USP Reference Standards** ⟨11⟩

USP Glycyrrhizic Acid RS

Licorice Fluidextract—*see Licorice Fluidextract NF*

Ground Limestone

DEFINITION

Ground Limestone is a fine, white to off-white, microcrystalline powder mainly consisting of calcium carbonate. It is obtained by crushing, grinding, and classifying naturally occurring limestone, benefited by flotation and/or air classification. After drying at 200° for 4 h, it contains NLT 94.0% and NMT 100.5% of calcium carbonate ($CaCO_3$).

IDENTIFICATION

• **A. Identification Tests—General,** *Calcium* ⟨191⟩

Analysis: Add acetic acid to the Ground Limstone, and boil the resulting solution.

Acceptance criteria: The solution produces effervescence (presence of carbonate) after the addition of acetic acid. After boiling, it meets the requirements in the chapter.

• **B. Acid-Insoluble Substances:** It meets the requirements in the test for *Acid-Insoluble Substances*.

DS Monographs

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ASSAY

• **PROCEDURE**

Sample: 200 mg of Ground Limestone, previously dried at 200° for 4 h

Blank: Proceed as directed in the *Analysis*, except omit the test specimen.

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Direct titration

Titrant: 0.05 M edetate disodium VS

Endpoint detection: Visual

Analysis: Transfer the *Sample* to a 250-mL beaker. Moisten thoroughly with a few mL of water, and add, dropwise, sufficient 3 N hydrochloric acid to dissolve. Add 100 mL of water, 15 mL of 1 N sodium hydroxide, and 300 mg of hydroxy naphthol blue, and titrate with the *Titrant* until the solution is a distinct blue color. Perform a *Blank* determination.

Calculate the percentage of calcium carbonate ($CaCO_3$) in the *Sample* taken:

$$\text{Result} = \{[(V_S - V_B) \times M \times F]/W\} \times 100$$

V_S = *Titrant* volume consumed by the *Sample* (mL)
V_B = *Titrant* volume consumed by the *Blank* (mL)
M = actual molarity of the *Titrant* (mM/mL)
F = equivalency factor, 100.1 mg/mM
W = *Sample* weight (mg)

Acceptance criteria: 94.0%–100.5%

IMPURITIES

• **ARSENIC,** *Method I* ⟨211⟩

Test preparation: Slowly dissolve 1.0 g in 15 mL of hydrochloric acid, and dilute with water to 55 mL.

Analysis: Proceed as directed in the chapter, except omit the addition of 20 mL of 7 N sulfuric acid.

Acceptance criteria: It meets the requirements in the chapter (NMT 3 ppm).

• **LEAD** ⟨251⟩

Test preparation: Mix 1.0 g with 5 mL of water, slowly add 8 mL of 3 N hydrochloric acid, evaporate on a steam bath to dryness, and dissolve the residue in 5 mL of water.

Acceptance criteria: NMT 3 ppm

• **LIMIT OF MAGNESIUM AND ALKALI SALTS**

Sample: 1.0 g

Analysis: Mix the *Sample* with 40 mL of water. Carefully add 5 mL of hydrochloric acid, heat the solution, and boil for 1 min. Rapidly add 40 mL of oxalic acid TS, and stir vigorously until precipitation is well established. Add immediately to the warm mixture 2 drops of methyl red TS and then 6 N ammonium hydroxide, dropwise, until the mixture is just alkaline. Cool to room temperature. Transfer to a 100-mL graduated cylinder, dilute with water to 100 mL, mix, and allow to stand for 4 h or overnight. Filter, and to 50 mL of the clear filtrate in a platinum dish add 0.5 mL of sulfuric acid, and evaporate the mixture on a steam bath to a small volume. Carefully heat over a free flame to dryness, and continue heating to complete decomposition and volatilization of ammonium salts. Ignite the residue to constant weight.

Acceptance criteria: NMT 3.5%. The weight of the residue is NMT 17.5 mg.

• **LIMIT OF FLUORIDE**

[NOTE—Prepare and store all solutions in plastic containers.]

Buffer solution: 294 mg/mL of sodium citrate dihydrate in water

Standard stock solution: 1.1052 mg/mL of USP Sodium Fluoride RS in water

Standard solution: Transfer 20.0 mL of *Standard stock solution* to a 100-mL volumetric flask containing 50.0 mL of *Buffer solution*, dilute with water to volume, and mix. Each mL of *Standard solution* contains 100 µg of fluoride ion.

Sample solution: Transfer 2.0 g of Ground Limestone to a beaker containing a plastic-coated stirring bar. Add 20 mL of water and 4.0 mL of hydrochloric acid, and stir until dissolved. Add 50.0 mL of *Buffer solution* and sufficient water to make 100 mL.

Electrode system: Use a fluoride-specific ion-indicating electrode and a silver–silver chloride reference electrode connected to a pH meter capable of measuring potentials with a minimum reproducibility of ± 0.2 mV (see *pH* ⟨791⟩).

Analysis

Samples: *Standard solution* and *Sample solution*

Standard response line: Transfer 50.0 mL of *Buffer solution* and 4.0 mL of hydrochloric acid to a beaker, and add water to make 100 mL. Add a plastic-coated stirring bar, insert the electrodes into the solution, stir for 15 min, and read the potential, in mV. Continue stirring, and at 5-min intervals add 100, 100, 300, and 500 µL of *Standard solution*, reading the potential 5 min after each addition. Plot the logarithms of the cumulative fluoride ion concentrations (0.1, 0.2, 0.5, and 1.0 µg/mL) versus potential, in mV.

Rinse and dry the electrodes, insert them into the *Sample solution*, stir for 5 min, and read the potential, in mV. From the measured potential and the *Standard response line* determine the concentration, C (in µg/mL), of fluoride ion in the *Sample solution*.

Calculate the content of fluoride in the portion of Ground Limestone taken:

$$\text{Result} = (C \times V)/W$$

C = concentration of fluoride ion, obtained from the *Standard response line*, in the *Sample solution* (µg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Ground Limestone taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 50 ppm

SPECIFIC TESTS

• **ACID-INSOLUBLE SUBSTANCES**

Sample: 5.0 g

Analysis: Mix the *Sample* with 25 mL of water. Add 25 mL of hydrochloric acid, dropwise, with agitation, until it ceases to cause effervescence. Add water to make the mixture measure 200 mL, and filter. Wash the insoluble residue with water until the last washing shows no chloride, and ignite and weigh the residue.

Acceptance criteria: 0.2%–2.5%. The weight of the residue is between 10 and 125 mg.

• **LOSS ON DRYING** ⟨731⟩**:** Dry a sample at 200° for 4 h: it loses NMT 2.0% of its weight.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Sodium Fluoride RS

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Conjugated Linoleic Acids–Free Fatty Acids

DEFINITION

Conjugated Linoleic Acids–Free Fatty Acids are derived from plant seed oils such as safflower oil. They consist of free fatty acids of which NLT 78% are conjugated linoleic acids (CLAs). They contain a 1:1 ratio of the active conjugated linoleic isomers (C18:2 *cis*-9, *trans*-11 and C18:2 *trans*-10, *cis*-12). Tocopherol may be added as an antioxidant.

IDENTIFICATION

• **A.** It meets the requirements in *Specific Tests* for *Fats and Fixed Oils, Procedures, Fatty Acid Composition.*

IMPURITIES

• **LIMIT OF BENZO[*a*]PYRENE**

[NOTE—An equivalent method such as the one described in ISO 22959 can be used instead.]

[**CAUTION**—Benzo[*a*]pyrene is a known carcinogen. Carry out all work in an appropriate hood.]

Mobile phase: Acetonitrile and water (88:12)

Standard stock solution: 0.5 mg/mL of benzo[*a*]pyrene in toluene. Store in the dark at 4°. Discard it after 6 months.

Standard solutions: Prepare four solutions having concentrations of 0.004, 0.02, 0.1, and 0.2 µg/mL of benzo[*a*]pyrene, by diluting the *Standard stock solution* with acetonitrile.

Sample solution: Transfer about 0.5 g of Conjugated Linoleic Acids–Free Fatty Acids to a 10-mL centrifuge tube. Add 3 mL of *n*-hexane to the tube and mix to dissolve the sample. Transfer this solution to a freshly conditioned solid-phase extraction (SPE) column.[1] [NOTE—The SPE column is conditioned by first washing the column with 5 mL of dichloromethane followed by 5 mL of *n*-hexane.] Rinse the sample tube with 2 mL of *n*-hexane and transfer to the SPE column. Wash the extraction column with 10 mL of *n*-hexane. Elute the benzo[*a*]pyrene with 5 mL of dichloromethane, and collect the eluate in a 10-mL centrifuge tube. Evaporate the eluate in a water bath at 35° under a gentle stream of nitrogen until the tube is completely dry. Dissolve the residue with about 1 mL of petroleum ether and transfer the solution quantitatively to a preweighed 2-mL vial. Evaporate the solution in the 2-mL vial until almost dry. Rinse the tube with 1 mL of petroleum ether and transfer the solution to the vial. Continue evaporating the solution until the vial is completely dry. Weigh the vial and determine the mass of the residue. The mass should be NMT 45 mg; otherwise repeat the *Sample solution* preparation steps. Cap the vial and store at 4°. Before use, dissolve the residue with a 300-µL mixture of tetrahydrofuran and acetonitrile (1:1). Pass the solution through a filter of 0.45-µm pore size.

Blank: Prepare as directed for the *Sample solution* but without the Conjugated Linoleic Acids–Free Fatty Acids.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Fluorescence

Excitation wavelength: 384 nm

Emission wavelength: 406 nm

Columns

Guard: 4.6-mm × 7.5-cm; 5-µm packing L1

Analytical: 4.6-mm × 25-cm; 5-µm packing L1

Flow rate: 1 mL/min

Injection volume: 10 µL. [NOTE—If an autosampler is used, the sample loop should be flushed with acetonitrile between injections.]

Analysis

Samples: *Standard solutions*, *Sample solution*, and *Blank*

Inject the *Standard solutions* and construct a four-point calibration curve using the peak area versus concentration (ng/µL). Inject each *Blank* and *Sample solution*, and determine its benzo[*a*]pyrene concentration (ng/µL) from the standard calibration curve.

Calculate the benzo[*a*]pyrene content, in µg/kg, in the portion of Conjugated Linoleic Acids–Free Fatty Acids taken:

$$\text{Result} = (C - C_0) \times (V/M)$$

C = concentration of benzo[*a*]pyrene obtained from the calibration curve for the oil sample (ng/µL)

C_0 = concentration of benzo[*a*]pyrene obtained from the calibration curve for the blank (ng/µL)

V = volume of acetonitrile and tetrahydrofuran (1:1) added to the vial, 300 µL

M = mass of the oil sample (g)

Acceptance criteria: NMT 2 µg/kg

SPECIFIC TESTS

• **WATER DETERMINATION** ⟨921⟩, *Method I, Method Ia*: NMT 0.1%

• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Acid Value*: 195–204

• **FATS AND FIXED OILS,** ⟨401⟩, *Procedures, Peroxide Value*: NMT 5.0

• **FATS AND FIXED OILS,** ⟨401⟩, *Procedures, Fatty Acid Composition*

Standard solution: Prepare as directed for the *Test Solution*, except use 100 mg of USP Conjugated Linoleic Acids–Free Fatty Acids RS.

Sample solution: Prepare as directed in the *Standard solution*, except replace USP Conjugated Linoleic Acids–Free Fatty Acids RS with Conjugated Linoleic Acids–Free Fatty Acids.

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for the fatty acid components are shown in *Table 1*.]

Suitability requirements

Resolution: NLT 1.5 between CLA isomer *c*9, *t*11 and CLA isomer *t*10, *c*12

Chromatogram similarity: The chromatogram of the *Standard solution* is similar to the reference chromatogram provided with the lot of USP Conjugated Linoleic Acids–Free Fatty Acids RS being used.

Acceptance criteria: Conjugated Linoleic Acids–Free Fatty Acids exhibit the composition profile of fatty acids in *Table 1*.

Table 1

Fatty Acid	Shorthand Notation	Relative Retention Time	Area Percentage
Palmitic acid	16:0	0.82	≤9
Stearic acid	18:0	0.92	≤5
Oleic acid	18:1	0.93	≤20
Linoleic acid	18:2	0.96	≤3
Conjugated linoleic acid	18:2	—	≥78

[1] Chromspher PI 120Å PAH, HPLC column, 3.0 mm × 8 cm, 5 µm, Agilent, part number CP28159; www.agilent.com.

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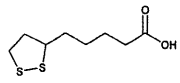
Table 1 *(continued)*

Fatty Acid	Shorthand Notation	Relative Retention Time	Area Percentage
CLA isomer *c*9, *t*11	18:2	1.00	≥37.5
CLA isomer *t*10, *c*12	18:2	1.01	≥37.5
CLA isomers *trans trans*	18:2	1.03	≤2.0

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers. Store in a cool, dry place, and protect from light, heat, and air.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Conjugated Linoleic Acids–Free Fatty Acids RS

Alpha Lipoic Acid



$C_8H_{14}O_2S_2$ 206.33

Thioctic acid;
1,2-Dithiolane-3-pentanoic acid;
1,2-Dithiolane-3-valeric acid [1077-28-7].

DEFINITION

Alpha Lipoic Acid contains NLT 99.0% and NMT 101.0% of $C_8H_{14}O_2S_2$, calculated on the dried basis.

IDENTIFICATION

- **A.** The retention time of the peak for alpha lipoic acid of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

Change to read:

- **B. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

ASSAY

- **PROCEDURE**

Buffer solution: 0.68 g/L of monobasic potassium phosphate
Mobile phase: Methanol, *Buffer solution*, and acetonitrile (58:46:9). Adjust with phosphoric acid solution (8.3 in 100) to a pH of 3.0–3.1.
Standard solution: 1.0 mg/mL of USP Alpha Lipoic Acid RS in *Mobile phase*
Sample solution: 1.0 mg/mL of Alpha Lipoic Acid in *Mobile phase*
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 215 nm
Column: 4.6-mm × 250-mm; packing L1
Column temperature: 35°
Flow rate: 1.2 mL/min
Injection size: 20 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Column efficiency: NLT 10,000 theoretical plates
Tailing factor: NMT 2.0 for the alpha lipoic acid peak
Relative standard deviation: NMT 2.0% for alpha lipoic acid
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of alpha lipoic acid ($C_8H_{14}O_2S_2$) in the portion of Alpha Lipoic Acid taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP Alpha Lipoic Acid RS in the *Standard solution* (mg/mL)
C_U = concentration of Alpha Lipoic Acid in the *Sample solution* (mg/mL)

Acceptance criteria: 99.0%–101.0% on the dried basis

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩: Less than 0.1%
- **CHROMATOGRAPHIC PURITY, PROCEDURE 1**

Buffer solution, Mobile phase, Standard solution, Sample solution, and **Chromatographic system:** Proceed as directed in the *Assay*.
Diluted standard solution: Dilute the *Standard solution* (1 in 1000) with *Mobile phase*.
System suitability
Sample: *Diluted standard solution*
Suitability requirements
Signal-to-noise ratio: NLT 10
Relative standard deviation: NMT 10.0%
Analysis
Sample: *Sample solution*
Calculate the percentage of each impurity in the portion of Alpha Lipoic Acid taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of each individual impurity from the *Sample solution*
r_T = sum of the responses of all the peaks from the *Sample solution*

Acceptance criteria
Individual impurities: NMT 0.1%
Total impurities: NMT 2.0%

- **CHROMATOGRAPHIC PURITY, PROCEDURE 2**

[NOTE—Use low-actinic glassware.]
Standard solution A: 40.0 mg/mL of USP Alpha Lipoic Acid RS in dimethylformamide
Standard solution B: 20.0 mg/mL of USP Alpha Lipoic Acid RS in dimethylformamide, prepared from the dilution of *Standard solution A*
Standard solution C: 10.0 mg/mL of USP Alpha Lipoic Acid RS in dimethylformamide, prepared from the dilution of *Standard solution B*
Sample solution: 40.0 mg/mL of Alpha Lipoic Acid in dimethylformamide
Chromatographic system
(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
Mode: TLC
Adsorbent: 0.25-mm layer of chromatographic silica gel mixture
Application volume: 5 µL
Developing solvent system: *n*-Propyl alcohol, ethyl acetate, water, and 25% ammonia water (40:40:10:5). Allow the chamber to become saturated for at least 1 h.
Iodine vapor–saturated chamber: Transfer 4 g of iodine crystals to a small watch glass, and place in a

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chromatographic chamber. Allow the chamber to become saturated for at least 2 h.

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Proceed as directed in the chapter, except to develop until the solvent front has moved 10 cm. Remove the plate, and allow to air-dry until the ammonia disappears completely. Heat at 50° for 20 min, cool the plate, and place in the *Iodine vapor–saturated chamber* until the spots are visible. The R_F value for the alpha lipoic acid spot is 0.25–0.30 and for the polymeric lipoic acid spot is 0.

Acceptance criteria: No spot other than the alpha lipoic acid spot from the *Sample solution* is more intense than the spot at $R_F = 0$ from *Standard solution A*.

SPECIFIC TESTS

- **Melting Range or Temperature** ⟨741⟩**:** 60.0°–62.0°
- **Optical Rotation,** *Specific Rotation* ⟨781S⟩

Sample solution: 50 mg/mL of Alpha Lipoic Acid, in dehydrated alcohol

Acceptance criteria: –1.0° to +1.0°

- **Loss on Drying** ⟨731⟩**:** Dry a sample in vacuum at 40° for 3 h: it loses NMT 0.2% of its weight.

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in well-closed containers.
- **USP Reference Standards** ⟨11⟩

USP Alpha Lipoic Acid RS

Alpha Lipoic Acid Capsules

DEFINITION

Alpha Lipoic Acid Capsules contain NLT 90.0% and NMT 115.0% of the labeled amount of $C_8H_{14}O_2S_2$.

IDENTIFICATION

- The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Alpha Lipoic Acid*.

STRENGTH

- **Content of Alpha Lipoic Acid**

Mobile phase: 0.025 M phosphoric acid and acetonitrile (62:38)

Standard solution: 0.05 mg/mL of USP Alpha Lipoic Acid RS in acetonitrile and water (1:1)

Sample solution A (for hard gelatin Capsules)**:** Empty and mix thoroughly the contents of NLT 20 Capsules. Transfer a portion of the powder, equivalent to 100 mg of alpha lipoic acid, to a suitable container. Add 70 mL of a mixture of acetonitrile and water (1:1), and shake for 45 min by mechanical means. Transfer to a 100-mL volumetric flask, dilute with the mixture of acetonitrile and water (1:1) to volume, and filter a portion of this preparation, discarding the first 5 mL of the filtrate. Transfer 5.0 mL of the remaining filtrate to a 100-mL volumetric flask, and dilute with acetonitrile and water (1:1) to volume.

Sample solution B (for soft gelatin Capsules)**:** Using a suitable cutting instrument, open a number of Capsules equivalent to 500 mg of alpha lipoic acid from a counted number of opened Capsules. Transfer the contents and the shells to a suitable container with stopper, add 500.0 mL of a mixture of acetonitrile and water (1:1), and shake for 45 min by mechanical means. Filter a portion of this preparation, discarding the first 5 mL of the filtrate. Transfer 5.0 mL of the remaining filtrate to a 100-mL volumetric flask, and dilute with acetonitrile and water (1:1) to volume.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 220 nm

Column: 3.9-mm × 30-cm; packing L1

Flow rate: 1.5 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Column efficiency: NLT 1300 theoretical plates

Tailing factor: NMT 1.2 for alpha lipoic acid

Relative standard deviation: NMT 1.0%

Analysis

Samples: *Standard solution* and appropriate *Sample solution*

Calculate the percentage of alpha lipoic acid in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from *Sample solution A* or *Sample solution B*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Alpha Lipoic Acid RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of alpha lipoic acid in *Sample solution A* or *Sample solution B* (mg/mL)

Acceptance criteria: 90.0%–115.0%

PERFORMANCE TESTS

- **Disintegration and Dissolution of Dietary Supplements** ⟨2040⟩**:** Meet the requirements for *Dissolution*

Medium: Water; 900 mL

Apparatus 1 (for hard gelatin Capsules)**:** 100 rpm

Apparatus 2 (for soft gelatin Capsules)**:** 75 rpm

Time: 60 min

Standard solution: 1 mg/mL of USP Alpha Lipoic Acid RS in a mixture of acetonitrile and water (1:1). Dilute with water to obtain a concentration of 0.02 mg/mL.

Sample solution: Withdraw a portion of the solution under test, and filter, discarding the first portion of the filtrate. Transfer an aliquot to a volumetric flask, and dilute with water to volume to obtain a solution having an expected concentration of 0.02 mg/mL of alpha lipoic acid.

Mobile phase and **Chromatographic system:** Proceed as directed in the test for *Content of Alpha Lipoic Acid*.

Injection size: 50 µL

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of alpha lipoic acid ($C_8H_{14}O_2S_2$) dissolved:

$$\text{Result} = (r_U/r_S) \times (V \times C \times D/L) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

V = volume of dissolution *Medium*, 900 mL

C = concentration of USP Alpha Lipoic Acid RS in the *Standard solution* (mg/mL)

D = dilution factor of the sample

L = label claim of alpha lipoic acid (mg/Capsule)

Tolerances: NLT 70% of the labeled amount of $C_8H_{14}O_2S_2$ is dissolved.

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- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩: Meet the requirements

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Alpha Lipoic Acid RS

Alpha Lipoic Acid Tablets

DEFINITION
Alpha Lipoic Acid Tablets contain NLT 90.0% and NMT 115.0% of the labeled amount of $C_8H_{14}O_2S_2$.

IDENTIFICATION
- The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Alpha Lipoic Acid.*

STRENGTH
- **CONTENT OF ALPHA LIPOIC ACID**

Mobile phase: 0.025 M phosphoric acid and acetonitrile (62:38)
Standard solution: 0.05 mg/mL of USP Alpha Lipoic Acid RS in acetonitrile and water (1:1)
Sample solution: Transfer the equivalent of 100 mg of alpha lipoic acid from NLT 20 finely powdered Tablets to a suitable container. Add 70 mL of a mixture of acetonitrile and water (1:1), and shake for 45 min by mechanical means. Transfer to a 100-mL volumetric flask, dilute with the mixture of acetonitrile and water (1:1) to volume, and filter a portion of this preparation, discarding the first 5 mL of the filtrate. Transfer 5.0 mL of the remaining filtrate to a 100-mL volumetric flask, and dilute with acetonitrile and water (1:1) to volume.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability.*)
Mode: LC
Detector: UV 220 nm
Column: 3.9-mm × 30-cm; packing L1
Flow rate: 1.5 mL/min
Injection size: 20 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Column efficiency: NLT 1300 theoretical plates
Tailing factor: NMT 1.2 for alpha lipoic acid
Relative standard deviation: NMT 1.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of alpha lipoic acid ($C_8H_{14}O_2S_2$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP Alpha Lipoic Acid RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of alpha lipoic acid in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–115.0%

PERFORMANCE TESTS
- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** ⟨2040⟩: Meet the requirements for *Dissolution*

Medium: Water; 900 mL
Apparatus 2: 75 rpm
Time: 60 min
Standard stock solution: 1 mg/mL of USP Alpha Lipoic Acid RS in a mixture of acetonitrile and water (1:1)
Standard solution: 0.02 mg/mL from the *Standard stock solution* in water
Sample solution: Withdraw a portion of the solution under test, and filter, discarding the first portion of the filtrate. Transfer an aliquot to a volumetric flask, and dilute with water to volume to obtain a solution having an expected concentration of 0.02 mg/mL of alpha lipoic acid.
Mobile phase and **Chromatographic system:** Proceed as directed in the test for *Content of Alpha Lipoic Acid.*
Injection size: 50 µL
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of alpha lipoic acid ($C_8H_{14}O_2S_2$) dissolved:

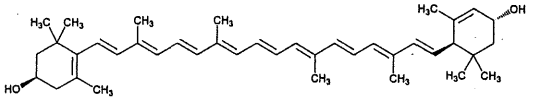
$$\text{Result} = (r_U/r_S) \times (V \times C \times D/L) \times 100$$

r_U = peak area from the *Sample solution*
r_S = peak area from the *Standard solution*
V = volume of dissolution *Medium*, 900 mL
C = concentration of USP Alpha Lipoic Acid RS in the *Standard solution* (mg/mL)
D = dilution factor of the sample
L = label claim of alpha lipoic acid (mg/Tablet)

Tolerances: NLT 70% of the labeled amount of alpha lipoic acid ($C_8H_{14}O_2S_2$) is dissolved.
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩: Meet the requirements

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **LABELING:** Tablets that are coated are so labeled.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Alpha Lipoic Acid RS

Lutein



$C_{40}H_{56}O_2$ 568.87
β-ε-Carotene-3,3′-diol (3*R*,3′*R*,6′*R*) [127-40-2].

DEFINITION
Lutein is the purified fraction obtained from saponification of the oleoresin of *Tagetes erecta* L. It contains NLT 80.0% of total carotenoids calculated as lutein ($C_{40}H_{56}O_2$). It contains NLT 74.0% of lutein and NMT 8.5% of zeaxanthin, both calculated as lutein ($C_{40}H_{56}O_2$) on the anhydrous basis.

Supplements

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IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS ⟨197⟩,** *Ultraviolet-Visible Spectroscopy*: **197U▲** (CN 1-May-2020)

Wavelength range: 300–700 nm
Sample solution: Prepare as directed for the *Sample solution* in the test for *Content of Total Carotenoids*.
Acceptance criteria: It meets the requirements in the chapter. The absorbance ratio A_{446}/A_{474} is 1.09–1.14.

- **B.** The retention time for the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Lutein*.

COMPOSITION

- **CONTENT OF TOTAL CAROTENOIDS**

[NOTE—Use low-actinic glassware.]
Diluent: Hexanes, acetone, toluene, and dehydrated alcohol (10:7:7:6)
Sample stock solution: 0.3 mg/mL of Lutein in *Diluent*
Sample solution: 3.0 µg/mL of Lutein in dehydrated alcohol from dilution of *Sample stock solution*
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: UV-Vis
Analytical wavelength: 446 nm
Blank: Dehydrated alcohol
Analysis
Sample: *Sample solution*
Calculate the percentage of total carotenoids (*T*) as lutein ($C_{40}H_{56}O_2$) in the portion of Lutein taken:

$$\text{Result} = A/(F \times C)$$

A = absorbance of the *Sample solution*
F = coefficient of extinction ($E^{1\%}$) of lutein in alcohol (100 mL · g^{-1} · cm^{-1}), 2550
C = concentration of lutein in the *Sample solution* (g/mL)

Acceptance criteria: NLT 80.0%

- **CONTENT OF LUTEIN**

Mobile phase: Hexane and ethyl acetate (3:1)
Standard solution: 150 µg/mL of USP Lutein RS in *Mobile phase*
Sample solution: Transfer 1 mL of the *Sample stock solution* from the test for *Content of Total Carotenoids*, and evaporate under a stream of nitrogen to dryness. Add 1 mL of *Mobile phase*, and sonicate to dissolve.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV-Vis at 446 nm
Column: 4.6-mm × 25-cm; 5-µm packing L3
Flow rate: 1.5 mL/min
Injection size: 10 µL
System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for lutein and zeaxanthin are about 1.0 and 1.05, respectively.]
Suitability requirements
Resolution: NLT 1.0 between lutein and zeaxanthin
Tailing factor: NMT 2.0
Relative standard deviation: NMT 2.0%
Analysis
Sample: *Sample solution*
Calculate the percentage of the lutein peak as the total detected area in the portion of Lutein taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of lutein
r_T = sum of the responses of all the peaks

Acceptance criteria: NLT 85%
Calculate the percentage of lutein in the portion of Lutein taken:

$$\text{Result} = (r_U/r_T) \times T$$

r_U = individual peak response
r_T = sum of the responses of all the peaks
T = percentage of total carotenoids as determined in the test for *Content of Total Carotenoids*

Acceptance criteria: NLT 74.0% of lutein on the anhydrous basis

- **ZEAXANTHIN AND OTHER RELATED COMPOUNDS**

[NOTE—Use low-actinic glassware.]
Mobile phase, Standard solution, Sample solution, and **Chromatographic system:** Proceed as directed in *Content of Lutein*.
Analysis
Sample: *Sample solution*
Calculate the percentage of zeaxanthin as the total detected area in the portion of Lutein taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of zeaxanthin
r_T = sum of the responses of all the peaks

Acceptance criteria: NMT 9.0%
Calculate the percentage of zeaxanthin in the portion of Lutein taken:

$$\text{Result} = (r_U/r_T) \times T$$

r_U = peak response of zeaxanthin
r_T = sum of the responses of all the peaks
T = percentage of total carotenoids as determined in the test for *Content of Total Carotenoids*

Calculate the percentage of other related compounds in the portion of Lutein taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = individual peak response of any other peak in the chromatogram (excluding zeaxanthin and lutein)
r_T = sum of the responses of all the peaks

Acceptance criteria: NMT 8.5% of zeaxanthin; NMT 1.0% of any other single related compound on the anhydrous basis

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩: NMT 2.0%
- **LEAD** ⟨251⟩: NMT 1 ppm

SPECIFIC TESTS

- **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in hermetically sealed, light- and oxygen-resistant containers. Store in a cool place.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Lutein RS

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Lutein Capsules

DEFINITION

Lutein Capsules contain NLT 95.0% and NMT 130.0% of the labeled amount of lutein ($C_{40}H_{56}O_2$). It may contain NMT 9% of zeaxanthin of the total carotenoid content.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Ultraviolet-Visible Spectroscopy*: **197U**▲ (CN 1-May-2020)

Sample solution: Prepare as directed for the *Sample solution* in the test for *Content of Total Carotenoids*.
Wavelength range: 300–700 nm
Ratio: A_{446}/A_{474}, 1.09–1.14

- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Lutein and Zeaxanthin*.

COMPOSITION

- **CONTENT OF TOTAL CAROTENOIDS**

Diluent: Hexanes, acetone, toluene, and dehydrated alcohol (10:7:7:6)
Sample stock solution: Weigh NLT 10 Capsules in a tared weighing bottle. With a sharp blade or by other appropriate means, carefully open the Capsules, without loss of the shell material, and transfer as much as possible of the combined Capsule contents to a suitable container. Remove any adhering substance from the emptied Capsules and shell remains by washing with several small portions of *n*-hexane. Discard the washings, and allow the empty Capsules and shell remains to dry in a current of dry air until the odor of *n*-hexane is no longer perceptible. Weigh the empty Capsules and shell remains in the original tared weighing bottle, and calculate the average net weight per Capsule by difference. Dissolve an accurately weighed portion of the combined Capsule contents in *Diluent* by stirring with a magnetic bar for 30 min, and dilute with *Diluent* to a designated volume to obtain a solution with a nominal concentration of 0.2 mg/mL of lutein.
Sample solution: Dilute a portion of the *Sample stock solution* with dehydrated alcohol to prepare a solution with a nominal concentration of 2.0 µg/mL of lutein.
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Analytical wavelength: 446 nm
Cell path: 1 cm
Blank: Dehydrated alcohol
Analysis
Sample: *Sample solution*
Calculate the content of total carotenoids as lutein ($C_{40}H_{56}O_2$), (T), in mg/Capsule, in the Capsules taken:

$$\text{Result} = [(A \times D \times V)/(F \times W)] \times A_w$$

A = absorbance of the *Sample solution*
D = dilution to obtain the *Sample solution* from *Sample stock solution*
V = volume of the *Sample solution* (mL)
F = absorptivity of the lutein in alcohol, 255.0 (mL/mg · cm)
W = weight of capsule contents to prepare the *Sample stock solution* (mg)
A_w = average weight of capsule contents (mg/Capsule)

- **CONTENT OF LUTEIN AND ZEAXANTHIN**

Mobile phase: Hexane and ethyl acetate (3:1)
Standard solution: 150 µg/mL of USP Lutein RS in *Mobile phase*
Sample solution: Transfer 1 mL of the *Sample stock solution* from the test for *Content of Total Carotenoids*, and evaporate under a stream of nitrogen to dryness. Add 1 mL of *Mobile phase*, and sonicate to dissolve.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV-Vis at 446 nm
Column: 4.6-mm × 25-cm; 5-µm packing L3
Flow rate: 1.5 mL/min
Injection volume: 10 µL
System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for lutein and zeaxanthin are about 1.0 and 1.05, respectively.]
Suitability requirements
Resolution: NLT 1.0 between lutein and zeaxanthin
Tailing factor: NMT 2.0
Relative standard deviation: NMT 2.0%
Analysis
Sample: *Sample solution*
Calculate the percentage of the labeled amount of lutein in the Capsules taken:

$$\text{Result} = [(r_U/r_T) \times T] \times (100/L)$$

r_U = peak response of lutein
r_T = sum of the responses of all the peaks
T = content of total carotenoids as determined in the test for *Content of Total Carotenoids* (mg/Capsule)
L = labeled amount of lutein (mg/Capsule)

Calculate the percentage of zeaxanthin relative to the total carotenoid content in the Capsules taken:

$$\text{Result} = (r_Z/r_T) \times 100$$

r_Z = peak response of zeaxanthin
r_T = sum of the responses of all the peaks

Acceptance criteria: 95.0%–130.0% of the labeled amount of lutein; NMT 9.0% of zeaxanthin in the total carotenoid content

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** ⟨2040⟩: Meet the requirements for *Disintegration*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩: Meet the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** Label it to indicate the amount of lutein and the amount of zeaxanthin per Capsule.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Lutein RS

Lutein Preparation

DEFINITION

Lutein Preparation is a combination of Lutein with one or more inert substances. It may be in a solid or a liquid form. It

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contains NLT 95.0% and NMT 130.0% of the labeled amount of lutein, calculated as $C_{40}H_{56}O_2$ on the anhydrous basis. It contains NLT 85.0% of lutein and NMT 9.0% of zeaxanthin of the total carotenoid content.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS ⟨197⟩,** *Ultraviolet-Visible Spectroscopy*: **197U▲** (CN 1-May-2020)

Analytical wavelength: 300–700 nm
Sample solution: Prepare as directed for the *Sample solution* in the test for *Content of Total Carotenoids*.
Ratio: A_{446}/A_{474}, 1.09–1.14

- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Lutein*.

COMPOSITION

- **CONTENT OF TOTAL CAROTENOIDS**

Diluent: Hexanes, acetone, toluene, and dehydrated alcohol (10:7:7:6)
Sample stock solution A (for solid lutein preparations labeled as containing gelatin): Transfer an amount of Preparation, equivalent to 3.5 mg of lutein, to a 50-mL centrifuge tube. Add 15 mL of warm water, 60 units of bacterial alkaline protease preparation, and 1 mg of bromelain. Cap and sonicate for 20 min with occasional swirling. Cool to room temperature, and add 20.0 mL of methylene chloride. Shake for 1 min, and centrifuge for 5 min at 2000 rpm. Remove the upper aqueous phase, and add 2–3 g of anhydrous sodium sulfate to the remaining red layer.
Sample stock solution B (for other solid lutein preparations) : Transfer an amount of Preparation, equivalent to 1.5 mg of lutein, to a 50-mL centrifuge tube. Add 15 mL of warm water, cap, and sonicate for 30 min with occasional swirling. Cool to room temperature, and add 30.0 mL of ethyl acetate and 2–3 g of sodium chloride. Shake for 1 min, and centrifuge for 5 min at 2000 rpm. Use the upper orange-red layer.
Sample stock solution C (for liquid lutein suspensions in oil) : Transfer a weighed amount of Preparation equivalent to 20 mg of lutein to a 100-mL volumetric flask, and dilute with *Diluent* to volume. Add a magnetic bar, and stir for 30 min.
Sample solution: Transfer 1.0 mL of *Sample stock solution A*, or 1.0 mL of *Sample stock solution B*, or 1.0 mL of *Sample stock solution C* into a 100-mL volumetric flask, and dilute with dehydrated alcohol to volume.
Spectrometric conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Analytical wavelength: 446 nm
Cell path: 1 cm
Blank: Dehydrated alcohol
Analysis
Sample: *Sample solution*
Calculate the percentage of total carotenoids (T) as lutein ($C_{40}H_{56}O_2$) in the Preparation:

$$\text{Result} = (A \times V \times D \times 100)/(F \times W)$$

A = absorbance of the *Sample solution*
F = absorptivity of the lutein in alcohol, 255.0 (mL/mg · cm)
V = volume of organic solvent (20.0 mL for *Sample stock solution A*, 30.0 mL for *Sample stock solution B*, and 100.0 mL for *Sample stock solution C*) used in preparing the *Sample stock solutions*
D = dilution factor used to prepare the *Sample solution* from *Sample stock solutions*
W = weight of Preparation taken to prepare the *Sample stock solutions* (mg)

- **CONTENT OF LUTEIN**

Diluent: Hexanes, acetone, toluene, and dehydrated alcohol (10:7:7:6)
Mobile phase: Hexane and ethyl acetate (75:25)
Standard solution: 150 µg/mL of USP Lutein RS in *Mobile phase*
Sample solution: Transfer 1.0 mL of *Sample stock solution A*, or 1.0 mL of *Sample stock solution B*, or 2.0 mL of *Sample stock solution C* from the test for *Content of Total Carotenoids* into a suitable vial. Evaporate the solvent to dryness under a stream of nitrogen. Add 1.0 mL of *Mobile phase*, and sonicate to dissolve.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 446 nm
Column: 4.6-mm × 25-cm; 5-µm packing L3
Flow rate: 1.5 mL/min
Injection size: 10 µL
System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for lutein and zeaxanthin are about 1.0 and 1.05, respectively.]
Suitability requirements
Resolution: NLT 1.0 between lutein and zeaxanthin
Tailing factor: NMT 2
Relative standard deviation: NMT 2.0%
Analysis
Sample: *Sample solution*
Calculate the percentage of lutein relative to total carotenoids in the Preparation taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = individual peak response of lutein
r_T = sum of the responses of all the peaks

Calculate the percentage of lutein in the Preparation taken:

$$\text{Result} = (r_U/r_T) \times T$$

r_U = individual peak response of lutein in the *Sample solution*
r_T = sum of the responses of all the peaks
T = percentage of total carotenoids as determined in the test for *Content of Total Carotenoids*

Acceptance criteria: NLT 85.0% of lutein in the total carotenoid content, and the Preparation contains 95.0%–130.0% of the labeled amount of lutein, calculated as $C_{40}H_{56}O_2$, on the anhydrous basis.

- **ZEAXANTHIN AND OTHER RELATED COMPOUNDS**

Solvent, Mobile phase, Standard solution, Sample solution, and **Chromatographic system:** Proceed as directed in the test for *Content of Lutein*.
Analysis
Sample: *Sample solution*
Injection size: 10 µL
Calculate the percentage of zeaxanthin relative to total carotenoids in the Preparation taken:

$$\text{Result} = (r_U/r_T) \times 100$$

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r_U = individual peak response of zeaxanthin
r_T = sum of the responses of all the peaks

Acceptance criteria
Zeaxanthin: NMT 9.0%
Any other single related compound: NMT 1.0%
Total related compounds (including zeaxanthin): NMT 15.0%

IMPURITIES
INORGANIC IMPURITIES
- **Residue on Ignition** ⟨281⟩: NMT 2.0%
- **Lead** ⟨251⟩: NMT 1 ppm

SPECIFIC TESTS
- **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 10.0%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tightly sealed, light- and oxygen-resistant containers. Store in a cool place.
- **LABELING:** The label states that this article is not intended for direct administration to humans or to animals.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Lutein RS

Lycopene

$C_{40}H_{56}$ 536.88
[502-65-8].

DEFINITION
Lycopene is a mixture of geometrical isomers of lycopene. It contains NLT 96.0% and NMT 101.0% of lycopene ($C_{40}H_{56}$), calculated on the dried basis.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Ultraviolet-Visible Spectroscopy*: **197U** ▲(CN 1-May-2020)
  Analytical wavelength: 300–700 nm
  Sample solution: Prepare as directed for the *Sample solution* in the test for *Content of Lycopene*.
  Acceptance criteria: Meets the requirements. The absorbance ratio A_{476}/A_{508} is 1.10–1.14.
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of all-E-Lycopene, 5Z-Lycopene, and Related Compounds*.

COMPOSITION
- **CONTENT OF LYCOPENE**
  Sample stock solution: Transfer 25 mg of Lycopene and 25 mg of butylated hydroxytoluene into a 100-mL volumetric flask. Add 60 mL of methylene chloride, and sonicate to dissolve. Dilute with methylene chloride to volume.
  Sample solution: Transfer 2.0 mL of the *Sample stock solution* into a 200-mL volumetric flask, and dilute with cyclohexane to volume.
  Instrumental conditions
  (See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
  Mode: UV-Vis
  Analytical wavelength: 476 nm
  Blank: Cyclohexane
  Analysis
  Sample: *Sample solution*

  Calculate the percentage of lycopene ($C_{40}H_{56}$) in the portion of Lycopene taken:

$$\text{Result} = [A_U/(a \times C_U)] \times 100$$

A_U = absorbance of the *Sample solution*
a = absorptivity of the pure lycopene in cyclohexane, 331 ($mL \cdot mg^{-1} \cdot cm^{-1}$)
C_U = concentration of lycopene in the *Sample solution* (mg/mL)

Acceptance criteria: 96.0%–101.0% on the dried basis

Change to read:

- **CONTENT OF ALL-*E*-LYCOPENE, *5Z*-LYCOPENE, AND RELATED COMPOUNDS**
  Mobile phase: *tert*-Butyl methyl ether, methanol, and tetrahydrofuran (784:665:74)
  Standard solution: Transfer a weighed quantity of ▲USP Lycopene Preparation RS, ▲(USP 1-May-2019) equivalent to approximately 5 mg of lycopene, into a 250-mL volumetric flask. Add about 60 units of bacterial alkaline protease preparation or another suitable enzyme, and about 25 mg of butylated hydroxytoluene. Add 2.5 mL of ammonium hydroxide diluted in water (2 in 100), and mix. Place in an ultrasonic bath at 50° for 10 min, rotate the flask occasionally to avoid having the material stick to the glass surface, and continue until the material is dispersed with no lumps. Add 5 mL of tetrahydrofuran, 40 mL of dehydrated alcohol, and mix. Place in an ultrasonic bath for about 1 min. Cool to room temperature, and dilute with *tert*-butyl methyl ether to volume. Shake vigorously, then allow the precipitate to settle. Filter the supernatant.
  Sample stock solution: Transfer 15 mg of Lycopene to a 25-mL volumetric flask, and dissolve in tetrahydrofuran containing 50 mg/L of butylated hydroxytoluene. Dilute with the same solvent to volume.
  Sample solution: Pipet 2 mL of the *Sample stock solution* into a 50-mL volumetric flask, and add 8 mL of tetrahydrofuran. Dilute with *tert*-butyl methyl ether to volume.
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: LC
  Detector: UV 472 nm
  Column: 4.6-mm × 25-cm, 5-µm packing L62; second column connected in series, 4.6-mm × 25-cm, 3-µm packing L62. [NOTE—New columns may require conditioning.]
  Flow rate: 1 mL/min
  Injection volume: 10 µL
  System suitability
  Sample: *Standard solution*
  [NOTE—The relative retention times for all-*E*-lycopene and 5*Z*-lycopene are 1.0 and about 1.07, respectively.]
  Suitability requirements
  Resolution: NLT 1.0 between all-*E*-lycopene and 5*Z*-lycopene
  Tailing factor: 0.8–2.0 for all-*E*-lycopene
  Relative standard deviation: NMT 2.0% for all-*E*-lycopene
  Analysis
  Sample: *Sample solution*
  Calculate the percentage of all-*E*-lycopene in the portion of Lycopene taken:

$$\text{Result} = (r_E/r_T) \times T$$

DS Monographs

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r_E = peak response of the all-*E*-lycopene isomer
r_T = sum of all the peak responses
T = percentage of total lycopene isomers obtained in the test for *Content of Lycopene*

Acceptance criteria: NLT 70.0% of all-*E*-lycopene
Calculate the percentage of the 5*Z*-lycopene isomer in the portion of Lycopene taken:

$$\text{Result} = (r_{5Z}/r_T) \times T$$

r_{5Z} = peak response of the 5*Z*-lycopene isomer
r_T = sum of all the peak responses
T = percentage of total lycopene isomers obtained in the test for *Content of Lycopene*

Acceptance criteria: NMT 23.0% of the 5*Z*-lycopene isomer
Calculate the percentage of related compounds in the portion of Lycopene taken:

$$\text{Result} = (r_S/r_T) \times T$$

r_S = sum of all the peak responses except for all-*E*-lycopene and 5*Z*-lycopene
r_T = sum of all the peak responses
T = percentage of total lycopene isomers obtained in the test for *Content of Lycopene*

Acceptance criteria: NMT 9.0% of other related compounds calculated as lycopene

IMPURITIES

• **Residue on Ignition** ⟨281⟩: NMT 0.2%

SPECIFIC TESTS

• **Loss on Drying** ⟨731⟩
Analysis: Dry over phosphorus pentoxide under vacuum at 40° for 4 h.
Acceptance criteria: NMT 0.2%

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Preserve in tight, light-resistant containers, under inert gas, and store in a cool place.
• **Labeling:** Label it to indicate whether the article is obtained from natural sources or is prepared synthetically. If obtained from natural sources, label it to indicate the natural source, including its Latin binomial.

Change to read:

• **USP Reference Standards** ⟨11⟩
▲USP Lycopene Preparation RS▲ (USP 1-May-2019)

Lycopene Preparation

DEFINITION

Lycopene Preparation is a combination of Lycopene with one or more inert substances and suitable antioxidants. It may be in a solid or oily liquid form. It contains NLT 95.0% and NMT 120.0% of the labeled amount of lycopene ($C_{40}H_{56}$), calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

• **A. ▲Spectroscopic Identification Tests** ⟨197⟩, *Ultraviolet-Visible Spectroscopy*: **197U▲** (CN 1-May-2020)
Analytical wavelength: 300–700 nm
Sample solution: Prepare as directed for the *Sample solution* in the test for *Content of Lycopene*.
Acceptance criteria: Meets the requirements. The absorbance ratio A_{476}/A_{508} is 1.10–1.14 in cyclohexane. The absorbance ratio A_{472}/A_{504} is 1.09–1.13 in isopropyl alcohol.

COMPOSITION

• **Content of Lycopene**
Procedure for oily preparations
Sample stock solution: Transfer a weighed quantity of oily Preparation containing 25 mg of lycopene to a 100-mL volumetric flask. Add 25 mg of butylated hydroxytoluene and 60 mL of methylene chloride, and sonicate to dissolve. Dilute with methylene chloride to volume.
Sample solution: 2.0 mL of the *Sample stock solution* diluted with cyclohexane to 200.0 mL
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: UV-Vis
Analytical wavelength: 476 nm
Blank: Cyclohexane
Analysis
Sample: *Sample solution*
Calculate the percentage of the labeled amount of lycopene ($C_{40}H_{56}$) in the portion of Preparation taken:

$$\text{Result} = [A_U/(a \times C_U)] \times 100$$

A_U = absorbance of the *Sample solution*
a = absorptivity of the pure lycopene in cyclohexane, 331 ($mL \cdot mg^{-1} \cdot cm^{-1}$)
C_U = nominal concentration of lycopene in the *Sample solution* (mg/mL)

Acceptance criteria: 95.0%–120.0% on the anhydrous basis
Procedure for solid preparations
Sample stock solution: Transfer a weighed quantity of solid Preparation, equivalent to approximately 5 mg of lycopene, into a 200-mL volumetric flask. Add about 60 units of bacterial alkaline protease preparation, or another suitable enzyme, and about 25 mg of butylated hydroxytoluene. Add 2.5 mL of ammonium hydroxide diluted in water (2 in 100), and mix. Place in an ultrasonic bath at 50° for 10 min, rotate the flask occasionally to avoid having the material stick to the glass surface, and continue until the material is dispersed with no lumps. Add 5 mL of tetrahydrofuran and 40 mL of dehydrated alcohol, and mix. Place in an ultrasonic bath for about 1 min. Cool to room temperature, and dilute with *tert*-butyl methyl ether to volume. Shake vigorously, then allow to stand until the solid has settled.
Sample solution: 2.0 mL of the *Sample stock solution* diluted with isopropyl alcohol to 25.0 mL
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: UV-Vis
Analytical wavelength: 472 nm
Blank: Isopropyl alcohol
Analysis
Sample: *Sample solution*

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Calculate the percentage of the labeled amount of lycopene ($C_{40}H_{56}$) in the portion of Preparation taken:

$$\text{Result} = [A_U/(a \times C_U)] \times 100$$

A_U = absorbance of the *Sample solution*
a = absorptivity of the pure lycopene in isopropyl alcohol, 320 (mL · mg^{-1} · cm^{-1})
C_U = nominal concentration of lycopene in the *Sample solution* (mg/mL)

Acceptance criteria: 95.0%–120.0% on the anhydrous basis

Change to read:

- **CONTENT OF ALL-*E*-LYCOPENE, 5*Z*-LYCOPENE, AND RELATED COMPOUNDS**

Mobile phase: *tert*-Butyl methyl ether, methanol, and tetrahydrofuran (784:665:74)

Standard solution: Transfer a weighed quantity of ▲USP Lycopene Preparation RS,▲ (USP 1-May-2019) equivalent to approximately 5 mg of lycopene, into a 250-mL volumetric flask. Add about 60 units of bacterial alkaline protease preparation, or another suitable enzyme, and about 25 mg of butylated hydroxytoluene. Add 2.5 mL of ammonium hydroxide diluted in water (2 in 100), and mix. Place in an ultrasonic bath at 50° for 10 min, rotate the flask occasionally to avoid having the material stick to the glass surface, and continue until the material is dispersed with no lumps. Add 5 mL of tetrahydrofuran and 40 mL of dehydrated alcohol, and mix. Place in an ultrasonic bath for about 1 min. Cool to room temperature, and dilute with *tert*-butyl methyl ether to volume. Shake vigorously, then allow the precipitate to settle. Filter the supernatant.

Sample solution for oily preparations: Transfer a quantity of oily Preparation, equivalent to 15 mg of lycopene, to a 25-mL volumetric flask, and dissolve in tetrahydrofuran containing 50 mg/L of butylated hydroxytoluene. Dilute with the same solvent to volume. Pipet 2 mL of this solution into a 50-mL volumetric flask, and add 8 mL of tetrahydrofuran. Dilute with *tert*-butyl methyl ether to volume.

Sample solution for solid preparations: Transfer a weighed quantity of solid Preparation, equivalent to approximately 5 mg of lycopene, into a 250-mL volumetric flask. Add about 60 units of bacterial alkaline protease preparation, or another suitable enzyme, and about 25 mg of butylated hydroxytoluene. Add 2.5 mL of ammonium hydroxide diluted in water (2 in 100), and mix. Place in an ultrasonic bath at 50° for 10 min, rotate the flask occasionally to avoid having the material stick to the glass surface, and continue until the material is dispersed with no lumps. Add 5 mL of tetrahydrofuran and 40 mL of dehydrated alcohol, and mix. Place in an ultrasonic bath for about 1 min. Cool to room temperature, and dilute with *tert*-butyl methyl ether to volume. Shake vigorously, then allow the precipitate to settle. Filter the supernatant.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 472 nm
Column: 4.6-mm × 25-cm, 5-µm packing L62; second column connected in series, 4.6-mm × 25-cm, 3-µm packing L62. [NOTE—New columns may require conditioning.]
Flow rate: 1 mL/min
Injection volume: 10 µL

System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for all-*E*-lycopene and 5*Z*-lycopene are 1.0 and about 1.07, respectively.]
Suitability requirements
Resolution: NLT 1.0 between all-*E*-lycopene and 5*Z*-lycopene
Tailing factor: 0.8–2.0 for all-*E*-lycopene
Relative standard deviation: NMT 2.0% for all-*E*-lycopene

Analysis
Sample: Relevant *Sample solution*
Calculate the percentage of all-*E*-lycopene in the portion of Preparation taken:

$$\text{Result} = (r_E/r_T) \times 100$$

r_E = peak response of the all-*E*-lycopene isomer
r_T = sum of all the peak responses

Acceptance criteria: NLT 65.0% of all-*E*-lycopene
Calculate the percentage of the 5*Z*-lycopene isomer in the portion of Preparation taken:

$$\text{Result} = (r_{5Z}/r_T) \times 100$$

r_{5Z} = peak response for the 5*Z*-lycopene isomer
r_T = sum of all the peak responses

Acceptance criteria: NMT 23.0% of the 5*Z*-lycopene isomer
Calculate the percentage of related compounds in the portion of Preparation taken:

$$\text{Result} = (r_S/r_T) \times 100$$

r_S = sum of the responses of all peaks except the peak for all-*E*-lycopene and the peak for 5*Z*-lycopene
r_T = sum of all the peak responses

Acceptance criteria: NMT 14% of other related compounds calculated as lycopene

SPECIFIC TESTS

- **WATER DETERMINATION** ⟨921⟩, *Method I*: NMT 8.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers under inert gas. Store the oily Preparation in a cool place and the solid Preparation at controlled room temperature.
- **LABELING:** Label it to state the name and content of added antioxidants and inert substances. Label it to indicate whether the article is prepared with lycopene from natural sources or with synthetic lycopene. If prepared with lycopene from natural sources, label it to indicate the natural source, including its Latin binomial.

Change to read:

- **USP REFERENCE STANDARDS** ⟨11⟩
▲USP Lycopene Preparation RS▲ (USP 1-May-2019)

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Tomato Extract Containing Lycopene

DEFINITION

Tomato Extract Containing Lycopene is an ethyl acetate extract of the natural tomato lipids. It is produced from the pulp of ripe fruits of *Lycopersicon esculentum* Mill. (Fam. Solanaceae), after removing the tomato water-soluble fraction. It contains NLT 95.0% and NMT 105.0% of the labeled amount of lycopene ($C_{40}H_{56}$). It contains NLT 4.7% and NMT 12.0% of lycopene, NLT 0.8% of the combined amount of phytofluene ($C_{40}H_{62}$) and phytoene ($C_{40}H_{64}$), NLT 0.2% of beta carotene ($C_{40}H_{56}$), and NLT 1.0% of tocopherols ($C_{28}H_{48}O_2$) on the anhydrous basis. Tocopherols may be added as antioxidants.

IDENTIFICATION

• **A. Presence of Lycopene, Phytofluene, and Phytoene**

Analysis: Proceed as directed in the test for *Content of Other Carotenoids and Tocopherols*.

Acceptance criteria: The retention times of the lycopene, phytofluene, and phytoene peaks of the *Sample solution* correspond to those of the *Standard solution*.

• **B. Ratio of all-*E*-Lycopene and 5*Z*-Lycopene**

Butylated hydroxytoluene stock solution: Prepare as directed in the test for *Content of Lycopene*.

Mobile phase: 0.05% diisopropylethylamine in *n*-hexane; sonicate for 3–4 min.

Sample solution: Prepare as directed in the test for *Content of Lycopene*, except dilute with *n*-hexane (5:100)

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV-Vis 472 nm

Column: Two 4.0-mm × 25-cm; 5-µm packing L3 (300-Å pore size), connected in a series

Column temperature: 22°

Flow rate: 0.5 mL/min

Injection volume: 10 µL

[NOTE—The peak for all-*E*-lycopene elutes in 30–45 min.]

[NOTE—The relative retention times for all-*E*-lycopene and 5*Z*-lycopene are 1.00 and in the range of 1.04–1.10, respectively.]

Analysis

Sample: *Sample solution*

Measure the areas of the 2 major peaks, and calculate their area ratio:

$$\text{Result} = r_{U1}/r_{U2}$$

r_{U1} = peak area of 5*Z*-lycopene

r_{U2} = peak area of all-*E*-lycopene

Acceptance criteria: NMT 0.10 for the area ratio

COMPOSITION

• **Content of Lycopene**

Butylated hydroxytoluene stock solution: 5 mg/mL of butylated hydroxytoluene in methylene chloride.

[NOTE—This solution can be stored protected from light for up to 3 months.]

Mobile phase: Acetonitrile, methylene chloride, *n*-hexane, and methanol (850:25:25:100). Add 0.05% of diisopropylethylamine, mix, and sonicate for 3–4 min.

Diluent: Acetonitrile, methylene chloride, *n*-hexane, butylated hydroxytoluene, and methanol (600: 150: 100: 0.5: 150). Add 0.05% of diisopropylethylamine, mix, and sonicate for 3–4 min.

Standard solution: Transfer a weighed quantity of USP Tomato Extract Containing Lycopene RS, equivalent to about 6 mg of lycopene, to a 100-mL volumetric flask, and dissolve in 1 mL of *Butylated hydroxytoluene stock solution* and 9 mL of methylene chloride using a sonicator. Dilute with *Diluent* to volume to obtain a solution with a known concentration of about 0.06 mg/mL of lycopene.

Sample stock solution: Warm the Tomato Extract Containing Lycopene to 50° in a water bath. Mix well with a glass rod or a spatula. Weigh and dissolve a quantity of 1–1.2 g of the sample in 10 mL of *Butylated hydroxytoluene stock solution* and 30 mL of methylene chloride. Sonicate the solution for 1 min to dissolve the sample completely. Cool to room temperature, and dilute with methylene chloride to 100 mL.

Sample solution: Dilute the *Sample stock solution* with *Diluent* (1 in 10).

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV-Vis 472 nm

Column: 4.6-mm × 25-cm; 5-µm packing L7

Column temperature: 39 ± 1°

Flow rate: 0.7 mL/min

Injection volume: 10 µL

System suitability

Sample: *Standard solution*

[NOTE—The retention time for lycopene is about 6 min.]

Suitability requirements

Relative standard deviation: NMT 2.0% for the lycopene peak area

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the responses of the major lycopene peaks. Calculate the percentage of lycopene in the portion of Tomato Extract Containing Lycopene taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times 100$$

r_U = peak area of lycopene from the *Sample solution*

r_S = peak area of lycopene from the *Standard solution*

C_S = concentration of lycopene in the *Standard solution* (mg/mL)

V = volume of the *Sample stock solution* (mL)

W = weight of Tomato Extract Containing Lycopene taken to prepare the *Sample stock solution* (mg)

D = dilution factor used to prepare the *Sample solution* from the *Sample stock solution*

Acceptance criteria: 95.0%–105.0% of the labeled amount of lycopene; 4.7%–12.0% of lycopene

• **Content of Other Carotenoids and Tocopherols (Phytofluene, Phytoene, Beta Carotene, and Tocopherols)**

Butylated hydroxytoluene stock solution, Diluent, Standard solution, and Sample solution: Prepare as directed in the test for *Content of Lycopene*.

Mobile phase: Acetonitrile, methylene chloride, *n*-hexane, and methanol (19:1:1:19). Add 0.05% of diisopropylethylamine, mix, and sonicate for 3–4 min.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV-Vis; 472 nm for lycopene, 450 nm for beta carotene, 350 nm for phytofluene, and 288 nm for phytoene and tocopherol

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 39 ± 1°

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Flow rate: 0.6 mL/min
Injection volume: 10 µL
System suitability
Sample: *Standard solution*
[NOTE—The relative retention times are about 0.6 for the tocopherol isomers, 1.0 for all-*E*-lycopene, 1.5–1.7 for the beta carotene isomers, 1.6–1.8 for the phytofluene isomers, and 1.8–2.2 for phytoene.]
Suitability requirements
Relative standard deviation: NMT 2.0% for all-*E*-lycopene
Chromatogram similarity: The chromatogram of the *Standard solution* is similar to the reference chromatogram provided with the lot of USP Tomato Extract Containing Lycopene RS being used.
Analysis
Samples: *Standard solution* and *Sample solution*
Identify the locus of the peaks of the lycopene isomers, beta carotene isomers, phytofluene isomers, and phytoene by comparison with the reference chromatogram provided with the corresponding lot of USP Tomato Extract Containing Lycopene RS. Measure the sum of the peak responses of the lycopene isomers at 472 nm in the *Standard solution*. [NOTE—The lycopene isomers may be resolved in more than one peak in this chromatographic system.] In the *Sample solution*, measure the sum of the peak responses of the beta carotene isomers at 450 nm, the sum of the peak responses of the phytofluene isomers at 350 nm, the peak response of phytoene at 288 nm, and the sum of the peak responses of all tocopherols at 288 nm.
Calculate the percentage of beta carotene in the portion of Tomato Extract Containing Lycopene taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times F \times 100$$

r_U = sum of the peak responses of the beta carotene isomers at 450 nm from the *Sample solution*
r_S = peak area of lycopene at 472 nm from the *Standard solution*
C_S = concentration of lycopene in the *Standard solution* (mg/mL)
V = volume of the *Sample stock solution* (mL)
W = weight of Tomato Extract Containing Lycopene taken to prepare the *Sample stock solution* (mg)
D = dilution factor used to prepare the *Sample solution* from the *Sample stock solution*
F = absorptivity ratio of pure lycopene to pure beta carotene, 345/259.2

Calculate the percentage of phytofluene in the portion of Tomato Extract Containing Lycopene taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times F \times 100$$

r_U = sum of the peak responses of the phytofluene isomers at 350 nm from the *Sample solution*
r_S = sum of the peak responses of the lycopene isomers at 472 nm from the *Standard solution*
C_S = concentration of lycopene in the *Standard solution* (mg/mL)
V = volume of the *Sample stock solution* (mL)
W = weight of Tomato Extract Containing Lycopene taken to prepare the *Sample stock solution* (mg)
D = dilution factor used to prepare the *Sample solution* from the *Sample stock solution*
F = absorptivity ratio of pure lycopene to pure phytofluene, 345/135

Calculate the percentage of phytoene in the portion of Tomato Extract Containing Lycopene taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times F \times 100$$

r_U = area of the phytoene peak response at 288 nm from the *Sample solution*
r_S = sum of the peak responses of the lycopene isomers at 472 nm from the *Standard solution*
C_S = concentration of lycopene in the *Standard solution* (mg/mL)
V = volume of the *Sample stock solution* (mL)
W = weight of Tomato Extract Containing Lycopene taken to prepare the *Sample stock solution* (mg)
D = dilution factor used to prepare the *Sample solution* from the *Sample stock solution*
F = absorptivity ratio of pure lycopene to pure phytoene, 345/125

Calculate the percentage of tocopherols in the portion of Tomato Extract Containing Lycopene taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times F \times 100$$

r_U = sum of the peak responses of all the tocopherol peaks at 288 nm from the *Sample solution*
r_S = sum of the peak responses of the lycopene isomers at 472 nm from the *Standard solution*
C_S = concentration of lycopene in the *Standard solution* (mg/mL)
V = volume of the *Sample stock solution* (mL)
W = weight of Tomato Extract Containing Lycopene taken to prepare the *Sample stock solution* (mg)
D = dilution factor used to prepare the *Sample solution* from the *Sample stock solution*
F = absorptivity ratio of pure lycopene to the average absorptivity of tocopherols, 345/8.5

Acceptance criteria: NLT 0.8% of the combined amount of phytofluene ($C_{40}H_{62}$) and phytoene ($C_{40}H_{64}$); NLT 0.2% of beta carotene ($C_{40}H_{56}$); and NLT 1.0% of tocopherols ($C_{28}H_{48}O_2$) on the anhydrous basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Test for Aflatoxins*: NMT 4 ng/g of total aflatoxins B1, B2, G1, and G2; NMT 2 ng/g of aflatoxin B1

Change to read:

- **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020) : Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 2×10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species, Test for Absence of Escherichia coli,* and *Test for Absence of Staphylococcus aureus*: Meets the requirements

SPECIFIC TESTS

- **CLARITY OF SOLUTION**

Analysis: Warm the sample to 50° in a water bath. Mix well with a glass rod or a spatula, and transfer 1 g of the Extract directly into a 100-mL volumetric flask. Add 50 mL of methylene chloride, and sonicate the solution for 1 min to completely dissolve the sample. Bring to room

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temperature, and dilute with methylene chloride to volume.
Acceptance criteria: The solution is clear: no deposit or turbidity is formed.
• **VISCOSITY—ROTATIONAL METHODS** ⟨912⟩
Analysis: Equilibrate the Tomato Extract Containing Lycopene at 37° in a 30-mL glass vial. Determine the viscosity using a rotational viscometer equipped with a spindle (No. 6) having a cylinder, 1.47 cm in diameter and 0.16 cm high, attached to a shaft 0.32 cm in diameter, with a distance of 3.02 cm from the top of the cylinder to the lower tip of the shaft. The spindle is rotating at the appropriate speed and immersion depth to obtain a scale reading of 10%–90% of full scale.
Calculate the viscosity, in centipoises, by multiplying the scale reading by the constant for the spindle and speed used.
Acceptance criteria: NMT 5000 centipoises
• **WATER DETERMINATION** ⟨921⟩, *Method I, Method Ia*: NMT 0.8%
• **PARTICLE SIZE DISTRIBUTION**
(See *Optical Microscopy* ⟨776⟩.)
Analysis: Transfer 1 drop to a microscope slide, and spread evenly. Isopropanol may be used as a diluent, if necessary. Examine the slide under a microscope equipped with a calibrated ocular micrometer, using 450× magnification. Scan the slide, and note the size of the individual particles.
Acceptance criteria: NLT 98% of the particles are less than 20 µm in length when measured along the longest axis; NLT 60% of the particles are less than 5 µm; and NLT 40% of the particles are less than 2 µm.

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store in a cool place.
• **LABELING:** Label it to state the content of lycopene as a percentage and to state that the material should be heated to 50° and mixed before use. Label it to indicate the Latin binomial and the part of the plant from which the article is derived.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Tomato Extract Containing Lycopene RS

Lysine Acetate—*see Lysine Acetate General Monographs*

Lysine Hydrochloride—*see Lysine Hydrochloride General Monographs*

Lysine Hydrochloride Tablets

DEFINITION
Lysine Hydrochloride Tablets contain NLT 90.0% and NMT 120.0% of the labeled amount of $C_6H_{14}N_2O_2 \cdot HCl$, as L-lysine hydrochloride.

IDENTIFICATION
• **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**
Standard solution: 0.4 mg/mL of USP L-Lysine Hydrochloride RS in water
Sample solution: A filtered solution in water, equivalent to 0.4 mg/mL of lysine hydrochloride from powdered Tablets
Chromatographic system
(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
Mode: TLC
Adsorbent: 0.25-mm layer of chromatographic silica gel
Application volume: 10 µL
Developing solvent system: Isopropyl alcohol and ammonium hydroxide (70:30)
Spray reagent: 2 mg/mL of ninhydrin in a mixture of butyl alcohol and 2 N acetic acid (95:5)
Analysis
Samples: *Standard solution* and *Sample solution*
Develop the plate, and dry at 100°–105° until the ammonia disappears completely. Spray with *Spray reagent*, and heat at 100°–105° for 15 min. Examine the plate under white light.
Acceptance criteria: The R_F value of the principal spot from the *Sample solution* corresponds to that from the *Standard solution*.

STRENGTH
• **PROCEDURE**
Sample: A portion of the powder from NLT 20 finely powdered Tablets, equivalent to 75 mg of lysine hydrochloride
Blank: Proceed as directed in the *Analysis* without the *Sample*.
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.1 N perchloric acid VS
Endpoint detection: Potentiometric or visual
Analysis: Dissolve the *Sample* in 5 mL of mercuric acetate TS with gentle heating. Cool, then add 50 mL of glacial acetic acid. Add 3 drops of crystal violet TS when needed. Titrate with *Titrant*. Perform the *Blank* determination.
Calculate the percentage of the labeled amount of L-lysine hydrochloride ($C_6H_{14}N_2O_2 \cdot HCl$) in the portion of *Sample* taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

V_S = *Titrant* volume consumed by the *Sample* (mL)
V_B = *Titrant* volume consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 91.33 mg/mEq
W = nominal amount of L-lysine hydrochloride in the *Sample* taken (mg)

Acceptance criteria: 90.0–120.0%

PERFORMANCE TESTS
• **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩: Meet the requirements for *Disintegration*
Time: 20 min
• **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩ : Meet the requirements

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at controlled room temperature.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP L-Lysine Hydrochloride RS

Magnesium Gluconate—*see Magnesium Gluconate General Monographs*

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Magnesium Gluconate Tablets—*see Magnesium Gluconate Tablets General Monographs*

Magnesium Oxide Capsules—*see Magnesium Oxide Capsules General Monographs*

Magnesium Oxide Tablets—*see Magnesium Oxide Tablets General Monographs*

Manganese Gluconate—*see Manganese Gluconate General Monographs*

Malabar-Nut-Tree, Leaf

DEFINITION

Malabar-Nut-Tree, Leaf, known in commerce as vasaka, consists of the dried leaves of *Justicia adhatoda* L. [syn. *Adhatoda vasica* Nees] (Fam. Acanthaceae). It contains NLT 0.6% of vasicine, calculated on the dried basis.

IDENTIFICATION

- **A.** Malabar-Nut-Tree, Leaf meets the requirements in *Specific Tests, Botanical Characteristics.*
- **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution A: 0.5 mg/mL of USP Vasicine RS in methanol. Sonicate to dissolve if necessary.

Standard solution B: 50 mg/mL of USP Powdered Malabar-Nut-Tree, Leaf Extract RS in methanol. Sonicate for about 15 min, centrifuge, and use the supernatant.

Sample solution: Transfer about 2.0 g of Malabar-Nut-Tree, Leaf, finely powdered and accurately weighed, to a 250-mL round-bottom flask fitted with a reflux condenser. Add 50 mL of methanol, reflux for 15 min, cool to room temperature, and decant the supernatant. Repeat until the extract is colorless. Combine the extracts, filter, and concentrate under vacuum. Transfer into a 25-mL volumetric flask, and adjust to volume with methanol. [NOTE—Save the filtrate for use in the test for *Content of Vasicine.*]

Adsorbent: Chromatographic silica gel with an average particle size of 10–15 µm (TLC plates)

Application volume: 10 µL, as 4-mm bands

Developing solvent system: Ethyl acetate, methanol, and ammonia (8: 2: 0.2)

Analysis

Samples: *Standard solution A, Standard solution B,* and *Sample solution*

Apply the *Samples* as bands. Using a saturated chamber, develop the chromatograms until the solvent front has moved up about three-fourths of the length of the plate. Remove the plate from the chamber, dry, and examine under UV at 254 nm.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits a quenching zone at an R_F value of approximately 0.35 for vasicine, corresponding to a zone in the chromatogram of *Standard solution A.* The chromatogram of the *Sample solution* also exhibits an additional quenching zone at an R_F value of approximately 0.53 for vasicinone, corresponding to a similar zone in the chromatogram of *Standard solution B.* Other minor zones may be observed in the *Sample solution* and *Standard solution B* chromatograms.

- **C. HPLC:** The chromatogram of the *Sample solution* from the test for *Content of Vasicine* shows a main peak at a retention time corresponding to that of vasicine in the chromatogram of *Standard solution A.* Identify other peaks in the *Sample solution* by comparison with the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Malabar-Nut-Tree, Leaf Extract RS being used. The *Sample solution* shows an additional peak corresponding to vasicinone.

COMPOSITION

- **CONTENT OF VASICINE**

Buffer solution: Dissolve 1.36 g of anhydrous potassium dihydrogen phosphate in 900 mL of water. Add 2.0 mL of phosphoric acid. Dilute with water to 1000 mL, and filter.

Mobile phase: *Buffer solution,* acetonitrile, and tetrahydrofuran (92:5:3)

Standard solution A: 0.1 mg/mL of USP Vasicine RS in methanol. Sonicate to dissolve if necessary.

Standard solution B: 5.0 mg/mL of USP Powdered Malabar-Nut-Tree, Leaf Extract RS in methanol. Sonicate for about 15 min. Before injection, pass through a membrane filter of 0.45-µm or finer pore size.

Sample solution: Dilute the *Sample solution,* prepared as directed in *Identification* test *B* (1:5), with methanol. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, and discard the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability.*)

Mode: LC

Detector: UV 280 nm

Column: 4.6-mm × 25-cm; 5-µm packing L10

Flow rate: 1.0 mL/min

Injection volume: 20 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

[NOTE—The approximate relative retention times of the vasicine and vasicinone peaks are 1.00 and 1.23, respectively.]

Suitability requirements

Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Malabar-Nut-Tree, Leaf Extract RS being used.

Resolution: NLT 2.0 between the vasicine and vasicinone peaks, *Standard solution B*

Relative standard deviation: NMT 2.0% determined from the vasicine peak in repeated injections, *Standard solution A*

Analysis

Samples: *Standard solution A, Standard solution B,* and *Sample solution*

Using the chromatogram of *Standard solution A, Standard solution B,* and the reference chromatogram provided with the lot of USP Powdered Malabar-Nut-Tree, Leaf Extract RS being used, identify the retention times of the peaks corresponding to vasicine and vasicinone.

Calculate the percentage of vasicine in the portion of Malabar-Nut-Tree, Leaf taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of vasicine from the *Sample solution*

r_S = peak area of vasicine from *Standard solution A*

C_S = concentration of USP Vasicine RS in *Standard solution A* (mg/mL)

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V = volume of the *Sample solution* (mL)
W = weight of Malabar-Nut-Tree, Leaf taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 0.6% on the dried basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria do not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: Leaves, simple, green to dull grayish-brown, minutely pubescent, lanceolate to ovate-lanceolate, tapering base and slightly acuminate apex, 10–20 cm long, 3–10 cm broad, petioles 2–8 cm long, and 8–10 pairs of reticulate lateral veins; characteristic odor, and bitter taste. Pharmacopeial article consists of dry, brittle, and grayish-brown leaves.

Microscopic

Transverse section of leaves: Upper epidermal cells uniform in size and sinuous in outline, while lower epidermal cells vary in size and are less wavy; 4–6 layers of collenchymal cells below epidermis in the midrib region; shows two layers of palisade cells with elongated cystoliths, which are absent in epidermal cells (a diagnostic feature that is not seen in the leaves of *Ailanthus excelsa*, a potential adulterant); globules of oil dispersed in palisade and spongy layers; numerous stomata diacytic or anomocytic; glandular and non-glandular trichomes are visible on both epidermal layers.

- **LOSS ON DRYING** ⟨731⟩

Sample: 1.0 g of finely powdered Malabar-Nut-Tree, Leaf
Analysis: Dry the *Sample* at 105° for 3 h.
Acceptance criteria: NMT 12.0%

- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩

Sample: 1.0 g of finely powdered Malabar-Nut-Tree, Leaf
Acceptance criteria: NMT 21%

- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 2%
- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩**:** NMT 2.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Powdered Malabar-Nut-Tree, Leaf Extract RS
USP Vasicine RS

Powdered Malabar-Nut-Tree, Leaf

DEFINITION

Powdered Malabar-Nut-Tree, Leaf is Malabar-Nut-Tree, Leaf reduced to a powder or very fine powder. It contains NLT 0.6% of vasicine, calculated on the dried basis.

IDENTIFICATION

- **A.** Powdered Malabar-Nut-Tree, Leaf meets the requirements in *Specific Tests, Botanical Characteristics.*
- **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution A: 0.5 mg/mL of USP Vasicine RS in methanol. Sonicate to dissolve if necessary.
Standard solution B: 50 mg/mL of USP Powdered Malabar-Nut-Tree, Leaf Extract RS in methanol. Sonicate for about 15 min, centrifuge, and use the supernatant.
Sample solution: Transfer about 2.0 g of Powdered Malabar-Nut-Tree, Leaf, accurately weighed, to a 250-mL round-bottom flask fitted with a reflux condenser. Add 50 mL of methanol, reflux for 15 min, cool to room temperature, and decant the supernatant. Repeat until the extract is colorless. Combine the extracts, filter, and concentrate under vacuum. Transfer into a 25-mL volumetric flask, and adjust to volume with methanol. [NOTE—Save the filtrate for use in the test for *Content of Vasicine.*]
Adsorbent: Chromatographic silica gel with an average particle size of 10–15 µm (TLC plates)
Application volume: 10 µL, as 4-mm bands
Developing solvent system: Ethyl acetate, methanol, and ammonia (8: 2: 0.2)
Analysis
Samples: *Standard solution A, Standard solution B,* and *Sample solution*
Apply the *Samples* as bands. Using a saturated chamber, develop the chromatograms until the solvent front has moved up about three-fourths of the length of the plate. Remove the plate from the chamber, dry, and examine under UV at 254 nm.
Acceptance criteria: The chromatogram of the *Sample solution* exhibits a quenching zone at an R_F value of approximately 0.35 for vasicine, corresponding to a zone in the chromatogram of *Standard solution A.* The chromatogram of the *Sample solution* also exhibits an additional quenching zone at an R_F value of approximately 0.53 for vasicinone, corresponding to a similar zone in the chromatogram of *Standard solution B.* Other minor zones may be observed in the *Sample solution* and *Standard solution B* chromatograms.

- **C. HPLC:** The chromatogram of the *Sample solution* from the test for *Content of Vasicine* shows a main peak at a retention time corresponding to that of vasicine in the chromatogram of *Standard solution A.* Identify other peaks in the *Sample solution* by comparison with the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Malabar-Nut-Tree, Leaf Extract RS being used. The *Sample solution* shows an additional peak corresponding to vasicinone.

COMPOSITION

- **CONTENT OF VASICINE**

Buffer solution: Dissolve 1.36 g of anhydrous potassium dihydrogen orthophosphate in 900 mL of HPLC grade water. Add 2.0 mL of phosphoric acid. Dilute with water to 1000 mL, and filter.
Mobile phase: *Buffer solution,* acetonitrile, and tetrahydrofuran (92:5:3)

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Standard solution A: 0.1 mg/mL of USP Vasicine RS in methanol. Sonicate to dissolve if necessary.
Standard solution B: 5.0 mg/mL of USP Powdered Malabar-Nut-Tree, Leaf Extract RS in methanol. Sonicate for about 15 min. Before injection, pass through a membrane filter of 0.45-µm or finer pore size.
Sample solution: Dilute the *Sample solution*, prepared as directed in *Identification* test *B* (1:5), with methanol. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, and discard the first few mL of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 280 nm
Column: 4.6-mm × 25-cm; 5-µm packing L10
Flow rate: 1.0 mL/min
Injection volume: 20 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
[NOTE—The approximate relative retention times of the vasicine and vasicinone peaks are 1.00 and 1.23, respectively.]
Suitability requirements
Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Malabar-Nut-Tree, Leaf Extract RS being used.
Resolution: NLT 2.0 between the vasicine and vasicinone peaks, *Standard solution B*
Relative standard deviation: NMT 2.0% determined from the vasicine peak in repeated injections, *Standard solution A*
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Using the chromatogram of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Malabar-Nut-Tree, Leaf Extract RS being used, identify the retention times of the peaks corresponding to vasicine and vasicinone.
Calculate the percentage of vasicine in the portion of Powdered Malabar-Nut-Tree, Leaf taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of vasicine from the *Sample solution*
r_S = peak area of vasicine from *Standard solution A*
C_S = concentration of USP Vasicine RS in *Standard solution A* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Powdered Malabar-Nut-Tree, Leaf taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 0.6% on the dried basis

CONTAMINANTS
- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria do not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS
- **BOTANICAL CHARACTERISTICS**
Macroscopic: Greenish-brown powder; characteristic odor; and bitter taste
Microscopic: It shows parenchyma cells with elongated cystoliths; oil globules; epidermal cells with numerous stomata diacytic or anomocytic, glandular and nonglandular, 1- to 3-celled, thin-walled, warty trichomes.
- **LOSS ON DRYING** ⟨731⟩
Sample: 1.0 g of Powdered Malabar-Nut-Tree, Leaf
Analysis: Dry the *Sample* at 105° for 3 h.
Acceptance criteria: NMT 12.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩
Sample: 1.0 g of Powdered Malabar-Nut-Tree, Leaf
Acceptance criteria: NMT 21.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 2.0%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Powdered Malabar-Nut-Tree, Leaf Extract RS
USP Vasicine RS

Powdered Malabar-Nut-Tree, Leaf Extract

DEFINITION
Powdered Malabar-Nut-Tree, Leaf Extract is prepared from Malabar-Nut-Tree, Leaf using suitable solvents such as water, methanol, or a mixture of these solvents. The ratio of plant material to extract is between 8:1 and 5:1. It contains NLT 90.0% and NMT 110.0% of the labeled amount of vasicine. It may contain suitable added substances as carriers.

IDENTIFICATION
- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**
Standard solution A: 0.5 mg/mL of USP Vasicine RS in methanol. Sonicate to dissolve if necessary.
Standard solution B: 50 mg/mL of USP Powdered Malabar-Nut-Tree, Leaf Extract RS in methanol. Sonicate for about 15 min, centrifuge, and use the supernatant.
Sample solution: 50 mg/mL of Powdered Malabar-Nut-Tree, Leaf Extract in methanol. Sonicate for about 15 min, centrifuge, and use the supernatant.
Adsorbent: Chromatographic silica gel with an average particle size of 10–15 µm (TLC plates)
Application volume: 10 µL, as 4-mm bands
Developing solvent system: A mixture of ethyl acetate, methanol, and ammonia (8: 2: 0.2)
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Apply the *Samples* as bands to a suitable thin-layer chromatographic plate (see *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*). Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about three-fourths of the plate. Remove the plate from the chamber, dry, and examine under UV at 254 nm.
Acceptance criteria: The chromatogram of the *Sample solution* exhibits a quenching zone at an RF value of

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approximately 0.35 for vasicine, corresponding to a zone in the chromatogram of *Standard solution A*. The chromatogram of the *Sample solution* also exhibits an additional quenching zone at an RF value of approximately 0.53 for vasicinone, corresponding to a similar zone in the chromatogram of *Standard solution B*. Other minor zones may be observed in the *Sample solution* and *Standard solution B* chromatograms.

- **B. HPLC:** The chromatogram of the *Sample solution* from the test for *Content of Vasicine* shows a main peak at a retention time corresponding to that of vasicine in the chromatogram of *Standard solution A*. Identify other peaks in the *Sample solution* by comparison with the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Malabar-Nut-Tree, Leaf Extract RS being used. The *Sample solution* shows an additional peak corresponding to vasicinone.

COMPOSITION

- **CONTENT OF VASICINE**

Buffer solution: Dissolve 1.36 g of anhydrous potassium dihydrogen phosphate in 900 mL of HPLC grade water. Add 2.0 mL of phosphoric acid. Dilute with water to 1000 mL, and filter.

Mobile phase: *Buffer solution*, acetonitrile, and tetrahydrofuran (92:5:3)

Standard solution A: 0.1 mg/mL of USP Vasicine RS in methanol. Sonicate to dissolve if necessary.

Standard solution B: 5.0 mg/mL of USP Powdered Malabar-Nut-Tree, Leaf Extract RS in methanol. Sonicate for about 15 min. Before injection, pass through a membrane filter of 0.45-µm or finer pore size.

Sample solution: 5.0 mg/mL of Powdered Extract in methanol. Sonicate for about 15 min. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, and discard the first part of the filtrate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 280 nm
Column: 4.6-mm × 25-cm; 5-µm packing L10
Flow rate: 1.0 mL/min
Injection size: 20 µL

System suitability
Samples: *Standard solution A* and *Standard solution B*
[NOTE—The approximate relative retention times for vasicine and vasicinone peaks are 1.00 and 1.23, respectively.]
Suitability requirements
Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Malabar-Nut-Tree, Leaf Extract RS being used.
Resolution: NLT 2.0 between the vasicine and vasicinone peaks, *Standard solution B*
Relative standard deviation: NMT 2.0% determined from the vasicine peak in repeated injections, *Standard solution A*

Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Using the chromatogram of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Malabar-Nut-Tree, Leaf Extract RS being used, identify the retention times of the peaks corresponding to vasicine and vasicinone.
Calculate the percentage of vasicine in the portion of Powdered Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of vasicine from the *Sample solution*
r_S = peak response of vasicine from *Standard solution A*
C_S = concentration of USP Vasicine RS in *Standard solution A* (mg/mL)
C_U = concentration of Powdered Malabar-Nut-Tree, Leaf Extract in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–110.0% of the labeled amount of vasicine

CONTAMINANTS

Change to read:

- **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020) **:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

- **LOSS ON DRYING** ⟨731⟩**:** Dry 1.0 g of Powdered Extract at 105° for 3 h: it loses NMT 5.0% of its weight.
- **OTHER REQUIREMENTS:** It meets the requirements of the test for *Residual Solvents* in *Botanical Extracts* ⟨565⟩.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived. It meets other labeling requirements in *Botanical Extracts* ⟨565⟩.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Powdered Malabar-Nut-Tree, Leaf Extract RS
USP Vasicine RS

Maritime Pine

DEFINITION

Maritime Pine consists of the bark of stems of *Pinus pinaster* Aiton (*Pinus maritima* Poir.) Fam. Pinaceae. It contains NLT 8.0% and NMT 12.0% of procyanidins, calculated on the dried basis.
[NOTE—This article is intended to be used in the preparation of extracts only and is not for direct human consumption.]

IDENTIFICATION

- **A. PRESENCE OF PROCYANIDINS**

Sample: Pulverize 1 g of the dried Maritime Pine. Use 10 mg.
Analysis: Add the *Sample* to 1 mL of methanol, and add 6 mL of a mixture of butanol and hydrochloric acid (95:5). Heat for 2 min in a water bath.
Acceptance criteria: The solution turns red.

- **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution: 25 mg/mL of USP Maritime Pine Extract RS in alcohol

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[NOTE—Retain a portion of this solution for use in *Identification* test *C*.]

Sample solution: Add 2 g of the powdered dried material to 20 mL of water. Place in a water bath for 20 min, and centrifuge. Extract the supernatant with 40 mL of ethyl acetate. Evaporate the ethyl acetate layer to dryness under a stream of nitrogen, with gentle heating. Dissolve the residue so obtained in 0.25 mL of alcohol.

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture

Application volume: 5 µL

Developing solvent system: Ethyl acetate, formic acid, and water (50:5:3)

Spray reagent: Alcohol and phosphoric acid (1:1), containing 1% of vanillin

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the chromatograms, dry the plate with the aid of a current of air, spray the plate with the *Spray reagent*, and heat at 115° for 15 min.

Acceptance criteria: The chromatogram of the *Sample solution* presents three red bands appearing in the middle third of the chromatogram corresponding to two dimeric procyanidins and catechin at the same R_F of similar bands in the *Standard solution*. The chromatogram of the *Sample solution* also exhibits a blue band between the upper band due to upper dimeric procyanidins and the band due to catechin, at the same R_F of a similar band found in the chromatogram of the *Standard solution*.

• **C. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution A: Use the *Standard solution*, prepared as directed for *Identification* test *B*.

Standard solution B: 1 mg/mL each of ferulic acid and protocatechuic acid

Sample solution: Use the *Sample solution*, prepared as directed for *Identification* test *B*.

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture

Application volume: 10 µL

Developing solvent system: Methylene chloride, methanol, glacial acetic acid, and water (80:15:2:2)

Spray reagent: 5% ferric chloride solution in methanol

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatogram, dry the plate at 110°, and examine the plate under short-wavelength and long-wavelength UV light. The chromatograms of *Standard solution A* and *Standard solution B* exhibit bands in the middle third and upper third that correspond to protocatechuic acid and ferulic acid, respectively. Spray the plate with the *Spray reagent*, and heat at 115° for 15 min. The bands due to ferulic acid and protocatechuic acid turn grayish green. Grayish-green bands become visible in the chromatogram of *Standard solution A* above and below protocatechuic acid, indicating the presence of caffeic acid and catechin, respectively.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits bands due to catechin, protocatechuic acid, caffeic acid, and ferulic acid that correspond in color and R_F values to those in the chromatogram of *Standard solution A* and *Standard solution B*.

COMPOSITION

• **CONTENT OF PROCYANIDINS**

Reagent solution A: Butanol and hydrochloric acid (95:5) [NOTE—Prepare this solution on the day of use.]

Reagent solution B: Dissolve 2 g of ferric ammonium sulfate in a mixture of 100 mL of water and 17.5 mL of hydrochloric acid. [NOTE—This solution can be used within 15 days of preparation.]

Standard solution: 95 µg/mL of procyanidins from USP Maritime Pine Extract RS in methanol

Sample stock solution: Dry crushed Maritime Pine at 110° for 3 h. Place 1.9 g of the crushed material in a 20-mL vial, and add 10 mL of methanol. Crimp the vial, and sonicate for 2 min. Heat in boiling water for 10 min. Cool to room temperature, allow the sediment to settle, and transfer the supernatant to a 100-mL volumetric flask, passing it through a filter having a 0.45-µm pore size. Wash the sediment two times with 10 mL of methanol, and transfer the solution into the same 100-mL volumetric flask, again passing it through a filter having a 0.45-µm pore size. Dilute with methanol to volume.

Sample solution: Dilute the *Sample stock solution* with methanol (1 in 20).

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Mode: Vis

Wavelength: 551 nm

Analysis

Samples: *Standard solution* and *Sample solution*

Transfer 1.0 mL each of the *Standard solution*, *Sample solution*, and methanol to three separate 10-mL vials. To each vial add 6.0 mL of *Reagent solution A* and 0.25 mL of *Reagent solution B*. Seal the vials with crimp caps. Mix, and heat in a water bath for 40 min. Quickly cool to room temperature in an ice bath. Quantitatively transfer these solutions, with the aid of *Reagent solution A*, to three separate 10-mL volumetric flasks, and dilute with *Reagent solution A* to volume.

Determine the absorbance of the solutions obtained from the *Standard solution* and the *Sample solution*, using the methanol-containing solution as a blank.

Calculate the percentage of total procyanidins in the portion of Maritime Pine taken:

$$\text{Result} = (A_U/A_S) \times C_S \times (V/W) \times D \times P$$

A_U = absorbance of the solution from the *Sample solution*

A_S = absorbance of the solution from the *Standard solution*

C_S = concentration of the USP Maritime Pine Extract RS in the *Standard solution* (mg/mL)

V = volume of the *Sample stock solution* (mL)

W = weight of Maritime Pine taken to prepare the *Sample stock solution* (mg)

D = dilution factor to prepare the *Sample solution* from *Sample stock solution*, 20

P = percentage of procyanidines in the USP Maritime Pine Extract RS

Acceptance criteria: 8.0%–12.0% on the dried basis

SPECIFIC TESTS

• **BOTANICAL CHARACTERISTICS**

Macroscopic: Bark pieces are typically 1–3 cm thick. The inner bark is plane to slightly concave, whitish to light brown, striped longitudinally; shiny and of slightly irregular surface, only a few millimeters thick. Abrupt change to a sequence of hard, convex, nearly parallel layers alternating with smooth, light brown layers. Up to 50 or more layers

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present, depending on the age of the bark. Outer surface of bark is dark reddish brown composed of irregular scaly patches, with deep V-shaped fissures. Outer surface may also be gray, gray-green, or green-yellow due to presence of lichens.

Microscopic (transverse section of bark): Light inner bark has irregular lateral stripes consisting of 3–5 cell layers of long, slender sieve cells with large pitted horizontal cell walls and large polygonal parenchyma cells containing single, irregular, rounded starch grain, 3–15 mm wide. Lateral stripes are separated from each other by ray parenchyma cells. Ray parenchyma cells are homogeneous in appearance, 1–4 cell layers thick and 4–20 cell layers high, each cell containing single, irregular, rounded starch grain, 3–15 mm wide. Cylindrical parenchyma cells with thin cell walls arranged in vertical rows with calcium oxalate prisms are also present. Outer part of the inner bark contains plate-shaped cells of undifferentiated periderm and older periderm with multiple layers of phellogen. The phellogen grows 3–7 rows of phellum to the exterior and 2–4 rows of small cell phelloderm to the interior. The oldest and outermost part of the bark is composed of lignified sections of phelloderm and phellum cells, 15–35 mm thick, separated by collapsed phellogen. Phelloderm and phellum cells are up to 100 mm wide, square, rectangular, polygonal, or irregularly shaped. The cell walls are colorless. Phelloderm cells are moderately pitted with a reddish-brown content. Phellum cells have a thicker cell wall of strongly pitted, undulated contour, and a yellowish-brown to brownish-red content. Radially between layers of phelloderm and phellum are layers of ray parenchyma cells, 5–8 cells thick, rounded to radially stretched, thin walled, strongly pitted with collapsed cells and dead sieve cells.

- **Articles of Botanical Origin,** *Foreign Organic Matter* ⟨561⟩: NMT 5%
- **Articles of Botanical Origin,** *Total Ash* ⟨561⟩: NMT 1.5%
- **Articles of Botanical Origin,** *Water Content* ⟨561⟩: NMT 35.0%

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Store at 25°, excursion permitted between 15° and 30°. Preserve in a well-closed container, and protect from moisture and excessive heat.
- **Labeling:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP Reference Standards** ⟨11⟩
  USP Maritime Pine Extract RS

Maritime Pine Extract

DEFINITION

Maritime Pine Extract is prepared from the pulverized Maritime Pine using suitable solvents. It contains NLT 65% and NMT 75% of procyanidins, calculated on the dried basis.

IDENTIFICATION

- **A. Presence of Procyanidins**
  Sample solution: Dissolve 50 mg of Extract in 6 mL of a mixture of butanol and hydrochloric acid (95:5).
  Analysis: Heat for 2 min in a water bath.
  Acceptance criteria: The solution turns red.
- **B. Thin-Layer Chromatographic Identification Test**
  Standard solution A: 25 mg/mL of USP Maritime Pine Extract RS in methanol
  Standard solution B: 1 mg/mL each of ferulic acid and protocatechuic acid in methanol
  Sample solution: 25 mg/mL of Extract in methanol
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
  Adsorbent: 0.25-mm layer of chromatographic silica gel mixture
  Application volume: 5 µL
  Developing solvent system: Methylene chloride, methanol, glacial acetic acid, and water (80:15:2:2)
  Spray reagent: 5% ferric chloride solution in methanol
  Analysis
  Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
  Develop the chromatogram, dry the plate at 110°, and examine the plate under short-wavelength and long-wavelength UV light. The chromatograms of *Standard solution A* and *Standard solution B* exhibit bands in the middle third and upper third that correspond to protocatechuic acid and ferulic acid, respectively. Spray the plate with the *Spray reagent*, and heat at 115° for 15 min. The bands due to ferulic acid and protocatechuic acid turn grayish green. Grayish-green bands become visible in the chromatogram of *Standard solution A* above and below protocatechuic acid, indicating the presence of caffeic acid and catechin, respectively.
  Acceptance criteria: The chromatogram of the *Sample solution* exhibits bands due to catechin, protocatechuic acid, caffeic acid, and ferulic acid that correspond in color and R_F values to those in the chromatogram of *Standard solution A* and *Standard solution B*.
- **C. Thin-Layer Chromatographic Identification Test**
  Standard solution: Use *Standard solution A*, prepared as directed for *Identification* test *B*.
  Sample solution: Use the *Sample solution*, prepared as directed for *Identification* test *B*.
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
  Adsorbent: 0.25-mm layer of chromatographic silica gel mixture
  Application volume: 5 µL
  Developing solvent system: Ethyl acetate, formic acid, and water (50:5:3)
  Spray reagent: Phosphoric acid and alcohol (1:1), containing 1% of vanillin
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Proceed as directed in the chapter, except to dry the plate with the aid of a current of air, spray the plate with the *Spray reagent*, and heat at 115° for 15 min. Three red bands appear in the middle third of the chromatogram of the *Standard solution* corresponding to two dimeric procyanidins and catechin. The chromatogram of the *Standard solution* also exhibits a blue band between the upper band due to upper dimeric procyanidins and the band due to catechin.
  Acceptance criteria: The chromatogram of the *Sample solution* contains bands that correspond to those found in the chromatogram of the *Standard solution*.
- **D. HPLC Identification Test**
  Solution A: Methanol
  Solution B: 1 mg/mL of phosphoric acid in water
  Mobile phase: See *Table 1*.

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Table 1

Time (min)	Solution A (%)	Solution B (%)
0	8	92
40	34	66
45	2	98
50	2	98
52	8	92
57	8	92

Standard solution: 2 mg/mL of USP Maritime Pine Extract RS in *Solution A*. Pass through a membrane having a 0.45-µm or finer pore size.
Sample solution: Add 20 mg of Extract to 10 mL of *Solution A*, and sonicate for 10 min to dissolve. Pass through a membrane having a 0.45-µm or finer pore size, discarding the first 4 mL of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 280 nm
Column: 4.6-mm × 15-cm; base-deactivated packing L7, having less than 5-µm particle size
Column temperature: 40°
Flow rate: 1 mL/min
Injection size: 10 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Chromatogram similarity: The chromatogram is similar to the reference chromatogram provided with the lot of USP Maritime Pine Extract RS being used.
Resolution: NLT 3.0 between taxifolin and ferulic acid
Tailing factor: NMT 2.0 for taxifolin
Analysis
Samples: *Standard solution* and *Sample solution*
Identify the peaks for catechin, caffeic acid, taxifolin, and ferulic acid by comparison of the chromatogram of the *Standard solution* with the reference chromatogram.
Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks for catechin, caffeic acid, taxifolin, and ferulic acid at the retention times corresponding to those in the chromatogram of the *Standard solution*.

COMPOSITION

• **Content of Procyanidins**
Reagent solution A: Butanol and hydrochloric acid (95:5) [Note—Prepare this solution on the day of use.]
Reagent solution B: Dissolve 2 g of ferric ammonium sulfate in a mixture of 100 mL of water and 17.5 mL of hydrochloric acid. [Note—This solution can be used within 15 days of preparation.]
Standard solution: 95 µg/mL of procyanidins from USP Maritime Pine Extract RS in methanol
Sample stock solution: Transfer 250 mg of Extract to a 100-mL volumetric flask. Dissolve with methanol, and dilute with the same solvent to volume.
Sample solution: Dilute the *Sample stock solution* with methanol (1 in 20).
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: Vis
Wavelength: 551 nm
Analysis
Samples: *Standard solution* and *Sample solution*
Transfer 1.0 mL each of the *Standard solution*, *Sample solution*, and methanol to three separate 10-mL vials. To each vial add 6.0 mL of *Reagent solution A* and 0.25 mL of *Reagent solution B*. Seal the vials with crimp caps. Mix, and heat in a water bath for 40 min. Quickly cool to room temperature in an ice bath. Quantitatively transfer these solutions, with the aid of *Reagent solution A*, to three separate 10-mL volumetric flasks, and dilute with *Reagent solution A* to volume.
Determine the absorbance of the solutions obtained from the *Standard solution* and the *Sample solution*, using the methanol-containing solution as a blank.
Calculate the percentage of total procyanidins in the portion of Extract taken:

$$\text{Result} = (A_U/A_S) \times C_S \times (V/W) \times D \times P$$

A_U = absorbance of the solution from the *Sample solution*
A_S = absorbance of the solution from the *Standard solution*
C_S = concentration of the USP Maritime Pine Extract RS in the *Standard solution* (mg/mL)
V = volume of the *Sample stock solution* (mL)
W = weight of Maritime Pine Extract taken to prepare the *Sample stock solution* (mg)
D = dilution factor to prepare the *Sample solution* from *Sample stock solution*, 20
P = percentage of procyanidines in the USP Maritime Pine Extract RS

Acceptance criteria: 65%–75% on the dried basis

CONTAMINANTS

Change to read:

• **▲Botanical Extracts** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020)
: Meets the requirements
• **Microbial Enumeration Tests** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
• **Absence of Specified Microorganisms** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

• **Loss on Drying** ⟨731⟩**:** Dry 1.0 g of Extract for 3 h at 110°: it loses NMT 8.0% of its weight.
• **Articles of Botanical Origin,** *Total Ash* ⟨561⟩**:** NMT 0.7%
• **Limit of Water-Insoluble Substances**
Analysis: Weigh 0.50 g of Extract, and stir in 50 mL of water at 20° for 15 min. Pass through a fine sintered glass filter, previously weighed. Dry the filter at 110° for 3 h, cool to room temperature, and weigh the filter. Calculate the amount of water-insoluble material.
Acceptance criteria: NMT 10% of the amount of Extract taken

ADDITIONAL REQUIREMENTS

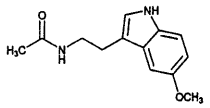
• **Packaging and Storage:** Preserve in tight containers, and store at 25°, excursion permitted between 15° and 30°. Protect from light.
• **Labeling:** The label states the Latin binomial and, following the official name of the article, the part of the plant from which the article was prepared, in addition to the information required for *Botanical Extracts* ⟨565⟩, *Labeling*.

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• **USP REFERENCE STANDARDS** ⟨11⟩
USP Maritime Pine Extract RS

Melatonin



$C_{13}H_{16}N_2O_2$ 232.28
N-Acetyl-5-methoxytryptamine;
N-(2-(5-Methoxy-1*H*-indol-3-yl)ethyl) acetamide [73-31-4].

DEFINITION
Melatonin contains NLT 98.5% and NMT 101.5% of melatonin ($C_{13}H_{16}N_2O_2$), calculated on the dried basis.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

Change to read:

• **B. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Ultraviolet-Visible Spectroscopy*: **197U**▲ (CN 1-May-2020)
Analytical wavelength: 277 nm
Sample solution: 10 µg/mL of Melatonin in isopropyl alcohol
Acceptance criteria: Meets the requirements. Absorptivities, calculated on the dried basis, do not differ by more than 3.0%.

• **C. HPLC IDENTIFICATION TEST**
Analysis: Proceed as directed in the *Assay*.
Acceptance criteria: The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*.

ASSAY

• **PROCEDURE**
Buffer: 0.5 g/L of monobasic potassium phosphate in water. Adjust with phosphoric acid to a pH of 3.5, and filter.
Mobile phase: Acetonitrile and *Buffer* (25:75)
System suitability solution: 0.1 mg/mL of USP Melatonin RS and 0.02 mg/mL USP Melatonin Related Compound A RS in *Mobile phase*
Standard solution: 0.1 mg/mL of USP Melatonin RS in *Mobile phase*
Sample solution: 0.1 mg/mL of Melatonin in *Mobile phase*
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 222 nm
Column: 4.6-mm × 15-cm; 5-µm packing L1
Flow rate: 1.0 mL/min
Injection size: 10 µL
System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The relative retention times for melatonin related compound A and melatonin are 0.4 and 1.0, respectively.]
Suitability requirements
Resolution: NLT 4 between melatonin and melatonin related compound A, *System suitability solution*
Relative standard deviation: NMT 2.0%, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of melatonin in the portion of Melatonin taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP Melatonin RS in the *Standard solution* (mg/mL)
C_U = concentration of Melatonin in the *Sample solution* (mg/mL)

Acceptance criteria: 98.5–101.5% on the dried basis

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%

• **CHLORIDE AND SULFATE,** *Chloride* ⟨221⟩
Standard: 0.10 mL of 0.020 N hydrochloric acid
Sample: 0.36 g of Melatonin
Acceptance criteria: NMT 0.02%

• **RELATED COMPOUNDS**
Solution A: Acetonitrile
Solution B: Use *Buffer*, prepared as directed in the *Assay*.
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	25	75
7	25	75
15	80	20
18	25	75
25	25	75

Diluent: Mixture of *Solution A* and *Solution B* (25:75)
System suitability solution: 0.1 mg/mL of USP Melatonin RS and 0.02 mg/mL of USP Melatonin Related Compound A RS in *Diluent*
Standard solution: 5 µg/mL of USP Melatonin RS in *Diluent*
Sample solution: 1 mg/mL of Melatonin in *Diluent*
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 222 nm
Column: 4.6-mm × 15-cm; 5-µm packing L1
Flow rate: 1.0 mL/min
Injection size: 10 µL
System suitability
Sample: *System suitability solution*
[NOTE—The relative retention times for melatonin related compound A and melatonin are 0.4 and 1.0, respectively.]
Suitability requirements
Resolution: NLT 4.0 between melatonin and melatonin related compound A
Relative standard deviation: NMT 2.0% for the melatonin peak
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of each individual impurity in the portion of Melatonin taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

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r_U = peak response of each individual impurity from the *Sample solution*
r_S = peak response of melatonin from the *Standard solution*
C_S = concentration of USP Melatonin RS in the *Standard solution* (mg/mL)
C_U = concentration of Melatonin in the *Sample solution* (mg/mL)

Acceptance criteria
Individual impurities: NMT 0.1%
Total impurities: NMT 1.0%

SPECIFIC TESTS
• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry a sample at 80° in a vacuum for 3 h.
Acceptance criteria: NMT 1.0%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Melatonin RS
USP Melatonin Related Compound A RS
2-(5-Methoxy-1*H*-indol-3-yl)ethanamine.
$C_{11}H_{14}N_2O$ 190.24

Melatonin Tablets

DEFINITION
Melatonin Tablets contain NLT 90.0% and NMT 110.0% of the labeled amount of melatonin ($C_{13}H_{16}N_2O_2$).

IDENTIFICATION
• **A.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in *Strength*.

STRENGTH
• **PROCEDURE**
Buffer: 0.5 g/L of monobasic potassium phosphate in water. Adjust with phosphoric acid to a pH of 3.5, and filter.
Mobile phase: Acetonitrile and *Buffer* (25:75)
System suitability solution: 0.1 mg/mL of USP Melatonin RS and 0.02 mg/mL of USP Melatonin Related Compound A RS in *Mobile phase*
Standard solution: 0.1 mg/mL of USP Melatonin RS in *Mobile phase*
Sample solution: Filtered portion of the solution in *Mobile phase*, equivalent to 0.1 mg/mL of Melatonin from NLT 20 finely powdered Tablets
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 222 nm
Column: 4.6-mm × 15-cm; 5-µm packing L1
Flow rate: 1.0 mL/min
Injection size: 10 µL
System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The relative retention times for melatonin related compound A and melatonin are 0.4 and 1.0, respectively.]
Suitability requirements
Resolution: NLT 4 between melatonin and melatonin related compound A, *System suitability solution*
Relative standard deviation: NMT 2.0%, *Standard solution*

Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of melatonin ($C_{13}H_{16}N_2O_2$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP Melatonin RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of melatonin in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–110.0%

PERFORMANCE TESTS
• **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** ⟨2040⟩**:** Meet the requirements for *Dissolution*
Medium: Water; 500 mL
Apparatus 2: 50 rpm
Time: 30 min
Standard solution: Dissolve a suitable amount of USP Melatonin RS in water to obtain a concentration similar to that expected in the *Sample solution*.
Sample solution: Filtered portion of the solution under test
Analysis: Proceed as directed in *Strength*, making any necessary adjustments.
Calculate the percentage of the labeled amount of melatonin ($C_{13}H_{16}N_2O_2$) dissolved:

$$\text{Result} = (r_U/r_S) \times (C_S \times V/L) \times 100$$

r_U = peak area from the *Sample solution*
r_S = peak area from the *Standard solution*
C_S = concentration of USP Melatonin RS in the *Standard solution* (mg/mL)
V = volume of *Medium*, 500 mL
L = labeled amount of melatonin (mg/Tablet)

Tolerances: NLT 75% of the labeled amount of melatonin ($C_{13}H_{16}N_2O_2$) is dissolved.
• **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩**:** Meet the requirements

IMPURITIES
• **RELATED COMPOUNDS**
Solution A: Acetonitrile
Solution B: Use *Buffer*, prepared as directed in *Strength*.
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	25	75
7	25	75
15	80	20
18	25	75
25	25	75

Diluent: Mixture of *Solution A* and *Solution B* (25:75)
System suitability solution: 0.1 mg/mL of USP Melatonin RS and 0.02 mg/mL of USP Melatonin Related Compound A RS in *Diluent*
Standard solution: 5 µg/mL of USP Melatonin RS in *Diluent*

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Sample solution: Filtered solution in *Diluent*, equivalent to 1 mg/mL of melatonin from NLT 20 finely powdered Tablets

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 222 nm
Column: 4.6-mm × 15-cm; 5-µm packing L1
Flow rate: 1.0 mL/min
Injection size: 10 µL

System suitability
Sample: *System suitability solution*
[NOTE—The relative retention times for melatonin related compound A and melatonin are 0.4 and 1.0, respectively.]
Suitability requirements
Resolution: NLT 4.0 between melatonin and melatonin related compound A
Relative standard deviation: NMT 2.0% for the melatonin peak

Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of each individual impurity in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

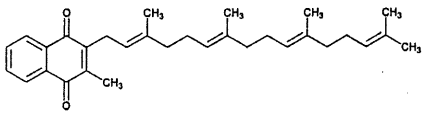
r_U = peak response of each individual impurity from the *Sample solution*
r_S = peak response of melatonin from the *Standard solution*
C_S = concentration of USP Melatonin RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of melatonin in the *Sample solution* (mg/mL)

Acceptance criteria
Individual impurities: NMT 0.1%
Total impurities: NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** The label states the quantity of melatonin in mg/Tablet.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Melatonin RS
  USP Melatonin Related Compound A RS
  2-(5-Methoxy-1*H*-indol-3-yl)ethanamine.
  $C_{11}H_{14}N_2O$ 190.24

Menaquinone-4



$C_{31}H_{40}O_2$ 444.65
2-Methyl-3-[(2*E*,6*E*,10*E*)-3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraen-1-yl]naphthalene-1,4-dione;
Menatetrenone [863-61-6].

DEFINITION

Menaquinone-4 contains NLT 98.0% and NMT 102.0% of menaquinone-4 ($C_{31}H_{40}O_2$), calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

- **A.** **▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197A**▲ (CN 1-May-2020)
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

ASSAY

- **PROCEDURE**

Mobile phase: Methanol
[NOTE—Protect the *Standard solution* and *Sample solution* from light and inject immediately after preparation.]
Standard solution: 0.05 mg/mL of USP Menaquinone-4 RS in isopropyl alcohol
System suitability solution: 0.05 mg/mL of USP Phytonadione RS in *Standard solution*
Sample stock solution: Transfer 50 mg of Menaquinone-4 to a 50-mL volumetric flask. Dissolve in isopropyl alcohol and dilute with the same solvent to volume.
Sample solution: 0.05 mg/mL of Menaquinone-4 in isopropyl alcohol prepared from the *Sample stock solution*
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 270 nm
Column: 4.6-mm × 15-cm; 5-µm packing L1
Column temperature: 40°
Flow rate: 1.0 mL/min
Injection volume: 20 µL
Run time: NLT 3 times the retention time of menaquinone-4

System suitability
Sample: *System suitability solution*
[NOTE—The relative retention times for menaquinone-4 and phytonadione are 1.0 and 1.7, respectively.]
Suitability requirements
Resolution: NLT 4 between menaquinone-4 and phytonadione
Relative standard deviation: NMT 2.0% for menaquinone-4

Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of menaquinone-4 ($C_{31}H_{40}O_2$) in the portion of Menaquinone-4 taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of menaquinone-4 from the *Sample solution*
r_S = peak response of menaquinone-4 from the *Standard solution*
C_S = concentration of USP Menaquinone-4 RS in the *Standard solution* (mg/mL)
C_U = concentration of Menaquinone-4 in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–102.0% on the anhydrous basis

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%
- **ISOMERIC PURITY**

Standard solution: 0.2 mg/mL of USP Menaquinone-4 RS in hexane
Sample solution: 10 mg/mL of Menaquinone-4 in hexane

DS Monographs

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Chromatographic system
(See *Chromatography* ⟨621⟩, *General Procedures, Thin-Layer Chromatography.*)
Mode: TLC
Adsorbent: 0.2- to 0.3-mm layer of chromatographic silica gel mixture
Application volume: 10 µL
Developing solvent system: Hexane and butyl ether (17:3)
Analysis
Samples: *Standard solution* and *Sample solution*
[NOTE—The relative R_F values of *cis*-menaquinone-4 and *trans*-menaquinone-4 are about 1.1 and 1.0, respectively.]
Proceed as directed in the chapter and develop the chromatogram until the solvent front has moved about 75% of the length of the plate. Dry the plate in air and examine under short-wavelength UV light.
Acceptance criteria: NMT 2.0%. Any spot corresponding to the relative R_F value of 1.1 in the *Sample solution* is neither larger nor more intense than the principal spot from the *Standard solution.*

• **ORGANIC IMPURITIES**
Mobile phase, System suitability solution, Sample stock solution, Chromatographic system, and **System suitability:** Proceed as directed in the *Assay.*
Standard solution: 1 µg/mL of USP Menaquinone-4 RS in isopropyl alcohol
Sample solution: Use the *Sample stock solution* from the *Assay.*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of each impurity in the portion of Menaquinone-4 taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

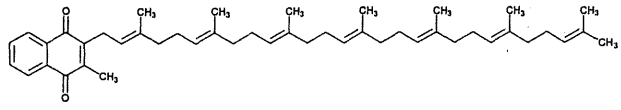
r_U = peak response of any impurity from the *Sample solution*
r_S = peak response of menaquinone-4 from the *Standard solution*
C_S = concentration of USP Menaquinone-4 RS in the *Standard solution* (mg/mL)
C_U = concentration of Menaquinone-4 in the *Sample solution* (mg/mL)

Acceptance criteria
Any individual impurity: NMT 0.1%
Total impurities: NMT 1.0%

SPECIFIC TESTS
• **WATER DETERMINATION** ⟨921⟩, *Method I, Method Ia*: NMT 0.5%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Store in a tight container in a cool place. Protect from light.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Menaquinone-4 RS
USP Phytonadione RS

Menaquinone-7



$C_{46}H_{64}O_2$ 649.00
(*all-E*)-2-(3,7,11,15,19,23,27-Heptamethyl-2,6,10,14,18,22,26-octacosaheptaenyl)-3-methyl-1,4-naphthalenedione [2124-57-4].

DEFINITION
Menaquinone-7 contains NLT 96.0% and NMT 101.0% of menaquinone-7 ($C_{46}H_{64}O_2$) and NMT 3% of menaquinone-6 ($C_{41}H_{56}O_2$), calculated on the as-is basis.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197A▲** (CN 1-May-2020)

Change to read:

• **B. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Ultraviolet-Visible Spectroscopy*: **197U▲** (CN 1-May-2020)
[NOTE—Use low-actinic glassware.]
Analytical wavelength: 200–450 nm
Sample solution: 40 µg/mL of Menaquinone-7 in dehydrated alcohol
Acceptance criteria: Meets the requirements
• **C.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay.*

ASSAY
• **PROCEDURE**
[NOTE—Protect the *Standard solution* and *Sample solution* from light, and inject immediately after preparation.]
Mobile phase: Dehydrated alcohol and water (97:3)
Standard solution: Transfer 25 mg of USP Menaquinone-7 RS into a 50-mL volumetric flask, add 1 mL of tetrahydrofuran, and dilute with dehydrated alcohol to volume. Transfer 5.0 mL of this solution into a 25-mL volumetric flask, and dilute with dehydrated alcohol to volume.
Sample solution: Transfer 25 mg of Menaquinone-7 into a 50-mL volumetric flask, add 1 mL of tetrahydrofuran, and dilute with dehydrated alcohol to volume. Transfer 5.0 mL of this solution into a 25-mL volumetric flask, and dilute with dehydrated alcohol to volume.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability.*)
Mode: LC
Detector: UV 268 nm
Column: 4.6-mm × 10-cm; 2.6-µm packing L1
Column temperature: 25°
Flow rate: 0.7 mL/min
Injection volume: 10 µL
Run time: NLT 3 times the retention time of menaquinone-7
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 3.0% from 6 replicate injections
Analysis
Samples: *Standard solution* and *Sample solution*

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[NOTE—The relative retention times for menaquinone-6 and menaquinone-7 are 0.81 and 1.00, respectively.]
Calculate the percentage of menaquinone-7 ($C_{46}H_{64}O_2$) in the portion of Menaquinone-7 taken:

$$\text{Result} = (r_{U7}/r_S) \times (C_S/C_U) \times 100$$

r_{U7} = peak response of menaquinone-7 from the *Sample solution*
r_S = peak response of menaquinone-7 from the *Standard solution*
C_S = concentration of USP Menaquinone-7 RS in the *Standard solution* (mg/mL)
C_U = concentration of Menaquinone-7 in the *Sample solution* (mg/mL)

Calculate the percentage of menaquinone-6 ($C_{41}H_{56}O_2$) in the portion of Menaquinone-7 taken:

$$\text{Result} = (r_{U6}/r_S) \times (C_S/C_U) \times 100$$

r_{U6} = peak response of menaquinone-6 from the *Sample solution*
r_S = peak response of menaquinone-7 from the *Standard solution*
C_S = concentration of USP Menaquinone-7 RS in the *Standard solution* (mg/mL)
C_U = concentration of Menaquinone-7 in the *Sample solution* (mg/mL)

Acceptance criteria
Menaquinone-7: 96.0%–101.0% on the as-is basis
Menaquinone-6: NMT 3% on the as-is basis

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 0.2%
- **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩

Acceptance criteria
Arsenic: NMT 2.0 µg/g
Cadmium: NMT 1.0 µg/g
Lead: NMT 3.0 µg/g
Mercury: NMT 0.1 µg/g

- **ISOMERIC PURITY**

Mobile phase: Water, dehydrated alcohol, methanol, and tetrahydrofuran (1:15:80:10)
Sample solution: [NOTE—Protect the solution from light and inject immediately after preparation.] Transfer 40 mg of Menaquinone-7 into a 100-mL volumetric flask, add 2 mL of tetrahydrofuran, and shake to dissolve the sample. Dilute with dehydrated alcohol to volume. Transfer 1.0 mL of this solution into a 10-mL volumetric flask, and dilute with dehydrated alcohol to volume. Pass the solution through a membrane filter of 0.45-µm pore size.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 268 nm
Column: 4.6-mm × 25-cm; 5-µm packing L62
Column temperature: 25°
Flow rate: 0.8 mL/min
Injection volume: 20 µL
Run time: NLT 1.5 times the retention time of *all-trans*-menaquinone-7
System suitability
Sample: *Sample solution*
[NOTE—The relative retention times of *all-trans*-menaquinone-7 and *cis*-menaquinone-7 are 1.0 and 1.1, respectively.]
Suitability requirements
Resolution: NLT 1.5 between *all-trans*-menaquinone-7 and *cis*-menaquinone-7
Analysis
Sample: *Sample solution*
Calculate the percentage of *cis*-menaquinone-7 in the portion of Menaquinone-7 taken:

$$\text{Result} = [r_C/(r_T + r_C)] \times 100$$

r_C = peak response of *cis*-menaquinone-7 from the *Sample solution*
r_T = peak response of *all-trans*-menaquinone-7 from the *Sample solution*

Acceptance criteria: NMT 2% of *cis*-menaquinone-7

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total bacterial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species, Test for Absence of Staphylococcus aureus,* and *Test for Absence of Escherichia coli*: Meets the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Store in a tight container, in a cool and dry place. Protect from light.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Menaquinone-7 RS

Menaquinone-7 Capsules

DEFINITION

Menaquinone-7 Capsules contain NLT 90.0% and NMT 110.0% of the labeled amount of menaquinone-7 ($C_{46}H_{64}O_2$).

IDENTIFICATION

- **A.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in *Method 1* or *Method 2* of the test for *Content of Menaquinone-7*.

STRENGTH

- **CONTENT OF MENAQUINONE-7, *Method 1***

[NOTE—Menaquinone-7 is extremely light sensitive. Protect samples from light.]
Diluent: 0.1 mg/mL of butylated hydroxytoluene in hexane
Mobile phase: Methanol and ethyl acetate. See *Table 1* for gradient.

Table 1

Time (min)	Flow Rate (mL/min)	Methanol (%)	Ethyl Acetate (%)
0	1.00	94	6
5.5	1.00	94	6
6.5	1.25	80	20
13	1.25	80	20
14	1.25	94	6
15	1.00	94	6
25	1.00	94	6

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Standard stock solution: Transfer 20 mg of USP Menaquinone-7 RS into a 50-mL volumetric flask, dilute with hexane to volume, and mix well. [NOTE—The solution can be kept in the freezer for 1 month.]
Standard solution: Transfer 1.0 mL of the *Standard stock solution* into a 10-mL volumetric flask, dilute with *Diluent* to volume, and mix well.
Sample solution A (for hard shell capsules): Empty and mix thoroughly the contents of NLT 30 Capsules. Transfer a portion of the powder, nominally equivalent to about 1.0 mg of menaquinone-7, to a 25-mL volumetric flask. Add 10 mL of *Diluent*, and shake for 2 min. Dilute with *Diluent* to volume, mix, and pass through a membrane filter of 0.45-µm pore size.
Sample solution B (for soft shell capsules): Weigh NLT 30 Capsules. With a sharp blade carefully open the Capsules, without loss of the shell material, and transfer as much as possible of the combined Capsule contents to a suitable container. Remove any adhering substances from the emptied Capsules and shell remains by washing with several small portions of hexane. Discard the washings, and allow the empty Capsules and shell remains to dry in a current of dry air until the odor of hexane is no longer perceptible. Weigh the empty Capsules and shell remains, and calculate the average net weight per Capsule by difference. Transfer accurately weighed combined Capsule contents equivalent to about 1.0 mg of menaquinone-7 to a 25-mL volumetric flask, add 10 mL of *Diluent*, and mix on a vortex mixer for 15 s. Dilute with *Diluent* to volume, mix, and pass through a membrane filter of 0.45-µm pore size.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 268 nm
Column: 4.6-mm × 25-cm; 4-µm packing L87. [NOTE—A suitable column is Synergy Max-RP from www.phenomenex.com.]
Column temperature: 35°
Flow rate: See *Table 1*.
Injection volume: 15 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 3.0% for six sequential injections
Retention time: NMT 0.5 min difference between the first and last injection for a total of six injections
Analysis
Samples: *Standard solution* and appropriate *Sample solution*
Calculate the percentage of the labeled amount of menaquinone-7 ($C_{46}H_{64}O_2$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of menaquinone-7 from the *Sample solution*
r_S = peak response of menaquinone-7 from the *Standard solution*
C_S = concentration of USP Menaquinone-7 RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of menaquinone-7 in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–110.0%

• **CONTENT OF MENAQUINONE-7, *Method 2***
[NOTE—Menaquinone-7 is extremely light sensitive. Protect samples from light, and inject immediately after preparation.]
Mobile phase: Ethanol and water (97:3)
Standard stock solution: Transfer 25 mg of USP Menaquinone-7 RS into a 50-mL volumetric flask, and add 1 mL of tetrahydrofuran. Dilute with dehydrated alcohol to volume, and mix well. [NOTE—The solution can be kept in the freezer for 1 month.]
Standard solution: 10 µg/mL of menaquinone-7 in dehydrated alcohol from the *Standard stock solution*
Sample solution: Treat Capsules as directed for *Sample solution A* or *Sample solution B* in *Method 1*. Transfer accurately weighed Capsule contents equivalent to about 0.25 mg of menaquinone-7 to a 25-mL volumetric flask, and add 2 mL of tetrahydrofuran. Dilute with dehydrated alcohol to volume, shake for 2 min, and pass through a membrane filter of 0.45-µm pore size.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 268 nm
Column: 4.6-mm × 10-cm; 2.6-µm packing L1
Column temperature: 25°
Flow rate: 0.7 mL/min
Injection volume: 10 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 3.0% for six replicate injections
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of menaquinone-7 ($C_{46}H_{64}O_2$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of menaquinone-7 from the *Sample solution*
r_S = peak response of menaquinone-7 from the *Standard solution*
C_S = concentration of USP Menaquinone-7 RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of menaquinone-7 in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–110.0%

PERFORMANCE TESTS

• **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩**:** Meet the requirements for *Disintegration*
• **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩ **:** Meet the requirements

CONTAMINANTS

• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 3×10^3 cfu/g, and the total combined molds and yeasts count does not exceed 3×10^2 cfu/g.
• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meet the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Store in tight, light-resistant containers, in a cool, dry place.

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- **LABELING:** The label states the content of menaquinone-7 in µg/Capsule. It also states the assay method used if *Method 1* is not used.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Menaquinone-7 RS

Menaquinone-7 Preparation

DEFINITION

Change to read:

Menaquinone-7 Preparation is a combination of ▲▲ (USP 1-Dec-2019) menaquinone-7 ($C_{46}H_{64}O_2$) ▲or concentrated menaquinone-7▲ (USP 1-Dec-2019) extract with one or more inert substances. It may be in a solid or liquid form. It contains NLT 90% and NMT 120% of the labeled amount (ppm) of menaquinone-7 calculated on the dried basis for the solid form and on the as-is basis for the liquid ▲and microencapsulated powder forms.▲ (USP 1-Dec-2019)

IDENTIFICATION

Change to read:

- **A. THIN-LAYER CHROMATOGRAPHY**
  Standard solution: Transfer 10 mg of USP Menaquinone-7 RS into a 10-mL volumetric flask, add 1 mL of tetrahydrofuran, and dissolve in and dilute with the same solvent to volume.
  ▲**Sample solution:**▲ (USP 1-Dec-2019) Prepare the appropriate solution as follows.
  For solid Preparation: Transfer an amount of Preparation, equivalent to about 1 mg of menaquinone-7, to a 2-mL centrifuge tube, and add 1 mL of tetrahydrofuran. Shake, and allow the mixture to settle (if necessary, centrifuge). Use the supernatant for the test.
  ▲▲ (USP 1-Dec-2019)
  For liquid Preparation (oil suspension): Transfer an amount of Preparation, equivalent to about 4 mg of menaquinone-7, to a 10-mL centrifuge tube, and add 4 mL of tetrahydrofuran. Shake, and allow the mixture to settle (if necessary, centrifuge). Use the supernatant for the test.
  ▲**For microencapsulated powder Preparation:** Transfer an amount of Preparation, equivalent to about 1 mg of menaquinone-7, to a 50-mL centrifuge tube, and add 10 mL of water. Heat to 50° over a water bath, and shake well. Add 10 mL of ethyl acetate, shake well, and allow the mixture to settle. Use the supernatant for the test.▲ (USP 1-Dec-2019)
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *General Procedures, Thin-Layer Chromatography*.)
  Mode: HPTLC
  Adsorbent: Nano silica gel F_{254} for HPTLC[1]
  Application volume: 5 µL
  Developing solvent system: Hexane and ethyl acetate (95:5)
  Spray reagent: Transfer 1 g of cerium sulfate and 21 g of ammonium molybdate into a 500-mL volumetric flask, dissolve with 33 mL of 98% (w/w) sulfuric acid, and dilute with deionized water to volume. Stir to complete dissolution. Cool, and store the solution at 0°. Allow the *Spray reagent* to come to room temperature before use.
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Develop the chromatogram in the *Developing solvent system* until the solvent front has moved about three-fourths the length of the plate. Remove the plate from the chamber, and air-dry. Examine the plate under visible light and short-wavelength UV light, and record the spots. Spray the plate with the *Spray reagent*, and heat at 100°–110° for 5 min. Examine the plate under white light, and record the spots.
  Acceptance criteria: Under visible light and short-wavelength UV light, the spots from the *Sample solution* correspond in color (light yellow), shape, and R_F value to those from the *Standard solution*. After applying the *Spray reagent*, the spots from the *Sample solution*, under white light, correspond in color (dark blue), shape, and R_F value to those from the *Standard solution*.
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Menaquinone-7*.

COMPOSITION

Change to read:

- **CONTENT OF MENAQUINONE-7▲** ▲ (USP 1-Dec-2019)
  [NOTE—Use low-actinic glassware.]
  Mobile phase: Dehydrated alcohol and water (97:3)
  [NOTE—Protect the *Standard solution* and *Sample solution* from light, and inject immediately after preparation.]
  Standard solution: Transfer 20 mg of USP Menaquinone-7 RS into a 50-mL volumetric flask, add 1.0 mL of tetrahydrofuran, and dilute with dehydrated alcohol to volume. Transfer 1.0 mL of this solution into a 10-mL volumetric flask, and dilute with dehydrated alcohol to volume.
  ▲**Sample solution:**▲ (USP 1-Dec-2019) Prepare the appropriate solution as follows.
  For solid Preparation: Transfer an accurately weighed solid Preparation, equivalent to about 0.8 mg of menaquinone-7, to a 25-mL volumetric flask, add 2 mL of tetrahydrofuran, and dilute with dehydrated alcohol to volume. Shake the suspension for 2 min, and pass through a membrane filter of 0.45-µm pore size.
  ▲▲ (USP 1-Dec-2019)
  For liquid Preparation (oil suspension): Transfer an accurately weighed liquid Preparation, equivalent to about 1.0 mg of menaquinone-7, to a 25-mL volumetric flask, add 2 mL of tetrahydrofuran, and dilute with dehydrated alcohol to volume. Pass this solution through a membrane filter of 0.45-µm pore size.
  ▲**For microencapsulated powder Preparation:** Transfer an accurately weighed microencapsulated powder Preparation, equivalent to about 0.6 mg of menaquinone-7, to a 50-mL centrifuge tube, add 10 mL of water, shake the suspension well, and sonicate for 15 min at 40°–50° by shaking well every 5 min. Cool the solution to room temperature over an ice bath and add 25 mL of ethyl acetate, shake the suspension well, and sonicate for another 15 min by shaking well every 5 min. Allow the suspension to cool to room temperature and separate the phases by centrifuging or by allowing it to settle for 30 min. Pass the supernatant through a membrane filter of 0.45-µm pore size.▲ (USP 1-Dec-2019)
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: LC

[1] Suitable nano silica gel F_{254} on HPTLC plates are available from Sigma-Aldrich, Fluka #09916-25EA.

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Detector: UV 268 nm
Column: 4.6-mm × 10-cm; 2.6-µm packing L1
Column temperature: 25°
Flow rate: 0.7 mL/min
Injection volume: 10 µL
Run time: NLT 3 times the retention time of menaquinone-7

System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 3.0% from 6 replicate injections

Analysis
Samples: *Standard solution* and *Sample solution*
▲▲ (USP 1-Dec-2019)
Calculate the content of menaquinone-7 ($C_{46}H_{64}O_2$), in ppm, in the portion of Preparation taken:

$$\text{Result} = (r_U/r_S) \times (C_S/W) \times V$$

r_U = peak response of menaquinone-7 from the *Sample solution*
r_S = peak response of menaquinone-7 from the *Standard solution*
C_S = concentration of USP Menaquinone-7 RS in the *Standard solution* (µg/mL)
W = weight of Preparation used to prepare the *Sample solution* (g)
V = volume of the *Sample solution* (mL)

▲▲ (USP 1-Dec-2019)
Acceptance criteria: ▲▲ (USP 1-Dec-2019) 90.0%–120.0% of the labeled amount (ppm) for the solid Preparation on the dried basis; 90.0%–120.0% of the labeled amount (ppm) for the liquid Preparation ▲and the microencapsulated powder Preparation▲ (USP 1-Dec-2019) on the as-is basis
▲▲ (USP 1-Dec-2019)

IMPURITIES

• **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩
Acceptance criteria
Arsenic: NMT 2.0 µg/g
Cadmium: NMT 1.0 µg/g
Lead: NMT 3.0 µg/g
Mercury: NMT 0.1 µg/g

Change to read:

• **ISOMERIC PURITY**
Mobile phase: Water, dehydrated alcohol, methanol, and tetrahydrofuran (1:15:80:10)
Sample solution: Prepare ▲the appropriate *Sample solution*▲ (USP 1-Dec-2019) as described in *Content of Menaquinone-7*. [NOTE—Protect the solution from light, and inject immediately after preparation.]
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 268 nm
Column: 4.6-mm × 25-cm; 5-µm packing L62
Column temperature: 25°
Flow rate: 0.8 mL/min
Injection volume: 20 µL
Run time: NLT 1.5 times the retention time of *all-trans*-menaquinone-7

System suitability
Sample: ▲*Sample solution*▲ (USP 1-Dec-2019)
[NOTE—The relative retention times for *all-trans*-menaquinone-7 and *cis*-menaquinone-7 are 1.0 and 1.1, respectively.]
Suitability requirements
Resolution: NLT 1.5 between *all-trans*-menaquinone-7 and *cis*-menaquinone-7

Analysis
Sample: *Sample solution*
Calculate the percentage of *cis*-menaquinone-7 in the portion of Preparation taken:

$$\text{Result} = [r_C/(r_T + r_C)] \times 100$$

r_C = peak response of *cis*-menaquinone-7 from the *Sample solution*
r_T = peak response of *all-trans*-menaquinone-7 from the *Sample solution*

Acceptance criteria: NMT 2.0% of *cis*-menaquinone-7

Add the following:

▲• **LIMIT OF MENAQUINONE-6**
Mobile phase, Standard solution, Sample solution, Chromatographic system, and System suitability: Proceed as directed in *Content of Menaquinone-7*.

Analysis
Samples: *Standard solution* and *Sample solution*
[NOTE—The relative retention times for menaquinone-6 and menaquinone-7 are 0.81 and 1.00, respectively.]
Calculate the content of menaquinone-6 ($C_{41}H_{56}O_2$), in ppm, in the portion of Preparation taken:

$$\text{Result} = (r_U/r_S) \times (C_S/W) \times V$$

r_U = peak response of menaquinone-6 from the *Sample solution*
r_S = peak response of menaquinone-7 from the *Standard solution*
C_S = concentration of USP Menaquinone-7 RS in the *Standard solution* (µg/mL)
W = weight of Preparation used to prepare the *Sample solution* (g)
V = volume of *Sample solution* (mL)

Acceptance criteria: NMT 10% of the labeled amount (ppm) of menaquinone-7 for the solid Preparation on the dried basis; NMT 10% of the labeled amount (ppm) of menaquinone-7 for the liquid Preparation and the microencapsulated powder Preparation on the as-is basis▲ (USP 1-Dec-2019)

SPECIFIC TESTS

• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total bacterial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.
• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species, Test for Absence of Escherichia coli*, and *Test for Absence of Staphylococcus aureus*: Meets the requirements
• **LOSS ON DRYING** ⟨731⟩ (for solid Preparation)
Analysis: Dry at 110° to a constant weight.
Acceptance criteria: NMT 5.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Store in a tight container, in a cool and dry place. Protect from light.

DS Monographs

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- **LABELING:** The label states the name and content of any carriers and antioxidants added to the formulation, and the content of menaquinone-7.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Menaquinone-7 RS

Menaquinone-7 Tablets

DEFINITION
Menaquinone-7 Tablets contain NLT 90.0% and NMT 110.0% of the labeled amount of menaquinone-7 ($C_{46}H_{64}O_2$).

IDENTIFICATION
- **A.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in *Method 1* or *Method 2* of the test for *Content of Menaquinone-7*.

STRENGTH
- **CONTENT OF MENAQUINONE-7, *Method 1***
  [NOTE—Menaquinone-7 is extremely light sensitive. Protect samples from light.]
  Diluent: 0.1 mg/mL of 2,6-di-*tert*-butyl-4methyl-phenol (BHT) in hexane
  Mobile phase: Methanol and ethyl acetate. See *Table 1* for gradient.

Table 1

Time (min)	Flow Rate (mL/min)	Methanol (%)	Ethyl Acetate (%)
0	1.00	94	6
5.5	1.00	94	6
6.5	1.25	80	20
13	1.25	80	20
14	1.25	94	6
15	1.00	94	6
25	1.00	94	6

Standard stock solution: Transfer 20 mg of USP Menaquinone-7 RS into a 50-mL volumetric flask, dilute with hexane to volume, and mix well. [NOTE—The solution can be kept in the freezer for 1 month.]
Standard solution: Transfer 1.0 mL of the *Standard stock solution* into a 10-mL volumetric flask, dilute with *Diluent* to volume, and mix well.
Sample solution: Transfer a portion from NLT 30 finely powdered Tablets, nominally equivalent to about 1.0 mg of menaquinone-7, to a 25-mL volumetric flask, add 10 mL of *Diluent*, and shake for 2 min. Dilute with *Diluent* to volume, mix, and pass through a membrane filter of 0.45-µm pore size.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 268 nm
Column: 4.6-mm × 25-cm; 4-µm packing L87. [NOTE—A suitable column is Synergy Max-RP from www.phenomenex.com.]
Column temperature: 35°
Flow rate: See *Table 1*.
Injection volume: 15 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 3.0% for six replicate injections
Retention time: NMT 0.5 min difference between the first and last injection for a total of six injections
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of menaquinone-7 ($C_{46}H_{64}O_2$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of menaquinone-7 from the *Sample solution*
r_S = peak response of menaquinone-7 from the *Standard solution*
C_S = concentration of USP Menaquinone-7 RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of menaquinone-7 in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–110.0%
- **CONTENT OF MENAQUINONE-7, *Method 2***
  [NOTE—Menaquinone-7 is extremely light sensitive. Protect samples from light, inject immediately after preparation, and inject only once.]
  Mobile phase: Ethanol and water (97:3)
  Standard stock solution: Transfer 25 mg of USP Menaquinone-7 RS into a 50-mL volumetric flask, add 0.5 mL of tetrahydrofuran, dilute with dehydrated alcohol to volume, and mix well. [NOTE—The solution can be kept in the freezer for 1 month.]
  Standard solution: 10 µg/mL of menaquinone-7 in dehydrated alcohol from the *Standard stock solution*
  Sample solution: Transfer a portion from NLT 20 finely powdered Tablets, nominally equivalent to about 0.25 mg of menaquinone-7, to a 25-mL volumetric flask, add 2 mL of tetrahydrofuran, and dilute with dehydrated alcohol to volume. Shake for 2 min, and pass through a membrane filter of 0.45-µm pore size.
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: UPLC
  Detector: UV 268 nm
  Column: 4.6-mm × 10-cm; 2.6-µm packing L1
  Column temperature: 25°
  Flow rate: 0.7 mL/min
  Injection volume: 10 µL
  System suitability
  Sample: *Standard solution*
  Suitability requirements
  Relative standard deviation: NMT 3.0% for six replicate injections
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Calculate the percentage of the labeled amount of menaquinone-7 ($C_{46}H_{64}O_2$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of menaquinone-7 from the *Sample solution*
r_S = peak response of menaquinone-7 from the *Standard solution*

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C_S = concentration of USP Menaquinone-7 RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of menaquinone-7 in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–110.0%

PERFORMANCE TESTS
- **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩**:** Meet the requirements for *Disintegration*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩ **:** Meet the requirements

CONTAMINANTS
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meet the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Store in tight, light-resistant containers, in a cool, dry place.
- **LABELING:** The label states the content of menaquinone-7 in µg/Tablet. It also states the assay method used if *Method 1* is not used.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Menaquinone-7 RS

Bacillus subtilis subsp. *subtilis* Menaquinone-7 Extract

DEFINITION
Bacillus subtilis subsp. *subtilis* Menaquinone-7 Extract is the product obtained by supercritical carbon dioxide (CO_2) extraction of cultures of *Bacillus subtilis* subsp. *subtilis* strains that are capable of fermenting soybean protein isolate (soybean peptone) from soybeans to produce natto. The Extract is a brown oil, consisting mainly of fat (>97%), that contains NLT 1.5% and NMT 5.0% of menaquinone-7 ($C_{46}H_{64}O_2$), and NLT 0.014% and NMT 0.15% of menaquinone-6 ($C_{41}H_{56}O_2$). It contains no organic solvents.

IDENTIFICATION
- **A. THIN-LAYER CHROMATOGRAPHY**
  Standard solution: Transfer 10 mg of USP Menaquinone-7 RS to a 10-mL volumetric flask, add 1 mL of tetrahydrofuran, and dissolve and dilute with the same solvent to volume.
  Sample solution: Transfer 1.0 g of Extract to a 10-mL volumetric flask, and add 8 mL of tetrahydrofuran. Shake and allow the mixture to settle, centrifuge if necessary, and use the supernatant.
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography.*)
  Mode: HPTLC
  Adsorbent: Nano silica gel F_{254} for HPTLC[1]
  Application volume: 5 µL
  Developing solvent system: Hexane and ethyl acetate (95:5)
  Spray reagent: Transfer 0.2 g of cerium sulfate and 4.2 g of ammonium molybdate to a 100-mL volumetric flask. Dissolve with 6.6 mL of 98% (w/w) sulfuric acid, and dilute with deionized water to volume. Stir to complete dissolution. Cool, and store the solution at 0°. Allow the spray reagent to achieve room temperature before use.
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Develop the chromatogram in the *Developing solvent system* until the solvent front has moved about three-fourths the length of the plate. Remove the plate from the chamber, and air-dry. Examine the plate under visible light and short-wavelength UV light, and record the spots. Spray the plate with the *Spray reagent*, and heat at 100°–110° for 5 min. Examine the plate under white light, and record the spots.
  Acceptance criteria: Under visible light and short-wavelength UV light, the spots from the *Sample solution* correspond in color (light yellow), shape, and R_F value to those from the *Standard solution*. After applying the *Spray reagent*, under white light, the spots from the *Sample solution* correspond in color (dark blue), shape, and R_F value to those from the *Standard solution*. [NOTE—The *Sample solution* should also contain intense spots at lower R_F values due to the brown oil and its components.]
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Menaquinone-7 and Menaquinone-6*.

COMPOSITION
- **CONTENT OF MENAQUINONE-7 AND MENAQUINONE-6**
  [NOTE—Use low-actinic glassware.]
  Mobile phase: Dehydrated alcohol and water (97:3)
  [NOTE—Protect the following *Standard solution* and *Sample solution* from light, and inject immediately after preparation.]
  Standard solution: Transfer 12.5 mg of USP Menaquinone-7 RS into a 25-mL volumetric flask, add 0.5 mL of tetrahydrofuran, and dilute with dehydrated alcohol to volume. Transfer 5.0 mL of this solution into a 25-mL volumetric flask, and dilute with dehydrated alcohol to volume.
  Sample solution: Transfer 100 mg of Extract, accurately weighed, to a 25-mL volumetric flask, and add 2 mL of tetrahydrofuran. Dissolve and dilute with dehydrated alcohol to volume. Pass this solution through a filter of 0.45-µm pore size.
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability.*)
  Mode: LC
  Detector: UV 268 nm
  Column: 4.6-mm × 10-cm; 2.6-µm packing L1
  Column temperature: 25°
  Flow rate: 0.7 mL/min
  Injection volume: 10 µL
  Run time: At least 3 times the retention time of menaquinone-7
  System suitability
  Sample: *Standard solution*
  [NOTE—The relative retention times for menaquinone-6 and menaquinone-7 are 0.81 and 1.00, respectively.]
  Suitability requirements
  Relative standard deviation: NMT 3.0% for six replicate injections
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Calculate the percentage of menaquinone-7 ($C_{46}H_{64}O_2$) in the portion of Extract taken:

$$\text{Result} = (r_{U7}/r_S) \times (C_S/C_U) \times 100$$

[1] Suitable nano silica gel F_{254} on HPTLC plates is available from Sigma-Aldrich, Fluka #09916-25EA.

DS Monographs

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r_{U7} = peak response of menaquinone-7 from the *Sample solution*
r_S = peak response of menaquinone-7 from the *Standard solution*
C_S = concentration of USP Menaquinone-7 RS in the *Standard solution* (mg/mL)
C_U = concentration of Extract in the *Sample solution* (mg/mL)

Calculate the percentage of menaquinone-6 ($C_{41}H_{56}O_2$) in the portion of Extract taken:

$$\text{Result} = (r_{U6}/r_S) \times (C_S/C_U) \times 100$$

r_{U6} = peak response of menaquinone-6 from the *Sample solution*
r_S = peak response of menaquinone-7 from the *Standard solution*
C_S = concentration of USP Menaquinone-7 RS in the *Standard solution* (mg/mL)
C_U = concentration of Extract in the *Sample solution* (mg/mL)

Acceptance criteria
Menaquinone-7: 1.5%–5.0%
Menaquinone-6: 0.014%–0.15%

IMPURITIES
• **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩
Acceptance criteria
Arsenic: NMT 2.0 µg/g
Cadmium: NMT 1.0 µg/g
Lead: NMT 3.0 µg/g
Mercury: NMT 0.1 µg/g
• **ISOMERIC PURITY**
Mobile phase: Methanol, tetrahydrofuran, dehydrated alcohol, and water (80:10:15:1)
Sample solution [NOTE—Protect the solution from light, and inject immediately after preparation.] Transfer 100 mg of Extract into a 25-mL volumetric flask, and add 2 mL of tetrahydrofuran. Dissolve and dilute with dehydrated alcohol to volume. Pass through a filter of 0.45-µm pore size.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 268 nm
Column: 4.6-mm × 25-cm; 5-µm packing L62
Column temperature: 25°
Flow rate: 0.8 mL/min
Injection volume: 20 µL
System suitability
Sample: *Sample solution*
[NOTE—The relative retention times for all-*trans*-menaquinone-7 and *cis*-menaquinone-7 are 1.0 and 1.1, respectively. The retention time for all-*trans*-menaquinone-7 is about 19 min.]
Suitability requirements
Resolution: NLT 1.5 between all-*trans*-menaquinone-7 and *cis*-menaquinone-7
Analysis
Sample: *Sample solution*
Calculate the percentage of *cis*-menaquinone-7 in the portion of Extract taken:

$$\text{Result} = [r_C/(r_T + r_C)] \times 100$$

r_C = peak response of *cis*-menaquinone-7 from the *Sample solution*
r_T = peak response of all-*trans*-menaquinone-7 from the *Sample solution*

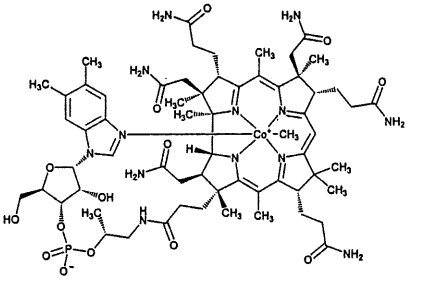
Acceptance criteria: NMT 2.0%

SPECIFIC TESTS
• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total bacterial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.
• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species, *Staphylococcus aureus*, and *Escherichia coli*.

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Store in a tight container, in a cool and dry place. Protect from light.
• **LABELING:** The label states the name and content of any carriers and antioxidants added to the material, and the content of menaquinone-7. The label also states that menaquinone-7 is a form of vitamin K_2.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Menaquinone-7 RS

Methionine—*see Methionine General Monographs*

Methylcobalamin



$C_{63}H_{91}CoN_{13}O_{14}P$ 1344.40
Coα-[α-5,6-dimethyl-1*H*-benzoimidazol-1-yl]-Coβ-methylcobamide [13422-55-4].

DEFINITION
Methylcobalamin contains NLT 98.0% and NMT 102.0% of methylcobalamin ($C_{63}H_{91}CoN_{13}O_{14}P$), calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Ultraviolet-Visible Spectroscopy*: **197U**▲ (CN 1-May-2020)
Sample solution: 50 µg/mL in phosphate buffer, pH 7.0 [NOTE—Use low-actinic glassware, and keep the solutions from exposure to light.]
Wavelength range: 200–700 nm
Acceptance criteria: The absorption spectrum exhibits maxima at 267 ± 2 nm, 342 ± 2 nm, and 522 ± 2 nm.
• **B. COBALT**
Sample: 1 mg of Methylcobalamin
Analysis: Fuse the *Sample* with 50 mg of potassium pyrosulfate in a porcelain or silica crucible. Cool, break up the mass with a glass rod, add 3 mL of water, and boil until

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dissolved. Add 1 drop of phenolphthalein TS, and add 2.5 N sodium hydroxide dropwise until a pink color appears. Add 0.5 g of sodium acetate, 0.5 mL of 1 N acetic acid, and 0.5 mL of a 2-mg/mL solution of nitroso R salt. Add 0.5 mL of hydrochloric acid, and boil for 1 min.

Acceptance criteria: A red or orange-red color appears immediately after the addition of nitroso R salt. The red or orange-red color persists after boiling with the addition of hydrochloric acid.

- **C.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

ASSAY

- **PROCEDURE**

Buffer solution: 3.1 g/L of sodium dihydrogen phosphate dihydrate in water. Adjust with phosphoric acid (1 in 100) to a pH of 3.5.

Mobile phase: To 200 mL of acetonitrile add 800 mL of *Buffer solution*, then add 3.76 g of sodium 1-hexane sulfonate, and mix to dissolve.

[NOTE—Use low-actinic glassware, and keep the following solutions from exposure to light.]

System suitability solution: 0.05 mg/mL each of cyanocobalamin and hydroxocobalamin acetate from USP Cyanocobalamin (Crystalline) RS and USP Hydroxocobalamin Acetate RS in *Mobile phase*

Standard solution: 1 mg/mL of USP Methylcobalamin RS in *Mobile phase*

Sample solution: 1 mg/mL of Methylcobalamin in *Mobile phase*

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 266 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 40°

Flow rate: 0.6 mL/min, or adjust to have methylcobalamin elute at about 12 min.

Injection volume: 10 µL

System suitability

Samples: *System suitability solution* and *Standard solution*

[NOTE—The relative retention times for cyanocobalamin and hydroxocobalamin are 0.8 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 3 between the cyanocobalamin and hydroxocobalamin peaks, *System suitability solution*

Column efficiency: NLT 6000 theoretical plates, *Standard solution*

Relative standard deviation: NMT 2.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of methylcobalamin ($C_{63}H_{91}CoN_{13}O_{14}P$) in the portion of sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Methylcobalamin RS in the *Standard solution* (mg/mL)

C_U = concentration of Methylcobalamin in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–102.0% on the anhydrous basis

IMPURITIES

- **RELATED COMPOUNDS**

Buffer solution, Mobile phase, System suitability solution, and **Chromatographic system:** Proceed as directed in the *Assay* except for the *Run time*.

[NOTE—Use low-actinic glassware, and keep the following solutions from exposure to light.]

Sample solution: 1 mg/mL of Methylcobalamin in *Mobile phase*

Quantitative limit solution: 1 µg/mL of Methylcobalamin in *Mobile phase*, diluted from the *Sample solution*

Run time: NLT 2.5 times the retention time of methylcobalamin

System suitability

Samples: *System suitability solution* and *Quantitative limit solution*

Suitability requirements

Resolution: NLT 3 between the cyanocobalamin and hydroxocobalamin peaks, *System suitability solution*

Signal-to-noise ratio: NLT 5.0 for the major peak, *Quantitative limit solution*

Analysis

Sample: *Sample solution*

Calculate the percentage of individual impurities in the portion of Methylcobalamin taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of each impurity from the *Sample solution*

r_T = sum of all the peak responses from the *Sample solution*

Acceptance criteria

Individual impurities: NMT 0.5%

Total impurities: NMT 2.0%

SPECIFIC TESTS

- **WATER DETERMINATION** ⟨921⟩, *Method I, Method Ia*: NMT 12.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at controlled room temperature.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Cyanocobalamin (Crystalline) RS

USP Hydroxocobalamin Acetate RS

USP Methylcobalamin RS

Methylcobalamin Tablets

DEFINITION

Methylcobalamin Tablets contain NLT 90.0% and NMT 125.0% of the labeled amount of methylcobalamin ($C_{63}H_{91}CoN_{13}O_{14}P$).

IDENTIFICATION

- **A.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in *Strength*.

STRENGTH

- **PROCEDURE**

Buffer solution: 3.1 g/L of sodium dihydrogen phosphate dihydrate in water. Adjust with phosphoric acid (1 in 100) to a pH of 3.5.

Mobile phase: Transfer 200 mL of acetonitrile to a 1-L volumetric flask and dilute with *Buffer solution* to volume.

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Then add 3.76 g of sodium 1-hexane sulfonate, and mix to dissolve.
[NOTE—Use low-actinic glassware, and keep the following solutions from exposure to light.]
System suitability solution: 0.05 mg/mL of cyanocobalamin from USP Cyanocobalamin (Crystalline) RS and 0.05 mg/mL of USP Hydroxocobalamin Acetate RS in *Mobile phase*
Standard solution: 100 µg/mL of USP Methylcobalamin RS in *Mobile phase*
Sample solution: Finely powder NLT 30 Tablets. Transfer a portion of the powder, nominally equivalent to 5 mg of methylcobalamin, to a 50-mL volumetric flask, add a suitable amount of *Mobile phase*, swirl gently, and dilute with *Mobile phase* to volume. Shake vigorously for 10 min and immediately pass through a nylon membrane filter of 0.2-µm pore size.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 266 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 40°
Flow rate: 0.6 mL/min
Injection volume: 50 µL
System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The relative retention times for cyanocobalamin and hydroxocobalamin are 0.8 and 1.0, respectively.]
Suitability requirements
Resolution: NLT 3 between the cyanocobalamin and hydroxocobalamin peaks, *System suitability solution*
Column efficiency: NLT 6000 theoretical plates, *Standard solution*
Relative standard deviation: NMT 2.0%, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of methylcobalamin ($C_{63}H_{91}CoN_{13}O_{14}P$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP Methylcobalamin RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of methylcobalamin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0%

PERFORMANCE TESTS
• **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩, *Disintegration*: Meet the requirements
• **WEIGHT VARIATION** ⟨2091⟩: Meet the requirements

CONTAMINANTS
• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic microbial count does not exceed 3×10^3 cfu/g, and the total combined molds and yeasts count does not exceed 3×10^2 cfu/g.
• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Escherichia coli*: Meet the requirements

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Cyanocobalamin (Crystalline) RS
USP Hydroxocobalamin Acetate RS
USP Methylcobalamin RS

Methylsulfonylmethane



$C_2H_6O_2S$ 94.13
Dimethyl sulfone;
Sulfonylbismethane [67-71-0].

DEFINITION
Methylsulfonylmethane contains NLT 98.0% and NMT 102.0% of methylsulfonylmethane ($C_2H_6O_2S$), calculated on the anhydrous basis. The chromatographic purity is NLT 99.8%.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
• **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

ASSAY
• **PROCEDURE**
Diluent: Transfer 950 mL of methanol to a 1-L volumetric flask. Add 0.60 mL of di(ethylene glycol) methyl ether, and dilute with methanol to volume.
Standard solution: 0.4 mg/mL of USP Methylsulfonylmethane RS in *Diluent*. Sonicate at 50° for 1 min, and allow to cool to room temperature.
Sample solution: 0.4 mg/mL of Methylsulfonylmethane in *Diluent*. Sonicate at 50° for 1 min, and allow to cool to room temperature.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.53-mm × 30-m capillary column coated with a 5-µm phase G2
Temperature
Column: 120°
Injector: 250°
Detector: 250°
Carrier gas: Helium
Flow rate: 5 mL/min
Split ratio: 2:1
Injection size: 1 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 2.0% for the peak response ratio of methylsulfonylmethane to di(ethylene glycol) methyl ether from replicate injections
Analysis
Samples: *Standard solution* and *Sample solution*

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Calculate the percentage of methylsulfonylmethane ($C_2H_6O_2S$) in the portion of Methylsulfonylmethane taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of methylsulfonylmethane to di(ethylene glycol) methyl ether from the *Sample solution*
R_S = peak response ratio of methylsulfonylmethane to di(ethylene glycol) methyl ether from the *Standard solution*
C_S = concentration of USP Methylsulfonylmethane RS in the *Standard solution* (mg/mL)
C_U = concentration of Methylsulfonylmethane in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–102.0% on the anhydrous basis

IMPURITIES

- **CHROMATOGRAPHIC PURITY AND LIMIT OF DIMETHYL SULFOXIDE**

Standard stock solution: 1.0 mg/mL of USP Dimethyl Sulfoxide RS in methanol
Sensitivity check solution: 2.0 µg/mL from the *Standard stock solution* in methanol
System suitability solution: 0.1 mg/mL of USP Dimethyl Sulfoxide RS and 0.4 mg/mL of USP Methylsulfonylmethane RS in methanol. In a 50-mL volumetric flask dissolve 20 mg of USP Methylsulfonylmethane RS in 5 mL of the *Standard stock solution*, and dilute with methanol to volume.
Sample solution: 2 mg/mL of Methylsulfonylmethane in methanol. Sonicate at 50° for 1 min, and allow to cool to room temperature.
Chromatographic system: Proceed as directed in the *Assay*.
System suitability
Samples: *Sensitivity check solution* and *System suitability solution*
Suitability requirements
Signal-to-noise ratio: NLT 10 for dimethyl sulfoxide peak, *Sensitivity check solution*
Resolution: NLT 2.0 between dimethyl sulfoxide and methylsulfonylmethane, *System suitability solution*
Analysis
Sample: *Sample solution*
Calculate the percentage of each impurity in the portion of Methylsulfonylmethane taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = response of each impurity in the *Sample solution*
r_T = sum of the responses of all of the peaks other than the solvent peak

Acceptance criteria
Individual impurities: NMT 0.1% of dimethyl sulfoxide; NMT 0.05% of any other individual impurity
Total impurities: NMT 0.2% for all impurities, including dimethyl sulfoxide

SPECIFIC TESTS

- **MELTING RANGE OR TEMPERATURE** ⟨741⟩**:** 108.5°–110.5°
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count is NMT 10^3 cfu/g or mL, and the total combined molds and yeasts count is NMT 10^2 cfu/g or mL.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements for absence of *Escherichia coli* in 10 g.
- **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 0.1%. [NOTE—500 mg of methylsulfonylmethane may be required for this analysis.]

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Dimethyl Sulfoxide RS
USP Methylsulfonylmethane RS
Dimethyl sulfone.
$C_2H_6O_2S$ 94.13

Methylsulfonylmethane Tablets

DEFINITION

Methylsulfonylmethane Tablets contain NLT 90.0% and NMT 110.0% of the labeled amount of methylsulfonylmethane ($C_2H_6O_2S$).

IDENTIFICATION

- **A.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Procedure* for *Strength*.

STRENGTH

- **PROCEDURE**

Diluent: Transfer 950 mL of methanol to a 1-L volumetric flask. Add 0.60 mL of diethylene glycol methyl ether, and dilute with methanol to volume.
Standard solution: 0.4 mg/mL of USP Methylsulfonylmethane RS in *Diluent*. Sonicate at 50° for 1 min, and allow to cool to room temperature.
Sample solution: Finely powder NLT 20 Tablets. Dissolve a portion of the finely powdered material, equivalent to 1 Tablet, in *Diluent*, and sonicate for 15 min at 50°. Allow to cool to room temperature, dilute with *Diluent* to volume, and mix. Quantitatively dilute with *Diluent* to obtain a final concentration of 0.4 mg/mL of methylsulfonylmethane. Transfer 1 mL of the suspension to a 1.5-mL microcentrifuge tube, and centrifuge for 20 s. Use the supernatant.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.53-mm × 30-m capillary; 5-µm phase G2 coating
Temperature
Column: 120°
Injector: 250°
Detector: 250°
Carrier gas: Helium
Flow rate: 5 mL/min
Injection size: 1 µL
Injector type: Split ratio, 2:1
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 2.0% for the peak response ratio of methylsulfonylmethane to diethylene glycol methyl ether from replicate injections
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amountof of methylsulfonylmethane ($C_2H_6O_2S$) in the portion of Tablets taken:

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$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = internal standard ratio (peak response of methylsulfonylmethane to diethylene glycol methyl ether) from the *Sample solution*
R_S = internal standard ratio (peak response of methylsulfonylmethane to diethylene glycol methyl ether) from the *Standard solution*
C_S = concentration of USP Methylsulfonylmethane RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of methylsulfonylmethane in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–110.0%

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩**:** Meet the requirements for *Disintegration*; 30 min
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩ **:** Meet the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Methylsulfonylmethane RS
  Dimethyl sulfone.
  $C_2H_6O_2S$ 94.13

Milk Thistle

DEFINITION

Milk Thistle consists of the dried ripe fruit of *Silybum marianum* (L.) Gaertn. (Fam. Asteraceae), the pappus having been removed. It contains NLT 2.0% of silymarin, calculated as silybin ($C_{25}H_{22}O_{10}$), on the dried basis.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution: 1.0 mg/mL of USP Silydianin RS in methanol

Sample solution: Use the *Sample solution*, prepared as directed in the test for *Content of Silymarin*.

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Adsorbent: 0.25-mm layer of chromatographic silica gel, typically 20 cm long (TLC plates)

Application volume: 10 µL

Developing solvent system: Freshly prepared mixture of chloroform, acetone, and anhydrous formic acid (75: 16.5: 8.5)

Spray reagent A: 10-mg/mL solution of 2-aminoethyl diphenylborinate in methanol

Spray reagent B: 50-mg/mL solution of polyethylene glycol 4000 in alcohol

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the chromatograms until the solvent front has moved about three-fourths of the plate, and dry it for 30 min in a current of cold air. Spray the plate with *Spray reagent A*, allow to dry, and then spray with *Spray reagent B*. One hour later examine the plate under long-wave UV light (365 nm).

Acceptance criteria: The chromatogram of the *Sample solution* exhibits an intense green-blue fluorescent zone at an R_F value of about 0.5 (presence of silybin) and a gray-blue spot at an R_F value of about 0.4, corresponding to that observed in the chromatogram of the *Standard solution*. The chromatogram of the *Sample solution* may exhibit other colored zones: an intense green-blue zone at an R_F value of about 0.25 (presence of silychristin) and a red-orange zone at an R_F value of about 0.3 (presence of taxifolin).

- **B. HPLC IDENTIFICATION TEST**

Analysis: Proceed as directed in the test for *Content of Silymarin*.

Acceptance criteria: The retention times of the peaks for silydianin, silychristin, silybin A, silybin B, isosilybin A, and isosilybin B in the chromatogram of the *Sample solution* correspond to those in the chromatogram of the *Milk thistle standard solution*.

COMPOSITION

- **CONTENT OF SILYMARIN**

Solution A: Methanol, phosphoric acid, and water (20: 0.5: 80)

Solution B: Methanol, phosphoric acid, and water (80: 0.5: 20)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	85	15
5	85	15
20	55	45
40	55	45
41	85	15
55	85	15

Milk thistle standard solution: 0.7 mg/mL of USP Powdered Milk Thistle Extract RS in methanol. Sonicate for 20 min to dissolve. Pass through a membrane filter having a 0.45-µm or finer pore size. Dilute 1:5 with methanol to obtain a solution of 0.14 mg/mL of USP Powdered Milk Thistle Extract RS.

Silybin standard solutions: 0.20, 0.02, and 0.004 mg/mL of USP Silybin RS in methanol. Pass through a membrane filter having a 0.45-µm or finer pore size.

Sample stock solution: Transfer 10 g of finely powdered Milk Thistle to an extraction thimble, and cover with a small cotton ball. Transfer the thimble to a continuous-extraction apparatus fitted with a 250-mL round-bottom flask containing 150 mL of solvent hexane, and heat the flask on a heating mantle for 4 h. After the extraction, detach the round-bottom flask from the extraction apparatus, and discard the solvent hexane. Dry the extraction thimble to remove residual solvent hexane, and transfer the thimble to an extraction apparatus suitable for hot extraction and fitted with a 250-mL round-bottom flask containing 100 mL of ethyl acetate. [NOTE—Adjust the volume of ethyl acetate, if necessary, to sustain continuous extraction.] Heat the flask to achieve gentle reflux. After 8 h, transfer the extract quantitatively into a 100-mL volumetric flask, and dilute with methanol to volume.

Sample solution: Dilute the *Sample stock solution* 1:25 with methanol.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 288 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Column temperature: 40°

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Flow rate: 1 mL/min
Injection volume: 10 µL
System suitability
Sample: *Milk thistle standard solution*
[NOTE—For the relative retention times, see *Table 2*.]

Table 2

	Relative Retention Time
Silychristin	0.68
Silydianin	0.73
Silybin A	1.00
Silybin B	1.05
Dehydrosilybin	1.09
Isosilybin A	1.15
Isosilybin B	1.19

Suitability requirements
Chromatogram similarity: The chromatogram of the *Milk thistle standard solution* is similar to the reference chromatogram provided with the lot of USP Powdered Milk Thistle Extract RS being used.
Resolution: NLT 1.0 between silybin A and silybin B
Tailing factor: 0.8–2.0
Relative standard deviation: NMT 2.0% for the sum of peak responses due to silybin A and silybin B
Analysis
Samples: *Milk thistle standard solution*, *Silybin standard solutions*, and *Sample solution*
Identify the peaks due to silychristin, silydianin, silybin A, silybin B, isosilybin A, and isosilybin B by comparison with the chromatogram of the *Milk thistle standard solution*, and measure the peak areas of the relevant peaks. Plot the combined peak areas of silybin A and silybin B against the concentrations of USP Silybin RS in the corresponding *Silybin standard solutions*, and establish a calibration curve using least-squares regression.
Separately calculate the percentage of each relevant component of silymarin as silybin ($C_{25}H_{22}O_{10}$) in the portion of Milk Thistle taken:

$$\text{Result} = C \times (V/W) \times D \times 100$$

C = concentration of the relevant component in the *Sample solution* as interpolated from the calibration curve (mg/mL)
V = volume of the *Sample stock solution* (mL)
W = weight of the portion of Milk Thistle taken (mg)
D = dilution factor to prepare the *Sample solution* from *Sample stock solution*, 25

Calculate the content of silymarin, as a percentage, in the portion of Milk Thistle taken by adding the individual percentages.
Acceptance criteria: NLT 2.0% of silymarin, calculated as silybin ($C_{25}H_{22}O_{10}$), on the dried basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: The fruits (achenes) are elongated ovoid, slightly crooked, somewhat flattened, roughly 6–7 mm in length, up to 3 mm in width, and 1.5 mm in thickness, and have a projecting cartilaginous, glossy, yellowish edge on the upper surface and a grooved hilum at the base. The fruit coat is glossy brown-black or matte gray-brown with dark or pale gray streaks; it encloses the straight embryo having two thick, flattened cotyledons that contain fatty oil and aleurone granules.
Microscopic: The fruit wall epidermis consists of almost colorless palisade cells arranged at right angles to the surface. They have greatly thickened outer walls, into which the lumen continues for some distance in the form of a slit. Viewed from above under high magnification, the cells show only a slit-shaped lumen. They have thickened ridges that appear as nodular thickenings of the cell wall when viewed from above. The subepidermal layer of the fruit wall is made up of unlignified thin-walled parenchymal cells and constitutes a pigment layer. Colorless cells and groups of cells alternate with pigment cells, the latter varying in number; this gives the fruit wall its mottled appearance. The fruit wall tissue is about 8 cell layers thick, with stippled parenchymal cells elongated in the longitudinal axis of the fruit. The cells of the innermost layer of the fruit wall may be collapsed; they contain large cigar-shaped or monoclinic calcium oxalate prisms. The seed coat epidermis is formed from large yellow palisade cells. The cells have a narrow lumen, somewhat expanded at each end of the cell, and the cell walls show conspicuous lamination. The subepidermal cells of the seed coat consist of peculiar stippled cells; their lignified cell membranes have prominent, close-set ridges or thickenings ("net cells"). Next to them is a single layer of cells having tough, somewhat swollen walls and lipophilic contents (endosperm residue). The embryo consists of thin-walled cells that, in addition to small glands, contain clumps of crystals and fat droplets.

- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩**:** NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩
Sample: 1.0 g of finely powdered Milk Thistle
Acceptance criteria: NMT 8.0%
- **LOSS ON DRYING** ⟨731⟩
Sample: 1.0 g of finely powdered Milk Thistle
Analysis: Dry the *Sample* at 105° for 2 h.
Acceptance criteria: NMT 8.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Powdered Milk Thistle Extract RS
USP Silybin RS
USP Silydianin RS

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Powdered Milk Thistle

DEFINITION

Powdered Milk Thistle is Milk Thistle reduced to a fine or very fine powder. It contains NLT 2.0% of silymarin, calculated as silybin ($C_{25}H_{22}O_{10}$), on the dried basis.

IDENTIFICATION

• **A. Thin-Layer Chromatographic Identification Test**

Standard solution: 1.0 mg/mL of USP Silydianin RS in methanol

Sample solution: Use the *Sample solution*, prepared as directed in the test for *Content of Silymarin*.

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Adsorbent: 0.25-mm layer of chormatographic silica gel, typically 20 cm long (TLC plates)

Application volume: 10 µL

Developing solvent system: Freshly prepared mixture of chloroform, acetone, and anhydrous formic acid (75: 16.5: 8.5)

Spray reagent A: 10-mg/mL solution of 2-aminoethyl diphenylborinate in methanol

Spray reagent B: 50-mg/mL solution of polyethylene glycol 4000 in alcohol

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the chromatograms until the solvent front has moved about three-fourths of the plate, and dry it for 30 min in a current of cold air. Spray the plate with *Spray reagent A*, allow to dry, and then spray with *Spray reagent B*. One hour later, examine the plate under long-wave UV light (365 nm).

Acceptance criteria: The chromatogram of the *Sample solution* exhibits an intense green-blue fluorescent zone at an R_F value of about 0.5 (presence of silybin) and a gray-blue spot at an R_F value of about 0.4, corresponding to that observed in the chromatogram of the *Standard solution*. The chromatogram of the *Sample solution* may exhibit other colored zones: an intense green-blue zone at an R_F value of about 0.25 (presence of silychristin) and a red-orange zone at an R_F value of about 0.3 (presence of taxifolin).

• **B. HPLC Identification Test**

Analysis: Proceed as directed in the test for *Content of Silymarin*.

Acceptance criteria: The retention times of the peaks for silydianin, silychristin, silybin A, silybin B, isosilybin A, and isosilybin B in the chromatogram of the *Sample solution* correspond to those in the chromatogram of the *Milk thistle standard solution*.

COMPOSITION

• **Content of Silymarin**

Solution A: Methanol, phosphoric acid, and water (20: 0.5: 80)

Solution B: Methanol, phosphoric acid, and water (80: 0.5: 20)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	85	15
5	85	15
20	55	45
40	55	45
41	85	15
55	85	15

Milk thistle standard solution: 0.7 mg/mL of USP Powdered Milk Thistle Extract RS in methanol. Sonicate for 20 min to dissolve. Pass through a membrane filter having a 0.45-µm or finer pore size. Dilute 1:5 with methanol to obtain a solution of 0.14 mg/mL of USP Powdered Milk Thistle Extract RS.

Silybin standard solutions: 0.20, 0.02, and 0.004 mg/mL of USP Silybin RS in methanol. Pass through a membrane filter having a 0.45-µm or finer pore size.

Sample stock solution: Transfer 10 g of Powdered Milk Thistle to an extraction thimble, and cover with a small cotton ball. Transfer the thimble to a continuous-extraction apparatus fitted with a 250-mL round-bottom flask containing 150 mL of solvent hexane, and heat the flask on a heating mantle for 4 h. After the extraction, detach the round-bottom flask from the extraction apparatus, and discard the solvent hexane. Dry the extraction thimble to remove residual solvent hexane, and transfer the thimble to an extraction apparatus suitable for hot extraction and fitted with a 250-mL round-bottom flask containing 100 mL of ethyl acetate. [Note—Adjust the volume of ethyl acetate, if necessary, to sustain continuous extraction.] Heat the flask to achieve gentle reflux. After 8 h, transfer the extract quantitatively into a 100-mL volumetric flask, and dilute with methanol to volume.

Sample solution: Dilute the *Sample stock solution* 1:25 with methanol.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 288 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Column temperature: 40°

Flow rate: 1 mL/min

Injection volume: 10 µL

System suitability

Sample: *Milk thistle standard solution*

[Note—For the relative retention times, see *Table 2*.]

Table 2

	Relative Retention Time
Silychristin	0.68
Silydianin	0.73
Silybin A	1.00
Silybin B	1.05
Dehydrosilybin	1.09
Isosilybin A	1.15
Isosilybin B	1.19

Suitability requirements

Chromatogram similarity: The chromatogram of the *Milk thistle standard solution* is similar to the reference chromatogram provided with the lot of USP Powdered Milk Thistle Extract RS being used.

Resolution: NLT 1.0 between silybin A and silybin B

Tailing factor: 0.8–2.0

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Relative standard deviation: NMT 2.0% for the sum of peak responses due to silybin A and silybin B

Analysis

Samples: *Milk thistle standard solution*, *Silybin standard solutions*, and *Sample solution*

Identify the peaks due to silychristin, silydianin, silybin A, silybin B, isosilybin A, and isosilybin B by comparison with the chromatogram of the *Milk thistle standard solution*, and measure the peak areas of the relevant peaks. Plot the combined peak areas of silybin A and silybin B against the concentrations of USP Silybin RS in the corresponding *Silybin standard solutions*, and establish a calibration curve using least-squares regression.

Separately calculate the percentage of each relevant component of silymarin as silybin ($C_{25}H_{22}O_{10}$) in the portion of Powdered Milk Thistle taken:

$$\text{Result} = C \times (V/W) \times D \times 100$$

C = concentration of the relevant component in the *Sample solution* as interpolated from the calibration curve (mg/mL)

V = volume of the *Sample stock solution* (mL)

W = weight of the portion of Powdered Milk Thistle taken (mg)

D = dilution factor to prepare the *Sample solution* from *Sample stock solution*, 25

Calculate the content of silymarin, as a percentage, in the portion of Powdered Milk Thistle taken by adding the individual percentages.

Acceptance criteria: NLT 2.0% of silymarin, calculated as silybin ($C_{25}H_{22}O_{10}$), on the dried basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS:** The powder is characterized by fragments of colorless cells, up to approximately 75 µm in length and 8 µm in width, from the palisade layer of the epidermis of the fruit wall, with their adherent pigment layer (they assume a red color in chloral hydrate preparations), and by gray pieces, viewed from above, with the slitlike lumen produced by the pronounced wall thickening or the nodes on the cell wall formed by the thickened ridges; fragments of the pigment layer viewed from above, with red coloration diffusing out of them in chloral hydrate preparations, pigment cells alternating with colorless parenchymal cells; conspicuously stippled colorless cells through which pigment cells are visible when viewed from above; cigar-shaped or monoclinic calcium oxalate prisms, lying free or in groups of cells; numerous fragments of the lemon-yellow palisade cells of the seed coat, up to roughly 150 µm in length, having a very narrow lumen and conspicuously stippled when viewed from above; pale yellowish fragments from the net cell layer together with portions of the embryo consisting of thin-walled cells with small glands and lipophilic substances.
- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩**:** NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩
  Sample: 1.0 g of Powdered Milk Thistle
  Acceptance criteria: NMT 8.0%
- **LOSS ON DRYING** ⟨731⟩
  Sample: 1.0 g of Powdered Milk Thistle
  Analysis: Dry the *Sample* at 105° for 2 h.
  Acceptance criteria: NMT 8.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant source from which the article was derived.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Powdered Milk Thistle Extract RS
  USP Silybin RS
  USP Silydianin RS

Powdered Milk Thistle Extract

DEFINITION

Powdered Milk Thistle Extract is prepared from Milk Thistle fruits or seeds by fat removal and subsequent extraction with suitable solvents. It contains NLT 90.0% and NMT 110.0% of the labeled amount of silymarin, calculated as silybin ($C_{25}H_{22}O_{10}$), on the dried basis, consisting of NLT 20.0% and NMT 45.0% for the sum of silydianin and silychristin, NLT 40.0% and NMT 65.0% for the sum of silybin A and silybin B, and NLT 10.0% and NMT 20.0% for the sum of isosilybin A and isosilybin B.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution: 1.0 mg/mL of USP Silydianin RS in methanol

Sample solution: 10 mg/mL of Powdered Extract in methanol, and allow to stand for 15 min before use.

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Adsorbent: 0.25-mm layer of chromatographic silica gel, typically 20 cm long

Application volume: 10 µL

Developing solvent system: Freshly prepared mixture of chloroform, acetone, and anhydrous formic acid (75: 16.5: 8.5)

Spray reagent A: 10-mg/mL solution of 2-aminoethyl diphenylborinate in methanol

Spray reagent B: 50-mg/mL solution of polyethylene glycol 4000 in alcohol

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the chromatograms until the solvent front has moved about three-fourths of the plate, and dry it for 30 min in a current of cold air. Spray the plate with *Spray reagent A*, allow to dry, and then spray with *Spray reagent B*. One h later, examine the plate under long-wavelength UV light.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits an intense green-blue fluorescent zone at an R_F value of about 0.5 (presence of silybin) and exhibits a gray-blue spot at an R_F value of about 0.4, corresponding to a spot observed in the chromatogram of the *Standard solution*. The chromatogram of the *Sample solution* may exhibit other colored zones: an intense green-blue zone at

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an R_F value of about 0.25 (presence of silychristin) and a red-orange zone at an R_F value of about 0.3 (presence of taxifolin).

• **B. HPLC IDENTIFICATION TEST**

Analysis: Proceed as directed in the test for *Content of Silymarin*.

Acceptance criteria: The retention times of the peaks for silydianin, silychristin, silybin A, silybin B, isosilybin A, and isosilybin B in the chromatogram of the *Sample solution* correspond to those in the chromatogram of the *Milk thistle standard solution*.

COMPOSITION

• **CONTENT OF SILYMARIN**

Solution A: Methanol, phosphoric acid, and water (20: 0.5: 80)

Solution B: Methanol, phosphoric acid, and water (80: 0.5: 20)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	85	15
5	85	15
20	55	45
40	55	45
41	85	15
55	85	15

Milk thistle standard solution: 0.7 mg/mL of USP Powdered Milk Thistle Extract RS in methanol. Sonicate for 20 min to dissolve.

Silybin standard solutions: 0.20, 0.02, and 0.004 mg/mL of USP Silybin RS in methanol. Pass through a membrane filter having a 0.45-µm or finer pore size.

Sample solution: Dissolve a sample of Extract in methanol to obtain a solution equivalent to 0.4 mg/mL of silymarin. Sonicate for 20 min, cool to 20°, and pass through a filter of 0.45-µm or finer pore size.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 288 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Column temperature: 40°

Flow rate: 1 mL/min

Injection size: 10 µL

System suitability

Sample: *Milk thistle standard solution*

[NOTE—For the relative retention times, see *Table 2*.]

Table 2

	Relative Retention Time
Silychristin	0.68
Silydianin	0.73
Silybin A	1.00
Silybin B	1.05
Dehydrosilybin	1.09
Isosilybin A	1.15
Tsosilybin B	1.19

Suitability requirements

Chromatogram similarity: The chromatogram from the *Milk thistle standard solution* is similar to the reference chromatogram provided with the lot of USP Powdered Milk Thistle Extract RS being used.

Resolution: NLT 1.0 between silybin A and silybin B

Tailing factor: 0.8–2.0

Relative standard deviation: NMT 2.0% for the sum of peak responses due to silybin A and silybin B

Analysis

Samples: *Milk thistle standard solution*, each of the *Silybin standard solutions*, and *Sample solution*

Identify the peaks due to silychristin, silydianin, silybin A, silybin B, isosilybin A, and isolybin B by comparison with the chromatogram of the *Milk thistle standard solution*, and measure the peak areas of the relevant peaks. Plot the areas of the sum of silybin A and silybin B peaks versus the concentration of USP Silybin RS in the *Silybin standard solutions*, and obtain a regression line for calibration.

Separately calculate the percentage of each relevant component of silymarin as silybin ($C_{25}H_{22}O_{10}$) in the portion of Powdered Extract taken:

$$\text{Result} = C \times (V/W) \times 100$$

C = concentration of the relevant component in the *Sample solution* as interpolated from the calibration graph (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of the portion of Powdered Extract taken (mg)

Calculate the percentage of the labeled amount of silymarin in the portion of Powdered Extract taken:

$$\text{Result} = \Sigma S/L \times 100$$

ΣS = sum of the percentages of the each relevant component of silymarin

L = labeled amount of silymarin, as a percentage, in the Powdered Extract

Acceptance criteria: 90.0%–110.0% of the labeled amount of silymarin, calculated as silybin ($C_{25}H_{22}O_{10}$), on the dried basis, consisting of: 20.0%–45.0% for the sum of silydianin and silychristin; 40.0%–65.0% for the sum of silybin A and silybin B; and 10.0%–20.0% for the sum of isosilybin A and isosilybin B

CONTAMINANTS

• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total bacterial count does not exceed 10^4/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the enterobacterial count does not exceed 10^3 cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli*.

• **OTHER REQUIREMENTS:** It meets the requirements in *Botanical Extracts* ⟨565⟩ for the tests in sections *Pesticide Residues* and *Residual Solvents*.

SPECIFIC TESTS

• **LOSS ON DRYING** ⟨731⟩**:** Dry a sample at 105° for 2 h: it loses NMT 5.0% of its weight.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, in a cool place.

• **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the

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article was prepared. It meets the requirements for *Botanical Extracts* ⟨565⟩, *Labeling.*

• **USP REFERENCE STANDARDS** ⟨11⟩
USP Powdered Milk Thistle Extract RS
USP Silybin RS
USP Silydianin RS

Milk Thistle Capsules

DEFINITION

Milk Thistle Capsules are prepared from Powdered Milk Thistle Extract. They contain NLT 90.0% and NMT 110.0% of the labeled amount of silymarin as silybin ($C_{25}H_{22}O_{10}$), calculated as the sum of silydianin, silychristin, silybin A, silybin B, isosilybin A, and isosilybin B.

IDENTIFICATION

• **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution: 1.0 mg/mL of USP Silydianin RS in methanol

Sample solution: Equivalent to 5 mg/mL of silymarin from powdered Capsule contents (finely powder the contents of NLT 20 Capsules) in methanol. Shake for 1 min, and sonicate for 10 min. Allow to stand for 15 min before use.

Chromatographic system
(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography.*)
Mode: TLC
Adsorbent: 0.25-mm layer of chromatographic silica gel, typically 20 cm long
Application volume: 10 µL
Developing solvent system: Freshly prepared mixture of chloroform, acetone, and anhydrous formic acid (75: 16.5: 8.5)
Spray reagent A: 10-mg/mL solution of 2-aminoethyl diphenylborinate in methanol
Spray reagent B: 50-mg/mL solution of polyethylene glycol 4000 in alcohol

Analysis
Samples: *Standard solution* and *Sample solution*
Develop the chromatograms until the solvent front has moved about three-fourths of the plate, and dry it for 30 min in a current of cold air. Spray the plate with *Spray reagent A*, allow to dry, and then spray with *Spray reagent B*. One h later, examine the plate under long-wavelength UV light.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits an intense green-blue fluorescent zone at an R_F value of 0.5 (presence of silybin) and exhibits a gray-blue spot at an R_F value of 0.4, corresponding to a spot observed in the chromatogram of the *Standard solution*. The chromatogram of the *Sample solution* may exhibit other colored zones: an intense green-blue zone at an R_F value of 0.25 (presence of silychristin) and a red-orange zone at an R_F value of 0.3 (presence of taxifolin).

• **B. HPLC IDENTIFICATION TEST**

Analysis: Proceed as directed for *Content of Silymarin.*

Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks for silydianin, silychristin, silybin A, silybin B, isosilybin A, and isosilybin B at retention times that correspond to those of the *Milk thistle standard solution.*

STRENGTH

• **CONTENT OF SILYMARIN**

Solution A: Methanol, phosphoric acid, and water (20: 0.5: 80)

Solution B: Methanol, phosphoric acid, and water (80: 0.5: 20)

Mobile phase: See *Table 1.*

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	85	15
5	85	15
20	55	45
40	55	45
41	85	15
55	85	15

Milk thistle standard solution: 0.7 mg/mL of USP Powdered Milk Thistle Extract RS in methanol. Sonicate for 20 min to dissolve.

Silybin standard solutions: 0.20, 0.02, and 0.004 mg/mL of USP Silybin RS in methanol. Pass through a membrane filter having a 0.45-µm or finer pore size.

Sample solution: Weigh and finely powder the contents of NLT 20 Capsules. Transfer an accurately weighed quantity of the powder equivalent to 100 mg of silymarin to a 100-mL volumetric flask, add 90 mL of methanol, and sonicate for 20 min with occasional shaking. Cool to 20°, and dilute with methanol to volume. Filter through a membrane with a 0.45-µm or finer pore size.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability.*)
Mode: LC
Detector: UV 288 nm
Column: 4.6-mm × 15-cm; 5-µm packing L1
Column temperature: 40°
Flow rate: 1 mL/min
Injection size: 10 µL

System suitability
Sample: *Milk thistle standard solution*
[NOTE—For the relative retention times, see *Table 2.*]

Table 2

Name	Relative Retention Time
Silychristin	0.68
Silydianin	0.73
Silybin A	1.00
Silybin B	1.05
Dehydrosilybin	1.09
Isosilybin A	1.15
Isosilybin B	1.19

Suitability requirements
Chromatogram similarity: The chromatogram from the *Milk thistle standard solution* is similar to the reference chromatogram provided with the lot of USP Powdered Milk Thistle Extract RS being used.
Resolution: NLT 1.0 between silybin A and silybin B
Tailing factor: 0.8–2.0
Relative standard deviation: NMT 2.0% for the sum of peak responses due to silybin A and silybin B

Analysis
Samples: *Milk thistle standard solution*, each of the *Silybin standard solutions*, and *Sample solution*
Identify the peaks due to silychristin, silydianin, silybin A, silybin B, isosilybin A, and isosilybin B by comparison of

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the chromatogram of the *Milk thistle standard solution* with the reference chromatogram, and measure the peak areas of the relevant peaks. Plot the areas of the sum of silybin A and silybin B peaks versus the concentration of USP Silybin RS in the *Silybin standard solutions*, and obtain a regression line for calibration.

Separately calculate the content, in mg, of each relevant component of silymarin as silybin ($C_{25}H_{22}O_{10}$) in the portion of contents of Capsules taken:

$$\text{Result} = C \times V$$

C = concentration of the relevant component in the *Sample solution* as interpolated in the calibration graph (mg/mL)
V = volume of the *Sample solution* (mL)

Calculate the content of silymarin, as a percentage, in the portion of Capsules taken:

$$\text{Result} = C_S \times (A_{WC}/W) \times (100/L)$$

C_S = sum of the contents of each relevant component of silymarin in the portion of content of Capsules taken (mg)
A_{WC} = average weight of the contents of Capsules (mg/Tablet)
W = weight of the portion of content of Capsules taken (mg)
L = labeled amount of silymarin (mg/Capsule)

Acceptance criteria: 90.0%–110.0%

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩**:** Meet the requirements for *Dissolution*
  Buffer: 6.9 g/L of monobasic sodium phosphate and 1.52 g/L of sodium hydroxide in water. Adjust to a pH of 7.5.
  Medium: *Buffer* containing 2% lauryl sulfate; 900 mL
  Apparatus 2: 100 rpm
  Time: 45 min
  Determine the amount of silymarin as silybin ($C_{25}H_{20}O_{10}$) dissolved by using the method in the test for *Content of Silymarin*, making any necessary modifications.
  Tolerances: NLT 75% of the labeled amount of silymarin as silybin ($C_{25}H_{22}O_{10}$) is dissolved.
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩**:** Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total bacterial count does not exceed 10^4 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the enterobacterial count does not exceed 10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** The label states the Latin binomial and, following the official name, the article from which the Capsules were prepared. The label also indicates the content of silymarin, in mg/Capsule.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Powdered Milk Thistle Extract RS
  USP Silybin RS
  USP Silydianin RS

Milk Thistle Tablets

DEFINITION

Milk Thistle Tablets are prepared from Powdered Milk Thistle Extract. They contain NLT 90.0% and NMT 110.0% of the labeled amount of silymarin as silybin ($C_{25}H_{22}O_{10}$), calculated as the sum of silydianin, silychristin, silybin A, silybin B, isosilybin A, and isosilybin B.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**
  Standard solution: 1.0 mg/mL of USP Silydianin RS in methanol
  Sample solution: Equivalent to 5 mg/mL of silymarin from finely powdered Tablets (NLT 20) in methanol. Shake for 1 min, and sonicate for 10 min. Allow to stand for 15 min before use.
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
  Mode: TLC
  Adsorbent: 0.25-mm layer of chormatographic silica gel, typically 20 cm long
  Application volume: 10 µL
  Developing solvent system: Freshly prepared mixture of chloroform, acetone, and anhydrous formic acid (75: 16.5: 8.5)
  Spray reagent A: 10-mg/mL solution of 2-aminoethyl diphenylborinate in methanol
  Spray reagent B: 50-mg/mL solution of polyethylene glycol 4000 in alcohol
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Develop the chromatograms until the solvent front has moved about three-fourths of the plate, and dry it for 30 min in a current of cold air. Spray the plate with *Spray reagent A*, allow to dry, and then spray with *Spray reagent B*. One h later, examine the plate under long-wavelength UV light.
  Acceptance criteria: The chromatogram of the *Sample solution* exhibits an intense green-blue fluorescent zone at an R_F value of 0.5 (presence of silybin) and exhibits a gray-blue spot at R_F value of 0.4, corresponding to a spot observed in the chromatogram of the *Standard solution*. The chromatogram of the *Sample solution* may exhibit other colored zones: an intense green-blue zone at an R_F value of 0.25 (presence of silychristin) and a red-orange zone at an R_F value of 0.3 (presence of taxifolin).
- **B. HPLC IDENTIFICATION TEST**
  Analysis: Proceed as directed for *Content of Silymarin*.
  Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks for silydianin, silychristin, silybin A, silybin B, isosilybin A, and isosilybin B at retention times that correspond to those of the *Milk thistle standard solution*.

STRENGTH

- **CONTENT OF SILYMARIN**
  Solution A: Methanol, phosphoric acid, and water (20: 0.5: 80)
  Solution B: Methanol, phosphoric acid, and water (80: 0.5: 20)
  Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	85	15
5	85	15

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Table 1 *(continued)*

Time (min)	Solution A (%)	Solution B (%)
20	55	45
40	55	45
41	85	15
55	85	15

Milk thistle standard solution: 0.7 mg/mL of USP Powdered Milk Thistle Extract RS in methanol. Sonicate for 20 min to dissolve.

Silybin standard solutions: 0.20, 0.02, and 0.004 mg/mL of USP Silybin RS in methanol. Pass through a membrane filter having a 0.45-µm or finer pore size.

Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer an accurately weighed quantity of the powder equivalent to 100 mg of silymarin to a 100-mL volumetric flask, add 90 mL of methanol, and sonicate for 20 min with occasional shaking. Cool to 20°, and dilute with methanol to volume. Filter through a membrane with a 0.45-µm or finer pore size.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 288 nm
Column: 4.6-mm × 15-cm; 5-µm packing L1
Column temperature: 40°
Flow rate: 1 mL/min
Injection size: 10 µL

System suitability
Sample: *Milk thistle standard solution*
[NOTE—For the relative retention times, see *Table 2*.]

Table 2

Name	Relative Retention Time
Silychristin	0.68
Silydianin	0.73
Silybin A	1.00
Silybin B	1.05
Dehydrosilybin	1.09
Isosilybin A	1.15
Isosilybin B	1.19

Suitability requirements
Chromatogram similarity: The chromatogram from the *Milk thistle standard solution* is similar to the reference chromatogram provided with the lot of USP Powdered Milk Thistle Extract RS being used.
Resolution: NLT 1.0 between silybin A and silybin B
Tailing factor: 0.8–2.0
Relative standard deviation: NMT 2.0% for the sum of peak responses due to silybin A and silybin B

Analysis
Samples: *Milk thistle standard solution*, each of the *Silybin standard solutions*, and *Sample solution*
Identify the peaks due to silychristin, silydianin, silybin A, silybin B, isosilybin A, and isosilybin B by comparison of the chromatogram of the *Milk thistle standard solution* with the reference chromatogram, and measure the peak areas of the relevant peaks. Plot the areas of the sum of silybin A and silybin B peaks versus the concentration of USP Silybin RS in the *Silybin standard solutions*, and obtain a regression line for calibration.
Separately calculate the content, in mg, of each relevant component of silymarin as silybin ($C_{25}H_{22}O_{10}$) in the portion of Tablets taken:

$$\text{Result} = C \times V$$

C = concentration of the relevant component in the *Sample solution* as interpolated in the calibration graph (mg/mL)
V = volume of the *Sample solution* (mL)

Calculate the content of silymarin, as a percentage, in the portion of Tablets taken:

$$\text{Result} = C_S \times (A_{WT}/W) \times (100/L)$$

C_S = sum of the contents of each relevant component of silymarin in the portion of Tablets taken (mg)
A_{WT} = average weight of the Tablets (mg/Tablet)
W = weight of the portion of Tablets taken (mg)
L = labeled amount of silymarin (mg/Tablet)

Acceptance criteria: 90.0%–110.0%

PERFORMANCE TESTS
• **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩**:** Meet the requirements for *Dissolution*
Buffer: 6.9 g/L of monobasic sodium phosphate and 1.52 g/L of sodium hydroxide in water. Adjust to a pH of 7.5.
Medium: *Buffer* containing 2% lauryl sulfate; 900 mL
Apparatus 2: 100 rpm
Time: 45 min
Determine the amount of silymarin as silybin ($C_{25}H_{20}O_{10}$) dissolved by using the method in the test for *Content of Silymarin*, making any necessary modifications.
Tolerances: NLT 75% of the labeled amount of silymarin as silybin ($C_{25}H_{22}O_{10}$) is dissolved.
• **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩**:** Meet the requirements

CONTAMINANTS
• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total bacterial count does not exceed 10^4 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the enterobacterial count does not exceed 10^2 cfu/g.
• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
• **LABELING:** The label states the Latin binomial and, following the official name, the article from which the Tablets were prepared. The label also indicates the content of silymarin, in mg/Tablet.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Powdered Milk Thistle Extract RS
USP Silybin RS
USP Silydianin RS

Minerals Capsules

DEFINITION
Minerals Capsules contain two or more minerals derived from substances generally recognized as safe, furnishing two or

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more of the following elements in ionizable form: boron, calcium, chromium, copper, fluorine, iodine, iron, magnesium, manganese, molybdenum, nickel, phosphorus, potassium, selenium, tin, vanadium, and zinc. Capsules contain NLT 90.0% and NMT 125.0% of the labeled amounts of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), potassium (K), and zinc (Zn); and NLT 90.0% and NMT 160.0% of the labeled amounts of boron (B), chromium (Cr), fluorine (F), iodine (I), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), and vanadium (V). They contain no vitamins. They may contain other labeled added substances in amounts that are unobjectionable.

STRENGTH

[NOTE—In the following assays, where more than one assay method is given for an individual ingredient, the requirements may be met by following any one of the specified methods, the method used being stated in the labeling only if *Method 1* is not used. Commercially available atomic absorption standard solutions for the minerals, where applicable, may be used where preparation of a *Standard stock solution* is described in the following assays. Use deionized water where water is specified. Where atomic absorption spectrophotometry is specified in the assay, the concentrations of the *Standard solutions* and the *Sample solutions* may be modified to fit the linear or working range of the instrument.]

• **CALCIUM, *Method 1***

Lanthanum chloride solution: 267 mg/mL of lanthanum chloride heptahydrate in 0.125 N hydrochloric acid

Calcium standard solution: 400 µg/mL of calcium. Dissolve 1.001 g of calcium carbonate, previously dried at 300° for 3 h and cooled in a desiccator for 2 h. Dissolve in 25 mL of 1 N hydrochloric acid. Boil to expel carbon dioxide, and dilute with water to 1000 mL.

Standard stock solution: 100 µg/mL of calcium from the *Calcium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Into separate 100-mL volumetric flasks pipet 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, and dilute with water to volume to obtain concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 µg/mL of calcium.

Polysorbate 80 solution: Dilute polysorbate 80 with alcohol (1 in 10)

Sample solution: Transfer 5 Capsules to a 100-mL volumetric flask. [NOTE—For hard gelatin Capsules, weigh NLT 20 Capsules. Open the Capsules, without loss of shell material, and transfer the contents to a suitable container. Remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and allow the Capsule shells to dry. Weigh the empty Capsule shells, calculate the net weight of the Capsule contents, and transfer a portion of the Capsule contents, equivalent to 5 Capsules, to a 100-mL volumetric flask.] Add 15 mL of water, 10 mL of 6 N hydrochloric acid, and 1 mL of *Polysorbate 80 solution* to the flask. Heat on a hot plate or steam bath, with intermittent swirling, until the Capsules are completely disintegrated or the contents are dissolved. Boil gently for an additional 15 min. Cool, dilute with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute this solution with 0.125 N hydrochloric acid to obtain a concentration of 2 µg/mL of calcium, adding 1 mL of *Lanthanum chloride solution* per 100 mL of the final volume.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Lamp: Calcium hollow-cathode

Flame: Nitrous oxide–acetylene

Analytical wavelength: Calcium emission line at 422.7 nm

Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution* per 100 mL

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of calcium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of calcium in the *Sample solution*.

Calculate the percentage of the labeled amount of calcium (Ca) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of calcium in the *Sample solution* (µg/mL)

C_U = nominal concentration of calcium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of calcium (Ca)

• **CHROMIUM, *Method 1***

Chromium standard solution: 1000 µg/mL of chromium from potassium dichromate, previously dried at 120° for 4 h, in water. Store in a polyethylene bottle.

Standard stock solution: 10 µg/mL of chromium from the *Chromium standard solution* diluted with 6 N hydrochloric acid and water (1 in 20)

Standard solutions: Transfer 10.0 and 20.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks, and transfer 15.0 and 20.0 mL of the *Standard stock solution* to separate 50-mL volumetric flasks. Dilute the contents of each of the four flasks with 0.125 N hydrochloric acid to volume to obtain concentrations of 1.0, 2.0, 3.0, and 4.0 µg/mL of chromium.

Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain 1 µg/mL of chromium and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Lamp: Chromium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Chromium emission line at 357.9 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of chromium, and draw the straight line best fitting the four plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of chromium in the *Sample solution*.

Calculate the percentage of the labeled amount of chromium (Cr) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of chromium in the *Sample solution* (µg/mL)

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C_U = nominal concentration of chromium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of chromium (Cr)

• **COPPER, *Method 1***

Copper standard solution: Dissolve 1.00 g of copper foil in a minimum volume of a 50% (v/v) solution of nitric acid, and dilute with a 1% (v/v) solution of nitric acid to 1000 mL. This solution contains 1000 µg/mL of copper.

Standard stock solution: 100 µg/mL of copper from the *Copper standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 200-mL volumetric flasks transfer 1.0, 2.0, 4.0, 6.0, and 8.0 mL of the *Standard stock solution*. Dilute with water to volume to obtain concentrations of 0.5, 1.0, 2.0, 3.0, and 4.0 µg/mL of copper.

Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain 2 µg/mL of copper and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Lamp: Copper hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Copper emission line at 324.7 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of copper, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of copper in the *Sample solution*.

Calculate the percentage of the labeled amount of copper (Cu) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of copper in the *Sample solution* (µg/mL)

C_U = nominal concentration of copper in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of copper (Cu)

• **FLUORIDE, *Method 1***

[NOTE—Store all solutions in plastic containers.]

3 M sodium acetate solution: Dissolve 408 g of sodium acetate in 600 mL of water in a 1000-mL volumetric flask. Allow the solution to equilibrate to room temperature, and dilute with water to volume. Adjust with a few drops of acetic acid to a pH of 7.0.

Sodium citrate solution: Dissolve 222 g of sodium citrate in 250 mL of water in a 1000-mL volumetric flask. Add 28 mL of perchloric acid, and dilute with water to volume.

Fluoride standard stock solution: 500 µg/mL of fluoride from a quantity of sodium fluoride, previously dried at 100° for 4 h and cooled in a desiccator, in water

Intermediate stock solution A: 100 µg/mL of fluoride from *Fluoride standard stock solution* diluted with water

Intermediate stock solution B: 10 µg/mL of fluoride from *Fluoride standard stock solution* diluted with water

Standard solutions: To five separate 100-mL volumetric flasks transfer 3.0, 5.0, and 10.0 mL of *Intermediate stock solution B* and 5.0 and 10.0 mL of *Intermediate stock solution A*. To each flask add 10.0 mL of 1 N hydrochloric acid, 25 mL of *3 M sodium acetate solution*, and 25.0 mL of *Sodium citrate solution*. Dilute the contents of each flask with water to volume to obtain concentrations of 0.3, 0.5, 1.0, 5.0, and 10.0 µg/mL of fluoride.

Sample solution: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the mixed Capsule contents, equivalent to 200 µg of fluoride, to a 100-mL volumetric flask. Add 10.0 mL of 1 N hydrochloric acid, 25.0 mL of *3 M sodium acetate solution*, and 25.0 mL of *Sodium citrate solution*. Dilute with water to volume.

Analysis

Samples: *Standard solutions* and *Sample solution*

To separate plastic beakers, each containing a plastic-coated stirring bar, transfer 50.0 mL each of the *Standard solutions* and the *Sample solution*. Measure the potentials (see *pH* ⟨791⟩), in mV, of the *Standard solutions* and the *Sample solution*, with a pH meter capable of a minimum reproducibility of ±0.2 mV and equipped with a fluoride-specific ion-indicating electrode and a calomel-reference electrode. [NOTE—When taking measurements, immerse the electrodes in the solution, stir on a magnetic stirrer with an insulated top until equilibrium is attained (1–2 min), and record the potential. Rinse and dry the electrodes between measurements, taking care to avoid damaging the crystal of the specific-ion electrode.]

Plot the logarithms of fluoride concentrations, in µg/mL, of the *Standard solutions* versus the potential, in mV. From the standard response curve so obtained and the measured potential of the *Sample solution*, determine the concentration, *C*, in µg/mL, of fluoride in the *Sample solution*.

Calculate the percentage of the labeled amount of fluorine (F) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of fluoride in the *Sample solution* (µg/mL)

C_U = nominal concentration of fluorine in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (F)

• **FLUORIDE, *Method 2***

[NOTE—Use plastic containers and deionized water throughout this procedure.]

pH 10.0 buffer: Add 214 mL of 0.1 N sodium hydroxide to 1000 mL of 0.05 M sodium bicarbonate.

Mobile phase: Alcohol, 0.1 N sulfuric acid, and water (20:5:175)

Standard stock solution: 220 µg/mL of USP Sodium Fluoride RS in water. This solution contains 100 µg/mL of fluoride.

Standard solution

[NOTE—Condition the solid-phase extraction column specified for use in the *Standard solution* and *Sample solution* in the following manner. Using a vacuum at a pressure not exceeding 5 mm of mercury, wash the column with 1 column volume of methanol followed by 1 column volume of *pH 10.0 buffer*. Do not allow the column top to dry. If the top of the column becomes dry, recondition the column.]

Transfer 10.0 mL of the *Standard stock solution* to a 100-mL volumetric flask. Add 75 mL of water, and adjust with 0.1 N sodium hydroxide to a pH of 10.4 ± 0.1. Dilute with water to volume. Filter, discarding the first 15 mL of the filtrate. Transfer 25.0 mL of the filtrate to a 50-mL volumetric flask. Add 15.0 mL of water, and adjust with

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0.1 N sodium hydroxide to a pH of 10.0. Dilute with *pH 10.0 buffer* to volume. Elute a portion of this solution through a 3-mL solid-phase extraction column containing L1 packing that is connected through an adaptor to a second solid-phase extraction column containing sulfonylpropyl strong cation-exchange packing. Discard the first 3 mL of the eluate, and collect the rest of the eluate in a suitable flask for injection into the chromatograph.

Sample solution: Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents to a 100-mL container. If necessary, remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to a nominal amount of 1 mg of fluorine, to a 100-mL volumetric flask. Add 15 mL of water, and shake vigorously. Rinse the sides of the flask with 15 mL of water, and allow to stand for 10 min. Dilute with water to 85 mL, adjust with 1 N sodium hydroxide to a pH of 10.4 ± 0.1, and dilute with water to 100 mL. Proceed as directed in *Standard solution*, beginning with "Filter, discarding the first 15 mL of the filtrate."

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Conductivity

Columns

Guard: 4.6-mm × 3-cm; packing L17

Analytical: 7.8-mm × 30-cm; packing L17

Flow rate: 0.5 mL/min

Injection volume: 100 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of fluoride.

Calculate the percentage of the labeled amount of fluorine (F) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of fluoride in the *Standard solution* (µg/mL)

C_U = nominal concentration of fluorine in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (F)

• **IODIDE**

Bromine water: To 20 mL of bromine in a glass-stoppered bottle add 100 mL of water. Insert the stopper into the bottle, and shake. Allow to stand for 30 min, and use the supernatant.

Analysis: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the contents, equivalent to 3 mg of iodine, to a nickel crucible. Add 5 g of sodium carbonate, 5 mL of 50% (w/v) sodium hydroxide solution, and 10 mL of alcohol, taking care that the entire specimen is moistened. Heat the crucible on a steam bath to evaporate the alcohol, then dry the crucible at 100° for 30 min to prevent spattering upon subsequent heating. Transfer the crucible with its contents to a furnace heated to 500°, and heat the crucible for 15 min. [NOTE—Heating at 500° is necessary to carbonize any organic matter present; a higher temperature may be used, if necessary, to ensure complete carbonization of all organic matter.] Cool the crucible, add 25 mL of water, cover the crucible with a watchglass, and boil gently for 10 min. Filter the solution, and wash the crucible with boiling water, collecting the filtrate and washings in a beaker. Add phosphoric acid until the solution is neutral to methyl orange, then add 1 mL excess of phosphoric acid. Add excess of *Bromine water*, and boil the solution gently until colorless and then for 5 min longer. Add a few crystals of salicylic acid, and cool the solution to 20°. Add 1 mL of phosphoric acid and 0.5 g of potassium iodide, and titrate the liberated iodine with 0.005 N sodium thiosulfate VS, adding starch TS when the liberated iodine color has nearly disappeared.

Calculate the percentage of the labeled amount of iodine (I) in the portion of Capsules taken:

$$\text{Result} = V \times N_A \times F \times I_{me} \times (A_W/W) \times (100/L)$$

V = volume of sodium thiosulfate consumed (mL)

N_A = actual normality of the sodium thiosulfate solution used (meq/mL)

F = correction factor to convert mg to µg, 1000 µg/mg

I_{me} = milliequivalent of iodine (I), 21.16 mg/mEq

A_W = average weight of the Capsules content

W = weight of the sample of Capsules content taken

L = labeled amount of iodine (µg/Capsule)

Acceptance criteria: 90.0%–160.0% of the labeled amount of iodine (I)

• **IRON, *Method 1***

Iron standard stock solution: Transfer 100 mg of iron powder to a 1000-mL volumetric flask. Dissolve in 25 mL of 6 N hydrochloric acid, dilute with water to volume, and mix.

Standard solutions: To separate 100-mL volumetric flasks transfer 2.0, 4.0, 5.0, 6.0, and 8.0 mL of *Iron standard stock solution*. Dilute the contents of each flask with water to volume to obtain concentrations of 2.0, 4.0, 5.0, 6.0, and 8.0 µg/mL of iron.

Polysorbate 80 solution: Prepare as directed in *Calcium, Method 1*.

Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain a nominal concentration of 5 µg/mL of iron and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Lamp: Iron hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Iron emission line at 248.3 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of iron, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of iron in the *Sample solution*.

Calculate the percentage of the labeled amount of iron (Fe) in the portion of Capsules taken:

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$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of iron in the *Sample solution* (µg/mL)
C_U = nominal concentration of iron in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of iron (Fe)

• **MAGNESIUM, *Method 1***

Lanthanum chloride solution: Prepare as directed in *Calcium, Method 1*.

Magnesium standard solution: Transfer 1.0 g of magnesium ribbon to a 1000-mL volumetric flask, dissolve in 50 mL of 6 N hydrochloric acid, dilute with water to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of magnesium.

Standard stock solution: 20 µg/mL of magnesium from *Magnesium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, and dilute with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.2, 0.3, 0.4, 0.5, and 0.6 µg/mL of magnesium.

Polysorbate 80 solution: Prepare as directed in *Calcium, Method 1*.

Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain 0.4 µg/mL of magnesium.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Lamp: Magnesium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Magnesium emission line at 285.2 nm

Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution* per 100 mL

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of magnesium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of magnesium in the *Sample solution*.

Calculate the percentage of the labeled amount of magnesium (Mg) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of magnesium in the *Sample solution* (µg/mL)
C_U = nominal concentration of magnesium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of magnesium (Mg)

• **MANGANESE, *Method 1***

Manganese standard stock solution: Transfer 1.00 g of manganese, weighed, to a 1000-mL volumetric flask. Dissolve in 20 mL of nitric acid, dilute with 6 N hydrochloric acid to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of manganese.

Standard stock solution: 50 µg/mL of manganese from *Manganese standard stock solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Standard stock solution*. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions with concentrations of 0.5, 0.75, 1.0, 1.5, and 2.0 µg/mL of manganese.

Polysorbate 80 solution: Prepare as directed in *Calcium, Method 1*.

Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain 1 µg/mL of manganese and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Lamp: Manganese hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Manganese emission line at 279.5 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of manganese, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of manganese in the *Sample solution*.

Calculate the percentage of the labeled amount of manganese (Mn) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of manganese in the *Sample solution* (µg/mL)
C_U = nominal concentration of manganese in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of manganese (Mn)

• **MOLYBDENUM, *Method 1***

Diluent: 20 mg/mL of ammonium chloride in water

Molybdenum standard solution: Transfer 1.0 g of molybdenum wire to a 1000-mL volumetric flask, and dissolve in 50 mL of nitric acid, warming if necessary. Dilute with water to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of molybdenum.

Standard stock solution: 100 µg/mL of molybdenum from *Molybdenum standard solution* diluted with water

Standard solutions: To separate 100-mL volumetric flasks transfer 2.0, 10.0, and 25.0 mL of the *Standard stock solution*, and add 5.0 mL of perchloric acid to each flask. Gently boil the solution in each flask for 15 min. Cool to room temperature, and dilute each with *Diluent* to volume to obtain concentrations of 5.0, 10.0, and 25.0 µg/mL of molybdenum.

Polysorbate 80 solution: Prepare as directed in *Calcium, Method 1*.

Sample solution: Proceed as directed in *Calcium, Method 1*, except take a number of Capsules or a portion of Capsule contents, equivalent to a nominal amount of 1000 µg of molybdenum, and make appropriate dilutions to obtain a final concentration of 10 µg/mL of molybdenum, omitting the addition of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Lamp: Molybdenum hollow-cathode

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Flame: Nitrous oxide–acetylene
Analytical wavelength: Molybdenum emission line at 313.3 nm
Blank: *Diluent* and perchloric acid (20:1)

Analysis
Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of molybdenum, and draw the straight line best fitting the three plotted points. From the graph so obtained, determine the concentration, C, in µg/mL, of molybdenum in the *Sample solution*.
Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of molybdenum in the *Sample solution* (µg/mL)
C_U = nominal concentration of molybdenum in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

• **MOLYBDENUM, *Method 2***
Sodium fluoride solution: Add 200 mL of water to 10 g of sodium fluoride, stir until the solution is saturated, and filter. Store in a polyethylene bottle.
Ferrous sulfate solution: 4.98 mg/mL of ferrous sulfate in water
Potassium thiocyanate solution: 200 mg/mL of potassium thiocyanate in water
20% Stannous chloride solution: Transfer 40 g of stannous chloride to a beaker, add 20 mL of 6.5 N hydrochloric acid, and heat the solution until the stannous chloride is dissolved. Cool, and dilute with water to 100 mL.
Diluted stannous chloride solution: *20% Stannous chloride solution* diluted with water (1 in 25). Prepare this solution fresh at the time of use.
Standard solution: 20 µg/mL of molybdenum from ammonium molybdate, in water
Sample: Remove the contents of a counted number of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Use a quantity of Capsule contents, equivalent to a nominal amount of 40 µg of molybdenum.
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: UV-Vis
Cell: 1 cm
Analytical wavelength: 465 nm
Blank: Amyl alcohol

Analysis
Samples: *Standard solution* and *Sample*
Transfer the *Sample* and 2.0 mL of the *Standard solution* to separate 200-mL beakers. Add 20 mL of nitric acid to each beaker. Cover each beaker with a watchglass, and boil slowly on a hot plate for 45 min. Cool to room temperature. Add 6 mL of perchloric acid, cover the beakers with a watchglass, and continue the heating until digestion is complete, as indicated when the liquid becomes colorless or pale yellow. Evaporate the solutions in the beakers to dryness. Rinse the sides of the beakers and the watchglasses with water, and add more water to each beaker to complete 50 mL in each beaker. Gently boil the water solutions for a few min. Cool to room temperature. Add 2 drops of methyl orange TS, and neutralize with ammonium hydroxide. Add 8.2 mL of hydrochloric acid. Quantitatively transfer the contents of the beakers to separate 100-mL volumetric flasks, rinse the beakers with water, transfer the rinsings to the corresponding volumetric flasks, and dilute with water to volume. Transfer 50.0 mL of each solution to separatory funnels. To each separatory funnel add 1.0 mL of *Sodium fluoride solution*, 0.5 mL of *Ferrous sulfate solution*, 4.0 mL of *Potassium thiocyanate solution*, 1.5 mL of *20% Stannous chloride solution*, and 15.0 mL of amyl alcohol, and shake the separatory funnel for 1 min. Allow the layers to separate, and discard the aqueous layers. Add 25 mL of *Diluted stannous chloride solution* to each separatory funnel, and shake gently for 15 s. Allow the layers to separate, and discard the aqueous layers. Transfer the organic layers from each separatory funnel to a centrifuge tube, and centrifuge at 2000 rpm for 10 min. Determine the absorbances of the organic phases from the *Standard solution* and the *Sample*, and correct with the *Blank*.
Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Capsules taken:

$$\text{Result} = (A_U/A_S) \times [(V \times C_S)/C_U] \times 100$$

A_U = absorbance of the solution from the *Sample*
A_S = absorbance of the solution from the *Standard solution*
V = volume of the *Standard solution* analyzed, 2.0 mL
C_S = concentration of molybdenum in the *Standard solution* (µg/mL)
C_U = nominal concentration of molybdenum in the *Sample* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

• **PHOSPHORUS, *Method 1***
Sulfuric acid solution: Cautiously add sulfuric acid to water (37.5: 100), and mix.
Ammonium molybdate solution: 50 mg/mL of ammonium molybdate in *Sulfuric acid solution* and water (2:3). [NOTE—Dissolve in water first, then dilute with *Sulfuric acid solution* to volume.]
Hydroquinone solution: 5 mg/mL of hydroquinone in water. Add 1 drop of sulfuric acid per 100 mL of solution.
Sodium bisulfite solution: 200 mg/mL of sodium bisulfite in water
Phosphorus standard stock solution: Weigh 4.395 g of monobasic potassium phosphate, previously dried at 105° for 2 h and stored in a desiccator, and transfer to a 1000-mL volumetric flask. Dissolve in water, add 6 mL of sulfuric acid as a preservative, dilute with water to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of phosphorus.
Standard solution: 20 µg/mL of phosphorus from *Phosphorus standard stock solution* diluted with water
Sample solution: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the Capsule contents, equivalent to a nominal amount of 100 mg of phosphorus, to 25 mL of nitric acid, and digest on a hot plate for 30 min. Add 15 mL of hydrochloric acid, and continue the digestion to the cessation of brown fumes. Cool, and transfer the contents of the flask to a 500-mL volumetric flask with the aid of small portions of water. Dilute with water to volume. Transfer 10.0 mL of this solution to a 100-mL volumetric flask, and dilute with water to volume.
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: UV-Vis
Cell: 1 cm

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Analytical wavelength: 650 nm

Analysis

Samples: *Standard solution* and *Sample solution*

To three separate 25-mL volumetric flasks transfer 5.0 mL each of the *Standard solution*, the *Sample solution*, and water to provide the blank. To each of the three flasks add 1.0 mL each of *Ammonium molybdate solution*, *Hydroquinone solution*, and *Sodium bisulfite solution*, and swirl to mix. Dilute the contents of each flask with water to volume, and allow the flasks to stand for 30 min. Determine the absorbances of the solutions, against the blank.

Calculate the percentage of the labeled amount of phosphorus (P) in the portion of Capsules taken:

$$\text{Result} = (A_U/A_S) \times (C_S/C_U) \times 100$$

A_U = absorbance of the *Sample solution*
A_S = absorbance of the *Standard solution*
C_S = concentration of phosphorus in the *Standard solution* (µg/mL)
C_U = nominal concentration of phosphorus in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of phosphorus (P)

• **POTASSIUM**

Potassium standard solution: 100 µg/mL of potassium from potassium chloride, previously dried at 105° for 2 h, in water

Standard stock solution: 10 µg/mL of potassium from *Potassium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Transfer 5.0, 10.0, 15.0, 20.0, and 25.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions containing 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of potassium.

Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain 1 µg/mL of potassium and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Lamp: Potassium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Potassium emission line at 766.5 nm

Blank: Water

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of potassium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of potassium in the *Sample solution*.

Calculate the percentage of the labeled amount of potassium (K) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of potassium in the *Sample solution* (µg/mL)
C_U = nominal concentration of potassium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of potassium (K)

• **SELENIUM, *Method 1***

Diluent: Prepare as directed in *Molybdenum, Method 1*.

Selenium standard solution: [**CAUTION**—Selenium is toxic; handle it with care.] Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness. Add 2 mL of water, and evaporate to dryness. Repeat the addition of water and the evaporation to dryness three times. Dissolve the residue in 3 N hydrochloric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume, to obtain a concentration of 1000 µg/mL of selenium.

Standard stock solution: 100 µg/mL of selenium from *Selenium standard solution* diluted with water

Standard solutions: To separate 100-mL volumetric flasks transfer 5.0, 10.0, and 25.0 mL of the *Standard stock solution*, and add 5.0 mL of perchloric acid to each flask. Gently boil the solutions for 15 min, cool to room temperature, and dilute each with *Diluent* to volume to obtain solutions containing 5.0, 10.0, and 25.0 µg/mL of selenium.

Sample solution: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the Capsule content, equivalent to a nominal amount of 1000 µg of selenium, to a suitable flask, and add 12 mL of nitric acid. [NOTE—The volume of nitric acid may be varied to ensure that the powder is uniformly dispersed.] Carefully swirl the flask to disperse the sample specimen. Sonicate for 10 min or until the sample specimen is completely dissolved. Gently boil the solution for 15 min, and cool to room temperature. Carefully add 8 mL of perchloric acid to the flask, heat the flask until perchloric acid fumes appear, and swirl the flask to dissipate the fumes. Repeat the heating and swirling until the fumes appear again. Cool to room temperature. Transfer the contents of the flask to a 50-mL volumetric flask with the aid of the *Diluent*, and dilute with *Diluent* to volume.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Lamp: Selenium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Selenium emission line at 196.0 nm

Blank: *Diluent* and perchloric acid (20:1)

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of selenium, and draw the straight line best fitting the three plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of selenium in the *Sample solution*.

Calculate the percentage of the labeled amount of selenium (Se) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of selenium in the *Sample solution* (µg/mL)
C_U = nominal concentration of selenium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of selenium (Se)

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• **SELENIUM, *Method 2***

Hydrochloric acid solution: Hydrochloric acid diluted with water (1 in 10)

50% Ammonium hydroxide solution: Ammonium hydroxide diluted with water (1 in 2)

Reagent A: 9 mg/mL of edetate disodium and 25 mg/mL of hydroxylamine hydrochloride in water. [NOTE—Dissolve edetate disodium in a portion of water first, add hydroxylamine hydrochloride, then dilute with water to volume.]

Reagent B: Transfer 200 mg of 2,3-diaminonaphthalene to a 250-mL separatory funnel, and add 200 mL of 0.1 N hydrochloric acid. Wash the solution with three 40-mL portions of cyclohexane, and discard the cyclohexane layer. Filter the solution into a brown bottle, and cover the solution with a 1-cm layer of cyclohexane. This solution is stable for 1 week if stored in a refrigerator.

Standard stock solution: [**CAUTION**—Selenium is toxic; handle it with care.] Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness, add 2 mL of water, and evaporate to dryness. Repeat the addition of water and evaporation to dryness three times. Dissolve the residue in 3 N hydrochloric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume to obtain a solution with a concentration of 1000 µg/mL of selenium. Dilute a volume of the solution with 0.125 N hydrochloric acid to obtain a concentration of 2.0 µg/mL of selenium.

Standard solution: Transfer 10.0 mL of the *Standard stock solution* to a glass-stoppered flask. Add 1 mL of perchloric acid and 1 mL of *Hydrochloric acid solution*, and dilute with water to 20 mL.

Sample solution: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the Capsule contents, equivalent to a nominal amount of 20 µg of selenium, to a suitable flask. Add 10 mL of nitric acid, and warm gently on a hot plate. Continue heating until the initial nitric acid reaction has subsided, then add 3 mL of perchloric acid. [**CAUTION**—Exercise care at this stage because the perchloric acid reaction becomes vigorous.] Continue heating on the hot plate until the appearance of white fumes of perchloric acid or until the digest begins to darken. Add 0.5 mL of nitric acid and resume heating, adding additional amounts of nitric acid if further darkening occurs. Digest for 10 min after the first appearance of perchloric acid fumes or until the digest becomes colorless. Cool the flask. Add 2.5 mL of *Hydrochloric acid solution*, and return the flask to the hot plate to expel residual nitric acid. Heat the mixture for 3 min after it begins to boil. Cool the flask to room temperature, and dilute with water to 20 mL.

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Mode: UV

Cell: 1 cm

Analytical wavelength: 380 nm

Blank: 1 mL of perchloric acid and 1 mL of *Hydrochloric acid solution* diluted with water to 20 mL

Analysis

Samples: *Standard solution* and *Sample solution*

Treat the *Sample solution*, *Standard solution*, and *Blank* as follows. Add 5 mL of *Reagent A* to each flask, and swirl gently to mix. Adjust the solution in each flask with *50% Ammonium hydroxide solution* to a pH of 1.1 ± 0.1. Add 5 mL of *Reagent B* to each flask, and swirl gently to mix. Place the flasks in a water bath maintained at 50°, and equilibrate for 30 min, taking care that the flasks are covered to protect them from light. Cool to room temperature, and transfer the contents of each flask to separate separatory funnels. Transfer 10.0 mL of cyclohexane to each separatory funnel, and extract vigorously for 1 min. Discard the aqueous layer. Transfer the cyclohexane layer to a centrifuge tube, and centrifuge at 1000 rpm for 1 min to remove any remaining water. Determine the absorbances of the solutions from the *Samples* against the solution from the *Blank*.

Calculate the percentage of the labeled amount of selenium (Se) in the portion of Capsules taken:

$$\text{Result} = (A_U/A_S) \times [(V \times C_S)/C_U] \times 100$$

A_U = absorbances of the cyclohexane layer from the *Sample solution*

A_S = absorbances of the cyclohexane layer from the *Standard solution*

V = volume of the *Standard stock solution* used to prepare the *Standard solution*, 10 mL

C_S = concentration of selenium in the *Standard stock solution* (µg/mL)

C_U = nominal amount of selenium in the *Sample solution* (µg)

Acceptance criteria: 90.0%–160.0% of the labeled amount of selenium (Se)

• **ZINC, *Method 1***

Zinc standard solution: 1000 µg/mL of zinc from zinc oxide dissolved in 5 M hydrochloric acid (3.89 mg/mL), and diluted with water to final volume. [NOTE—Dissolve in 5 M hydrochloric acid by warming, if necessary, cool, and then dilute to final volume.]

Standard stock solution: 50 µg/mL of zinc from *Zinc standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Transfer 1.0, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of zinc.

Polysorbate 80 solution: Prepare as directed in *Calcium, Method 1*.

Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain a nominal concentration of 2 µg/mL of zinc and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Lamp: Zinc hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Zinc emission line at 213.8 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of zinc, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of zinc in the *Sample solution*.

Calculate the percentage of the labeled amount of zinc (Zn) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of zinc in the *Sample solution* (µg/mL)

C_U = nominal concentration of zinc in the *Sample solution* (µg/mL)

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Acceptance criteria: 90.0%–125.0% of the labeled amount of zinc (Zn)

- **BORON, NICKEL, TIN,** and **VANADIUM, *Method 1*; CALCIUM, CHROMIUM, COPPER, IRON, MAGNESIUM, MANGANESE, PHOSPHORUS,** and **ZINC, *Method 2*; MOLYBDENUM** and **SELENIUM, *Method 3***

Stock aqua regia solution: Prepare a mixture of hydrochloric acid and nitric acid (3:1) by adding the nitric acid to the hydrochloric acid. [NOTE—Periodically vent the solution in an appropriate fume hood.]

Diluent: Prepare a mixture of *Stock aqua regia solution* and water (1:9) by adding 1 volume of *Stock aqua regia solution* to 2 volumes of water. Dilute with additional water to volume, and mix well.

System suitability solution: Prepare a mixture of 1000 mg/L of yttrium in 5% (v/v) nitric acid solution and 1000 mg/L of scandium in 5% (v/v) nitric acid solution with *Diluent* (1:1:198), and mix.

Standard stock solution 1 (Ca, Cu, Fe, Mg, Mn, P, and Zn) : [NOTE—It is only necessary to include the minerals of interest in the solution.] Using commercially available element standard (single- or multi-element) solutions in 5% (v/v) nitric acid solution, pipet the appropriate amount of element standard solution into a volumetric flask, and dilute with 5% (v/v) nitric acid solution to obtain a solution with final concentrations of about 1000 mg/L of calcium, 100 mg/L of copper, 250 mg/L of iron, 500 mg/L of magnesium, 100 mg/L of manganese, 800 mg/L of phosphorus, and 250 mg/L of zinc.

Standard stock solution 2 (B, Cr, Mo, Ni, Se, Sn, and V): [NOTE—It is only necessary to include the minerals of interest in the solution.] Using commercially available element standard (single- or multi-element) solutions in 20% (v/v) hydrochloric acid solution, pipet the appropriate amount of element standard solution into a volumetric flask, and dilute with 20% (v/v) hydrochloric acid solution to obtain a solution with final concentrations of about 200 mg/L of boron, and 100 mg/L each of chromium, molybdenum, nickel, selenium, tin, and vanadium.

Standard solutions: Prepare a mixture of *Standard stock solution 1* and *Standard stock solution 2*, as required, in *Diluent* to prepare a six-point calibration curve to bracket the concentration range of each mineral of interest.

Sample solution: Weigh, then transfer 5 Capsules to a 250-mL volumetric flask, and heat gently on a hot plate until the contents begin to release. Cautiously add 25 mL of *Stock aqua regia solution* in 5-mL increments, and swirl. Heat, continue to swirl until the Capsules dissolve into the acid, immediately remove from the heat source, and add 150 mL of water. Cool, and dilute with water to volume. Filter about 30 mL into a centrifuge tube using a nylon syringe filter of 5-µm pore size. If necessary, make any further adjustments using the *Diluent*.

Instrumental conditions

(See *Plasma Spectrochemistry* ⟨730⟩.)

Mode: Inductively coupled plasma spectrometry using a spectrometer set to measure the emission of each mineral of interest at about the corresponding wavelength. [NOTE—The operating conditions may be developed and optimized based on the manufacturer's recommendation. The wavelengths selected should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision.]

System suitability

[NOTE—Analyze the *System suitability solution*, and obtain the response as directed in *Analysis*.]

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the emission of each mineral of interest in the *Standard solutions* and *Sample solution* with an inductively coupled plasma system using the *Diluent* as the blank. Plot the emission of the *Standard solutions* versus the concentration, in mg/L, of the minerals of interest, and draw the straight line best fitting the plotted points. From the graph so obtained, determine the concentration, *C*, in mg/L, for each mineral of interest in the *Sample solution*.

Calculate the percentage of the labeled amount for each mineral taken:

$$\text{Result} = C \times (V/W) \times F \times (T_W/L) \times 100$$

C	= measured concentration of the relevant element in the *Sample solution* (mg/L)
V	= volume of the *Sample solution* (L)
W	= sample weight (mg)
F	= dilution factor of the *Sample solution*
T_W	= average Capsule weight (mg)
L	= labeled amount of the relevant element (mg/Capsule)

Acceptance criteria: 90%–125% of the labeled amount of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), and zinc (Zn); 90%–160% of the labeled amount of boron (B), chromium (Cr), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), and vanadium (V)

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** ⟨2040⟩**:** Meet the requirements for *Dissolution*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩**:** Meet the requirements

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 3×10^3 cfu/g, and the combined molds and yeasts count does not exceed 3×10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meet the requirements of the tests for absence of *Salmonella* species and for absence of *Escherichia coli*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** The label states that the product is Minerals Capsules. The label also states the salt form of the mineral used as the source of each element. Where more than one assay method is given for a particular mineral, the labeling states the assay method used only if *Method 1* is not used.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Sodium Fluoride RS

Minerals Tablets

DEFINITION

Minerals Tablets contain two or more minerals derived from substances generally recognized as safe, furnishing two or more of the following elements in ionizable form: boron, calcium, chromium, copper, fluorine, iodine, iron, magnesium, manganese, molybdenum, nickel, phosphorus, potassium, selenium, tin, vanadium, and zinc. Tablets contain NLT 90.0% and NMT 125.0% of the labeled

Supplement 2

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amounts of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), potassium (K), and zinc (Zn); and NLT 90.0% and NMT 160.0% of the labeled amounts of boron (B), chromium (Cr), fluorine (F), iodine (I), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), and vanadium (V). They contain no vitamins. They may contain other labeled added substances in amounts that are unobjectionable.

STRENGTH

[NOTE—In the following assays, where more than one assay method is given for an individual ingredient, the requirements may be met by following any one of the specified methods, the method used being stated in the labeling only if *Method 1* is not used. Commercially available atomic absorption standard solutions for the minerals, where applicable, may be used where preparation of a *Standard stock solution* is described in the following assays. Use deionized water where water is specified. Where atomic absorption spectrophotometry is specified in the assay, the concentrations of the *Standard solutions* and the *Sample solution* may be modified to fit the linear or working range of the instrument.]

• **CALCIUM, *Method 1***

Lanthanum chloride solution: 267 mg/mL of lanthanum chloride heptahydrate in 0.125 N hydrochloric acid

Calcium standard solution: 400 µg/mL of calcium. Dissolve 1.001 g of calcium carbonate, previously dried at 300° for 3 h and cooled in a desiccator for 2 h. Dissolve in 25 mL of 1 N hydrochloric acid. Boil to expel carbon dioxide, and dilute with water to 1000 mL.

Standard stock solution: 100 µg/mL of calcium from the *Calcium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Into separate 100-mL volumetric flasks pipet 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, and dilute with water to volume to obtain concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 µg/mL of calcium.

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 5 Tablets, to a porcelain crucible. Heat the crucible in a muffle furnace maintained at 550° for 6–12 h, and cool. Add 60 mL of hydrochloric acid, and boil gently on a hot plate or steam bath for 30 min, intermittently rinsing the inner surface of the crucible with 6 N hydrochloric acid. Cool, and quantitatively transfer the contents of the crucible to a 100-mL volumetric flask. Rinse the crucible with small portions of 6 N hydrochloric acid, and add the rinsings to the flask. Dilute with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute this solution quantitatively with 0.125 N hydrochloric acid to obtain a nominal concentration of 2 µg/mL of calcium, adding 1 mL of *Lanthanum chloride solution* per 100 mL of the final volume.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Lamp: Calcium hollow-cathode

Flame: Nitrous oxide–acetylene

Analytical wavelength: Calcium emission line at 422.7 nm

Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution* per 100 mL

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of calcium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of calcium in the *Sample solution*.

Calculate the percentage of the labeled amount of calcium (Ca) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of calcium in the *Sample solution* (µg/mL)

C_U = nominal concentration of calcium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of calcium (Ca)

• **CHROMIUM, *Method 1***

Chromium standard solution: 1000 µg/mL of chromium from potassium dichromate, previously dried at 120° for 4 h, in water. Store in a polyethylene bottle.

Standard stock solution: 10 µg/mL of chromium from the *Chromium standard solution* diluted with 6 N hydrochloric acid and water (1 in 20)

Standard solutions: Transfer 10.0 and 20.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks, and transfer 15.0 and 20.0 mL of the *Standard stock solution* to separate 50-mL volumetric flasks. Dilute the contents of each of the four flasks with 0.125 N hydrochloric acid to volume to obtain concentrations of 1.0, 2.0, 3.0, and 4.0 µg/mL of chromium.

Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain 1 µg/mL of chromium and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Lamp: Chromium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Chromium emission line at 357.9 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of chromium, and draw the straight line best fitting the four plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of chromium in the *Sample solution*.

Calculate the percentage of the labeled amount of chromium (Cr) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of chromium in the *Sample solution* (µg/mL)

C_U = nominal concentration of chromium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of chromium (Cr)

• **COPPER, *Method 1***

Copper standard solution: Dissolve 1.00 g of copper foil in a minimum volume of a 50% (v/v) solution of nitric acid, and dilute with a 1% (v/v) solution of nitric acid to 1000 mL. This solution contains 1000 µg/mL of copper.

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Standard stock solution: 100 µg/mL of copper from the *Copper standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 200-mL volumetric flasks transfer 1.0, 2.0, 4.0, 6.0, and 8.0 mL of the *Standard stock solution*. Dilute with water to volume to obtain concentrations of 0.5, 1.0, 2.0, 3.0, and 4.0 µg/mL of copper.

Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain 2 µg/mL of copper and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Lamp: Copper hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Copper emission line at 324.7 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of copper, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of copper in the *Sample solution*.

Calculate the percentage of the labeled amount of copper (Cu) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of copper in the *Sample solution* (µg/mL)

C_U = nominal concentration of copper in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of copper (Cu)

• **FLUORIDE, *Method 1***

[NOTE—Store all solutions in plastic containers.]

3 M sodium acetate solution: Dissolve 408 g of sodium acetate in 600 mL of water in a 1000-mL volumetric flask. Allow the solution to equilibrate to room temperature, and dilute with water to volume. Adjust with a few drops of acetic acid to a pH of 7.0.

Sodium citrate solution: Dissolve 222 g of sodium citrate in 250 mL of water in a 1000-mL volumetric flask. Add 28 mL of perchloric acid, and dilute with water to volume.

Fluoride standard stock solution: 500 µg/mL of fluoride from a quantity of sodium fluoride, previously dried at 100° for 4 h and cooled in a desiccator, in water

Intermediate stock solution A: 100 µg/mL of fluoride from the *Fluoride standard stock solution* diluted with water

Intermediate stock solution B: 10 µg/mL of fluoride from the *Fluoride standard stock solution* diluted with water

Standard solutions: To five separate 100-mL volumetric flasks transfer 3.0, 5.0, and 10.0 mL of *Intermediate stock solution B* and 5.0 and 10.0 mL of *Intermediate stock solution A*. To each flask add 10.0 mL of 1 N hydrochloric acid, 25 mL of *3 M sodium acetate solution*, and 25.0 mL of *Sodium citrate solution*. Dilute the contents of each flask with water to volume to obtain concentrations of 0.3, 0.5, 1.0, 5.0, and 10.0 µg/mL of fluoride.

Sample solution: Transfer a quantity of the finely powdered Tablets, equivalent to a nominal amount of 200 µg of fluoride, to a 100-mL volumetric flask. Add 10.0 mL of 1 N hydrochloric acid, 25.0 mL of *3 M sodium acetate solution*, and 25.0 mL of *Sodium citrate solution*, and dilute with water to 100 mL.

Analysis

Samples: *Standard solutions* and *Sample solution*

To separate plastic beakers, each containing a plastic-coated stirring bar, transfer 50.0 mL each of the *Standard solutions* and the *Sample solution*. Measure the potentials (see *pH* ⟨791⟩), in mV, of the *Standard solutions* and the *Sample solution*, with a pH meter capable of a minimum reproducibility of ±0.2 mV and equipped with a fluoride-specific ion-indicating electrode and a calomel reference electrode. [NOTE—When taking measurements, immerse the electrodes in the solution, stir on a magnetic stirrer with an insulated top until equilibrium is attained (1–2 min), and record the potential. Rinse and dry the electrodes between measurements, taking care to avoid damaging the crystal of the specific-ion electrode.]

Plot the logarithms of fluoride concentrations, in µg/mL, of the *Standard solutions* versus the potential, in mV. From the standard response curve so obtained and the measured potential of the *Sample solution*, determine the concentration, *C*, in µg/mL, of fluoride in the *Sample solution*.

Calculate the percentage of the labeled amount of fluorine (F) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of fluoride in the *Sample solution* (µg/mL)

C_U = nominal concentration of fluorine in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (F)

• **FLUORIDE, *Method 2***

[NOTE—Use plastic containers and deionized water throughout this procedure.]

pH 10.0 buffer: Add 214 mL of 0.1 N sodium hydroxide to 1000 mL of 0.05 M sodium bicarbonate.

Mobile phase: Alcohol, 0.1 N sulfuric acid, and water (20:5:175)

Standard stock solution: 220 µg/mL of USP Sodium Fluoride RS in water. This solution contains 100 µg/mL of fluoride.

Standard solution

[NOTE—Condition the solid-phase extraction column specified for use in the *Standard solution* and the *Sample solution* in the following manner. Using a vacuum at a pressure not exceeding 5 mm of mercury, wash the column with 1 column volume of methanol followed by 1 column volume of *pH 10.0 buffer*. Do not allow the column top to dry. If the top of the column becomes dry, recondition the column.]

Transfer 10.0 mL of the *Standard stock solution* to a 100-mL volumetric flask. Add 75 mL of water, and adjust with 0.1 N sodium hydroxide to a pH of 10.4 ± 0.1. Dilute with water to volume. Filter, discarding the first 15 mL of the filtrate. Transfer 25.0 mL of the filtrate to a 50-mL volumetric flask. Add 15.0 mL of water, and adjust with 0.1 N sodium hydroxide to a pH of 10.0. Dilute with *pH 10.0 buffer* to volume. Elute a portion of this solution through a 3-mL solid-phase extraction column containing L1 packing that is connected through an adaptor to a second solid-phase extraction column containing sulfonylpropyl strong cation-exchange packing. Discard the first 3 mL of the eluate, and collect the rest of the eluate in a suitable flask for injection into the chromatograph.

DS Monographs

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Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of powdered Tablets, equivalent to a nominal amount of 1 mg of fluorine, to 15 mL of water, and shake vigorously. Rinse the sides of the flask with 15 mL of water, and allow to stand for 10 min. Dilute with water to 85 mL, adjust with 1 N sodium hydroxide to a pH of 10.4 ± 0.1, and dilute with water to 100 mL. Proceed as directed in *Standard solution*, beginning with "Filter, discarding the first 15 mL of the filtrate."

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Conductivity

Columns

Guard: 4.6-mm × 3-cm; packing L17

Analytical: 7.8-mm × 30-cm; packing L17

Flow rate: 0.5 mL/min

Injection volume: 100 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of fluoride. Calculate the percentage of the labeled amount of fluorine (F) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of fluoride in the *Standard solution* (µg/mL)

C_U = nominal concentration of fluorine in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (F)

• **IODIDE**

Bromine water: To 20 mL of bromine in a glass-stoppered bottle add 100 mL of water. Insert the stopper into the bottle, and shake. Allow to stand for 30 min, and use the supernatant.

Analysis

Sample: Tablets

Transfer a portion of finely powdered Tablets, equivalent to a nominal amount of 3 mg of iodide, to a nickel crucible. Add 5 g of sodium carbonate, 5 mL of 50% (w/v) sodium hydroxide solution, and 10 mL of alcohol, taking care that the entire specimen is moistened. Heat the crucible on a steam bath to evaporate the alcohol, then dry the crucible at 100° for 30 min to prevent spattering upon subsequent heating. Transfer the crucible with its contents to a furnace heated to 500°, and heat the crucible for 15 min. [NOTE—Heating at 500° is necessary to carbonize any organic matter present; a higher temperature may be used, if necessary, to ensure complete carbonization of all organic matter.] Cool the crucible, add 25 mL of water, cover the crucible with a watchglass, and boil gently for 10 min. Filter the solution, and wash the crucible with boiling water, collecting the filtrate and washings in a beaker. Add phosphoric acid until the solution is neutral to methyl orange, then add 1 mL excess of phosphoric acid. Add excess of *Bromine water*, and boil the solution gently until colorless and then for 5 min longer. Add a few crystals of salicylic acid, and cool the solution to 20°. Add 1 mL of phosphoric acid and 0.5 g of potassium iodide, and titrate the liberated iodine with 0.005 N sodium thiosulfate VS, adding starch TS when the liberated iodine color has nearly disappeared.

Calculate the percentage of the labeled amount of iodine (I) in the portion of Tablets taken:

$$\text{Result} = V \times N_A \times F \times I_{me} \times (A_W/W) \times (100/L)$$

V = volume of sodium thiosulfate consumed (mL)

N_A = actual normality of the sodium thiosulfate solution used (meq/mL)

F = correction factor to convert mg to µg, 1000 µg/mg

I_{me} = milliequivalent of I, 21.16 mg/mEq

A_W = average weight of the Tablets

W = weight of the portion of Tablets taken

L = labeled amount of iodine (µg/Tablet)

Acceptance criteria: 90.0%–160.0% of the labeled amount of iodine (I)

• **IRON, *Method 1***

Iron standard stock solution: Transfer 100 mg of iron powder to a 1000-mL volumetric flask. Dissolve in 25 mL of 6 N hydrochloric acid, dilute with water to volume, and mix.

Standard solutions: To separate 100-mL volumetric flasks transfer 2.0, 4.0, 5.0, 6.0, and 8.0 mL of *Iron standard stock solution*. Dilute the contents of each flask with water to volume to obtain concentrations of 2.0, 4.0, 5.0, 6.0, and 8.0 µg/mL of iron.

Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain a nominal concentration of 5 µg/mL of iron and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Lamp: Iron hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Iron emission line at 248.3 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of iron, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of iron in the *Sample solution*.

Calculate the percentage of the labeled amount of iron (Fe) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of iron in the *Sample solution* (µg/mL)

C_U = nominal concentration of iron in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of iron (Fe)

• **MAGNESIUM, *Method 1***

Lanthanum chloride solution: Prepare as directed in *Calcium, Method 1*.

Magnesium standard solution: Transfer 1.0 g of magnesium ribbon to a 1000-mL volumetric flask, dissolve in 50 mL of 6 N hydrochloric acid, dilute with water to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of magnesium.

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Standard stock solution: 20 µg/mL of magnesium from the *Magnesium standard solution* diluted with 0.125 N hydrochloric acid
Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 2.5, and 3.0 mL of *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, and dilute with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.2, 0.3, 0.4, 0.5, and 0.6 µg/mL of magnesium.
Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain a nominal concentration of 0.4 µg/mL of magnesium.
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Lamp: Magnesium hollow-cathode
Flame: Air–acetylene
Analytical wavelength: Magnesium emission line at 285.2 nm
Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution* per 100 mL
Analysis
Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of magnesium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of magnesium in the *Sample solution*.
Calculate the percentage of the labeled amount of magnesium (Mg) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of magnesium in the *Sample solution* (µg/mL)
C_U = nominal concentration of magnesium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of magnesium (Mg)

• **MANGANESE, *Method 1***
Manganese standard stock solution: Transfer 1.00 g of manganese, weighed, to a 1000-mL volumetric flask. Dissolve in 20 mL of nitric acid, dilute with 6 N hydrochloric acid to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of manganese.
Standard stock solution: 50 µg/mL of manganese from the *Manganese standard stock solution* diluted with 0.125 N hydrochloric acid
Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Standard stock solution*. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions with concentrations of 0.5, 0.75, 1.0, 1.5, and 2.0 µg/mL of manganese.
Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain 1 µg/mL of manganese and omit the use of the *Lanthanum chloride solution*.
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Lamp: Manganese hollow-cathode
Flame: Air–acetylene
Analytical wavelength: Manganese emission line at 279.5 nm
Blank: 0.125 N hydrochloric acid
Analysis
Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of manganese, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of manganese in the *Sample solution*.
Calculate the percentage of the labeled amount of manganese (Mn) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of manganese in the *Sample solution* (µg/mL)
C_U = nominal concentration of manganese in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of manganese (Mn)

• **MOLYBDENUM, *Method 1***
Diluent: 20 mg/mL of ammonium chloride in water
Molybdenum standard solution: Transfer 1.0 g of molybdenum wire to a 1000-mL volumetric flask, and dissolve in 50 mL of nitric acid, warming if necessary. Dilute with water to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of molybdenum.
Standard stock solution: 100 µg/mL of molybdenum from the *Molybdenum standard solution* diluted with water
Standard solutions: To separate 100-mL volumetric flasks transfer 2.0, 10.0, and 25.0 mL of the *Standard stock solution*, and add 5.0 mL of perchloric acid to each flask. Gently boil the solution in each flask for 15 min. Cool to room temperature, and dilute each with *Diluent* to volume to obtain concentrations of 5.0, 10.0, and 25.0 µg/mL of molybdenum.
Sample solution: Transfer a portion of the powder, equivalent to a nominal amount of 1000 µg of molybdenum, to a suitable flask, and add 12 mL of nitric acid. [NOTE—The volume of nitric acid may be varied to ensure that the powder is uniformly dispersed.] Carefully swirl the flask to disperse the test specimen. Sonicate for 10 min, or until the test specimen is completely dissolved. Gently boil the solution for 15 min, and cool to room temperature. Carefully add 8 mL of perchloric acid, heat until perchloric acid fumes appear, and swirl the flask to dissipate the fumes. Repeat the heating and swirling until the fumes appear again. Cool to room temperature. Quantitatively transfer the contents of the flask to a 100-mL volumetric flask with the aid of the *Diluent*, and dilute with *Diluent* to volume.
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Lamp: Molybdenum hollow-cathode
Flame: Nitrous oxide–acetylene
Analytical wavelength: Molybdenum emission line at 313.3 nm
Blank: *Diluent* and perchloric acid (20:1)
Analysis
Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of molybdenum, and draw the straight line best fitting the three plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of molybdenum in the *Sample solution*.

USP Monographs

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Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of molybdenum in the *Sample solution* (µg/mL)
C_U = nominal concentration of molybdenum in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

• **MOLYBDENUM, *Method 2***

Sodium fluoride solution: Add 200 mL of water to 10 g of sodium fluoride, stir until the solution is saturated, and filter. Store in a polyethylene bottle.

Ferrous sulfate solution: 4.98 mg/mL of ferrous sulfate in water

Potassium thiocyanate solution: 200 mg/mL of potassium thiocyanate in water

20% Stannous chloride solution: Transfer 40 g of stannous chloride to a beaker, add 20 mL of 6.5 N hydrochloric acid, and heat the solution until the stannous chloride is dissolved. Cool, and dilute with water to 100 mL.

Diluted stannous chloride solution: *20% Stannous chloride solution* diluted with water (1 in 25). Prepare this solution fresh at the time of use.

Standard solution: 20 µg/mL of molybdenum from ammonium molybdate, in water

Sample: A portion of finely powdered Tablets, equivalent to a nominal amount of 40 µg of molybdenum

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Mode: UV-Vis

Cell: 1 cm

Analytical wavelength: 465 nm

Blank: Amyl alcohol

Analysis

Samples: *Standard solution* and *Sample*

Transfer the *Sample* and 2.0 mL of the *Standard solution* to separate 200-mL beakers. Add 20 mL of nitric acid to each beaker. Cover each beaker with a watchglass, and boil slowly on a hot plate for 45 min. Cool to room temperature. Add 6 mL of perchloric acid, cover the beakers with a watchglass, and continue the heating until digestion is complete, as indicated when the liquid becomes colorless or pale yellow. Evaporate the solutions in the beakers to dryness. Rinse the sides of the beakers and the watchglasses with water, and add more water to complete 50 mL in each beaker. Gently boil the water solution for a few min. Cool to room temperature. Add 2 drops of methyl orange TS, and neutralize with ammonium hydroxide. Add 8.2 mL of hydrochloric acid. Quantitatively transfer the contents of the beakers to separate 100-mL volumetric flasks, rinse the beakers with water, transfer the rinsings to the corresponding volumetric flasks, and dilute with water to volume. Transfer 50.0 mL of each solution to separatory funnels. To each separatory funnel add 1.0 mL of *Sodium fluoride solution*, 0.5 mL of *Ferrous sulfate solution*, 4.0 mL of *Potassium thiocyanate solution*, 1.5 mL of *20% Stannous chloride solution*, and 15.0 mL of amyl alcohol, and shake the separatory funnel for 1 min. Allow the layers to separate, and discard the aqueous layers. Add 25 mL of *Diluted stannous chloride solution* to each separatory funnel, and shake gently for 15 s. Allow the layers to separate, and discard the aqueous layers. Transfer the organic layers from each separatory funnel to a centrifuge tube, and centrifuge at 2000 rpm for 10 min. Determine the absorbances of the organic phases from the *Standard solution* and the *Sample*, and correct with the *Blank*.

Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Tablets taken:

$$\text{Result} = (A_U/A_S) \times [(V \times C_S)/M_U] \times 100$$

A_U = absorbance of the solution from the *Sample*
A_S = absorbance of the solution from the *Standard solution*
V = volume of the *Standard solution* analyzed, 2.0 mL
C_S = concentration of molybdenum in the *Standard solution* (µg/mL)
M_U = nominal amount of molybdenum in the *Sample* (µg)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

• **PHOSPHORUS, *Method 1***

Sulfuric acid solution: Cautiously add sulfuric acid to water (37.5: 100), and mix.

Ammonium molybdate solution: 50 mg/mL of ammonium molybdate in *Sulfuric acid solution* and water (2:3). [NOTE—Dissolve in water first, then dilute with *Sulfuric acid solution* to volume.]

Hydroquinone solution: 5 mg/mL of hydroquinone in water. Add 1 drop of sulfuric acid per 100 mL of solution.

Sodium bisulfite solution: 200 mg/mL of sodium bisulfite in water

Phosphorus standard stock solution: Weigh 4.395 g of monobasic potassium phosphate, previously dried at 105° for 2 h and stored in a desiccator, and transfer to a 1000-mL volumetric flask. Dissolve in water, add 6 mL of sulfuric acid as a preservative, dilute with water to volume, and mix to obtain a solution with a concentration of 100 µg/mL of phosphorus.

Standard solution: 20 µg/mL of phosphorus from *Phosphorus standard stock solution* diluted with water

Sample solution: [NOTE—Finely powder and weigh a counted number of Tablets.] Transfer a portion of the powder, equivalent to a nominal amount of 100 mg of phosphorus, to 25 mL of nitric acid, and digest on a hot plate for 30 min. Add 15 mL of hydrochloric acid, and continue the digestion to the cessation of brown fumes. Cool, and transfer the contents of the flask to a 500-mL volumetric flask with the aid of small portions of water. Dilute with water to volume. Transfer 10.0 mL of this solution to a 100-mL volumetric flask, and dilute with water to volume.

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Mode: UV-Vis

Cell: 1 cm

Analytical wavelength: 650 nm

Analysis

Samples: *Standard solution* and *Sample solution*

To three separate 25-mL volumetric flasks transfer 5.0 mL each of the *Standard solution*, the *Sample solution*, and water to provide the blank. To each of the three flasks add 1.0 mL each of *Ammonium molybdate solution*, *Hydroquinone solution*, and *Sodium bisulfite solution*, and swirl to mix. Dilute the contents of each flask with water to volume, and allow the flasks to stand for 30 min. Determine the absorbances of the solutions against the blank.

Calculate the percentage of the labeled amount of phosphorus (P) in the portion of Tablets taken:

$$\text{Result} = (A_U/A_S) \times (C_S/C_U) \times 100$$

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A_U = absorbance of the *Sample solution*
A_S = absorbance of the *Standard solution*
C_S = concentration of phosphorus in the *Standard solution* (µg/mL)
C_U = nominal concentration of phosphorus in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of phosphorus (P)

• **POTASSIUM**

Potassium standard solution: 100 µg/mL of potassium from potassium chloride, previously dried at 105° for 2 h, in water

Standard stock solution: 10 µg/mL of potassium from the *Potassium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Transfer 5.0, 10.0, 15.0, 20.0, and 25.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions containing 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of potassium.

Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain a nominal concentration of 1 µg/mL of potassium and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Lamp: Potassium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Potassium emission line at 766.5 nm

Blank: Water

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of potassium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of potassium in the *Sample solution*.

Calculate the percentage of the labeled amount of potassium (K) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of potassium in the *Sample solution* (µg/mL)
C_U = nominal concentration of potassium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of potassium (K)

• **SELENIUM, *Method 1***

Diluent: Prepare as directed in *Molybdenum, Method 1*.

Selenium standard solution: [**CAUTION**—Selenium is toxic; handle it with care.] Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness. Add 2 mL of water, and evaporate to dryness. Repeat the addition of water and the evaporation to dryness three times. Dissolve the residue in 3 N hydrochloric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume, to obtain a concentration of 1000 µg/mL of selenium.

Standard stock solution: 100 µg/mL of selenium from the *Selenium standard solution* diluted with water

Standard solutions: To separate 100-mL volumetric flasks transfer 5.0, 10.0, and 25.0 mL of the *Standard stock solution*, and add 5.0 mL of perchloric acid to each flask. Gently boil the solutions for 15 min, cool to room temperature, and dilute each with *Diluent* to volume to obtain solutions with concentrations of 5.0, 10.0, and 25.0 µg/mL of selenium.

Sample solution: Transfer a portion of the powder, equivalent to a nominal amount of 1000 µg of selenium, to a suitable flask, and add 12 mL of nitric acid. [NOTE—The volume of nitric acid may be varied to ensure that the powder is uniformly dispersed.] Carefully swirl the flask to disperse the sample specimen. Sonicate for 10 min or until the sample specimen is completely dissolved. Gently boil the solution for 15 min, and cool to room temperature. Carefully add 8 mL of perchloric acid to the flask, heat the flask until perchloric acid fumes appear, and swirl the flask to dissipate the fumes. Repeat the heating and swirling until the fumes appear again. Cool to room temperature. Transfer the contents of the flask to a 50-mL volumetric flask with the aid of the *Diluent*, and dilute with *Diluent* to volume.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Lamp: Selenium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Selenium emission line at 196.0 nm

Blank: *Diluent* and perchloric acid (20:1)

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of selenium, and draw the straight line best fitting the three plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of selenium in the *Sample solution*.

Calculate the percentage of the labeled amount of selenium (Se) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of selenium in the *Sample solution* (µg/mL)
C_U = nominal concentration of selenium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of selenium (Se)

• **SELENIUM, *Method 2***

Hydrochloric acid solution: Hydrochloric acid diluted with water (1 in 10)

50% Ammonium hydroxide solution: Ammonium hydroxide diluted with water (1 in 2)

Reagent A: 9 mg/mL of edetate disodium and 25 mg/mL of hydroxylamine hydrochloride in water. [NOTE—Dissolve edetate disodium in a portion of water first, add hydroxylamine hydrochloride, and then dilute with water to volume.]

Reagent B: Transfer 200 mg of 2,3-diaminonaphthalene to a 250-mL separatory funnel, and add 200 mL of 0.1 N hydrochloric acid. Wash the solution with three 40-mL portions of cyclohexane, and discard the cyclohexane layer. Filter the solution into a brown bottle, and cover the solution with a 1-cm layer of cyclohexane. This solution is stable for 1 week if stored in a refrigerator.

Standard stock solution: [**CAUTION**—Selenium is toxic; handle it with care.] Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness, add 2 mL of water, and evaporate to dryness. Repeat the

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addition of water and evaporation to dryness three times. Dissolve the residue in 3 N hydrochloric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume to obtain a solution with a concentration of 1000 µg/mL of selenium. Dilute a volume of the solution with 0.125 N hydrochloric acid to obtain a concentration of 2.0 µg/mL of selenium.

Standard solution: Transfer 10.0 mL of the *Standard stock solution* to a glass-stoppered flask. Add 1 mL of perchloric acid and 1 mL of *Hydrochloric acid solution*, and dilute with water to 20 mL.

Sample solution: Transfer a portion of finely powdered Tablets, equivalent to a nominal amount of 20 µg of selenium, to a suitable flask. Add 10 mL of nitric acid, and warm gently on a hot plate. Continue heating until the initial nitric acid reaction has subsided, then add 3 mL of perchloric acid. [**CAUTION**—Exercise care at this stage because the perchloric acid reaction becomes vigorous.] Continue heating on the hot plate until the appearance of white fumes of perchloric acid or until the digest begins to darken. Add 0.5 mL of nitric acid, and resume heating, adding additional amounts of nitric acid if further darkening occurs. Digest for 10 min after the first appearance of perchloric acid fumes or until the digest becomes colorless. Cool the flask, add 2.5 mL of *Hydrochloric acid solution*, and return the flask to the hot plate to expel residual nitric acid. Heat the mixture for 3 min after it begins to boil. Cool the flask to room temperature, and dilute with water to 20 mL.

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Mode: UV

Cell: 1 cm

Analytical wavelength: 380 nm

Blank: 1 mL of perchloric acid and 1 mL of *Hydrochloric acid solution* diluted with water to 20 mL

Analysis

Samples: *Standard solution* and *Sample solution*

Treat the *Sample solution*, *Standard solution*, and *Blank* as follows. Add 5 mL of *Reagent A* to each flask, and swirl gently to mix. Adjust the solution in each flask with *50% Ammonium hydroxide solution* to a pH of 1.1 ± 0.1. Add 5 mL of *Reagent B* to each flask, and swirl gently to mix. Place the flasks in a water bath maintained at 50°, and equilibrate for 30 min, taking care that the flasks are covered to protect them from light. Cool to room temperature, and transfer the contents of each flask to separate separatory funnels. Transfer 10.0 mL of cyclohexane to each separatory funnel, and extract vigorously for 1 min. Discard the aqueous layer. Transfer the cyclohexane layer to a centrifuge tube, and centrifuge at 1000 rpm for 1 min to remove any remaining water. Determine the absorbances of the solutions from the *Samples* against the solution from the *Blank*.

Calculate the percentage of the labeled amount of selenium (Se) in the portion of Tablets taken:

$$\text{Result} = (A_U/A_S) \times [(V \times C_S)/M_U] \times 100$$

A_U = absorbance of the cyclohexane layer from the *Sample solution*

A_S = absorbance of the cyclohexane layer from the *Standard solution*

V = volume of the *Standard stock solution* used to prepare the *Standard solution*, 10 mL

C_S = concentration of selenium in the *Standard stock solution* (µg/mL)

M_U = nominal amount of selenium in the *Sample solution* (µg)

Acceptance criteria: 90.0%–160.0% of the labeled amount of selenium (Se)

• **ZINC, *Method 1***

Zinc standard stock solution: 1000 µg/mL of zinc from zinc oxide dissolved in 5 M hydrochloric acid (3.89 mg/mL), diluted with water to final volume. [NOTE—Dissolve in 5 M hydrochloric acid by warming, if necessary, cool, and then dilute to final volume.]

Standard stock solution: 50 µg/mL of zinc from the *Zinc standard stock solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Transfer 1.0, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of zinc.

Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain a nominal concentration of 2 µg/mL of zinc and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Lamp: Zinc hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Zinc emission line at 213.8 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of zinc, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C, in µg/mL, of zinc in the *Sample solution*.

Calculate the percentage of the labeled amount of zinc (Zn) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of zinc in the *Sample solution* (µg/mL)

C_U = nominal concentration of zinc in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of zinc (Zn)

• **BORON, NICKEL, TIN,** and **VANADIUM, *Method 1*; CALCIUM, CHROMIUM, COPPER, IRON, MAGNESIUM, MANGANESE, PHOSPHORUS,** and **ZINC, *Method 2*; MOLYBDENUM** and **SELENIUM, *Method 3***

Stock aqua regia solution: Prepare a mixture of hydrochloric acid and nitric acid (3:1) by adding the nitric acid to the hydrochloric acid. [NOTE—Periodically vent the solution in an appropriate fume hood.]

Diluent: Prepare a mixture of *Stock aqua regia solution* and water (1:9) by adding 1 volume of *Stock aqua regia solution* to 2 volumes of water. Dilute with additional water to volume, and mix well.

System suitability solution: Prepare a mixture of 1000 mg/L of yttrium in 5% (v/v) nitric acid solution and 1000 mg/L of scandium in 5% (v/v) nitric acid solution with *Diluent* (1:1:198), and mix.

Standard stock solution 1 (Ca, Cu, Fe, Mg, Mn, P, and Zn): [NOTE—It is only necessary to include the minerals of interest in the solution.] Using commercially available element standard (single- or multi-element) solutions in 5% (v/v) nitric acid solution, pipet the appropriate amount of element standard solution into a volumetric flask, and dilute

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with 5% (v/v) nitric acid solution to obtain a solution with final concentrations of about 1000 mg/L of calcium, 100 mg/L of copper, 250 mg/L of iron, 500 mg/L of magnesium, 100 mg/L of manganese, 800 mg/L of phosphorus, and 250 mg/mL of zinc.

Standard stock solution 2 (B, Cr, Mo, Ni, Se, Sn, and V): [NOTE—It is only necessary to include the minerals of interest in the solution.] Using commercially available element standard (single- or multi-element) solutions in 20% (v/v) hydrochloric acid solution, pipet the appropriate amount of element standard solution into a volumetric flask, and dilute with 20% (v/v) hydrochloric acid solution to obtain a solution with final concentrations of about 200 mg/L of boron, and 100 mg/L of chromium, molybdenum, nickel, selenium, tin, and vanadium each.

Standard solutions: Prepare a mixture of *Standard stock solution 1* and *Standard stock solution 2*, as required, in *Diluent* to prepare a six-point calibration curve to bracket the concentration range of each mineral of interest.

Sample solution 1 (for Tablets containing minerals found in ***Standard stock solution 1*** and ***Standard stock solution 2***): Weigh and finely powder NLT 20 Tablets. Transfer a portion, equal to 3.5 times the average Tablet weight, to a 250-mL volumetric flask. Slowly add 25 mL of *Stock aqua regia solution* in 5-mL increments, followed by mixing. [NOTE—If the sample contains a carbonate, bubbling will occur. Wait until bubbling ends to proceed.] Bring the solution to a boil on a hot plate. Continue to heat gently until fumes cease (about 1 h). [NOTE—If the sample contains selenium, digest for NMT 15 min.] Remove from heat, cool, and dilute with water to volume. Filter about 30 mL into a centrifuge tube using a 5-µm pore size nylon syringe filter. If necessary, make any further dilutions using the *Diluent*.

Sample solution 2 (for Tablets containing minerals found only in ***Standard stock solution 2***): Weigh and finely powder NLT 20 Tablets. Transfer a portion, equal to 3.5 times the average Tablet weight, to a 250-mL volumetric flask. Slowly add 25 mL of *Stock aqua regia solution* in 5-mL increments, followed by mixing. [NOTE—If the sample contains a carbonate, bubbling will occur. Wait until bubbling ends to proceed.] Bring the solution to a boil on a hot plate. Continue to heat gently until fumes cease (about 1 h). [NOTE—If the sample contains selenium, digest for NMT 15 min.] Remove from heat, cool, and dilute with water to volume. Filter about 30 mL into a centrifuge tube using a 5-µm pore size nylon syringe filter. If necessary, make any further dilutions using the *Diluent*.

Sample solution 3 (for Tablets containing minerals found only in ***Standard stock solution 1***): Weigh and finely powder NLT 20 Tablets. Transfer a weighed portion, equal to the average Tablet weight, to a 250-mL volumetric flask. Slowly add 25 mL of *Stock aqua regia solution* in 5-mL increments, followed by mixing. [NOTE—If the sample contains a carbonate, bubbling will occur. Wait until bubbling ends to proceed.] Bring the solution to a boil on a hot plate. Continue to heat gently (about 1 h) until fumes cease. Remove from heat, cool, and dilute with water to volume. Filter about 30 mL into a centrifuge tube using a 5-µm pore size nylon syringe filter. If necessary, make any further dilutions using the *Diluent*.

Instrumental conditions

(See *Plasma Spectrochemistry* ⟨730⟩.)

Mode: Inductively coupled plasma spectrometry using a spectrometer set to measure the emission of each mineral of interest at about the corresponding wavelength. [NOTE—The operating conditions may be developed and optimized based on the manufacturer's recommendation. The wavelengths selected should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision.]

System suitability

[NOTE—Analyze the *System suitability solution*, and obtain the response as directed for *Analysis*.]

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the emission of each mineral of interest in the *Standard solutions* and *Sample solution* with an inductively coupled plasma system using the *Diluent* as the blank. Plot the emission of the *Standard solutions* versus the concentration, in mg/L, of the minerals of interest, and draw the straight line best fitting the plotted points. From the graph so obtained, determine the concentration, *C*, in mg/L, for each mineral of interest in the *Sample solution*.

Calculate the percentage of the labeled amount for each mineral taken:

$$\text{Result} = C \times (V/W) \times F \times (T_W/L) \times 100$$

C = measured concentration of the relevant element in the *Sample solution* (mg/L)
V = volume of the *Sample solution* (L)
W = sample weight (mg)
F = dilution factor of the *Sample solution*
T_W = average Tablet weight (mg)
L = labeled amount of the relevant element (mg/Tablet)

Acceptance criteria: 90.0%–125.0% of the labeled amount of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), and zinc (Zn); 90.0%–160.0% of the labeled amount of boron (B), chromium (Cr), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), and vanadium (V)

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** ⟨2040⟩**:** Meet the requirements for *Dissolution*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩**:** Meet the requirements

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 3×10^3 cfu/g, and the combined molds and yeasts count does not exceed 3×10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species *Escherichia coli*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** The label states that the product is Minerals Tablets. The label also states the salt form of the mineral used as the source of each element. Where more than one assay method is given for a particular mineral, the labeling states the assay method used only if *Method 1* is not used.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Sodium Fluoride RS

MSM—*see Methylsulfonylmethane in Dietary Supplements section*

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Nettle, Stinging—*see Stinging Nettle*

Niacin—*see Niacin General Monographs*

Niacin Tablets—*see Niacin Tablets General Monographs*

Niacinamide—*see Niacinamide General Monographs*

Niacinamide Tablets—*see Niacinamide Tablets General Monographs*

Oleovitamin A and D—*see Oleovitamin A and D General Monographs*

Oleovitamin A and D Capsules—*see Oleovitamin A and D Capsules General Monographs*

Olive Leaf

DEFINITION

Olive Leaf consists of the dried leaf of *Olea europaea* L. (Fam. Oleaceae). It contains NLT 6.0% of oleuropein ($C_{25}H_{32}O_{13}$), calculated on the dried basis.

IDENTIFICATION

• **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Flavonoids

Standard solution A: 1 mg/mL of USP Rutin RS and 2 mg/mL each of USP Verbascoside RS, USP Oleuropein RS, and USP Oleanolic Acid RS in methanol

Standard solution B: 25 mg/mL of USP Olive Leaf Dry Extract RS in methanol. Sonicate for 10 min, centrifuge, and use the supernatant.

Sample solution: Suspend about 500 mg of Olive Leaf, finely powdered, in 5 mL of methanol, and sonicate for 10 min. Centrifuge, and use the supernatant.

Chromatographic system

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plate)[1]

Application volume: 2 µL each of *Standard solution A*, *Standard solution B*, and *Sample solution* as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of 33%.

Temperature: Ambient temperature, not to exceed 30°

Developing solvent system: Ethyl formate, formic acid, toluene, and water (60:8:3:6)

Developing distance: 6 cm

Derivatization reagent A: 5 mg/mL of 2-aminoethyl diphenylborinate in ethyl acetate

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands and dry in air. Develop in a saturated chamber and dry in air. Heat at 100° for 3 min. While the plate is still warm, treat with *Derivatization reagent A*, and examine under long-wave UV light (365 nm).

[NOTE—Immediately after examination, use the same developed and derivatized plate for *Identification B*.]

System suitability: Under long-wave UV (365 nm), the derivatized chromatogram of *Standard solution B* displays, in its lower third, three yellowish bands: the first close to the application line; the second located just above, or coincident with, the rutin band in *Standard solution A*; the third, due to luteolin-7-*O*-glucoside, positioned above the light-blue band corresponding to verbascoside in *Standard solution A*. There is a bluish-green band just above the band due to rutin. A faint light-blue band may appear just below the verbascoside band. The bands due to verbascoside and luteolin-7-*O*-glucoside are clearly separated. Above the yellow band due to luteolin-7-*O*-glucoside, there is a faint blue zone and a faint yellow band.

Acceptance criteria: Under long-wave UV light (365 nm), the chromatogram of the *Sample solution* displays the bands similar in position and color to those seen in *Standard solution B*. Additional faint bands may be observed.

• **B. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Secoiridoids and Triterpenoids

[NOTE—Apply *Derivatization reagent B* to the plate used earlier in *Identification A*. Perform the second derivatization within 20 min of the first one.]

Chromatographic system

Derivatization reagent B: 85 mL of ice-cold methanol combined with 10 mL of glacial acetic acid, 5 mL of sulfuric acid, and 0.5 mL of *p*-anisaldehyde

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Treat the plate with *Derivatization reagent B*, dry in air, heat for 3 min at 100°, and immediately examine under white light.

System suitability: Under white light, the derivatized chromatogram of *Standard solution B* exhibits, in its lower third, a number of faint grayish-brown bands. In the middle of the chromatogram, an intense gray-brown band corresponding to oleuropein in *Standard solution A* appears. An intense violet band, corresponding to the oleanolic acid band in *Standard solution A*, is seen near the solvent front, and a weaker blue band due to maslinic acid just below it.

Acceptance criteria: Under white light, the chromatogram of the *Sample solution* displays the bands similar in position and color to those seen in *Standard solution B*. Additional bands may be observed.

COMPOSITION

• **CONTENT OF OLEUROPEIN**

Mobile phase: 0.1% phosphoric acid in water and acetonitrile (80:20)

Standard solution: 0.2 mg/mL of USP Oleuropein RS in *Mobile phase*

Sample solution: Transfer about 1.0 g of Olive Leaf, finely powdered and accurately weighed, to a 50-mL centrifuge tube, add 15 mL of *Mobile phase*, cap tightly, and sonicate for 20 min. Centrifuge, and transfer the supernatant to a

[1] Suitable commercially available plates are HPTLC Silica Gel 60 F_{254} from EMD Millipore (e.g., Part No. 1.05642.0001).

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50-mL volumetric flask. Repeat with two additional 15-mL aliquots of *Mobile phase*, and combine all supernatants into the same volumetric flask. Dilute with *Mobile phase* to volume, and mix well. Dilute the resulting solution 10-fold with *Mobile phase*. Pass through a PTFE filter of 0.45-µm or finer pore size, discarding the initial few milliliters of the filtrate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: HPLC
Detector: UV 230 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 25°
Flow rate: 1.5 mL/min
Injection volume: 20 µL

System suitability
Sample: *Standard solution*
Suitability requirements
Tailing factor: NMT 2.0 for the oleuropein peak
Relative standard deviation: NMT 2.0% determined for the oleuropein peak in replicate injections

Analysis
Samples: *Standard solution* and *Sample solution*
Using the chromatogram of the *Standard solution*, identify the retention time of the peak corresponding to oleuropein in the *Sample solution*.
Calculate the percentage of oleuropein ($C_{25}H_{32}O_{13}$) in the portion of Olive Leaf taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times 100$$

r_U = peak area of oleuropein from the *Sample solution*
r_S = peak area of oleuropein from the *Standard solution*
C_S = concentration of USP Oleuropein RS in the *Standard solution* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Olive Leaf taken to prepare the *Sample solution* (mg)
D = dilution factor, 10

Acceptance criteria: NLT 6.0% on the dried basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Limits of Elemental Impurities*: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Pesticide Residue Analysis*: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^5 cfu/g, and the bile-tolerant Gram-negative bacterial count does not exceed 10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures*, *Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: The leaf is simple, subsessile, thick, coriaceous, lanceolate to obovate, 3–8 cm long, 0.5–2 cm broad; base cuneate; apex apiculate to mucronulate. The margins are entire and slightly revolute. Nervation of the leaf is reticular. The upper surface is grayish green to dark green, glabrous and shiny; the lower surface silvery (or reddish in Asian material of subsp. *cuspidata*), and tomentous (densely pilous), densely covered with a layer of peltate scales but without filamentous hairs.

Microscopic: The leaf has iso-bilateral structure. Both the adaxial epidermis and the abaxial epidermis are simple, with thick cuticle. The anticlinal walls of both adaxial and abaxial epidermal cells are straight. The adaxial epidermis has sporadic, and the abaxial epidermis numerous densely distributed, nonglandular, very large scutiform trichomes. The leaves are hypostomatic and their numerous stomata possess highly cutinized guard cells. The mesophyll is constituted of three layers of condensed long palisade cells located under the adaxial epidermis and one or two layers of short palisade cells on the abaxial epidermis. The central part of the leaf contains tiny cells of spongy parenchyma with large intercellulars. Present in the mesophyll are filiform sclereids, which are long, fiber-like, and sometimes branched. Microscopically, the olive leaf can be clearly identified by fragments of epidermis with long filiform sclereids and characteristic very large scutiform trichomes. Presence of fragments of epidermis with crypts may indicate adulteration with oleander leaves, while presence of numerous anomocytic stomata and large cluster crystals suggests adulteration with pittosporum.

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Foreign Organic Matter*: NMT 2.0%
- **LOSS ON DRYING** ⟨731⟩
Sample: 1.0 g of Olive Leaf, finely powdered
Analysis: Dry the *Sample* at 105° for 2 h.
Acceptance criteria: NMT 10.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Total Ash*
Sample: 1.0 g of Olive Leaf, finely powdered
Acceptance criteria: NMT 9.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the official name of the article and the corresponding Latin binomial.
- **USP REFERENCE STANDARDS** ⟨11⟩.
USP Oleanolic Acid RS
USP Oleuropein RS
USP Olive Leaf Dry Extract RS
USP Rutin RS
USP Verbascoside RS

Olive Leaf Dry Extract

DEFINITION

Olive Leaf Dry Extract is prepared from the dried leaf of *Olea europaea* L. (Fam. Oleaceae) by extraction with hydroalcoholic mixtures, ethyl acetate, or other suitable solvents. It contains NLT 90% and NMT 110% of the labeled amount of oleuropein ($C_{25}H_{32}O_{13}$), calculated on the dried basis. The ratio of starting plant material to extract is between 5:1 and 60:1. It may contain suitable added substances.

IDENTIFICATION

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Flavonoids
Standard solution A: 1 mg/mL of USP Rutin RS and 2 mg/mL each of USP Verbascoside RS, USP Oleuropein RS, and USP Oleanolic Acid RS in methanol
Standard solution B: 25 mg/mL of USP Olive Leaf Dry Extract RS in methanol. Sonicate for 10 min, centrifuge, and use the supernatant.
Sample solution: Suspend about 125 mg of Olive Leaf Dry Extract in 5 mL of methanol, and sonicate for 10 min. Centrifuge, and use the supernatant.

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Chromatographic system
Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plate)[1]
Application volume: 2 µL each of *Standard solution A*, *Standard solution B*, and *Sample solution* as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of 33%.
Temperature: Ambient temperature, not to exceed 30°
Developing solvent system: Ethyl formate, formic acid, toluene, and water (60:8:3:6)
Developing distance: 6 cm
Derivatization reagent A: 5 mg/mL of 2-aminoethyl diphenylborinate in ethyl acetate
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Apply the *Samples* as bands and dry in air. Develop in a saturated chamber and dry in air. Heat at 100° for 3 min. While the plate is still warm, treat with *Derivatization reagent A*, and examine under long-wave UV light (365 nm).
[NOTE—Immediately after examination, use the same developed and derivatized plate for *Identification B*.]
System suitability: Under long-wave UV (365 nm), the derivatized chromatogram of *Standard solution B* displays, in its lower third, three yellowish bands: the first close to the application line; the second located just above, or coincident with, the rutin band in *Standard solution A*; the third, due to luteolin-7-*O*-glucoside, positioned above the light-blue band corresponding to verbascoside in *Standard solution A*. There is a bluish-green band just above the band due to rutin. A faint light-blue band may appear just below the verbascoside band. The bands due to verbascoside and luteolin-7-*O*-glucoside are clearly separated. Above the yellow band due to luteolin-7-*O*-glucoside, there is a faint blue zone and a faint yellow band.
Acceptance criteria: Under long-wave UV light (365 nm), the chromatogram of the *Sample solution* displays the bands similar in position and color to those seen in *Standard solution B*. Additional faint bands may be observed.

• **B. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩
Secoiridoids and Triterpenoids
[NOTE—Apply *Derivatization reagent B* to the plate used earlier in *Identification A*. Perform the second derivatization within 20 min of the first one.]
Chromatographic system
Derivatization reagent B: 85 mL of ice-cold methanol combined with 10 mL of glacial acetic acid, 5 mL of sulfuric acid, and 0.5 mL of *p*-anisaldehyde
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Treat the plate with *Derivatization reagent B*, dry in air, heat for 3 min at 100°, and immediately examine under white light.
System suitability: Under white light, the derivatized chromatogram of *Standard solution B* exhibits, in its lower third, a number of faint grayish-brown bands. In the middle of the chromatogram, an intense gray-brown band corresponding to oleuropein in *Standard solution A* appears. An intense violet band, corresponding to the oleanolic acid band in *Standard solution A*, is seen near the solvent front, and a weaker blue band due to maslinic acid just below it.
Acceptance criteria: Under white light, the chromatogram of the *Sample solution* displays the bands similar in position and color to those seen in *Standard solution B*; however, the bands due to oleanolic and maslinic acids may be faint or absent. Additional bands may be observed.

[1] Suitable commercially available plates are HPTLC Silica Gel 60 F_{254} from EMD Millipore (e.g., Part No. 1.05642.0001).

COMPOSITION

• **CONTENT OF OLEUROPEIN**
Mobile phase: 0.1% phosphoric acid in water and acetonitrile (80:20)
Standard solution: 0.2 mg/mL of USP Oleuropein RS in *Mobile phase*
Sample solution: Accurately weigh the amount of Olive Leaf Dry Extract calculated to contain 20 mg of oleuropein into a 50-mL volumetric flask, add 25 mL of *Mobile phase*, and sonicate for 5 min. Dilute with *Mobile phase* to volume, and mix well. Pass through a PTFE filter of 0.45-µm or finer pore size, discarding the initial few milliliters of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: HPLC
Detector: UV 230 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 25°
Flow rate: 1.5 mL/min
Injection volume: 20 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Tailing factor: NMT 2.0 for the oleuropein peak
Relative standard deviation: NMT 2.0% determined for the oleuropein peak in replicate injections
Analysis
Samples: *Standard solution* and *Sample solution*
Using the chromatogram of the *Standard solution*, identify the retention time of the peak corresponding to oleuropein in the *Sample solution*.
Calculate the percentage of oleuropein ($C_{25}H_{32}O_{13}$) in the portion of Olive Leaf Dry Extract taken:

$$P = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of oleuropein from the *Sample solution*
r_S = peak area of oleuropein from the *Standard solution*
C_S = concentration of USP Oleuropein RS in the *Standard solution* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Olive Leaf Dry Extract taken to prepare the *Sample solution* (mg)

Calculate the percentage of the labeled amount of oleuropein ($C_{25}H_{32}O_{13}$) in the portion of Olive Leaf Dry Extract taken:

$$\text{Result} = (P/L) \times 100$$

P = oleuropein content as determined above
L = labeled amount of oleuropein

Acceptance criteria: 90.0%–110.0% on the dried basis

CONTAMINANTS

Change to read:

• **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues:* ▲ (CN 1-May-2020) Meets the requirements

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- **Botanical Extracts** ⟨565⟩, *General Pharmacopeial Requirements, Residual Solvents*: Meets the requirements
- **Microbial Enumeration Tests** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.
- **Absence of Specified Microorganisms** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS

- **Loss on Drying** ⟨731⟩
  Sample: 1 g of Olive Leaf Dry Extract
  Analysis: Dry the *Sample* at 105° for 2 h.
  Acceptance criteria: NMT 8.0%

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **Labeling:** The label states the official name of the article and the corresponding Latin binomial. The label also indicates the content of oleuropein, the extraction solvent used in preparation, and the ratio of the starting crude plant material to Dry Extract. It meets the labeling requirements of *Botanical Extracts* ⟨565⟩.
- **USP Reference Standards** ⟨11⟩
  USP Oleanolic Acid RS
  USP Oleuropein RS
  USP Olive Leaf Dry Extract RS
  USP Rutin RS
  USP Verbascoside RS

Olive Leaf Powder

DEFINITION

Olive Leaf Powder consists of the dried leaf of *Olea europaea* L. (Fam. Oleaceae) reduced to fine or very fine powder. It contains NLT 6.0% of oleuropein ($C_{25}H_{32}O_{13}$), calculated on the dried basis.

IDENTIFICATION

- **A. HPTLC for Articles of Botanical Origin** ⟨203⟩
  Flavonoids
  Standard solution A: 1 mg/mL of USP Rutin RS and 2 mg/mL each of USP Verbascoside RS, USP Oleuropein RS, and USP Oleanolic Acid RS in methanol
  Standard solution B: 25 mg/mL of USP Olive Leaf Dry Extract RS in methanol. Sonicate for 10 min, centrifuge, and use the supernatant.
  Sample solution: Suspend about 500 mg of Olive Leaf Powder in 5 mL of methanol, and sonicate for 10 min. Centrifuge, and use the supernatant.
  Chromatographic system
  Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plate)[1]
  Application volume: 2 µL each of *Standard solution A*, *Standard solution B*, and *Sample solution* as 8-mm bands
  Relative humidity: Condition the plate to a relative humidity of 33%.
  Temperature: Ambient temperature, not to exceed 30°
  Developing solvent system: Ethyl formate, formic acid, toluene, and water (60:8:3:6)
  Developing distance: 6 cm
  Derivatization reagent A: 5 mg/mL of 2-aminoethyl diphenylborinate in ethyl acetate
  Analysis
  Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
  Apply the *Samples* as bands and dry in air. Develop in a saturated chamber and dry in air. Heat at 100° for 3 min. While the plate is still warm, treat with *Derivatization reagent A*, and examine under long-wave UV light (365 nm).
  [Note—Immediately after examination, use the same developed and derivatized plate for *Identification B*.]
  System suitability: Under long-wave UV (365 nm), the derivatized chromatogram of *Standard solution B* displays, in its lower third, three yellowish bands: the first close to the application line; the second located just above, or coincident with, the rutin band in *Standard solution A*; the third, due to luteolin-7-*O*-glucoside, positioned above the light-blue band corresponding to verbascoside in *Standard solution A*. There is a bluish-green band just above the band due to rutin. A faint light-blue band may appear just below the verbascoside band. The bands due to verbascoside and luteolin-7-*O*-glucoside are clearly separated. Above the yellow band due to luteolin-7-*O*-glucoside, there is a faint blue zone and a faint yellow band.
  Acceptance criteria: Under long-wave UV light (365 nm), the chromatogram of the *Sample solution* displays the bands similar in position and color to those seen in *Standard solution B*. Additional faint bands may be observed.
- **B. HPTLC for Articles of Botanical Origin** ⟨203⟩
  Secoiridoids and Triterpenoids
  [Note—Apply *Derivatization reagent B* to the plate used earlier in *Identification A*. Perform the second derivatization within 20 min of the first one.]
  Chromatographic system
  Derivatization reagent B: 85 mL of ice-cold methanol combined with 10 mL of glacial acetic acid, 5 mL of sulfuric acid, and 0.5 mL of *p*-anisaldehyde
  Analysis
  Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
  Treat the plate with *Derivatization reagent B*, dry in air, heat for 3 min at 100°, and immediately examine under white light.
  System suitability: Under white light, the derivatized chromatogram of *Standard solution B* exhibits, in its lower third, a number of faint grayish-brown bands. In the middle of the chromatogram, an intense gray-brown band corresponding to oleuropein in *Standard solution A* appears. An intense violet band, corresponding to the oleanolic acid band in *Standard solution A*, is seen near the solvent front, and a weaker blue band due to maslinic acid just below it.
  Acceptance criteria: Under white light, the chromatogram of the *Sample solution* displays the bands similar in position and color to those seen in *Standard solution B*. Additional bands may be observed.

COMPOSITION

- **Content of Oleuropein**
  Mobile phase: 0.1% phosphoric acid in water and acetonitrile (80:20)
  Standard solution: 0.2 mg/mL of USP Oleuropein RS in *Mobile phase*
  Sample solution: Transfer about 1.0 g of Olive Leaf Powder, accurately weighed, to a 50-mL centrifuge tube, add 15 mL of *Mobile phase*, cap tightly, and sonicate for 20 min. Centrifuge, and transfer the supernatant to a 50-mL

[1] Suitable commercially available plates are HPTLC Silica Gel 60 F_{254} from EMD Millipore (e.g., Part No. 1.05642.0001).

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volumetric flask. Repeat with two additional 15-mL aliquots of *Mobile phase*, and combine all supernatants into the same volumetric flask. Dilute with *Mobile phase* to volume, and mix well. Dilute the resulting solution 10-fold with *Mobile phase*. Pass through a PTFE filter of 0.45-µm or finer pore size, discarding the initial few milliliters of the filtrate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: HPLC
Detector: UV 230 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 25°
Flow rate: 1.5 mL/min
Injection volume: 20 µL

System suitability
Sample: *Standard solution*
Suitability requirements
Tailing factor: NMT 2.0 for the oleuropein peak
Relative standard deviation: NMT 2.0% determined for the oleuropein peak in replicate injections

Analysis
Samples: *Standard solution* and *Sample solution*
Using the chromatogram of the *Standard solution*, identify the retention time of the peak corresponding to oleuropein in the *Sample solution*.
Calculate the percentage of oleuropein ($C_{25}H_{32}O_{13}$) in the portion of Olive Leaf Powder taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times 100$$

r_U = peak area of oleuropein from the *Sample solution*
r_S = peak area of oleuropein from the *Standard solution*
C_S = concentration of USP Oleuropein RS in the *Standard solution* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Olive Leaf Powder taken to prepare the *Sample solution* (mg)
D = dilution factor, 10

Acceptance criteria: NLT 6.0% on the dried basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Limits of Elemental Impurities*: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Pesticide Residue Analysis*: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^5 cfu/g, and the bile-tolerant Gram-negative bacterial count does not exceed 10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**
Macroscopic: The powder is yellowish green.
Microscopic: The powder shows fragments of abaxial epidermis with small anomocytic stomata and adaxial epidermis with thick-walled polygonal cells, palisade composed of three layers of cells, and spongy parenchyma composed of small cells. Fragments of the lamina show thick cuticle. Long filiform sclereids and very large characteristic scutiform trichomes are abundant. Presence of fragments of epidermis with crypts may indicate adulteration with oleander leaves, while presence of numerous anomocytic stomata and large cluster crystals suggests adulteration with pittosporum.
- **LOSS ON DRYING** ⟨731⟩
Sample: 1.0 g of Olive Leaf Powder
Analysis: Dry the *Sample* at 105° for 2 h.
Acceptance criteria: NMT 10.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Total Ash*
Sample: 1.0 g of Olive Leaf Powder
Acceptance criteria: NMT 9.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the official name of the article and the corresponding Latin binomial.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Oleanolic Acid RS
USP Oleuropein RS
USP Olive Leaf Dry Extract RS
USP Rutin RS
USP Verbascoside RS

Omega-3 Acid Triglycerides

DEFINITION

Omega-3 Acid Triglycerides is a mixture of mono-, di-, and triesters of omega-3 acids with glycerol containing mainly triesters and obtained either by esterification of concentrated and purified omega-3 acids with glycerol or by transesterification of the omega-3 acid ethyl esters with glycerol. The omega-3 acids are from the body oil of fish of the families Engraulidae, Carangidae, Clupeidae, Osmeridae, Salmonidae, and Scombridae and are defined as the following: alpha-linolenic acid (C18:3 n–3), moroctic acid (C18:4 n–3), eicosatetraenoic acid (C20:4 n–3), eicosapentaenoic acid (EPA) (C20:5 n–3), heneicosapentaenoic acid (C21:5 n–3), docosapentaenoic acid (C22:5 n–3), and docosahexaenoic acid (DHA) (C22:6 n–3). It contains NLT 58.0% of total omega-3 acids expressed as triglycerides and NLT the labeled amount of EPA and DHA, expressed as the free fatty acids. Suitable antioxidants in appropriate concentrations may be added.

IDENTIFICATION

- **A.** The retention times of the peaks for eicosapentaenoic acid methyl ester and docosahexaenoic acid methyl ester of the *Sample solution* in the tests for *Content of EPA and DHA* and *Content of Total Omega-3 Acids* correspond to those for the respective compounds of the *Standard solutions*. If either EPA or DHA is not claimed on the labeling, the peak corresponding to that omega-3 acid does not exceed 15.0% of the total detected area of the *Sample solution* in the test for *Content of EPA and DHA* and *Content of Total Omega-3 Acids*.
- **B.** The retention time of the peak corresponding to the triglycerides of the *Sample solution* corresponds to the triglycerides peak of the *System suitability solution* in the test for *Content of Oligomers and Partial Glycerides*.

COMPOSITION

- **CONTENT OF EPA AND DHA**
Analysis: Proceed as directed in *Fats and Fixed Oils* ⟨401⟩, *Omega-3 Fatty Acids Determination and Profile*.
Acceptance criteria: NLT the labeled amount, expressed as free acids

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• **CONTENT OF TOTAL OMEGA-3 ACIDS**

Analysis: Proceed as directed in *Fats and Fixed Oils* ⟨401⟩, *Omega-3 Fatty Acids Determination and Profile.*

Acceptance criteria: NLT 58.0% of total omega-3 acids, expressed as triglycerides

• **CONTENT OF OLIGOMERS AND PARTIAL GLYCERIDES**

Mobile phase: Use tetrahydrofuran.

Sample solution: 1.00 mg/mL of Omega-3 Acid Triglycerides in *Mobile phase*

System suitability solution: 0.5 mg/mL of monodocosahexaenoin, 0.3 mg/mL of didocosahexaenoin, and 0.2 mg/mL of tridocosahexaenoin in *Mobile phase.* [NOTE—Suitable grades of monodocosahexaenoin, didocosahexaenoin, and tridocosahexaenoin may be obtained from Nu-Chek Prep.]

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability.*)

Mode: LC

Detector: Differential refractometer

Columns: Three 7.8-mm × 30-cm; connected in series; packing L21, 7-µm. Two columns are 50 nm in pore size, and the other is 10 nm, arranged so that the 50-nm pore size columns are closer to the injector.

Flow rate: 0.8 mL/min

Injection size: 40 µL

System suitability

Sample: *System suitability solution*

Suitability requirements

Elution order: Tridocosahexaenoin, didocosahexaenoin, and monodocosahexaenoin

Resolution: NLT 2.0 between monodocosahexaenoin and didocosahexaenoin and NLT 1.0 between didocosahexaenoin and tridocosahexaenoin

Analysis

Sample: *Sample solution*

Measure the areas of the major peaks.

Calculate the percentage of oligomers in the portion of Omega-3 Acid Triglycerides taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = sum of the areas of the peaks with a retention time less than that of the triglyceride peak

r_T = sum of the areas of all peaks in the chromatogram

Calculate the percentage of partial glycerides (mono- and diglycerides) in the portion of Omega-3 Acid Triglycerides taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = sum of the areas of the peaks corresponding to diglycerides and monoglycerides

r_T = sum of the areas of all peaks in the chromatogram

Acceptance criteria: NMT 3.0% of oligomers and NMT 50.0% of partial glycerides

CONTAMINANTS

• **LIMIT OF ARSENIC**

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytef, and plastic vessels before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin. Select all reagents to have as low a content of arsenic as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytef, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

Solution A: Transfer 1 g of ultrapure palladium metal into a Teflon beaker. Add 20 mL of water and 10 mL of nitric acid, and warm on a hot plate to dissolve. Allow the solution to cool to room temperature, transfer it into a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution B: Transfer 1 g of ultrapure magnesium nitrate into a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it to a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution C: *Solution A*, *Solution B*, and 2% nitric acid (3:2:5). A volume of 5 µL provides 0.015 mg of palladium and 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (1:19)

Standard stock solution: Transfer 10.0 mL of *Standard Arsenic Solution*, prepared as directed in *Arsenic* ⟨211⟩, to a 100-mL volumetric flask. Add 40 mL of water and 5 mL of nitric acid, and dilute with water to volume. This solution contains 0.10 µg/mL of arsenic.

Standard solutions: Dilute the *Standard stock solution* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 µg/mL of arsenic.

Sample solution: For preparation of the *Sample solution*, use a microwave oven with a magnetron frequency of 2455 MHz and a selectable output power of 0–950 watts in 1% increments, equipped with advanced composite vessels with 100-mL polytef liners. Use rupture membranes to vent vessels should the pressure exceed 125 psi. The vessels fit into a turntable, and each vessel can be vented into an overflow container. Equip the microwave oven with an exhaust tube to ventilate fumes. [**CAUTION**—Wear proper eye protection and protective clothing and gloves.] Transfer approximately 500 mg of Omega-3 Acid Triglycerides, weighed to the nearest 0.1 mg, into a Teflon digestion vessel liner. Prepare samples in duplicate. Add 15 mL of nitric acid, and swirl gently. Cover the vessels with lids, leaving the vent fitting off. Predigest overnight under a hood. Place the rupture membrane in the vent fitting, and tighten the lid. Place all vessels on the microwave oven turntable. Connect the vent tubes to the vent trap, and connect the pressure-sensing line to the appropriate vessel. Initiate a two-stage digestion procedure by heating the microwave at 15% power for 15 min, followed by 25% power for 45 min. Remove the turntable of vessels from the oven, and allow the vessels to cool to room temperature. [NOTE—A cool water bath may be used to speed the cooling process.] Vent the vessels when they reach room temperature. Remove the lids, and slowly add 2 mL of 30% hydrogen peroxide to each. Allow the reactions to subside, and seal the vessels. Return the vessels on the turntable to the microwave oven, and heat for an additional 15 min at 30% power. Remove the vessels from the oven, and allow them to cool to room temperature. Transfer the cooled digests into 25-mL volumetric flasks, and dilute with water to volume.

Analysis: Program the graphite furnace as follows. Dry at 115°, using a 1-s ramp, a 65-s hold, and an argon flow of 300 mL/min; char the sample at 1000°, using a 1-s ramp, a 20-s hold, and an airflow of 300 mL/min; cool down, and purge the air from the furnace for 10 s, using a 20° set temperature and an argon flow of 300 mL/min; atomize at 2400°, using a 0-s ramp and a 5-s hold with the argon flow stopped; and clean out at 2600° with a 1-s ramp and a 5-s hold.

Separately inject equal volumes (20 µL) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by an injection of 5 µL of *Solution C* for each of the samples, into the graphite tube of a suitable graphite furnace

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atomic absorption spectrometer equipped with a hollow-cathode lamp for arsenic. Determine the peak area at the arsenic emission line at 193.7 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of arsenic, in µg/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in µg/mL, of arsenic in each mL of the *Sample solution* by interpolation from the regression line.

Calculate the content of arsenic in the portion of Omega-3 Acid Triglycerides taken:

$$\text{Result} = (C \times V)/W$$

C = concentration of arsenic, as obtained above, in the *Sample solution* (µg/mL)
V = final volume of the *Sample solution* (mL)
W = weight of Omega-3 Acid Triglycerides taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g

• **LIMIT OF LEAD**

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytef, and plastic vessels before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin. Select all reagents to have as low a content of lead as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytef, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

Solution A: 10 g of ultrapure monobasic ammonium phosphate in 1 mL of nitric acid and 40 mL of water to dissolve the phosphate. Dilute with deionized water to 100 mL.

Solution B: 1 g of ultrapure magnesium nitrate to a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it to a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution C: *Solution A*, *Solution B*, and 2% nitric acid (2:1:2). A volume of 5 µL provides 0.2 mg of phosphate plus 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (1:19)

Standard stock solution: Transfer 10.0 mL of lead nitrate stock solution TS to a 100-mL volumetric flask. Add 40 mL of water and 5 mL of nitric acid, and dilute with water to volume. Transfer 1.0 mL of this solution to a second 100-mL volumetric flask, add 50 mL of water and 1 mL of nitric acid, and dilute with water to volume. This solution contains 0.10 µg/mL of lead.

Standard solutions: Dilute the *Standard stock solution* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 µg/mL of lead.

Sample solution: Prepare as directed for *Sample solution* in the test for *Limit of Arsenic*.

Analysis: Program the graphite furnace as follows. Dry at 120°, using a 1-s ramp, a 55-s hold, and an argon flow of 300 mL/min; char the sample at 850°, using a 1-s ramp, a 30-s hold, and an airflow of 300 mL/min; cool down, and purge the air from the furnace for 10 s, using a 20° set temperature and an argon flow of 300 mL/min; atomize at 2100°, using a 0-s ramp and a 5-s hold with the argon flow stopped; and clean out at 2600° with a 1-s ramp and a 5-s hold.

Separately inject equal volumes (20 µL) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by an injection of 5 µL of *Solution C* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for lead. Determine the peak area at the lead emission line at 283.3 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of lead, in µg/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in µg/mL, of lead in each mL of the *Sample solution* by interpolation from the regression line.

Calculate the content of lead in the portion of Omega-3 Acid Triglycerides taken:

$$\text{Result} = (C \times V)/W$$

C = concentration of lead, as obtained above, in the *Sample solution* (µg/mL)
V = final volume of the *Sample solution* (mL)
W = weight of Omega-3 Acid Triglycerides taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g

• **LIMIT OF CADMIUM**

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytef, and plastic vessels before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin. Select all reagents to have as low a content of cadmium as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytef, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

Solution A: 10 g of ultrapure monobasic ammonium phosphate in 40 mL of water and 1 mL of nitric acid to dissolve the phosphate. Dilute with deionized water to 100 mL.

Solution B: Transfer 1 g of ultrapure magnesium nitrate to a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it to a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution C: *Solution A*, *Solution B*, and 2% nitric acid to volume (2:1:2). A volume of 5 µL provides 0.2 mg of phosphate and 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (1:19)

Standard stock solution A: 0.1372 mg/mL of cadmium nitrate

Standard stock solution B: *Standard stock solution A*, nitric acid, and water (2:1:97). This solution contains 0.10 µg/mL of cadmium. [NOTE—Before make up to final volume, dissolve in a portion of water and nitric acid.]

Standard solutions: Dilute *Standard stock solution B* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 µg/mL of cadmium.

Sample solution: Prepare as directed for *Sample solution* in the test for *Limit of Arsenic*.

Analysis: Program the graphite furnace as follows. Dry at 120°, using a 1-s ramp, a 55-s hold, and an argon flow of 300 mL/min; char the sample at 850°, using a 1-s ramp, a 30-s hold, and an airflow of 300 mL/min; cool down, and purge the air from the furnace for 10 s, using a 20° set temperature and an argon flow of 300 mL/min; atomize at 2400°, using a 0-s ramp and a 5-s hold with the argon flow stopped; and clean out at 2600° with a 1-s ramp and a 5-s hold.

Separately inject equal volumes (20 µL) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by an injection of 5 µL of *Solution C* for each of the samples,

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into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for cadmium. Determine the peak area at the cadmium emission line at 228.8 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of cadmium, in µg/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in µg/mL, of cadmium in each mL of the *Sample solution* by interpolation from the regression line.

Calculate the content of cadmium in the Omega-3 Acid Triglycerides taken:

$$\text{Result} = (C \times V)/W$$

C = concentration of cadmium, as obtained above, in the *Sample solution* (µg/mL)
V = final volume of the *Sample solution* (mL)
W = weight of Omega-3 Acid Triglycerides taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g

- **LIMIT OF MERCURY:** Proceed as directed in *Mercury* ⟨261⟩, *Method IIa*, except use a *Standard Mercury Solution* having the equivalent of 0.1 µg/mL of mercury.

Sample solution: Prepare as directed for the *Sample solution* in the test for *Limit of Arsenic*, combining the two duplicate cooled digests into 1.0 mL of *Potassium Permanganate Solution*.

Acceptance criteria: NMT 0.1 µg/g

- **LIMIT OF DIOXINS, FURANS, AND POLYCHLORINATED BIPHENYLS (PCBs)**

Analysis: Determine the content of polychlorinated dibenzo-para-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) by Method No. 1613, Revision B, of the Environmental Protection Agency. Determine the content of polychlorinated biphenyls (PCBs) by Method No. 1668, Revision A, of the Environmental Protection Agency.

Acceptance criteria: The sum of PCDDs and PCDFs is NMT 2.0 pg/g of World Health Organization (WHO) toxic equivalents. The sum of PCDDs, PCDFs, and dioxin-like PCBs (polychlorinated biphenyls, nonortho IUPAC congeners PCB-77, PCB-81, PCB-126, and PCB-169, and mono-ortho IUPAC congeners PCB-105, PCB-114, PCB-118, PCB-123, PCB-156, PCB-157, PCB-167, and PCB-189) is NMT 10.0 pg/g of WHO toxic equivalents.

SPECIFIC TESTS

- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩: NMT 3
- **FATS AND FIXED OILS,** *Anisidine Value* ⟨401⟩: NMT 30.0
- **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩: NMT 10.0
- **FATS AND FIXED OILS,** *Unsaponifiable Matter* ⟨401⟩: NMT 2.0%
- **ABSORBANCE**

Sample solution: 0.24 mg/mL in isooctane

Acceptance criteria: The absorbance is NMT 0.73, determined at 233 nm.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at controlled room temperature. It may be bottled or otherwise packaged in containers from which air has been expelled by production of a vacuum or by an inert gas.
- **LABELING:** The label states the average content of DHA and EPA as free acids, in mg/g, and the total content of omega-3 acids as free acids, in mg/g. It also states the name and concentration of any added antioxidant.

Add the following:

▲Omega-3 Free Fatty Acids

DEFINITION

Omega-3 Free Fatty Acids is a mixture of omega-3 acids in their free form. It consists mainly of alpha-linolenic acid (C18:3 n–3), moroctic acid (C18:4 n–3), eicosatetraenoic acid (C20:4 n–3), eicosapentaenoic acid (EPA) (C20:5 n–3), heneicosapentaenoic acid (C21:5 n–3), docosapentaenoic acid (C22:5 n–3), and docosahexaenoic acid (DHA) (C22:6 n–3). It contains NLT 80% (w/w) of total omega-3 free fatty acids. It contains NLT 45% (w/w) and NMT 65% (w/w) of EPA, and NLT 10% (w/w) and NMT 30% (w/w) of DHA. Suitable antioxidants in appropriate concentrations may be added.

IDENTIFICATION

- **A.** The retention times of the peaks of EPA methyl ester and DHA methyl ester of the *Sample solution* correspond to those of the respective compounds of the *Standard solution*, as obtained in the test for *Content of EPA, DHA, and Total Omega-3 Free Fatty Acids*.
- **B.** It meets the requirements of the test for *Content of EPA, DHA, and Total Omega-3 Free Fatty Acids*.

COMPOSITION

- **CONTENT OF EPA, DHA, AND TOTAL OMEGA-3 FREE FATTY ACIDS**

System suitability solution 1: Transfer 250 mg of USP Omega-3 Free Fatty Acids RS and 70 mg of USP Methyl Tricosanoate RS (internal standard) to a 100-mL flask suitable for heating under reflux. Add 8 mL of a 2% (w/v) solution of sodium hydroxide in methanol to the flask. Heat the mixture under reflux while stirring until the sample is dissolved. Add through the reflux condenser 10 mL of 14% boron trichloride–methanol solution. Continue to reflux the solution for NLT 2 min, and then cool to room temperature in a water bath. Add 10 mL of *n*-hexane and then 20–30 mL of 10% (w/v) sodium chloride solution. Cap the flask and shake vigorously for NLT 30 s. Allow the layers to separate and dry the upper layer over anhydrous sodium sulfate before analysis.

System suitability solution 2: Transfer 55 mg of DHA methyl ester and about 5 mg of tetracos-15-enoic acid methyl ester, accurately weighed, to a 10-mL volumetric flask. Dissolve in and dilute with *n*-hexane to volume.

Standard solution: Transfer 70 mg of USP Methyl Tricosanoate RS (internal standard), 60.0 mg of USP Docosahexaenoic Acid RS, and 90.0 mg of USP Eicosapentaenoic Acid RS to a 100-mL flask suitable for heating under reflux. Prepare as directed for *System suitability solution 1* starting with "Add 8 mL".

Sample solution: Prepare as directed for *System suitability solution 1* except replace the USP Omega-3 Free Fatty Acids RS with Omega-3 Free Fatty Acids.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.25-mm × 25-m; coated with a 0.20-µm film of phase G16

Temperatures

Injection port: 250°

Detector: 270°

Column: See *Table 1*.

DS Monographs

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Table 1

Initial Temperature (°)	Hold Time at 170° (min)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
170	2	3	240	2.5

Carrier gas: Helium
Flow rate: 1 mL/min
Injection volume: 1 µL
Injection type: Split; split ratio, 200:1

System suitability

Samples: *System suitability solution 1*, *System suitability solution 2*, and *Standard solution*

[NOTE—The relative retention times for DHA methyl ester and tetracos-15-enoic acid methyl ester from *System suitability solution 2* are 1.0 and about 1.01, respectively. The relative retention times for EPA methyl ester, methyl tricosanoate (internal standard), and DHA methyl ester from the *Standard solution* are 1.0, about 1.2, and about 1.3, respectively.]

Suitability requirements

Chromatogram similarity: The chromatogram of *System suitability solution 1* is similar to the reference chromatogram provided with the lot of USP Omega-3 Free Fatty Acids RS being used.

Resolution: NLT 1.2 between DHA methyl ester and tetracos-15-enoic acid methyl ester, *System suitability solution 2*

Relative standard deviation: NMT 2.0% for peak response ratios of the EPA methyl ester and DHA methyl ester to the internal standard, *Standard solution*

Analysis

Samples: *System suitability solution 1*, *Standard solution*, and *Sample solution*

Identify the relevant fatty acid methyl esters (see *Table 2*) in the chromatogram of the *Sample solution* by comparing the peaks with those in the chromatogram of *System suitability solution 1* and the reference chromatogram provided with the lot of USP Omega-3 Free Fatty Acids RS being used.

Separately calculate the percentage of EPA and DHA, as free fatty acids, in the portion of Omega-3 Free Fatty Acids taken:

$$\text{Result} = (R_U/R_S) \times (W_S/W_U) \times 100$$

R_U = peak response ratio of the EPA methyl ester or DHA methyl ester to the internal standard from the *Sample solution*
R_S = peak response ratio of the EPA methyl ester or DHA methyl ester to the internal standard from the *Standard solution*
W_S = weight of USP Eicosapentaenoic Acid RS or USP Docosahexaenoic Acid RS taken to prepare the *Standard solution* (mg)
W_U = weight of Omega-3 Free Fatty Acids taken to prepare the *Sample solution* (mg)

Calculate the percentage of the total omega-3 free fatty acids in the portion of Omega-3 Free Fatty Acids taken:

$$\text{Result} = EPA + DHA + [A_{n-3} \times (EPA + DHA)/(A_{EPA} + A_{DHA})]$$

EPA = content of EPA, as free fatty acid (%)
DHA = content of DHA, as free fatty acid (%)

A_{n-3} = sum of the peak areas corresponding to C18:3 n–3, C18:4 n–3, C20:4 n–3, C21:5 n–3, and C22:5 n–3 methyl esters in the *Sample solution*
A_{EPA} = peak area corresponding to the EPA methyl ester in the *Sample solution*
A_{DHA} = peak area corresponding to the DHA methyl ester in the *Sample solution*

Acceptance criteria: See *Table 2*.

Table 2

Name	Relative Retention Time	Acceptance Criteria, NLT (%, w/v)	Acceptance Criteria, NMT (%, w/v)
C18:3 n–3, FFA[a]	0.564	—	—
C18:4 n–3, FFA[b]	0.592	—	—
C20:4 n–3, FFA[c]	0.770	—	—
C20:5 n–3, FFA (EPA)[d]	0.793	45	65
C21:5 n–3, FFA[e]	0.887	—	—
C22:5 n–3, FFA[f]	0.973	—	—
C22:6 n–3, FFA (DHA)[g]	1.000	10	30
Total omega-3 FFA	—	80	—

[a] Alpha-linolenic free fatty acid.
[b] Moroctic free fatty acid.
[c] Eicosatetraenoic free fatty acid.
[d] Eicosapentaenoic free fatty acid.
[e] Heneicosapentaenoic free fatty acid.
[f] Docosapentaenoic free fatty acid (clupanodonic free fatty acid).
[g] Docosahexaenoic free fatty acid.

IMPURITIES

• CHOLESTEROL

Internal standard stock solution: 3 mg/mL of 5α-cholestane in *n*-hexane. [NOTE—Prepare fresh before use.]

Internal standard solution: 0.3 mg/mL of 5α-cholestane in *n*-hexane. [NOTE—Prepare fresh before use.]

Standard stock solution: 3.0 mg/mL of USP Cholesterol RS in *n*-hexane. [NOTE—This solution is stable for 6 months stored in a freezer.] Transfer 1.0 mL of this solution to a 10.0-mL volumetric flask. Dilute with *n*-hexane to volume. [NOTE—Prepare this solution fresh daily.]

Standard solution: Transfer 1.0 mL each of the *Standard stock solution* and the *Internal standard solution* to a 15-mL centrifuge tube. Evaporate to dryness at about 50° with a gentle stream of nitrogen. Add 0.5 mL of 50% (w/v) potassium hydroxide and 3 mL of alcohol, fill the tube with nitrogen, and cap. Heat the sample at 100° for 60 min using a heating block. Cool for about 10 min. Add 6 mL of water to the tube, and shake for 1 min. Extract the solution 4 times with 2.5-mL portions of ethyl ether, using a vortex mixer or suitable shaker for 1 min for each extraction. Transfer and combine the extracts into a large centrifuge tube, and wash with 5 mL of water, mixing completely with gentle inversion. Remove the water phase, and add 5 mL of 0.5 M potassium hydroxide to the ether phase, mixing carefully to avoid an emulsion. Remove the potassium hydroxide, and add another 5 mL of water, mixing carefully. Transfer the ether phase to a small centrifuge tube. [NOTE—If an emulsion has occurred, a small amount of sodium chloride may be added to obtain a separation of

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the phases.] Evaporate the ether phase to dryness under a stream of nitrogen with careful heating. Dissolve the sample in 600 µL of ethyl acetate, and mix well. Transfer 200 µL of this solution to a sample vial, and dilute with ethyl acetate to about 2 mL.

Alpha tocopherol stock solution: 1.5–2.0 mg/mL of USP Alpha Tocopherol RS in *n*-hexane. [NOTE—This solution is stable for 12 months stored in a freezer.]

System suitability solution: Mix 1.0 mL of the *Standard stock solution*, 1.0 mL of the *Internal standard stock solution*, and 2.0 mL of the *Alpha tocopherol stock solution* in a 50-mL volumetric flask. Evaporate to dryness with the aid of heat, and dilute with ethyl acetate to volume. Dilute 1.0 mL of this solution with ethyl acetate to 10.0 mL. [NOTE—This solution is stable for 6 months stored in a freezer.]

Sample solution: Transfer 100 mg of Omega-3 Free Fatty Acids to a 15-mL centrifuge tube. Add 1.0 mL of the *Internal standard solution*. Prepare as directed for the *Standard solution* beginning with "Evaporate to dryness".

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.25-mm × 30-m capillary; coated with a 0.25-µm film of phase G27
Temperatures
Injection port: 320°
Detector: 300°
Column: See *Table 3*.

Table 3

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
170	0	170	1
170	4	320	1.5

Carrier gas: Helium
Flow rate: 1.3 mL/min
Injection volume: 1 µL
Injection type: Splitless
System suitability
Sample: *System suitability solution*
Suitability requirements
Resolution: NLT 1.2 between alpha tocopherol and cholesterol
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the content of total cholesterol in the portion of Omega-3 Free Fatty Acids taken:

$$\text{Result} = (R_U/R_S) \times (W_S/W_U)$$

R_U = peak area ratio of cholesterol to the internal standard from the *Sample solution*
R_S = peak area ratio of cholesterol to the internal standard from the *Standard solution*
W_S = weight of USP Cholesterol RS taken to prepare the *Standard solution* (mg)
W_U = weight of Omega-3 Free Fatty Acids taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 3.0 mg/g

• **OLIGOMERS**
Mobile phase: Tetrahydrofuran
System suitability solution: Monodocosahexaenoin, didocosahexaenoin, and tridocosahexaenoin[1] in *Mobile phase*, with concentrations of about 0.5, 0.3, and 0.2 mg/mL, respectively.
Sample solution: 1.00 mg/mL of Omega-3 Free Fatty Acids in *Mobile phase*
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Differential refractometer
Columns: 3 in series, 7.8-mm × 30-cm; 5-µm packing L21, with the following pore sizes.
Column 1: 50 nm
Column 2: 10 nm
Column 3: 5 nm
Connection sequence: Injector–*Column 1*–*Column 2*–*Column 3*–*Detector*
Flow rate: 0.8 mL/min
Injection volume: 40 µL
System suitability
Sample: *System suitability solution*
Suitability requirements
Elution order: Tridocosahexaenoin, didocosahexaenoin, and monodocosahexaenoin
Resolution: NLT 2.0 between monodocosahexaenoin and didocosahexaenoin; NLT 1.0 between didocosahexaenoin and tridocosahexaenoin
Analysis
Sample: *Sample solution*
Measure the areas of the major peaks.
Calculate the percentage of oligomers in the portion of Omega-3 Free Fatty Acids taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak area of oligomers (sum of the areas of the peaks with a retention time less than that of triglyceride) from the *Sample solution*
r_T = sum of the areas of all the peaks from the *Sample solution*

Acceptance criteria: NMT 2.0% of oligomers

• **LIMIT OF DIOXINS, FURANS, AND POLYCHLORINATED BIPHENYLS (PCBS):** Determine the content of polychlorinated dibenzo-para-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) by Method No. 1613 revision B of the Environmental Protection Agency. Determine the content of PCBs by Method No. 1668 revision A of the Environmental Protection Agency.
Acceptance criteria: The sum of PCDDs and PCDFs is NMT 1 pg/g of WHO toxic equivalents. The sum of PCBs (IUPAC congeners PCB-28, PCB-52, PCB-101, PCB-118, PCB-138, PCB-153, and PCB-180) is NMT 0.5 ppm.

• **LIMIT OF TOTAL UNIDENTIFIED FATTY ACID METHYL ESTERS**
From the chromatogram obtained with the *Sample solution* in the test for *Content of EPA, DHA, and Total Omega-3 Free Fatty Acids*, determine the area of the largest single unidentified peak with a relative retention time different from those in *Table 4*.

Table 4

Identified Fatty Acid Methyl Ester	Relative Retention Time
C16:1 n–7	0.322

[1] Suitable grades of monodocosahexaenoin, didocosahexaenoin, and tridocosahexaenoin may be obtained from Nu-Chek Prep.

Supplements

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Table 4 *(continued)*

Identified Fatty Acid Methyl Ester	Relative Retention Time
Phytanic acid	0.369
C18:1 n–9	0.470
C18:2 n–6	0.509
C18:3 n–6	0.535
C18:3 n–3	0.564
C18:4 n–3	0.592
C18:4 n–1	0.602
Furan acid 5	0.676
C20:2 n–6	0.705
C20:3 n–6	0.710
C20:4 n–6	0.731
C20:4 n–3	0.770
EPA	0.793
C21:5 n–3	0.887
Furan acid 10	0.913
C22:5 n–6	0.939
C22:5 n–3	0.973
DHA	1.000

Calculate the content of unidentified fatty acid methyl esters in area percentage:

$$\text{Result} = 100 - (100 \times \Sigma Ai/r_T)$$

Ai = peak area of each identified fatty acid methyl ester in *Table 4*

r_T = sum of the areas of all peaks except solvents and butylated hydroxytoluene (BHT)

Acceptance criteria: The area of the largest single unidentified peak is NMT 0.5% of the total area. The total area of unidentified peaks as previously calculated is NMT 2%.

SPECIFIC TESTS

- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Acid Value*: 165–195
- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Anisidine Value*: NMT 15.0
- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Peroxide Value*: NMT 10.0
- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Ester Value*: NMT 15.0
- **ABSORBANCE**

Sample solution: 0.24 mg/mL in isooctane

Acceptance criteria: NMT 0.73, determined at 233 nm, with isooctane being used as the blank

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at controlled room temperature. It may be bottled or otherwise packaged in containers from which air has been expelled by production of a vacuum or by an inert gas.
- **LABELING:** The label states the average content of DHA and EPA as free acids, and the total content of omega-3 acids as free acids. It also states the name and concentration of any added antioxidant.

- **USP REFERENCE STANDARDS** ⟨11⟩

USP Cholesterol RS
USP Docosahexaenoic Acid RS
USP Eicosapentaenoic Acid RS
USP Methyl Tricosanoate RS
USP Omega-3 Free Fatty Acids RS
USP Alpha Tocopherol RS ▲ (USP 1-Dec-2019)

Panthenol—*see Panthenol General Monographs*

Phenylalanine—*see Phenylalanine General Monographs*

Phyllanthus amarus

DEFINITION

Phyllanthus amarus consists of the dried aerial parts of *Phyllanthus amarus* Schumach. (Fam. Euphorbiaceae). It contains NLT 0.25% of lignans, calculated as the sum of phyllanthin and hypophyllanthin on the dried basis.

IDENTIFICATION

- **A.** *Phyllanthus amarus* meets the requirements in *Specific Tests* for *Botanical Characteristics*.
- **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩

Standard solution A: 0.1 mg/mL of USP Phyllanthin RS in methanol

Standard solution B: 10 mg/mL of USP Powdered *Phyllanthus amarus* Extract RS in methanol. Sonicate for about 10 min, centrifuge, and use the supernatant.

Sample solution: Sonicate about 0.5 g of *Phyllanthus amarus*, finely powdered, in 5 mL of methanol for 10 min, centrifuge, and use the supernatant.

Adsorbent: Chromatographic silica gel with an average particle size of 10–15 µm (TLC plates) or 5 µm (HPTLC plates)

Application volume: 10 µL (TLC plates) or 4 µL (HPTLC plates)

Developing solvent system: Hexane and ethyl acetate (2:1)

Derivatization reagent: A solution of 10% sulfuric acid in methanol. [NOTE—Prepare fresh.]

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands. Use a saturated chamber. Develop until the solvent front has moved up about three-fourths of the plate. Remove the plate from the chamber, dry in air, treat with the *Derivatization reagent*, heat for 3 min at 120°, and examine under white light.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits a blue band in the lower third of the plate due to phyllanthin, corresponding in color and R_F to that in the chromatogram of *Standard solution A*; a violet band due to hypophyllanthin at an R_F higher than that of phyllanthin; a blue band at an R_F higher than that of hypophyllanthin; and a violet band in the upper third of the plate. Bands detected in the chromatogram of the *Sample solution* correspond in position and color to bands in the chromatogram of *Standard solution B*. Other minor bands

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may be observed in the chromatograms of the *Sample solution* and *Standard solution B*.

- **C. HPLC:** The chromatogram of the *Sample solution* obtained in the test for *Content of Lignans* shows a peak at a retention time corresponding to that of phyllanthin in the chromatogram of *Standard solution A*. Identify other lignan peaks in the chromatogram of the *Sample solution* by comparison with the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered *Phyllanthus amarus* Extract RS being used. The chromatogram of the *Sample solution* shows an additional peak corresponding to hypophyllanthin.

COMPOSITION

- **CONTENT OF LIGNANS**

Solution A: Dissolve 0.14 g of potassium dihydrogen phosphate in 900 mL of water, add 0.5 mL of phosphoric acid, dilute with water to 1000 mL, mix, filter, and degas.

Mobile phase: Acetonitrile and *Solution A* (4:6)

Standard solution A: 0.1 mg/mL of USP Phyllanthin RS in methanol

Standard solution B: Sonicate a portion of USP Powdered *Phyllanthus amarus* Extract RS in methanol to obtain a solution having a concentration of about 5.0 mg/mL. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.

Sample solution: Transfer about 3.0 g of *Phyllanthus amarus*, finely powdered and accurately weighed, to a 250-mL flask fitted with a reflux condenser. Add 50 mL of methanol, reflux for about 20 min, allow to settle, and decant the supernatant. Repeat until the extract is colorless. Combine the extracts, concentrate under vacuum, transfer quantitatively into a 100-mL volumetric flask, and adjust to volume with methanol. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 230 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Flow rate: 1.5 mL/min

Injection volume: 10 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered *Phyllanthus amarus* Extract RS being used.

Resolution: NLT 1.5 between the phyllanthin and hypophyllanthin peaks, *Standard solution B*

Tailing factor: NMT 1.5 for the phyllanthin peak, *Standard solution A*

Relative standard deviation: NMT 2.0% determined from the phyllanthin peak in replicate injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*. [NOTE—*Standard solution A*, *Standard solution B*, and the *Sample solution* are stable for 48 h at room temperature.]

Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered *Phyllanthus amarus* Extract RS being used, identify the retention times of the peaks corresponding to phyllanthin and hypophyllanthin.

Separately calculate the percentages of phyllanthin and hypophyllanthin in the portion of *Phyllanthus amarus* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*

r_S = peak area of phyllanthin from *Standard solution A*

C_S = concentration of USP Phyllanthin RS in *Standard solution A* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of *Phyllanthus amarus* taken to prepare the *Sample solution* (mg)

F = conversion factor: 1.00 for phyllanthin; 0.75 for hypophyllanthin

Acceptance criteria: Add the percentages of phyllanthin and hypophyllanthin: NLT 0.25% is found on the dried basis.

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacterial count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: Erect annual herb, 10- to 60-cm high; slender stem, leaves on main stem are reduced to scales; secondary branchlets, short, extend at right angles, each carrying 15–30 leaves; leaves have a green upper surface with raised midrib and a pale green lower surface with prominent midrib and secondary veins, simple, alternate, 3–11 mm long, 1.5–6 mm wide, short petiolate, elliptical-oblong to obovate, apex obtuse and often with small pointed tip, margin entire, base often slightly asymmetric; flowers minute, yellowish, greenish, or whitish, unisexual, axillary on secondary branchlets, 1–2 and sometimes 3 per axil, first 1–2 internodes of each branchlet bear 1–2 male flowers, the rest have male and female flowers; fruits are flattened spherical capsules, straw color, 3-loculed, about 2 mm in diameter; seeds usually 2 per locule, light brown, about 0.9 mm long, triangular with 6–7 longitudinal ribs and many transverse striations on the back. Pharmacopeial article is green to yellowish-green masses composed mostly of leaves, branchlets, and stem fragments; taste bitter.

Microscopic

Transverse section of stems: Epidermal layer; about 15 layers of cortex cells, thick walled, contain chloroplasts, some contain calcium oxalate crystals, inner 7–10 layers are made of thick-walled cells interrupted at regular interval by parenchyma cells; a layer of parenchyma cells containing starch grains; phloem 7–10 layers of thin-walled cells; groups of xylem vessels; pith, multilayer of thin-walled cells, few contain calcium oxalate crystals.

Transverse section of branchlets: The transverse section is round; 6–8 layers of cortex, thick-walled cells, most contain chloroplasts and a few calcium oxalate crystals, after 3–4 layers there is a layer of cells containing starch grains, followed by 2–3 layers of fiber cells interrupted by

USP Monographs

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cortex parenchyma; phloem 5–7 layers of thin-walled cells; groups of xylem vessels; pith, multilayer of thin-walled cells, containing chloroplasts.

Transverse section of leaves: Upper and lower epidermis; a single layer of palisade cells, which occupy nearly half of the space between each epidermis; 3–5 layers of parenchyma cells, a few contain crystals of calcium oxalate; vascular bundles are also present.

- **LOSS ON DRYING** ⟨731⟩
  Sample: 1.0 g of finely powdered *Phyllanthus amarus*
  Analysis: Dry the *Sample* at 105° for 2 h.
  Acceptance criteria: NMT 12.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩
  Sample: 1.0 g of finely powdered *Phyllanthus amarus*
  Acceptance criteria: NMT 8.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩
  Sample: 1.0 g of finely powdered *Phyllanthus amarus*
  Acceptance criteria: NMT 5.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩**:** NMT 2.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Phyllanthin RS
  USP Powdered *Phyllanthus amarus* Extract RS

Powdered *Phyllanthus amarus*

DEFINITION

Powdered *Phyllanthus amarus* is *Phyllanthus amarus* reduced to a powder or very fine powder. It contains NLT 0.25% of lignans, calculated as the sum of phyllanthin and hypophyllanthin, on the dried basis.

IDENTIFICATION

- **A.** Powdered *Phyllanthus amarus* meets the requirements in *Specific Tests* for *Botanical Characteristics*.
- **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩
  Standard solution A: 0.1 mg/mL of USP Phyllanthin RS in methanol
  Standard solution B: 10 mg/mL of USP Powdered *Phyllanthus amarus* Extract RS in methanol. Sonicate for about 10 min, centrifuge, and use the supernatant.
  Sample solution: Sonicate about 0.5 g of Powdered *Phyllanthus amarus* in 5 mL of methanol for 10 min, centrifuge, and use the supernatant.
  Adsorbent: Chromatographic silica gel with an average particle size of 10–15 µm (TLC plates) or 5 µm (HPTLC plates)
  Application volume: 10 µL (TLC plates) or 4 µL (HPTLC plates)
  Developing solvent system: Hexane and ethyl acetate (2:1)
  Derivatization reagent: A solution of 10% sulfuric acid in methanol. [NOTE—Prepare fresh.]
  Analysis
  Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
  Apply the *Samples* as bands. Use a saturated chamber. Develop until the solvent front has moved up about three-fourths of the plate. Remove the plate from the chamber, dry in air, treat with the *Derivatization reagent*, heat for 3 min at 120°, and examine under white light.
  Acceptance criteria: The chromatogram of the *Sample solution* exhibits a blue band in the lower third of the plate due to phyllanthin, corresponding in color and R_F to that in the chromatogram of *Standard solution A*; a violet band due to hypophyllanthin at an R_F higher than that of phyllanthin; a blue band at an R_F higher than that of hypophyllanthin; and a violet band in the upper third of the plate. Bands detected in the chromatogram of the *Sample solution* correspond in position and color to bands in the chromatogram of *Standard solution B*. Other minor bands may be observed in the chromatograms of the *Sample solution* and *Standard solution B*.
- **C. HPLC:** The chromatogram of the *Sample solution* obtained in the test for *Content of Lignans* shows a peak at a retention time corresponding to that of phyllanthin in the chromatogram of *Standard solution A*. Identify other lignan peaks in the chromatogram of the *Sample solution* by comparison with the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered *Phyllanthus amarus* Extract RS being used. The chromatogram of the *Sample solution* shows an additional peak corresponding to hypophyllanthin.

COMPOSITION

- **CONTENT OF LIGNANS**
  Solution A: Dissolve 0.14 g of potassium dihydrogen phosphate in 900 mL of water, add 0.5 mL of phosphoric acid, dilute with water to 1000 mL, mix, filter, and degas.
  Mobile phase: Acetonitrile and *Solution A* (4:6)
  Standard solution A: 0.1 mg/mL of USP Phyllanthin RS in methanol
  Standard solution B: Sonicate a portion of USP Powdered *Phyllanthus amarus* Extract RS in methanol to obtain a solution having a concentration of about 5.0 mg/mL. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.
  Sample solution: Transfer about 3.0 g of Powdered *Phyllanthus amarus*, accurately weighed, to a 250-mL flask fitted with a reflux condenser. Add 50 mL of methanol, reflux for about 20 min, allow to settle, and decant the supernatant. Repeat until the extract is colorless. Combine the extracts, concentrate under vacuum, transfer quantitatively into a 100-ml volumetric flask, and adjust to volume with methanol. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: LC
  Detector: UV 230 nm
  Column: 4.6-mm × 25-cm; 5-µm packing L1
  Flow rate: 1.5 mL/min
  Injection volume: 10 µL
  System suitability
  Samples: *Standard solution A* and *Standard solution B*
  Suitability requirements
  Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered *Phyllanthus amarus* Extract RS being used.
  Resolution: NLT 1.5 between the phyllanthin and hypophyllanthin peaks, *Standard solution B*
  Tailing factor: NMT 1.5 for the phyllanthin peak, *Standard solution A*

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Relative standard deviation: NMT 2.0% determined from the phyllanthin peak in replicate injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*. [NOTE—*Standard solution A*, *Standard solution B*, and the *Sample solution* are stable for 48 h at room temperature.]

Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered *Phyllanthus amarus* Extract RS being used, identify the retention times of the peaks corresponding to phyllanthin and hypophyllanthin.

Separately calculate the percentages of phyllanthin and hypophyllanthin in the portion of Powdered *Phyllanthus amarus* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*

r_S = peak area of phyllanthin from *Standard solution A*

C_S = concentration of USP Phyllanthin RS in *Standard solution A* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Powdered *Phyllanthus amarus* taken to prepare the *Sample solution* (mg)

F = conversion factor: 1.00 for phyllanthin; 0.75 for hypophyllanthin

Acceptance criteria: Add the percentages of phyllanthin and hypophyllanthin: NLT 0.25% is found on the dried basis.

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩**:** Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacterial count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: Greenish to greenish-brown in color; taste bitter

Microscopic: Fragments of epidermal cells of the leaves with wavy walls, showing anisocytic and paracytic stomata; parenchyma cells, some showing clusters of calcium oxalate crystals; narrow fibers from the stem; pitted vessels of the stem; fragments of the epicarp of the fruits showing anomocytic stomata

- **LOSS ON DRYING** ⟨731⟩

Sample: 1.0 g of Powdered *Phyllanthus amarus*
Analysis: Dry the *Sample* at 105° for 2 h.
Acceptance criteria: NMT 12.0%

- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩

Sample: 1.0 g of Powdered *Phyllanthus amarus*
Acceptance criteria: NMT 8.0%

- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩

Sample: 1.0 g of Powdered *Phyllanthus amarus*
Acceptance criteria: NMT 5.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Phyllanthin RS
USP Powdered *Phyllanthus amarus* Extract RS

Phytonadione—*see Phytonadione General Monographs*

Phytonadione Tablets—*see Phytonadione Tablets General Monographs*

Plant Stanol Esters

DEFINITION

Plant Stanol Esters consists mainly of the fatty acid esters of plant sitostanol (24-ethylcholestan-3β-ol) and plant campestanol (24-methylcholestan-3β-ol). The sum of campestanol and sitostanol is NLT 56% (w/w). It is produced from esterification of plant stanols with fatty acids from rapeseed oil, sunflower oil, corn oil, soybean oil, and their blends. It can be a liquid or solid depending on the temperature and on the fatty acids with which plant stanols are esterified. A suitable antioxidant may be added.

IDENTIFICATION

- **A.** It meets the acceptance criteria for the *Content of Total Stanols* and *Content of Unesterified Stanols* tests.
- **B.** The retention times of the campestanol trimethylsilyl (TMS) and sitostanol TMS peaks from the *Derivatized sample solution* in the test for *Content of Total Stanols* correspond to the campestanol TMS and sitostanol TMS peaks from the *Derivatized standard solution*.

COMPOSITION

- **CONTENT OF TOTAL STANOLS**

Silylation reagent: Bis(trimethylsilyl)-trifluoracetamid (BSTFA) with 1% trimethylchlorosilane (TMCS)[1]

Saponification solution: 2 M potassium hydroxide solution in ethanol (11.22 g of potassium hydroxide dissolved in 100 mL of ethanol)

Internal standard solution: 0.6 mg/mL of USP Epicoprostanol RS in *n*-propanol

Derivatized internal standard solution: Transfer 500 µL of the *Internal standard solution* into a 12-mL screw-cap test tube and evaporate to dryness in a vacuum oven or under nitrogen flow. Add 200 µL of *Silylation reagent* and 100 µL of pyridine to the test tube, mix well, and heat for 15 min at 70°. Cool the solution to room temperature before injecting into the gas chromatograph.

Standard solution: Melt a portion of USP Plant Stanol Esters RS in an oven or microwave oven to obtain a clear and homogenous liquid. Transfer 40–70 mg of the melted USP Plant Stanol Esters RS, accurately weighed, to a 25-mL volumetric flask and dissolve in tetrahydrofuran. Sonicate

[1] BSTFA + TMCS (99:1) is available from Sigma-Aldrich, Product# 33148; www.sigmaaldrich.com/catalog/product/supelco/33148.

DS Monographs

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the solution using an ultrasonic bath for about 15 min. After sonication, allow the flask to cool to ambient temperature (for about 1 h) before filling up to the mark with tetrahydrofuran, and mix well.

Derivatized standard solution: Transfer 500 µL each of the *Internal standard solution* and the well-mixed *Standard solution* to a 12-mL screw-cap test tube. Evaporate the sample to dryness either under moderate nitrogen flow or in a vacuum oven (temperature not higher than 80°). Add 2.5 mL of *Saponification solution* to the residue, close the test tube cap tightly, mix well on a vortex mixer, and reflux in an ultrasonic bath with a heater at a temperature of 65°–75° for 1 h. Add 2 mL of deionized water and 3 mL of *n*-heptane to the mixture, mix on a vortex mixer for 1 min, and wait until the layers separate. If necessary, centrifuge the test tubes to separate the layers. Transfer the *n*-heptane fraction to another test tube and repeat the extraction of the aqueous phase with 3 mL of *n*-heptane. Collect the *n*-heptane fractions containing plant stanols and plant sterols in the same test tube and evaporate to dryness under moderate nitrogen flow. During the evaporation, test tubes can be warmed (approximately up to 70°). Transfer 200 µL of *Silylation reagent* and 100 µL of pyridine to the test tube, mix well, and heat for 15 min at 70°. Cool the solution to room temperature before injecting into the gas chromatograph.

Sample solution: Prepare as directed for the *Standard solution* except replace the USP Plant Stanol Esters RS with Plant Stanol Esters.

Derivatized sample solution: Prepare as directed for the *Derivatized standard solution* except replace the *Standard solution* with *Sample solution.*

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability.*)

Mode: GC

Detector: Flame ionization

Column: 0.32-mm x 30-m fused silica capillary; bonded with a 0.25-µm film of phase G27

Temperatures

Injection port: 310°

Detector: 310°

Column: 300°

Carrier gas: Helium

Flow rate: 1 mL/min

Injection volume: 1 µL

Injection type: Split ratio, 50:1

System suitability

Samples: *Derivatized internal standard solution* and *Derivatized standard solution*

Suitability requirements

[NOTE—Locate the epicoprostanol (internal standard) peak in the chromatogram of the *Derivatized standard solution* by comparing its retention time with that of the epicoprostanol peak in the chromatogram of the *Derivatized internal standard solution*. The relative retention times of the components in the *Derivatized standard solution* are shown in *Table 1*.]

Resolution: NLT 1.0 between sitosterol and sitostanol, *Derivatized standard solution*

Relative standard deviation: NMT 2.0% for the epicoprostanol peak, *Derivatized internal standard solution*

Chromatogram similarity: The chromatogram obtained from the *Derivatized standard solution* is similar to the reference chromatogram provided with the lot of USP Plant Stanol Esters RS being used.

Table 1

Name	Relative Retention Time
Epicoprostanol	1.00
Cholestanol	1.17
Campesterol	1.34
Campestanol	1.37
Sitosterol	1.54
Sitostanol	1.57

Analysis

Sample: *Derivatized sample solution*

[NOTE—The response factor of epicoprostanol is equivalent to that of campestanol or sitostanol.]

Separately calculate the percentage (w/w) of campestanol and sitostanol in the portion of Plant Stanol Esters taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak response of campestanol or sitostanol from the *Derivatized sample solution*

r_S = peak response of epicoprostanol from the *Derivatized sample solution*

C_S = concentration of USP Epicoprostanol RS in the *Derivatized sample solution* (mg/mL)

C_U = concentration of Plant Stanol Esters in the *Derivatized sample solution* (mg/mL)

F = purity factor of USP Epicoprostanol RS (obtained from the RS label)

Acceptance criteria

Sum of campestanol and sitostanol: NLT 56% (w/w) of total stanols [sum of percentages (w/w) of campestanol and sitostanol]. The percentage of campestanol and sitostanol in total stanols is NMT 32% and NLT 68%, respectively.

• **CONTENT OF UNESTERIFIED STANOLS**

Silylation reagent, Internal standard solution, Derivatized internal standard solution, and **Chromatographic system:** Proceed as directed in the test for *Content of Total Stanols.*

Sample solution: Transfer 400 µL of the *Internal standard solution* to a 12-mL screw-cap test tube and evaporate to dryness either under moderate nitrogen flow or in a vacuum oven (temperature not higher than 80°). Melt a portion of Plant Stanol Esters in an oven or microwave oven to obtain a clear and homogenous liquid. Transfer 30–70 mg of the melted Plant Stanol Esters, accurately weighed, to the test tube containing the evaporated internal standard. Transfer 1 mL of a mixture of petroleum ether and diethyl ether (90:10) into the test tube and mix to dissolve (if necessary, use a vortex mixer and/or heat to help dissolve the sample).

Fractionation by solid phase extraction (SPE): Activate an SPE tube (NH_2 phase, 3-mL volume, 500-mg sorbent[2]) by rinsing it twice with 3 mL of a mixture of petroleum ether and diethyl ether (90:10). Allow the solvent to flow under gravity through the tube. After activation, transfer the *Sample solution* into the SPE tube. Wash stanol esters out of the SPE tube with 2 x 3 mL of a mixture of petroleum ether and diethyl ether (90:10). Elute the unesterified stanols and sterols from the SPE tube with 2 x 3 mL of acetone and collect the eluate into a test tube. Evaporate the free stanol/

[2] DSC-NH_2 SPE tube is available commercially from Sigma-Aldrich or other suppliers.

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sterol fraction to dryness under nitrogen flow. [NOTE—Do not allow the SPE tube to dry during the whole procedure.]

Derivatized sample solution: Transfer 200 µL of *Silylation reagent* and 100 µL of pyridine to the test tube containing free stanol/sterol obtained from the fractionation step above, mix well, and heat at 70° for 15 min. Cool the solution to room temperature before injecting into the gas chromatograph.

System suitability

Samples: *Derivatized internal standard solution* and *Derivatized sample solution*

Suitability requirements

[NOTE—Locate the epicoprostanol peak in the chromatogram of the *Derivatized sample solution* by comparing its retention time with that of the epicoprostanol peak in the chromatogram of the *Derivatized internal standard solution*. The relative retention times of the components in the *Derivatized sample solution* are shown in *Table 2*.]

Table 2

Name	Relative Retention Time
Epicoprostanol	1.00
Campestanol	1.37
Sitostanol	1.57

Analysis

Sample: *Derivatized sample solution*

[NOTE—The response factor of epicoprostanol is equivalent to that of campestanol or sitostanol.]

Separately calculate the percentage (w/w) of unesterified campestanol and unesterified sitostanol in the portion of Plant Stanol Esters taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak response of campestanol or sitostanol from the *Derivatized sample solution*

r_S = peak response of epicoprostanol from the *Derivatized sample solution*

C_S = concentration of USP Epicoprostanol RS in the *Derivatized sample solution* (mg/mL)

C_U = concentration of Plant Stanol Esters in the *Derivatized sample solution* (mg/mL)

F = purity factor of USP Epicoprostanol RS (obtained from the RS label)

Acceptance criteria: NMT 3% (w/w) of unesterified stanols (unesterified campestanol + unesterified sitostanol)

• **CONTENT OF PLANT STANOL ESTERS**

Calculate the percentage of plant stanol esters in the portion of Plant Stanol Esters taken:

$$\text{Result} = (S_T - S_U) \times F_W$$

S_T = sum of campestanol and sitostanol from the test for *Content of Total Stanols* (%)

S_U = sum of unesterified campestanol and unesterified sitostanol from the test for *Content of Unesterified Stanols* (%)

F_W = 1.63, molecular weight proportion of plant stanols from an average plant stanol fatty acid ester molecule for plant stanol ester esterified with fatty acids derived from main vegetable oil such as rapeseed oil, sunflower oil, corn oil, soybean oil, and their blends. The factor depends on the fatty acid composition of plant stanol esters.

Acceptance criteria: NLT 91% (w/w) of plant stanol esters

CONTAMINANTS

• **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩

Acceptance criteria

Arsenic: NMT 1.0 µg/g

Lead: NMT 1.0 µg/g

SPECIFIC TESTS

• **WATER DETERMINATION** ⟨921⟩, *Method I*: NMT 0.1%

• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Peroxide Value*: NMT 10.0

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed, light resistant containers. Store in a refrigerator.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Epicoprostanol RS

(3α,5β)-Cholestan-3-ol.

$C_{27}H_{48}O$ 388.67

USP Plant Stanol Esters RS

Potassium Citrate—*see Potassium Citrate General Monographs*

Potassium Citrate Tablets

DEFINITION

Potassium Citrate Tablets contain NLT 90.0% and NMT 110.0% of the labeled amount of potassium (K).

IDENTIFICATION

• **A.** The *Sample solution* for *Strength* produces line emissions or absorptions at the characteristic wavelengths for potassium.

• **B. IDENTIFICATION TESTS—GENERAL,** *Citrate* ⟨191⟩

Analysis: Grind a Tablet to a fine powder in a mortar. Transfer the powder to a centrifuge tube, add 2–5 mL of water, sonicate for 1 min, shake, and centrifuge.

Acceptance criteria: The supernatant meets the requirements for the test.

STRENGTH

• **CONTENT OF POTASSIUM, PROCEDURE 1**

[NOTE—A standard stock solution is commercially available at different potassium concentrations, which may be used for preparation of the *Standard stock solution*. Necessary volumetric adjustment can be made in the *Standard solution*. Concentrations of the *Standard solution* and the *Sample solution* may be modified to fit the linear or working range of the instrument.]

Standard stock solution: Solution of potassium chloride, previously dried at 105° for 2 h, in water containing 1000 mg/L of potassium.

Standard solution: To a 50-mL volumetric flask add 20 mL of water and 1 mL of nitric acid, and mix thoroughly. Pipet 10.0 mL of the *Standard stock solution* into a volumetric flask, and dilute with water to volume to obtain a solution having a known concentration of about 200 µg/mL of potassium.

Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer an accurately weighed portion of the powdered Tablets, equivalent to about 0.1 g of potassium, to a 50-mL

USP Monographs

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flask. Add 10 mL of nitric acid, and heat the solution to a gentle boil, during which fuming evolves. Boil the solution for an additional 30 min with constant swirling, during which time no fuming should be observed. Cool the solution to room temperature. Quantitatively transfer all of the solution to a 500-mL volumetric flask, dilute with water to volume, mix, and filter.

Inductively coupled plasma system
(See *Plasma Spectrochemistry* ⟨730⟩.)
Mode: Atomic emission spectroscopy
Analytical wavelength: 766.49 nm. [NOTE—The operating conditions may be developed and optimized based on the manufacturer's recommendation. A typical setting includes radio frequency (RF) power of about 1300 watts, argon torch flow of about 15 L/min, argon auxiliary flow of about 0.2 L/min, and a nebulizer flow rate of about 0.8 L/min.]
Blank: 2% nitric acid solution
Analysis
Samples: *Standard solution*, *Sample solution*, and *Blank*
Calculate the percentage of the labeled amount of potassium (K) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = response from the *Sample solution*
r_S = response from the *Standard solution*
C_S = concentration of potassium in the *Standard solution* (µg/mL)
C_U = nominal concentration of potassium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–110.0% of the labeled amount of potassium

• **CONTENT OF POTASSIUM, PROCEDURE 2**
Standard stock solution A: 100 µg/mL of potassium chloride, previously dried at 105° for 2 h, in water
Standard stock solution B: 10 µg/mL of potassium from *Standard stock solution A* in 0.125 N hydrochloric acid
Standard solutions: Transfer 5.0, 10.0, 15.0, 20.0, and 25.0 mL of *Standard stock solution B* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions containing 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of potassium.
Sample solution: Finely powder NLT 20 Tablets. Transfer an equivalent to 5 Tablets to a porcelain crucible. Heat the crucible in a muffle furnace maintained at 550° for 6–12 h, and cool. Add 60 mL of hydrochloric acid, and boil gently on a hot plate or steam bath for 30 min, intermittently rinsing the inner surface of the crucible with 6 N hydrochloric acid. Cool, and quantitatively transfer the contents of the crucible to a 100-mL volumetric flask. Rinse the crucible with small portions of 6 N hydrochloric acid, and add the rinsings to the flask. Dilute with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute this solution quantitatively with 0.125 N hydrochloric acid to obtain a nominal concentration of 2 µg/mL of potassium.
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Analytical wavelength: 766.5 nm
Lamp: Potassium hollow-catode
Flame: Air–acetylene
Blank: 0.125 N hydrochloric acid
Analysis
Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions, using the *Blank*. Plot the absorbances of the *Standard solutions* versus concentration, in µg/mL, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL of potassium in the *Sample solution*.
Calculate the percentage of the labeled amount of potassium (K) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = determined concentration of potassium in the *Sample solution* interpolated from the graph (µg/mL)
C_U = nominal concentration of potassium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–110.0% of the label claim

SPECIFIC TESTS
• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined yeast and mold count does not exceed 10^2 cfu/g.
• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meet the requirement of the test for absence of *Escherichia coli*

PERFORMANCE TESTS
• **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩
Medium: Water; 900 mL
Apparatus 2: 75 rpm
Time: 30 min
Analysis: Proceed as directed in *Content of Potassium, Procedure 1* or *Procedure 2*, for *Strength*, making any necessary volumetric adjustments.
Sample solution: If *Content of Potassium, Procedure 1* is used, pipet 10.0 mL of the filtered pooled solution under test to a 50-mL volumetric flask, and dilute with 2% nitric acid solution to 50 mL. If *Content of Potassium, Procedure 2* is used, dilute the filtered pooled solution under test with 0.125 N hydrochloric acid to a concentration falling within the range of the *Standard solutions*.
Calculate the percentage of the labeled amount of potassium (K) dissolved:

$$\text{Result} = (C \times D \times V/L) \times 100$$

C = measured concentration of potassium in the *Sample solution* (mg/mL)
D = dilution factor for the *Sample solution*
V = volume of *Medium*, 900 mL
L = label claim (mg/Tablet)

Tolerances: NLT 75% of the labeled amount of K is dissolved.
• **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩ **:** Meet the requirements

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers.
• **LABELING:** The label states the quantity of potassium in terms of mg/Tablet.

Potassium Gluconate—*see Potassium Gluconate General Monographs*

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Potassium Gluconate Oral Solution—*see Potassium Gluconate Oral Solution General Monographs*

Potassium Gluconate Tablets—*see Potassium Gluconate Tablets General Monographs*

Proline—*see Proline General Monographs*

Pygeum

DEFINITION

Pygeum consists of the bark of *Prunus africana* (Hook f.) Kalkman (*Pygeum africanum* Hook f.) (Fam. Rosaceae). It contains NLT 9.0% of extractable matter.

IDENTIFICATION

• **A. Thin-Layer Chromatography**

Standard solution A: 10 mg/mL of USP Pygeum Extract RS in chloroform

Standard solution B: 1 mg/mL of USP β-Sitosterol RS in chloroform

Sample solution: Transfer 10 g of the powdered plant material to a Soxhlet apparatus. Extract with 150 mL of methylene chloride for 4 h. Evaporate the extract under vacuum to dryness. Dissolve the residue with 10 mL of methylene chloride.

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Adsorbent: 0.25-mm layer of chromatographic silica gel (TLC plates)

Application volume: 10 µL

Developing solvent system: Methylene chloride

Derivatization reagent: Sulfuric acid and water (1:1)

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop in a saturated chamber for NLT 15 cm, and dry the plate in a stream of air. Treat with *Derivatization reagent*, heat the plate at 100° for 10 min, and examine under white light.

Acceptance criteria: The chromatogram of the *Sample solution* shows one red-violet zone turning to grayish-brown near the origin that corresponds in color and R_F value to that in the chromatogram of *Standard solution A*; one red-violet zone turning to grayish-brown at an R_F about 0.08, corresponding in color and R_F value to that in the chromatogram of *Standard solution B*; further upward, a grayish-brown zone may be present, corresponding in color and R_F value to that in the chromatogram of *Standard solution A*. Other colored zones of varying intensities may be observed in the chromatogram of the *Sample solution*.

COMPOSITION

• **Extractable Matter**

Analysis: Extract 2.00 g of the powdered material in a Soxhlet apparatus with 150 mL of alcohol for 6 h. Evaporate the solution to dryness under vacuum, and dry the residue at 105° for 24 h.

Acceptance criteria: NLT 9.0% of extractable matter

CONTAMINANTS

• **Articles of Botanical Origin,** *Limits of Elemental Impurities* ⟨561⟩: Meets the requirements

• **Articles of Botanical Origin,** *Pesticide Residue Analysis* ⟨561⟩: Meets the requirements

SPECIFIC TESTS

• **Botanical Characteristics**

Macroscopic: Bark pieces consist of long fragments of variable dimensions, from only a few cm to 1 m long with a thickness varying from a few mm to 1–2 cm. The color is brown, more or less dark on the external surface; light brown to red-brown on the internal surface. The external face of the bark presents a very dark and fissured rhytidome that in the samples of old trees is fragmented in more or less square plaques of about 1–5 cm. The thickness varies from 1 mm in young plants or branches to 5–8 mm in old plants. The outer surface may also be covered with whitish lichens or thin filamentous moss. The internal face of the bark, under the rhytidome, is clearer and has a reddish coloration, with a long fibrous break, from reddish to light brown or dark brown in color, often presenting concentric stratification cracks. The internal surface presents small wrinkles.

Microscopic: The transverse section of the bark presents a suberized bed of variable thickness depending on the age of the plant, consisting of multiple layers of small, square cells with the walls of moderate thickness. It presents a cortical parenchyma of more or less round cells, with a few apparent formations of very thin-walled sclereids, definitely sharp. Often, in the parenchyma, there are groups of cells containing oxalate druses; a few bigger cells with highly thickened walls can also be observed. It shows a liber with phloem zones and medullary rays. The phloemical portions contain groups of fibrous cells with highly thickened walls, as well as phloemical and parenchymal elements, sometimes containing druses of oxalate. The medullary rays are of conical shape, larger on the external surface and thinner on the internal side; they can also contain druses of oxalate.

• **Articles of Botanical Origin,** *Foreign Organic Matter* ⟨561⟩: NMT 5.0%

• **Loss on Drying** ⟨731⟩: Dry at 60° for 15 h; it loses NMT 10.0% of its weight.

• **Articles of Botanical Origin,** *Total Ash* ⟨561⟩: NMT 10.0%

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Preserve in well-closed containers, and store at room temperature.

• **Labeling:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.

• **USP Reference Standards** ⟨11⟩

USP Pygeum Extract RS

USP β-Sitosterol RS

Pygeum Extract

DEFINITION

Pygeum Extract is prepared from pulverized Pygeum, using suitable solvents. It contains NLT 90% and NMT 110% of the labeled amount of docosyl ferulate and NLT 90% and NMT 110% of the labeled amount of total sterols as β-sitosterol, calculated on the dried basis.

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IDENTIFICATION

• **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution A: 15 mg/mL of USP Pygeum Extract RS in chloroform

Standard solution B: 2 mg/mL of USP β-Sitosterol RS in chloroform

Sample solution: Dissolve 150 mg of Extract in 10 mL of chloroform.

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Adsorbent: 0.25-mm layer of chromatographic silica gel

Application volume: 10 µL

Developing solvent system: Methylene chloride in a saturated chamber

Spray reagent: Sulfuric acid and water (1:1)

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatograms to a length of NLT 15 cm, and dry the plate in a current of air. Spray the plate with *Spray reagent*, and heat the plate at 100° for 10 min. Examine the plate under white light.

Acceptance criteria: The chromatogram from the *Sample solution* shows one red-violet zone turning to grayish-brown near the origin that corresponds in color and R_F value to that of *Standard solution A*, and one red-violet zone turning to grayish-brown at an R_F of 0.08 corresponding in color and R_F value to that in the chromatogram of *Standard solution B*; above these spots a grayish-brown zone may be present, corresponding in color and R_F value to that of *Standard solution A*; and other colored zones of varying intensities may be observed in the *Sample solution*.

• **B. HPLC IDENTIFICATION TEST**

Analysis: Proceed as directed in the test for *Content of Docosyl Ferulate*.

Acceptance criteria: The chromatogram of the *Sample solution* presents a peak for docosyl ferulate that corresponds in retention time to the principal peak in the chromatogram of the *Standard solution*.

COMPOSITION

• **CONTENT OF STEROLS**

Derivatizing solution: Bis(trimethylsilyl)acetamide and trimethylchlorosilane (9:1)

Internal standard solution: 2 mg/mL of 5α-cholestane in chloroform

System suitability stock solution: 2 mg/mL each of campesterol, stigmasterol, and USP β-Sitosterol RS

System suitability solution: Mix 2.0 mL of the *System suitability stock solution* and 2.0 mL of the *Internal standard solution*, and dilute with chloroform to 10 mL. Evaporate 500 µL of this solution to dryness using a stream of nitrogen. Dissolve the residue in 80 µL of *Derivatizing solution* and 20 µL of pyridine. Allow to stand for NLT 10 min at room temperature.

Standard stock solution: 2.0 mg/mL of USP β-Sitosterol RS in chloroform

Standard solution: Mix 2.0 mL of the *Standard stock solution* and 2.0 mL of the *Internal standard solution*, and dilute with chloroform to 10 mL. Evaporate 500 µL of this solution to dryness using a stream of nitrogen. Dissolve the residue in 80 µL of *Derivatizing solution* and 20 µL of pyridine. Allow to stand for NLT 10 min at room temperature.

Sample solution: Transfer 100 mg of Extract into a 100-mL round-bottom flask. Add 2.0 mL of *Internal standard solution* and 20 mL of diluted hydrochloric acid. Attach a condenser, and reflux in a bath at 100° for 30 min. Cool the solution to room temperature, and adjust by the addition of about 5 mL of 10 N sodium hydroxide to a pH of 8. Extract twice using 50 mL of ether each time, wash the collected organic phases with 50 mL of water, and evaporate the organic phase to dryness under vacuum. Dissolve the residue with 4 mL of chloroform, and transfer to a cartridge containing 500 mg of packing L8[1] that has been conditioned with a 2-column volume of *n*-hexane. Collect the eluate. Elute twice with a 1-column volume of a mixture of chloroform and isopropanol (2:1). Combine the eluates, and evaporate to dryness. Dissolve the residue in 10 mL of chloroform. Evaporate 500 µL of this solution to dryness under a stream of nitrogen. Dissolve the residue with 80 µL of *Derivatizing solution* and 20 µL of pyridine. Allow to stand for NLT 10 min at room temperature.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.32-mm × 30-m capillary; G27 phase coating of 0.25-µm thickness

Temperature

Injector: 285°

Detector: 285°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
250	0	250	5
250	5	320	0

Carrier gas: Helium. [NOTE—The carrier gas flow rate should be adjusted to obtain a retention time of about 19 min for β-sitosterol.]

Makeup gas: Helium

Injection size: 2 µL

Injection type: Split injection system

Split ratio: 1:50

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for 5α-cholestane, campesterol, stigmasterol, and β-sitosterol are about 0.66, 0.94, 0.96, and 1.00, respectively.]

Suitability requirements

Resolution: NLT 2 between campesterol and stigmasterol

Column efficiency: NLT 150,000 theoretical plates for the 5α-cholestane peak

Tailing factor: NMT 2.0 for each relevant peak

Analysis

Samples: *Standard solution* and *Sample solution*

Identify the signals corresponding to the relevant analytes by comparison with the chromatograms obtained with the *System suitability solution*.

Separately calculate the individual percentages of campesterol, stigmasterol, and β-sitosterol, as β-sitosterol, respectively, in the portion of Pygeum Extract taken:

$$C_i = (R_U/R_S) \times C_S \times (V/W) \times 100$$

R_U = ratio of the appropriate sterol peak to the internal standard from the *Sample solution*

[1] A suitable cartridge is Chromabond NH2, manufactured by Macheray Nagel, or equivalent.

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R_S = ratio of the β-sitosterol peak to the 5α-cholestane internal standard from the *Standard solution*
C_S = concentration of β-sitosterol in the *Standard stock solution* (mg/mL)
V = volume of the *Standard stock solution* taken to prepare the *Standard solution* (mL)
W = weight of Pygeum Extract taken to prepare the *Sample solution* (mg)

Calculate the percentage of the labeled amount of total sterols as β-sitosterol:

$$\text{Result} = (\Sigma C_i / L) \times 100$$

C_i = individual percentage of each sterol as calculated above
L = labeled amount of total sterols in the Pygeum Extract taken

Acceptance criteria: 90%–110% of the labeled amount of total sterols as β-sitosterol on the dried basis

• **CONTENT OF DOCOSYL FERULATE**

Solution A: Methanol and water (95:5)
Solution B: Acetonitrile
Mobile phase: *Solution A* and *Solution B* (17:3)
Standard solution: Dissolve a quantity of USP Docosyl Ferulate RS in chloroform, and dilute with acetonitrile to obtain a concentration of 0.02 mg/mL. Pass through a filter of 0.45-µm or finer pore size.
Sample solution: To 250 mg of Extract add 5 mL of chloroform, and dilute with acetonitrile to 25 mL. Pass through a filter of 0.45-µm or finer pore size, discarding the first 4 mL of filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 323 nm
Column: 4-mm × 25-cm; packing L7
Column temperature: 25°
Flow rate: 1 mL/min
Injection size: 20 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Column efficiency: NLT 1700 theoretical plates for the docosyl ferulate peak
Tailing factor: NMT 2.0 for docosyl ferulate
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of docosyl ferulate (P) in the portion of Extract taken:

$$P = (r_U / r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area for docosyl ferulate from the *Sample solution*
r_S = peak area for docosyl ferulate from the *Standard solution*
C_S = concentration of USP Docosyl Ferulate RS in the *Standard solution* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Extract taken to prepare the *Sample solution* (mg)

Calculate the percentage of the labeled amount of docosyl ferulate:

$$\text{Result} = (P/L) \times 100$$

P = percent of docosyl ferulate in the portion of Extract taken
L = labeled amount of docosyl ferulate in the Pygeum Extract (%)

Acceptance criteria: 90%–110% of the labeled amount of docosyl ferulate on the dried basis

CONTAMINANTS

• **ARTICLES OF BOTANICAL ORIGIN,** *Test for Aflatoxins* ⟨561⟩**:** NMT 4 µg/kg of total aflatoxins B1, B2, G1, and G2; NMT 2 µg/kg of aflatoxin B1

Change to read:

• **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues:*▲ (CN 1-May-2020) Meets the requirement
• **BOTANICAL EXTRACTS,** *Preparations* ⟨565⟩**:** Meets the requirements in *General Pharmacopeial Requirements, Residual Solvents*
• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

• **LOSS ON DRYING** ⟨731⟩**:** Dry 1.0 g of Extract for 3 h at 110°: it loses NMT 10% of its weight.
• **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 0.5%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Store in tight containers, protected from light.
• **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. Label the content as a percentage of total sterols as β-sitosterol and the content as a percentage of docosyl ferulate. It also meets the requirements in *Botanical Extracts* ⟨565⟩, *Labeling*.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Docosyl Ferulate RS
USP Pygeum Extract RS
USP β-Sitosterol RS

Pygeum Capsules

DEFINITION

Pygeum Capsules contain Pygeum Extract. Capsules contain NLT 90.0% and NMT 110.0% of the labeled amount of Extract, calculated as sterols and docosyl ferulate.

IDENTIFICATION

• **A.** The retention times of the peaks for campesterol, stigmasterol, and β-sitosterol, of the *Sample solution*, correspond to those of the *Standard solution*, as obtained in the test for *Content of Sterols*.
• **B.** The retention time of the peak for docosyl ferulate in the *Sample solution* corresponds to that in the *Standard solution*, as obtained in the test for *Content of Docosyl Ferulate*.

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STRENGTH

• **CONTENT OF STEROLS**

Derivatizing solution: Bis(trimethylsilyl)acetamide and trimethylchlorosilane (9:1)

Internal standard solution: 2 mg/mL of 5α-cholestane in chloroform

System suitability stock solution: 2 mg/mL each of campesterol, stigmasterol, and USP β-Sitosterol RS

System suitability solution: Mix 2.0 mL of the *System suitability stock solution* and 2.0 mL of the *Internal standard solution*, and dilute with chloroform to 10 mL. Evaporate 500 µL of this solution to dryness using a stream of nitrogen. Dissolve the residue in 80 µL of *Derivatizing solution* and 20 µL of pyridine. Allow to stand for NLT 10 min at room temperature.

Standard stock solution: 2.0 mg/mL of USP β-Sitosterol RS in chloroform

Standard solution: Mix 2.0 mL of the *Standard stock solution* and 2.0 mL of the *Internal standard solution*, and dilute with chloroform to 10 mL. Evaporate 500 µL of this solution to dryness using a stream of nitrogen. Dissolve the residue in 80 µL of *Derivatizing solution* and 20 µL of pyridine. Allow to stand for NLT 10 min at room temperature.

Sample solution: Transfer a quantity of Capsules, equivalent to 100 mg of the labeled amount of Extract, into a 100-mL round-bottomed flask. Add 2.0 mL of the *Internal standard solution* and 20 mL of diluted hydrochloric acid. Attach a condenser, and reflux in a bath at 100° for 30 min. Cool the solution to room temperature, and adjust by the addition of about 5 mL of 10 N sodium hydroxide to a pH of 8. Extract twice using 50 mL of ether each time, wash the collected organic phases with 50 mL of water, and evaporate the organic phase to dryness under vacuum. Dissolve the residue with 4 mL of chloroform, and transfer to a cartridge containing 500 mg of packing L8 that has been conditioned with a 2-column volume of *n*-hexane. [NOTE—A suitable cartridge is Chromabond NH2, manufactured by Macheray Nagel, or equivalent.] Collect the eluate. Elute twice with a 1-column volume of a mixture of chloroform and isopropanol (2:1). Combine the eluates, and evaporate to dryness. Dissolve the residue in 10 mL of chloroform. Evaporate 500 µL of this solution to dryness under a stream of nitrogen. Dissolve the residue with 80 µL of *Derivatizing solution* and 20 µL of pyridine. Allow to stand for NLT 10 min at room temperature.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.32-mm × 30-m capillary column coated with a G27 phase of 0.25-µm thickness

Temperature

Detector: 285°

Injector: 285°

Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
250	0	250	5
250	5	320	as needed

Carrier gas: Helium. [NOTE—The carrier gas flow rate should be adjusted to obtain a retention time of 19 min for β-sitosterol.]

Makeup gas: Helium

Injection size: 2 µL

Injection type: Split injection system

Split ratio: 1:50

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for 5α-cholestane, campesterol, stigmasterol, and β-sitosterol are about 0.66, 0.94, 0.96, and 1.00, respectively.]

Suitability requirements

Resolution: NLT 2 between campesterol and stigmasterol

Column efficiency: NLT 150,000 theoretical plates for the 5α-cholestane peak

Tailing factor: NMT 2.0 for each relevant peak

Analysis

Samples: *Standard solution* and *Sample solution*

Identify the signals corresponding to the relevant analytes by comparison with the chromatograms obtained with the *System suitability solution*.

Calculate the percentages of the labeled amount of Extract as sterols in the portion of the Capsules taken:

$$\text{Result} = (\Sigma R_U/R_S) \times (C_S \times V/W) \times (A_W \times 100/L_E) \times 100/L$$

ΣR_U = ratio of the sum of peak responses for campesterol, stigmasterol, and β-sitosterol relative to the internal standard from the *Sample solution*

R_S = ratio of the β-sitosterol peak relative to the internal standard from the *Standard solution*

C_S = concentration of β-sitosterol in the *Standard stock solution* (mg/mL)

V = volume of the *Standard stock solution* used to prepare the *Standard solution* (2.0 mL)

W = weight of the sample of Capsules taken to prepare the *Sample solution* (mg)

A_W = average weight of the Capsules contents (mg)

L_E = content of sterols in 100 mg of the Extract used to prepare the Capsules (mg)

L = labeled amount of Extract per Capsule (mg/Capsule)

Acceptance criteria: 90%–110% of the labeled amount of Extract, calculated as sterols

• **CONTENT OF DOCOSYL FERULATE**

Solution A: Methanol and water (95:5)

Solution B: Acetonitrile

Mobile phase: *Solution A* and *Solution B* (17:3)

Standard solution: Dissolve a quantity of USP Docosyl Ferulate RS in chloroform, and dilute stepwise if necessary, with acetonitrile to obtain a concentration of 0.01 mg/mL. Pass through a membrane filter of 0.45-µm or finer pore size.

Sample solution: Weigh the contents of NLT 20 Capsules and transfer a quantity of the material, equivalent to 0.2 mg of docosyl ferulate, to a 50-mL beaker. Add 5 mL of chloroform and dissolve in an ultrasonic bath. Transfer to a 20-mL volumetric flask with the aid of NMT 2 mL of chloroform. Dilute with acetonitrile to volume and mix. Pass through a membrane filter of 0.45-µm or finer pore size.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 323 nm

Column: 4-mm × 25-cm; packing L7

Column temperature: 25°

Flow rate: 1 mL/min

Injection size: 320 µL

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System suitability
Sample: *Standard solution*
Suitability requirements
Column efficiency: NLT 1700 theoretical plates for the docosyl ferulate peak
Tailing factor: NMT 2.0 for docosyl ferulate
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the areas of the analyte peaks.
Calculate the percentage of the labeled amount of Extract as docosyl ferulate in the portion of the Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S \times V/W) \times (A_W \times 100/L_E) \times 100/L$$

r_U = peak response for docosyl ferulate from the *Sample solution*
r_S = peak response for docosyl ferulate from the *Standard solution*
C_S = concentration of USP Docosyl Ferulate RS in the *Standard solution* (mg/mL)
V = final volume of *Sample solution* (20.0 mL)
W = weight of the sample of Capsules taken to prepare the *Sample solution* (mg)
A_W = average weight of the Capsules contents (mg)
L_E = content of docosyl ferulate in 100 mg of the Extract used to prepare the Capsules (mg)
L = labeled amount of Extract per Capsule (mg/Capsule)

Acceptance criteria: 90%–110% of the labeled amount of Extract, calculated as docosyl ferulate and sterols (from *Content of Sterols*)

PERFORMANCE TESTS
- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** ⟨2040⟩**:** Meet the requirements for *Rupture Test for Soft Shell Capsules*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩**:** Meet the requirements

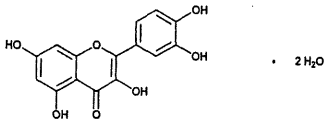
CONTAMINANTS
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total bacterial count does not exceed 1000 cfu/g. The total combined molds and yeasts count does not exceed 1000 cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** The Capsules meet the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the article from which the Capsules were prepared. The label also indicates the quantity of Extract, in mg/Capsule. Label the Capsules to indicate the quantity of sterols and docosyl ferulate in percentage of the Extract contained in the Capsules.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Docosyl Ferulate RS
  USP Pygeum Extract RS
  USP β-Sitosterol RS

Pyridoxine Hydrochloride—*see Pyridoxine Hydrochloride General Monographs*

Pyridoxine Hydrochloride Tablets—*see Pyridoxine Hydrochloride Tablets General Monographs*

Quercetin



$C_{15}H_{10}O_7$ 302.2
2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-1-benzopyran-4-one;
Quercetin dihydrate 338.2
[6151-25-3].

DEFINITION
Quercetin contains NLT 98.0% and NMT 102.0% of quercetin ($C_{15}H_{10}O_7$), calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)•
[NOTE—The substance is a dihydrate.]

Change to read:

- **B. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Ultraviolet-Visible Spectroscopy*: **197U**▲ (CN 1-May-2020)
Wavelength range: 210–450 nm
Sample solution: 10 µg/mL of Quercetin in methanol. Filter if necessary.
Acceptance criteria: The spectrum exhibits two absorption maxima at 255 nm and 371 nm. The absorptivity at the maximum at 371 nm is 75.5–80.0, calculated on the anhydrous basis.
- **C. HPLC IDENTIFICATION TEST**
Analysis: Proceed as directed in the *Assay*.
Acceptance criteria: The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

ASSAY
- **PROCEDURE**
Mobile phase: Methanol, water, and phosphoric acid (100:100:1)
System suitability solution: 0.02 mg/mL each of USP Quercetin RS, USP Kaempferol RS, and USP Isorhamnetin RS in methanol
Standard solution: Transfer 10 mg of USP Quercetin RS to a 50-mL volumetric flask, and add 20 mL of methanol to dissolve. Add 20 mL of water, mix, and dilute with methanol to volume.
Sample solution: Transfer 10 mg of Quercetin to a 50-mL volumetric flask, and add 20 mL of methanol to dissolve. Add 20 mL of water, mix, and dilute with methanol to volume.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 370 nm
Column: 4.6-mm × 25-cm; packing L1
Flow rate: 1.0 mL/min
Injection volume: 20 µL

DS Monographs

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System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The relative retention times for quercetin, kaempferolare, and isorhamnetin are 1.0, 1.8, and 2.0, respectively.]
Suitability requirements
Resolution: NLT 1.5 between kaempferol and isorhamnetin, *System suitability solution*
Column efficiency: NLT 2000 theoretical plates
Relative standard deviation: NMT 2.0%, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of Quercetin in the portion of Sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP Quercetin RS in the *Standard solution* (mg/mL)
C_U = concentration of Quercetin in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–102.0% on the anhydrous basis

IMPURITIES
• **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 0.1%
• **KAEMPFEROL AND OTHER RELATED COMPOUNDS**
Mobile phase, System suitability solution, Standard solution, Sample solution, and **System suitability:** Proceed as directed in the *Assay*.
Chromatographic system: Proceed as directed in the *Assay* but use UV detection at 270 nm.
Analysis
Sample: *Sample solution*
Calculate the percentage of each individual impurity in the portion of the *Sample* taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of each individual impurity from the *Sample solution*
r_T = sum of the responses of all the peaks from the *Sample solution*

Acceptance criteria
Kaempferol: NMT 0.5%
Individual impurities: NMT 0.1%
Total impurities: NMT 2.0%

SPECIFIC TESTS
• **WATER DETERMINATION,** *Method Ia* ⟨921⟩
Sample: 100 mg
Acceptance criteria: NMT 12.0%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed, tight, light-resistant containers.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Isorhamnetin RS
USP Kaempferol RS
USP Quercetin RS

Red Clover

DEFINITION
Red Clover consists of the aerial parts of *Trifolium pratense* L. (Fam. Fabaceae). It contains NLT 0.5% of isoflavones, calculated on the dried basis, as the sum of daidzein, genistein, formononetin, and biochanin A.

IDENTIFICATION
• **A.** Red Clover meets the requirements under *Specific Tests, Botanic Characteristics*.
• **B. THIN-LAYER CHROMATOGRAPHY**
Presence of biochanin A and formononetin
Solvent A: Methanol and water (7:3)
Solvent B: Methanol and water (6:4)
Standard solution A: 0.5 mg/mL of USP Biochanin A RS in methanol
Standard solution B: 0.5 mg/mL of USP Formononetin RS in methanol
Standard solution C: 10 mg/mL of USP Powdered Red Clover Extract RS in *Solvent A*. Shake to disperse, heat in a water bath at 60°–80° for 10 min, cool, centrifuge, and use the supernatant.
Sample solution: Transfer 0.5 g of finely pulverized Red Clover to a centrifuge tube, add 5 mL of *Solvent B*, shake to disperse, heat in a water bath at 60°–80° for 10 min, cool, centrifuge, and use the supernatant.
[NOTE—Reserve a portion of the supernatant for *Identification* test C.]
Chromatographic system
(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)
Application volume: 4 µL each of *Standard solution A*, *Standard solution B*, and *Sample solution*, and 3 µL of *Standard solution C*, as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
Developing solvent system: A mixture of ethyl acetate, toluene, and formic acid (30:70:1)
Developing distance: 6 cm
Derivatization reagent: 5 mg/mL of 2-aminoethyl diphenylborinate in ethyl acetate
Analysis
Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*
Apply the *Samples* as bands to a suitable high performance thin-layer chromatographic plate, and dry in air. Develop the chromatograms in a saturated chamber. Remove the plate from the chamber, heat at 100° for 5 min, derivatize the plate while still warm with the *Derivatization reagent*, dry in air, and examine under UV light at 365 nm.
System suitability: *Standard solution C* exhibits, at about the middle of the chromatogram, a greenish band corresponding to the band due to biochanin A in the chromatogram of *Standard solution A*, and a bluish band, at about one third of the chromatogram, corresponding to formononetin in the chromatogram of *Standard solution B*. Below the band due to formononetin, the *Standard solution C* chromatogram exhibits a red band in the lower third section of the chromatogram.
Acceptance criteria: The chromatogram of the *Sample solution* exhibits the following main bands similar in positions and colors to the corresponding bands in the chromatogram of *Standard solution C*: two greenish bands at about the middle of the chromatogram (distinction from white clover, soy, and alfalfa), one of

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these greenish bands corresponds to the band due to biochanin A in the chromatogram of *Standard solution A*; and a bluish band at about one third of the chromatogram, corresponding to the band due to formononetin in the chromatogram of *Standard solution B*; below the band due to formononetin, the *Sample solution* chromatogram exhibits a red band in the lower third section of the chromatogram.

• **C. Thin-Layer Chromatography**

Presence of flavone glycosides

Solvent A: Methanol and water (7:3)

Solvent B: Methanol and water (6:4)

Standard solution A: 0.1 mg/mL of USP Hyperoside RS in methanol

Standard solution B: 25 mg/mL of USP Powdered Red Clover Extract RS in *Solvent A*. Shake to disperse, heat in a water bath at 60°–80° for 10 min, cool, centrifuge, and use the supernatant.

Sample solution: Use the solution as prepared in *Identification* test *B*.

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)

Application volume: 4 µL of *Standard solution A*, 8 µL of *Standard solution B*, and 2 µL of *Sample solution*, as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

Developing solvent system: A mixture of ethyl acetate, formic acid, glacial acetic acid, and water (100:11:11:27)

Developing distance: 6 cm

Derivatization reagent: 5 mg/mL of 2-aminoethyl diphenylborinate in ethyl acetate

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands to a suitable high performance thin-layer chromatographic plate, and dry in air. Develop the chromatograms in a saturated chamber. Remove the plate from the chamber, heat at 100° for 5 min, derivatize the plate while still warm with *Derivatization reagent*, dry in air, and examine under UV light at 365 nm.

System suitability: *Standard solution B* exhibits, at about the middle of the chromatogram, two yellow bands, the lower band corresponds in color and R_F to the band due to hyperoside in the chromatogram of *Standard solution A*, the upper yellow band is due to isoquercitrin; two green bands, or a broad green band, above the yellow bands; and a blue band in the upper third section of the chromatogram. The yellow bands due to hyperoside and isoquercitrin are clearly separated.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits the following bands similar in positions and colors to the corresponding bands in the chromatogram of the *Standard solution B*: a yellow band at about the middle of the chromatogram corresponding to the band due to hyperoside in the chromatogram of *Standard solution A*; another yellow band, at an R_F slightly higher than that of hyperoside; two green bands, or a broad green band, above the yellow bands; and a blue band in the upper third section of the chromatogram.

• **D. HPLC Identification Test**

Analysis: Proceed as directed for *Content of Isoflavones*.

Using the values obtained in the test for *Content of Isoflavones*, calculate the ratio of 5,7-dihydroxyisoflavones to 7-hydroxyisoflavones:

$$\text{Result} = (B + G)/(D + F)$$

B = percentage of biochanin A
G = percentage of genistein
D = percentage of daidzein
F = percentage of formononetin

Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks for daidzein, genistein, formononetin, and biochanin A at retention times that correspond to those in the chromatogram of *Standard solution A*, and the ratio of 5,7-dihydroxyisoflavones to 7-hydroxyisoflavones is between 0.1 and 10.

COMPOSITION

• **Content of Isoflavones**

Solution A: Acetonitrile and water (1:3) containing 0.05% trifluoroacetic acid

Solution B: Acetonitrile containing 0.05% trifluoroacetic acid

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
2	100	0
2.5	87	13
7.5	80	20
7.8	73	27
8.0	55	45
11.0	50	50
13.0	40	60
15.0	26	74
16.0	0	100
18.1	100	0
23.0	100	0

Solvent: Alcohol and water (1:1)

Standard stock solution A: Transfer a quantity of USP Powdered Red Clover Extract RS, equivalent to 30 mg of the labeled content of isoflavones, to a 250-mL volumetric flask. Add 15 mL of dehydrated alcohol, sonicate until dissolved, and dilute with *Solvent* to volume.

Standard solution A: Evaporate 50 mL of *Standard stock solution A* to dryness under vacuum. Add 15 mL of 2 N hydrochloric acid, and heat in a water bath for 30 min. Quantitatively transfer the resulting solution, with the aid of 15 mL of alcohol, to a 50-mL volumetric flask, and dilute with *Solvent* to volume. Centrifuge, or filter through a membrane of 0.45-µm or finer pore size.

Standard solution B: 0.1 mg/mL of USP Formononetin RS, 0.02 mg/mL of USP Genistein RS, 0.02 mg/mL of USP Daidzein RS, and 0.1 mg/mL of USP Biochanin A RS in a mixture of *n*-propanol and water (1:1). Sonicate, and filter through a membrane of 0.45-µm or finer pore size.

Sample stock solution: Transfer about 2.5 g of ground Red Clover, accurately weighed, into a 120-mL flask with a stopper. Add exactly 100 mL of *Solvent*, close the flask, and shake on an orbital or wrist-action shaker for NLT 12 h.

Supplements

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Sample solution: Evaporate 50 mL of the *Sample stock solution* to dryness under vacuum at 40°. Add 15 mL of 2 N hydrochloric acid, and heat in a water bath for 30 min. Quantitatively transfer this solution, with the aid of 15 mL of alcohol, to a 50-mL volumetric flask, and dilute with *Solvent* to volume. Filter through a membrane of 0.45-µm or finer pore size, discarding the first 4 mL of the filtrate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; end-capped 5-µm packing L1
Column temperature: 45°
Flow rate: 1 mL/min
Injection volume: 10 µL

System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram from *Standard solution A* is similar to the reference chromatogram provided with the lot of USP Powdered Red Clover Extract RS being used.
Tailing factor: NMT 2.0 for the formononetin peak, *Standard solution B*
Relative standard deviation: NMT 2.0%, determined from the formononetin peak in repeated injections, *Standard solution B*

Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Identify the peaks corresponding to daidzein, genistein, formononetin, and biochanin A in the *Sample solution* chromatogram by comparison with the chromatogram obtained from *Standard solution A* and the reference chromatogram. Measure the areas of the analyte peaks.
Separately calculate the percentages of daidzein, genistein, formononetin, and biochanin A in the portion of Red Clover taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times 100$$

r_U = peak response for each relevant isoflavone from the *Sample solution*
r_S = peak response for daidzein, genistein, formononetin, or biochanin A from *Standard solution B*
C_S = concentration of daidzein, genistein, formononetin, or biochanin A in *Standard solution B* (mg/mL)
V = volume of the *Sample stock solution* (mL)
W = weight of Red Clover used to prepare the *Sample solution* (mg)
D = dilution factor to prepare the *Sample solution* from the *Sample stock solution*, 1

Acceptance criteria: Add the percentages of daidzein, genistein, formononetin, and biochanin A. NLT 0.5% of isoflavones on the dried basis.

CONTAMINANTS

• **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩
Acceptance criteria
Arsenic: NMT 1.0 µg/g
Cadmium: NMT 0.5 µg/g
Lead: NMT 5.0 µg/g
Mercury: NMT 1.0 µg/g

Change to read:

• **▲ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩▲ (CN 1-May-2019)**:** Meets the requirements
• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^6 cfu/g, the total combined molds and yeast count does not exceed 10^4 cfu/g, and the enterobacterial count is NMT 10^3 cfu/g.
• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

• **BOTANIC CHARACTERISTICS**
Macroscopic: Red Clover inflorescences are ovoid with a rounded summit, mostly 12–34 mm in length and width, usually on a very short stalk, shriveled, purplish, and more or less brown from drying, consisting of many papilionaceous flowers, crowded together and clothed at the base with broad, pointed, pale green ciliate stipules with darker veins. The flowers are up to 15 mm in length and have the following: five green, hairy, subulate calyx teeth, one longer than the other four; petals united into a more or less campanulate tube, somewhat recurved, and colorless with pinkish purple veins; diadelphous stamens; slender style; a faintly aromatic, somewhat tea-like odor; and a sweet, then slightly bitter taste. Leaves trifoliolate, petiolate, stipulate, with stipules fused to petiole; leaflets petiolulate, obovate to oblong-obovate or broadly elliptical.
Microscopic
Flower: Epidermis of calyx composed of polygonal cells with faintly striated cuticle and occasional anomocytic stomata on the outer epidermis only; covering trichomes abundant, uniseriate, with two small thin-walled basal cells, and a thick-walled tapering end cell, up to 1 mm in length with a warty cuticle; glandular trichomes present, particularly on the lower epidermis, each with a one- or two-celled stalk and a head composed of several cells arranged in two rows; epidermal cells of the corolla, papillose at the tip, elongated with slightly wavy walls and a strongly striated cuticle; vascular strands of corolla and calyx are surrounded by a crystal sheath containing prismatic crystals of calcium oxalate; fibrous layer of anthers; subspherical pollen grains, 20–48 µm in diameter with smooth exine, three distinct pores, and three furrows.
Transverse section of leaf: Dorsiventral; one to three rows of pallisade cells under the upper epidermis; mesophyll made of spongy parenchyma cells; lower epidermis; covering trichomes on both epidermis, and are similar to those found on the flowers; glandular trichomes, more close to the veins on the lower epidermis, and are similar to those found on the flowers; collateral vascular bundles, surrounded by a crystal sheath containing prismatic crystals of calcium oxalate.
Transverse section of stem: It shows ridges along the surface, with groups of collenchyma cells under the epidermal layer of the ridges; epidermis followed by a few layers of parenchyma cells; a wide central parenchymatous pith, surrounded by groups of vascular bundles that are separated by thick-wall, lignified parenchyma cells.
• **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩**:** NMT 2.0%
• **ARTICLES OF BOTANICAL ORIGIN,** *Water-Soluble Extractives, Method 2* ⟨561⟩**:** NLT 15.0%
• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry 1 g at 105° for 2 h.
Acceptance criteria: NMT 12.0%

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- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩: NMT 10.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩: NMT 2.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in a well-closed, light-resistant container, protected from moisture.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Biochanin A RS
  USP Daidzein RS
  USP Formononetin RS
  USP Genistein RS
  USP Hyperoside RS
  USP Powdered Red Clover Extract RS

Red Clover Aerial Parts Isoflavone Aglycones Dry Extract

DEFINITION

Red Clover Aerial Parts Isoflavone Aglycones Dry Extract is prepared from dried aerial parts of *Trifolium pratense* L. (Fam. Fabaceae) by extraction with hydroalcoholic mixtures or other suitable solvents. It contains NLT 36.0% and NMT 44.0% of isoflavones, predominantly as aglycones, calculated on the dried basis as the sum of daidzein, genistein, formononetin, and biochanin A. It contains NMT 1.0% of daidzein and NMT 1.0% of genistein, both on the dried basis. The ratio of 5,7-dihydroxyisoflavones to 7-hydroxyisoflavones is between 0.9 and 1.7. It may contain suitable excipients.

IDENTIFICATION

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Presence of biochanin A and formononetin

Solvent: Methanol and water (7:3)

Standard solution A: 1 mg/mL each of USP Biochanin A RS and USP Formononetin RS in methanol

Standard solution B: 10 mg/mL of USP Red Clover Aerial Parts Isoflavone Aglycones Dry Extract RS in *Solvent*. Shake to disperse, heat on a water bath at 60°–80° for 10 min, centrifuge, and use the supernatant.

Sample solution: 10 mg/mL of Red Clover Aerial Parts Isoflavone Aglycones Dry Extract in *Solvent*. Shake to disperse, heat on a water bath at 60°–80° for 10 min, centrifuge, and use the supernatant.

Chromatographic system

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plate)[1]

Application volume: 2 µL each of *Standard solution A*, *Standard solution B*, and *Sample solution*, as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

Temperature: Ambient, not to exceed 30°

Developing solvent system: Ethyl acetate, toluene, and formic acid (30:70:1)

Developing distance: 6 cm

Derivatization reagent: 5 mg/mL of 2-aminoethyl diphenylborinate in ethyl acetate

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* and dry in air. Develop in a saturated chamber. Remove the plate from the chamber, heat at 100° for 5 min, derivatize while warm with the *Derivatization reagent*, dry in air, and examine under UV light at 365 nm.

System suitability: *Standard solution A* exhibits a bluish band at about one-third of the chromatogram due to formononetin, and a greenish band due to biochanin A at about the middle of the chromatogram. *Standard solution B* exhibits prominent bands of similar color and position to those of formononetin and biochanin A in *Standard solution A*. A red band appears below the formononetin band in *Standard solution B*.

Acceptance criteria: The *Sample solution* exhibits the following prominent bands similar in position and color to those in *Standard solution B*: two greenish bands at about the middle of the chromatogram (a distinction from white clover, soy, and alfalfa), one of them corresponding to biochanin A in *Standard solution A*; a bluish band at about one-third of the chromatogram, corresponding to the formononetin band in *Standard solution B*; and a red band below that due to formononetin.

- **B. HPLC**

Analysis: Proceed as directed in the test for *Content of Isoflavones*.

Using the values obtained in the test for *Content of Isoflavones*, calculate the ratio of 5,7-dihydroxyisoflavones to 7-hydroxyisoflavones:

$$\text{Result} = (B + G)/(D + F)$$

B = percentage of biochanin A
G = percentage of genistein
D = percentage of daidzein
F = percentage of formononetin

Acceptance criteria: The *Sample solution* exhibits peaks for daidzein, genistein, formononetin, and biochanin A at retention times corresponding to those in *Standard solution A* and *Standard solution B*. The ratio of 5,7-dihydroxyisoflavones to 7-hydroxyisoflavones is between 0.9 and 1.7.

COMPOSITION

- **CONTENT OF ISOFLAVONES**[2]

Solution A: Acetonitrile and water (1:3) containing 0.05% trifluoroacetic acid

Solution B: Acetonitrile containing 0.05% trifluoroacetic acid

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
2	100	0
2.5	87	13
7.5	80	20
7.8	73	27
8.0	55	45

[1] A suitable commercially available plate is HPTLC Silica Gel 60 F_{254} from EMD Millipore (e.g., Part No. 1.05642.0001).

[2] Limits specified for the content of daidzein and genistein facilitate detection of material derived from other isoflavone-rich sources, notably, soy.

DS Monographs

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Table 1 *(continued)*

Time (min)	Solution A (%)	Solution B (%)
11.0	50	50
13.0	40	60
15.0	26	74
16.0	0	100
18.1	100	0
23.0	100	0

Diluent: Alcohol and water (1:1)
Standard solution A: Prepare a 0.10–0.15-mg/mL solution of USP Red Clover Aerial Parts Isoflavone Aglycones Dry Extract RS in *Diluent*, sonicate briefly, and filter through a membrane of 0.45-µm or finer pore size.
Standard solution B: 0.1 mg/mL of USP Formononetin RS, 0.02 mg/mL of USP Genistein RS, 0.02 mg/mL of USP Daidzein RS, and 0.1 mg/mL of USP Biochanin A RS in a mixture of *n*-propanol and water (1:1)
Sample solution: Accurately weigh a quantity of Red Clover Aerial Parts Isoflavone Aglycones Dry Extract, equivalent to 12 mg of the labeled content of isoflavones, and transfer to a 100-mL volumetric flask. Add 50 mL of *Diluent*, sonicate to disperse, and dilute with *Diluent* to volume. Before injection, pass through a filter of 0.45-µm or finer pore size.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; end-capped 5-µm packing L1
Column temperature: 45°
Flow rate: 1.0 mL/min
Injection volume: 10 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Tailing factor: NMT 2.0 for the formononetin peak, *Standard solution B*
Relative standard deviation: NMT 2.0%, determined from the formononetin peak in replicate injections, *Standard solution B*
Chromatogram similarity: The chromatogram of *Standard solution A* is similar to the reference chromatogram provided with the lot of USP Red Clover Aerial Parts Isoflavone Aglycones Dry Extract RS being used.
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Identify the peaks corresponding to daidzein, genistein, formononetin, and biochanin A in the *Sample solution* by comparison with the chromatograms obtained from *Standard solution A*, *Standard solution B*, and the reference chromatogram supplied.
Calculate the percentage of each isoflavone in the portion of Red Clover Aerial Parts Isoflavone Aglycones Dry Extract taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of a relevant isoflavone from the *Sample solution*
r_S = peak area of the corresponding isoflavone from *Standard solution B*
C_S = concentration of the corresponding isoflavone in *Standard solution B* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of the Dry Extract taken to prepare the *Sample solution* (mg)

Acceptance criteria: 36.0%–44.0% of isoflavones as the sum of daidzein, genistein, formononetin, and biochanin A on the dried basis; NMT 1.0% of daidzein and NMT 1.0% of genistein, both on the dried basis

CONTAMINANTS

Change to read:

- **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparatons, General Pharmacopeial Requirements, Pesticide Residues*:▲ (CN 1-May-2020) Meets the requirements
- **BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Residual Solvents*: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic microbial count is NMT 10^4 cfu/g, and the total combined molds and yeasts count is NMT 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS
- **LOSS ON DRYING** ⟨731⟩
Sample: 1.0 g of Dry Extract
Analysis: Dry the *Sample* at 105° for 2 h.
Acceptance criteria: NMT 3.0%
- **RESIDUE ON IGNITION** ⟨281⟩
Analysis: Ignite 1.0 g of Dry Extract.
Acceptance criteria: NMT 4.0%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, protected from moisture, in a cool place.
- **LABELING:** The label states the official name of the article and the corresponding Latin binominal. The label also indicates the content of isoflavones, their status as aglycones, the extraction solvent used for preparation, and the ratio of the starting crude plant material to the Dry Extract. It meets the requirements of *Botanical Extracts* ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Labeling*.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Biochanin A RS
USP Daidzein RS
USP Formononetin RS
USP Genistein RS
USP Red Clover Aerial Parts Isoflavone Aglycones Dry Extract RS

Powdered Red Clover

DEFINITION
Powdered Red Clover is Red Clover reduced to a powder or very fine powder. It contains NLT 0.5% of isoflavones, calculated on the dried basis as the sum of daidzein, genistein, formononetin, and biochanin A.

IDENTIFICATION
- **A.** Powdered Red Clover meets the requirements under *Specific Tests, Botanic Characteristics*.
- **B. THIN-LAYER CHROMATOGRAPHY**
Presence of biochanin A and formononetin
Solvent A: Methanol and water (7:3)

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Solvent B: Methanol and water (6:4)
Standard solution A: 0.5 mg/mL of USP Biochanin A RS in methanol
Standard solution B: 0.5 mg/mL of USP Formononetin RS in methanol
Standard solution C: 10 mg/mL of USP Powdered Red Clover Extract RS in *Solvent A*. Shake to disperse, heat in a water bath at 60°–80° for 10 min, cool, centrifuge, and use the supernatant.
Sample solution: Transfer 0.5 g of Powdered Red Clover to a centrifuge tube, and add 5 mL of *Solvent B*. Shake to disperse, heat in a water bath at 60°–80° for 10 min, cool, centrifuge, and use the supernatant. [NOTE—Reserve a portion of the supernatant for *Identification* test C.]
Chromatographic system
(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)
Application volume: 4 µL each of *Standard solution A*, *Standard solution B*, and *Sample solution*, and 3 µL of *Standard solution C*, as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
Developing solvent system: A mixture of ethyl acetate, toluene, and formic acid (30:70:1)
Developing distance: 6 cm
Derivatization reagent: 5 mg/mL of 2-aminoethyl diphenylborinate in ethyl acetate
Analysis
Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*
Apply the *Samples* as bands to a suitable HPTLC plate, and dry in air. Develop the chromatograms in a saturated chamber. Remove the plate from the chamber, heat at 100° for 5 min, derivatize the plate while still warm with *Derivatization reagent*, dry in air, and examine under UV light at 365 nm.
System suitability: *Standard solution C* exhibits, at about the middle of the chromatogram, a greenish band corresponding to the band due to biochanin A in the chromatogram of *Standard solution A*, and a bluish band, at about one third of the chromatogram, corresponding to formononetin in the chromatogram of *Standard solution B*. Below the band due to formononetin, the *Standard solution C* chromatogram exhibits a red band in the lower third section of the chromatogram.
Acceptance criteria: The chromatogram of the *Sample solution* exhibits the following main bands similar in positions and colors to the corresponding bands in the chromatogram of *Standard solution C*: two greenish bands at about the middle of the chromatogram (distinction from white clover, soy, and alfalfa), one of these greenish bands corresponds to the band due to biochanin A in the chromatogram of *Standard solution A*; and a bluish band at about one third of the chromatogram, corresponding to the band due to formononetin in the chromatogram of *Standard solution B*; below the band due to formononetin, the *Sample solution* chromatogram exhibits a red band in the lower third section of the chromatogram.

• **C. THIN-LAYER CHROMATOGRAPHY**
Presence of flavone glycosides
Solvent A: Methanol and water (7:3)
Solvent B: Methanol and water (6:4)
Standard solution A: 0.1 mg/mL of USP Hyperoside RS in methanol
Standard solution B: 25 mg/mL of USP Powdered Red Clover Extract RS in *Solvent A*. Shake to disperse, heat in a water bath at 60°–80° for 10 min, cool, centrifuge, and use the supernatant.
Sample solution: Use the solution as prepared in *Identification* test *B*.
Chromatographic system
(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)
Application volume: 4 µL of *Standard solution A*, 8 µL of *Standard solution B*, and 2 µL of *Sample solution*, as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
Developing solvent system: A mixture of ethyl acetate, formic acid, glacial acetic acid, and water (100:11:11:27)
Developing distance: 6 cm
Derivatization reagent: 5 mg/mL of 2-aminoethyl diphenylborinate in ethyl acetate
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Apply the *Samples* as bands to a suitable HPTLC plate, and dry in air. Develop the chromatograms in a saturated chamber. Remove the plate from the chamber, heat at 100° for 5 min, derivatize the plate while still warm with *Derivatization reagent*, dry in air, and examine under UV light at 365 nm.
System suitability: *Standard solution B* exhibits, at about the middle of the chromatogram, two yellow bands. The lower band corresponds in color and R_F to the band due to hyperoside in the chromatogram of *Standard solution A* and the upper yellow band is due to isoquercitrin. The solution also exhibits two green bands, or a broad green band, above the yellow bands, and a blue band in the upper third section of the chromatogram. The yellow bands due to hyperoside and isoquercitrin are clearly separated.
Acceptance criteria: The chromatogram of the *Sample solution* exhibits the following bands similar in positions and colors to the corresponding bands in the chromatogram of *Standard solution B*: a yellow band at about the middle of the chromatogram corresponding to the band due to hyperoside in the chromatogram of *Standard solution A*; another yellow band, at an R_F slightly higher than that of hyperoside; two green bands, or a broad green band, above the yellow bands; and a blue band in the upper third section of the chromatogram.

• **D. HPLC IDENTIFICATION TEST**
Analysis: Proceed as directed for the *Content of Isoflavones*. Using the values obtained in the test for *Content of Isoflavones*, calculate the ratio of 5,7-dihydroxyisoflavones to 7-hydroxyisoflavones:

$$\text{Result} = (B + G)/(D + F)$$

B = percentage of biochanin A
G = percentage of genistein
D = percentage of daidzein
F = percentage of formononetin

Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks for daidzein, genistein, formononetin, and biochanin A at retention times that correspond to those in the chromatogram of *Standard solution A*, and the ratio of 5,7-dihydroxyisoflavones to 7-hydroxyisoflavones is between 0.1 and 10.

DS Monographs

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COMPOSITION

• **CONTENT OF ISOFLAVONES**

Solution A: Acetonitrile and water (1:3) containing 0.05% trifluoroacetic acid

Solution B: Acetonitrile containing 0.05% trifluoroacetic acid

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
2	100	0
2.5	87	13
7.5	80	20
7.8	73	27
8.0	55	45
11.0	50	50
13.0	40	60
15.0	26	74
16.0	0	100
18.1	100	0
23.0	100	0

Solvent: Alcohol and water (1:1)

Standard stock solution A: Transfer a quantity of USP Powdered Red Clover Extract RS, equivalent to 30 mg of the labeled content of isoflavones, to a 250-mL volumetric flask. Add 15 mL of dehydrated alcohol, sonicate until dissolved, and dilute with *Solvent* to volume.

Standard solution A: Evaporate 50 mL of *Standard stock solution A* to dryness under vacuum. Add 15 mL of 2 N hydrochloric acid, and heat in a water bath for 30 min. Quantitatively transfer the resulting solution, with the aid of 15 mL of alcohol, to a 50-mL volumetric flask, and dilute with *Solvent* to volume. Centrifuge, or filter through a membrane of 0.45-µm or finer pore size.

Standard solution B: 0.1 mg/mL of USP Formononetin RS, 0.02 mg/mL of USP Genistein RS, 0.02 mg/mL of USP Daidzein RS, and 0.1 mg/mL of USP Biochanin A RS in a mixture of *n*-propanol and water (1:1). Sonicate, and filter through a membrane of 0.45-µm or finer pore size.

Sample stock solution: Transfer about 2.5 g of Powdered Red Clover, accurately weighed, into a 120-mL flask with a stopper. Add exactly 100 mL of *Solvent*, close the flask, and shake on an orbital or wrist-action shaker for NLT 12 h.

Sample solution: Evaporate 50 mL of the *Sample stock solution* to dryness under vacuum at 40°. Add 15 mL of 2 N hydrochloric acid, and heat in a water bath for 30 min. Quantitatively transfer this solution, with the aid of 15 mL of alcohol, to a 50-mL volumetric flask, and dilute with *Solvent* to volume. Filter through a membrane of 0.45-µm or finer pore size, discarding the first 4 mL of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; end-capped 5-µm packing L1

Column temperature: 45°

Flow rate: 1.0 mL/min

Injection volume: 10 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution A* is similar to the reference chromatogram provided with the lot of USP Powdered Red Clover Extract RS being used.

Tailing factor: NMT 2.0 for the formononetin peak, *Standard solution B*

Relative standard deviation: NMT 2.0%, determined from the formononetin peak in repeated injections, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Identify the peaks corresponding to daidzein, genistein, formononetin, and biochanin A in the *Sample solution* chromatogram by comparison with the chromatogram from *Standard solution A* and the reference chromatogram. Measure the areas of the analyte peaks.

Separately calculate the percentages of daidzein, genistein, formononetin, and biochanin A in the portion of Powdered Red Clover taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times 100$$

r_U = peak response for each relevant isoflavone from the *Sample solution*

r_S = peak response for daidzein, genistein, formononetin, or biochanin A from *Standard solution B*

C_S = concentration of daidzein, genistein, formononetin, or biochanin A in *Standard solution B* (mg/mL)

V = volume of the *Sample stock solution* (mL)

W = weight of Powdered Red Clover used to prepare the *Sample solution* (mg)

D = dilution factor to prepare the *Sample solution* from the *Sample stock solution*, 1

Acceptance criteria: Add the percentages of daidzein, genistein, formononetin, and biochanin A. NLT 0.5% of isoflavones on the dried basis.

CONTAMINANTS

• **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩

Acceptance criteria

Arsenic: NMT 1.0 µg/g

Cadmium: NMT 0.5 µg/g

Lead: NMT 5.0 µg/g

Mercury: NMT 1.0 µg/g

Change to read:

• **▲ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩▲ (CN 1-May-2019)**:** Meets the requirements

• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^6 cfu/g, the total combined molds and yeast count does not exceed 10^4 cfu/g, and the enterobacterial count is NMT 10^3 cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

• **BOTANIC CHARACTERISTICS:** Fragments of epidermis of calyx composed of polygonal cells with faintly striated cuticle and occasional anomocytic stomata; epidermal cells of the corolla, papillose at the tip, elongated with slightly wavy walls and a strongly striated cuticle; upper epidermal cells of leaflets with sinuous and slightly beaded anticlinal walls; lower epidermal cells of leaflets with sinuous to wavy

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walls; both epidermal cells show anomocytic stomata, covering trichomes, and glandular trichomes; covering trichomes, uniseriate, with two small thin-walled basal cells, and a thick-walled tapering end cell, up to 1 mm in length with a warty cuticle; glandular trichomes, each with a one- or two-celled stalk and a head composed of several cells arranged in two rows; pollen grains, smooth, nearly spheroidal, from 20 to 48 µm in diameter; sclerenchyma fibers with adherent crystal sheath containing prisms of calcium oxalate.

- **ARTICLES OF BOTANICAL ORIGIN,** *Water-Soluble Extractives, Method 2* ⟨561⟩: NLT 15.0%
- **LOSS ON DRYING** ⟨731⟩
  Sample: 1 g
  Analysis: Dry the *Sample* at 105° for 2 h.
  Acceptance criteria: NMT 12.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩: NMT 10.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩: NMT 2.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed, light-resistant containers, protected from moisture.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Biochanin A RS
  USP Daidzein RS
  USP Formononetin RS
  USP Genistein RS
  USP Hyperoside RS
  USP Powdered Red Clover Extract RS

Powdered Red Clover Extract

DEFINITION

Powdered Red Clover Extract is prepared from Red Clover by extraction with hydroalcoholic mixtures or other suitable solvents. It contains NLT 90.0% and NMT 110.0% of the labeled amount of isoflavones, calculated on the dried basis as the sum of daidzein, genistein, formononetin, and biochanin A. It may contain suitable added substances.

IDENTIFICATION

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩
  Presence of biochanin A and formononetin
  Solvent A: Methanol and water (7:3)
  Solvent B: Methanol and water (6:4)
  Standard solution A: 0.5 mg/mL of USP Biochanin A RS in methanol
  Standard solution B: 0.5 mg/mL of USP Formononetin RS in methanol
  Standard solution C: 10 mg/mL of USP Powdered Red Clover Extract RS in *Solvent A*. Shake to disperse, heat on a water bath at 60°–80° for 10 min, cool, centrifuge, and use the supernatant.
  Sample solution: 10 mg/mL of Powdered Extract in *Solvent A*. Shake to disperse, heat on a water bath at 60°–80° for 10 min, cool, centrifuge, and use the supernatant. [NOTE—Reserve a portion of the supernatant for *Identification* test *B*.]
  Chromatographic system
  Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plates)
  Application volume: 4 µL each of *Standard solution A*, *Standard solution B*, and the *Sample solution*, and 3 µL of *Standard solution C*, as 8-mm bands
  Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
  Temperature: Ambient, not to exceed 30°
  Developing solvent system: Ethyl acetate, toluene, and formic acid (30:70:1)
  Developing distance: 6 cm
  Derivatization reagent: 5 mg/mL of 2-aminoethyl diphenylborinate in ethyl acetate
  Analysis
  Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*
  Apply the *Samples* as bands and dry in air. Develop in a saturated chamber. Remove the plate from the chamber, heat at 100° for 5 min, treat while still warm with *Derivatization reagent*, dry in air, and examine under UV light at 365 nm.
  System suitability: *Standard solution C* exhibits, at about the middle of the chromatogram, a greenish band corresponding to biochanin A in *Standard solution A*, and a bluish band, at about one-third of the chromatogram, corresponding to formononetin in *Standard solution B*. Below the band due to formononetin, *Standard solution C* exhibits a red band.
  Acceptance criteria: The *Sample solution* exhibits the following bands similar in position and color to the corresponding bands in the chromatogram of *Standard solution C*: two greenish bands at about the middle of the chromatogram (a distinction from white clover, soy, and alfalfa), one of these greenish bands corresponding to biochanin A in *Standard solution A*; a bluish band at about one-third of the chromatogram, corresponding to the formononetin band in *Standard solution B*. Below the formononetin band, the *Sample solution* exhibits a red band.
- **B. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩
  Presence of flavone glycosides
  Solvent A: Methanol and water (7:3)
  Solvent B: Methanol and water (6:4)
  Standard solution A: 0.1 mg/mL of USP Hyperoside RS in methanol
  Standard solution B: 25 mg/mL of USP Powdered Red Clover Extract RS in *Solvent A*. Shake to disperse, heat on a water bath at 60°–80° for 10 min, cool, centrifuge, and use the supernatant.
  Sample solution: Use the solution prepared in *Identification* test *A*.
  Chromatographic system
  Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plates)
  Application volume: 4 µL of *Standard solution A*, 8 µL of *Standard solution B*, and 2 µL of the *Sample solution*, as 8-mm bands
  Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
  Temperature: Ambient, not to exceed 30°
  Developing solvent system: Ethyl acetate, formic acid, glacial acetic acid, and water (100:11:11:27)
  Developing distance: 6 cm
  Derivatization reagent: 5 mg/mL of 2-aminoethyl diphenylborinate in ethyl acetate
  Analysis
  Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
  Apply the *Samples* as bands and dry in air. Develop in a saturated chamber. Remove the plate from the chamber, heat at 100° for 5 min, treat while still warm with

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Derivatization reagent, dry in air, and examine under UV light at 365 nm.

System suitability: *Standard solution B* exhibits, at about the middle of the chromatogram, two yellow bands. The lower band corresponds in color and R_F to hyperoside in *Standard solution A*. The upper yellow band is due to isoquercitrin. The solution also exhibits two green bands or a broad green band, above the yellow bands, and a blue band in the upper third section of the chromatogram. The yellow bands due to hyperoside and isoquercitrin are clearly separated.

Acceptance criteria: The *Sample solution* exhibits the following bands similar in position and color to the corresponding bands in *Standard solution B*: a yellow band in about the middle of the chromatogram corresponding to hyperoside in *Standard solution A*; another yellow band, at an R_F slightly higher than that of hyperoside; two green bands or a broad green band above the yellow bands; and a blue band in the upper third section of the chromatogram.

• **C. HPLC**

Analysis: Proceed as directed in the test for *Content of Isoflavones*.

Using the values obtained in the test for *Content of Isoflavones*, calculate the ratio of 5,7-dihydroxyisoflavones to 7-hydroxyisoflavones:

$$\text{Result} = (B + G)/(D + F)$$

B = percentage of biochanin A
G = percentage of genistein
D = percentage of daidzein
F = percentage of formononetin

Acceptance criteria: The *Sample solution* exhibits peaks for daidzein, genistein, formononetin, and biochanin A at retention times that correspond to those in *Standard solution A*. The ratio of 5,7-dihydroxyisoflavones to 7-hydroxyisoflavones is between 0.1 and 10.0.

COMPOSITION

• **CONTENT OF ISOFLAVONES**

Solution A: Acetonitrile and water (1:3) containing 0.05% trifluoroacetic acid

Solution B: Acetonitrile containing 0.05% trifluoroacetic acid

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
2	100	0
2.5	87	13
7.5	80	20
7.8	73	27
8.0	55	45
11.0	50	50
13.0	40	60
15.0	26	74
16.0	0	100
18.1	100	0
23.0	100	0

Solvent: Alcohol and water (1:1)

Standard solution A: Suspend 10–15 mg of USP Powdered Red Clover Extract RS in 15 mL of 2 N hydrochloric acid, sonicate to disperse, and heat on a water bath for 30 min. Add 15 mL of alcohol and mix well. Centrifuge or filter through a membrane of 0.45-µm or finer pore size.

Standard solution B: 0.1 mg/mL of USP Formononetin RS, 0.02 mg/mL of USP Genistein RS, 0.02 mg/mL of USP Daidzein RS, and 0.1 mg/mL of USP Biochanin A RS in a mixture of *n*-propanol and water (1:1)

Sample solution: Accurately transfer a quantity of Powdered Extract, equivalent to 6 mg of the labeled amount of isoflavones, to a 50-mL volumetric flask. Add 15 mL of 2 N hydrochloric acid, sonicate to disperse, and heat on a water bath for 30 min. Allow to cool, add 15 mL of alcohol, and dilute with *Solvent* to volume. Centrifuge or pass through a filter of 0.45-µm or finer pore size.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; end-capped 5-µm packing L1

Column temperature: 45°

Flow rate: 1 mL/min

Injection volume: 10 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Tailing factor: NMT 2.0 for the formononetin peak, *Standard solution B*

Relative standard deviation: NMT 2.0%, determined from the formononetin peak in replicate injections, *Standard solution B*

Chromatogram similarity: The chromatogram of *Standard solution A* is similar to the reference chromatogram provided with the lot of USP Powdered Red Clover Extract RS being used.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Identify the peaks corresponding to daidzein, genistein, formononetin, and biochanin A in the *Sample solution* by comparison with the chromatogram from *Standard solution A* and the reference chromatogram. Measure the areas of the analyte peaks.

Calculate the percentage of each isoflavone in the portion of Powdered Extract taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of a relevant isoflavone from the *Sample solution*
r_S = peak area of a corresponding isoflavone from *Standard solution B*
C_S = concentration of a relevant isoflavone in *Standard solution B* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Powdered Extract used to prepare the *Sample solution* (mg)

Calculate the percentage of the labeled amount of isoflavones in the portion of Powdered Extract taken:

$$\text{Result} = (\Sigma P_i/L) \times 100$$

ΣP_i = total combined content of isoflavones as determined above
L = labeled amount of isoflavones

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Acceptance criteria: 90.0%–110.0% of the labeled amount of isoflavones as the sum of daidzein, genistein, formononetin, and biochanin A, on the dried basis

CONTAMINANTS

- **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩

Acceptance criteria

Arsenic: NMT 1.0 µg/g
Cadmium: NMT 0.5 µg/g
Lead: NMT 5.0 µg/g
Mercury: NMT 1.0 µg/g

- **BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*: Meets the requirements
- **BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Residual Solvents*: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic microbial count does not exceed 10^4 cfu/g and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS

- **LOSS ON DRYING** ⟨731⟩

Sample: 1 g
Analysis: Dry the *Sample* at 105° for 2 h.
Acceptance criteria: NMT 5.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, in a cool place.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. The label also indicates the content of isoflavones, the extracting solvent used for preparation, and the ratio of the starting crude plant material to Powdered Extract. It meets the requirements in *Botanical Extracts* ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Labeling*.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Biochanin A RS
USP Daidzein RS
USP Formononetin RS
USP Genistein RS
USP Hyperoside RS
USP Powdered Red Clover Extract RS

Red Clover Tablets

DEFINITION

Red Clover Tablets contain Powdered Red Clover Extract. Tablets contain NLT 90.0% and NMT 110.0% of the labeled amount of Powdered Extract, calculated as isoflavones.

IDENTIFICATION

- **A. HPLC IDENTIFICATION TEST**

Analysis: Proceed as directed for the *Content of Isoflavones*. Using the values obtained in the test for *Content of Isoflavones*, calculate the ratio of 5,7-dihydroxyisoflavones to 7-hydroxyisoflavones:

$$\text{Result} = (B + G)/(D + F)$$

B = percentage of biochanin A
G = percentage of genistein
D = percentage of daidzein
F = percentage of formononetin

Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks for daidzein, genistein, formononetin, and biochanin A at retention times that correspond to those in the chromatogram of *Standard solution A*, and the ratio of 5,7-dihydroxyisoflavones to 7-hydroxyisoflavones is between 0.1 and 10.

STRENGTH

- **CONTENT OF ISOFLAVONES**

Solution A: Acetonitrile and water (1:3) containing 0.05% trifluoroacetic acid
Solution B: Acetonitrile containing 0.05% trifluoroacetic acid
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
2	100	0
2.5	87	13
7.5	80	20
7.8	73	27
8.0	55	45
11.0	50	50
13.0	40	60
15.0	26	74
16.0	0	100
18.1	100	0
23.0	100	0

Solvent: Alcohol and water (1:1)
Standard stock solution: Transfer a quantity of USP Powdered Red Clover Extract RS, equivalent to 30 mg of the labeled content of isoflavones, to a 250-mL volumetric flask. Add 15 mL of dehydrated alcohol, sonicate until dissolved, and dilute with *Solvent* to volume.
Standard solution A: Evaporate 50 mL of the *Standard stock solution* to dryness under vacuum. Add 15 mL of 2 N hydrochloric acid, and heat in a water bath for 30 min. Quantitatively transfer the resulting solution, with the aid of 15 mL of alcohol, to a 50-mL volumetric flask, and dilute with *Solvent* to volume. Centrifuge, or filter through a membrane of 0.45-µm or finer pore size.
Standard solution B: 0.1 mg/mL of USP Formononetin RS, 0.02 mg/mL of USP Genistein RS, 0.02 mg/mL of USP Daidzein RS, and 0.1 mg/mL of USP Biochanin A RS in a mixture of *n*-propanol and water (1:1). Sonicate, and filter through a membrane of 0.45-µm or finer pore size.
Sample stock solution: Weigh NLT 20 Tablets, and pulverize. Transfer the equivalent of 40 mg of the labeled amount of isoflavones to a 250-mL volumetric flask. Add 15 mL of water, shake to disperse the powder, add 15 mL of dehydrated alcohol and 200 mL of *Solvent*, and sonicate for 30 min. If dark particles are present in the bottom of the flask, sonicate again for an additional 10 min or until they disappear. Cool to room temperature, dilute with *Solvent* to volume, and filter.
Sample solution: Transfer 50.0 mL of the resulting solution to a round-bottom flask, and evaporate to dryness under vacuum. Add 15 mL of 2 N hydrochloric acid, and heat in a

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water bath for 30 min. Quantitatively transfer this solution, with the aid of 15 mL of alcohol, to a 50-mL volumetric flask, and dilute with *Solvent* to volume. Pass 5 mL of the solution through a filter of 0.45-µm pore size, discarding the first 4 mL of the filtrate. Collect the remaining 1 mL of filtrate for testing.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; end-capped 5-µm packing L1
Column temperature: 45°
Flow rate: 1 mL/min
Injection volume: 10 µL

System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram from *Standard solution A* is similar to the reference chromatogram provided with the lot of USP Powdered Red Clover Extract RS being used.
Tailing factor: NMT 2.0 for the formononetin peak, *Standard solution B*
Relative standard deviation: NMT 2.0%, determined from the formononetin peak in repeated injections, *Standard solution B*

Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Identify the peaks corresponding to daidzein, genistein, formononetin, and biochanin A in the *Sample solution* chromatogram by comparison with the chromatogram obtained from *Standard solution A* and the reference chromatogram. Measure the areas of the analyte peaks.
Calculate the content of each isoflavone, in mg, in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times C_S \times V \times D$$

r_U = peak response for each relevant isoflavone from the *Sample solution*
r_S = peak response for daidzein, genistein, formononetin, or biochanin A from *Standard solution B*
C_S = concentration of daidzein, genistein, formononetin, or biochanin A in *Standard solution B* (mg/mL)
V = volume of *Sample stock solution* (mL)
D = dilution factor to prepare the *Sample solution* from the *Sample stock solution*, 1

Calculate the percentage of the labeled amount of Powdered Red Clover Extract taken:

$$\text{Result} = C_I \times (A_{WT}/W) \times (100/L_E) \times (100/L)$$

C_I = sum of the content of isoflavones in the portion of Tablets taken (mg)
A_{WT} = average weight of Tablets (mg)
W = weight of the powdered Tablets taken (mg)
L_E = labeled percentage of isoflavones in the Powdered Extract used to prepare the Tablets
L = label claim of Powdered Extract (mg/Tablet)

Acceptance criteria: 90.0%–110.0% as isoflavones

PERFORMANCE TESTS
• **Disintegration and Dissolution** ⟨2040⟩**:** Meet the requirements for disintegration in *Botanical Dosage Forms*
• **Weight Variation of Dietary Supplements** ⟨2091⟩ **:** Meet the requirements

SPECIFIC TESTS
• **Microbial Enumeration Tests** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^4 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the enterobacterial count is NMT 10^3 cfu/g.
• **Absence of Specified Microorganisms** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

ADDITIONAL REQUIREMENTS
• **Packaging and Storage:** Preserve in tight, light-resistant containers.
• **Labeling:** The label states the Latin binomial and, following the official name, the article from which Tablets were prepared. The label also indicates the quantity, in mg, of Powdered Extract per Tablet. Label Tablets to indicate the content, in mg, of isoflavones per 100 mg of Powdered Extract.
• **USP Reference Standards** ⟨11⟩
USP Biochanin A RS
USP Daidzein RS
USP Formononetin RS
USP Genistein RS
USP Powdered Red Clover Extract RS

Riboflavin—*see Riboflavin General Monographs*

Riboflavin Tablets—*see Riboflavin Tablets General Monographs*

Riboflavin 5′-Phosphate Sodium—*see Riboflavin 5′-Phosphate Sodium General Monographs*

Rhodiola crenulata Root and Rhizome

DEFINITION
Rhodiola crenulata Root and Rhizome consists of the dried roots and rhizomes of *Rhodiola crenulata* (Hook.f. & Thomson) H.Ohba (alt. name *Sedum crenulatum* Hook.f. & Thomson) (Fam. Crassulaceae), collected after the scape withers in autumn. It contains NLT 1.0% of total phenylethanoids calculated as the sum of salidroside ($C_{14}H_{20}O_7$) and tyrosol ($C_8H_{10}O_2$) on the dried basis, and NLT 0.6% of salidroside on the dried basis.

IDENTIFICATION
• **A. HPTLC for Articles of Botanical Origin** ⟨203⟩
Standard solution: 2.0 mg/mL of USP Salidroside RS and 1.0 mg/mL of USP Rosavin RS in methanol
Sample solution: 500 mg of *Rhodiola crenulata* Root and Rhizome, finely powdered, in 5 mL of methanol. Sonicate for 10 min, centrifuge, and use the supernatant.
Chromatographic system
Absorbent: Chromatographic silica gel with an average particle size of about 5 µm
Application volume: 5 µL, as 8-mm bands

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Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
Developing solvent system: Ethyl acetate, methanol, water, and formic acid (77:13:10:2)
Developing distance: 6 cm
Derivatization reagent: Dissolve 1 g of diphenylamine in 40 mL of acetone, add 1 mL of aniline, and mix. Carefully add 7.5 mL of phosphoric acid, and mix.

Analysis
Samples: *Standard solution* and *Sample solution*
Apply the *Samples* as bands and dry in air. Develop in a saturated chamber, remove the plate from the chamber, and dry in air. Treat with *Derivatization reagent*, heat at 120° for 5 min, and examine under white light.

System suitability: The *Standard solution* shows two bands in the lower half of the chromatogram. The band with a higher R_F is due to salidroside, and the band with a lower R_F is due to rosavin.

Acceptance criteria: The *Sample solution* exhibits a band corresponding in R_F and color to salidroside in the *Standard solution*. In the *Sample solution*, no band corresponding in R_F and color to rosavin is observed (a distinction from *Rhodiola rosea*). The *Sample solution* exhibits additional bands, including two bands below salidroside but above the position corresponding to rosavin, two yellow bands in the upper half of the chromatogram, and a couple of bands close to the starting position.

- **B. HPLC**

Analysis: Proceed as directed in the test for *Content of Phenylethanoids*.

Acceptance criteria: The *Sample solution* exhibits a peak with a retention time corresponding to salidroside in the *Standard solution*, a peak due to tyrosol with lower intensity than salidroside, and a peak due to gallic acid. The content ratio of salidroside to tyrosol is NLT 2.

COMPOSITION

- **CONTENT OF PHENYLETHANOIDS**

Solution A: 0.02% phosphoric acid in water
Solution B: Methanol and acetonitrile (9:1)
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	95	5
10	95	5
15	83	17
28	83	17
28.1	10	90
43	10	90
43.1	95	5
53	95	5

Solvent: Methanol and water (6:4)
Standard solution: 0.30 mg/mL of USP Salidroside RS in methanol
Sample solution: Accurately transfer about 500 mg of finely powdered *Rhodiola crenulata* Root and Rhizome to a suitable flask, accurately add 15.0 mL of *Solvent*, and tightly close. Weigh the filled flask with a precision of ±0.1 mg and sonicate for 30 min. Cool to room temperature and adjust to the initial weight by adding *Solvent* if needed. Before injection, pass through a suitable membrane filter of 0.45-µm or finer pore size and discard the first portion of the filtrate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 275 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 25°
Flow rate: 1.0 mL/min
Injection volume: 10 µL

System suitability
Samples: *Standard solution* and *Sample solution*
Suitability requirements
Resolution: NLT 1.5 between salidroside and tyrosol, *Sample solution*
Tailing factor: NMT 1.5 for salidroside, *Standard solution*
Relative standard deviation: NMT 2.0% for salidroside in repeated injections, *Standard solution*

Analysis
Samples: *Standard solution* and *Sample solution*
Using the chromatogram of the *Standard solution* and the relative retention times, identify the peaks corresponding to gallic acid, salidroside, and tyrosol in the *Sample solution*.
[NOTE—The approximate relative retention times for the peaks of gallic acid, salidroside, and tyrosol are 0.48, 1.00, and 1.05, respectively.]
Separately calculate the percentages of salidroside and tyrosol in the portion of *Rhodiola crenulata* Root and Rhizome taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of salidroside from the *Standard solution*
C_S = concentration of USP Salidroside RS in the *Standard solution* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of *Rhodiola crenulata* Root and Rhizome taken to prepare the *Sample solution* (mg)
F = conversion factors for the analytes: 1.00 for salidroside, 0.43 for tyrosol

Calculate the content of total phenylethanoids as the sum of the percentages of salidroside and tyrosol.

Acceptance criteria
Salidroside: NLT 0.6% on the dried basis
Total phenylethanoids: NLT 1.0% on the dried basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Limits of Elemental Impurities*: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Pesticide Residue Analysis*: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacterial count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures*, *Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: Rhizome cylindrical, stout, slightly curved, few branched, 5–20 cm long, 3–5 cm in diameter. Externally brown or chocolate brown, coarse with wrinkles; under the outer epidermis, a layer of yellow membranous

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epidermis with pink decorative pattern appears; some old scape persist with triangular or ovate membranous scales at the base; internode irregular, fracture pink to purplish red with a ring; texture light and lax. Taproot cylindrical, stout, about 20 cm long, and about 1.5 cm in diameter at the upper part. Lateral root 10–30 cm long, fracture orange-red or purplish red, sometimes with cracks.

Microscopic

Transverse section of root: Cork consists of 5–8 layers of cells, phelloderm cells are elliptic or sub-rounded. Stele is broad with numerous vascular bundles in 2–4 rings, peripheral vascular bundles of collateral type are relatively large, interior 2–3 rings of amphivasal-type vascular bundles are gradually smaller toward the inside.

Transverse section of rhizome: The old rhizomes contain 2–3 bands of cork. The young rhizomes do not contain a cork layer. Cork consists of several layers of cells and phelloderm is indistinct. Stele collateral vascular bundles are radially arranged in a ring. The outer and inner vascular tissues of each bundle are well-developed, parenchymatous cells in the middle, phloem and xylem are almost equal with obvious cambium. Medulla is broad, consists of parenchymatous cells with amphivasal vascular bundles randomly. The parenchymatous cells contain brown secretion.

- **Loss on Drying** ⟨731⟩
  Sample: Finely powdered
  Analysis: Dry the *Sample* at 105° for 5 h.
  Acceptance criteria: NMT 12.0%
- **Articles of Botanical Origin** ⟨561⟩, *Methods of Analysis, Foreign Organic Matter*: NMT 2.0%
- **Articles of Botanical Origin** ⟨561⟩, *Methods of Analysis, Alcohol-Soluble Extractives, Method 1*: NLT 25.0%
- **Articles of Botanical Origin** ⟨561⟩, *Methods of Analysis, Total Ash*: NMT 6.0%
- **Articles of Botanical Origin** ⟨561⟩, *Methods of Analysis, Acid-Insoluble Ash*: NMT 1.0%

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.
- **Labeling:** The label states the Latin binomial following the official name of the plant contained in the article.
- **USP Reference Standards** ⟨11⟩
  USP Rosavin RS
  USP Salidroside RS

Rhodiola crenulata Root and Rhizome Dry Extract

DEFINITION

Rhodiola crenulata Root and Rhizome Dry Extract is prepared from the dried roots and rhizomes of *Rhodiola crenulata* (Hook.f. & Thomson) H.Ohba (alt. name *Sedum crenulatum* Hook.f. & Thomson) (Fam. Crassulaceae), collected after the scape withers in autumn, by extraction with alcohol or a hydroalcoholic mixture. It contains NLT 90.0% and NMT 110.0% of the labeled amount of total phenylethanoids, calculated as the sum of salidroside ($C_{14}H_{20}O_7$) and tyrosol ($C_8H_{10}O_2$) on the dried basis, and NLT 2.0% of salidroside on the dried basis. It may contain suitable added substances as carriers.

IDENTIFICATION

- **A. HPTLC for Articles of Botanical Origin** ⟨203⟩
  Standard solution: 2.0 mg/mL of USP Salidroside RS and 1.0 mg/mL of USP Rosavin RS in methanol
  Sample solution: Sonicate 50 mg/mL of *Rhodiola crenulata* Root and Rhizome Dry Extract in methanol for 10 min, centrifuge, and use the supernatant.
  Chromatographic system
  Adsorbent: Chromatographic silica gel with an average particle size of about 5 µm
  Application volume: 5 µL, as 8-mm bands
  Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
  Developing solvent system: Ethyl acetate, methanol, water, and formic acid (77:13:10:2)
  Developing distance: 6 cm
  Derivatization reagent: Dissolve 1 g of diphenylamine in 40 mL of acetone, add 1 mL of aniline, and mix. Carefully add 7.5 mL of phosphoric acid, and mix.
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Apply the *Samples* as bands and dry in air. Develop in a saturated chamber, remove the plate from the chamber, and dry in air. Treat with *Derivatization reagent*, heat at 120° for 5 min, and examine under white light.
  System suitability: The *Standard solution* shows two bands in the lower half of the chromatogram. The band with a higher R_F is due to salidroside, and the band with a lower R_F is due to rosavin.
  Acceptance criteria: The *Sample solution* exhibits a band corresponding in R_F and color to salidroside in the *Standard solution*. In the *Sample solution*, no band corresponding in R_F and color to rosavin is observed (a distinction from *Rhodiola rosea*). The *Sample solution* exhibits additional bands, including two bands below salidroside but above the position corresponding to rosavin, some yellow bands in the upper half of the chromatogram, and a couple of bands close to the starting position.
- **B. HPLC**
  Analysis: Proceed as directed in the test for *Content of Phenylethanoids*.
  Acceptance criteria: The *Sample solution* exhibits a peak with a retention time corresponding to salidroside in the *Standard solution*, a peak due to tyrosol with lower intensity than salidroside, and a peak due to gallic acid.

COMPOSITION

- **Content of Phenylethanoids**
  Solution A: 0.02% phosphoric acid in water
  Solution B: Methanol and acetonitrile (9:1)
  Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	95	5
10	95	5
15	83	17
28	83	17
28.1	10	90
43	10	90
43.1	95	5
53	95	5

Solvent: Methanol and water (6:4)

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Standard solution: 0.30 mg/mL of USP Salidroside RS in methanol
Sample solution: Accurately transfer about 250 mg of *Rhodiola crenulata* Root and Rhizome Dry Extract to a suitable flask, accurately add 15.0 mL of *Solvent*, and tightly close. Weigh the filled flask with a precision of ±0.1 mg and sonicate for 30 min. Cool to room temperature and adjust to the initial weight by adding *Solvent* if needed. Before injection, pass through a suitable membrane filter of 0.45-µm or finer pore size and discard the first portion of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 275 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 25°
Flow rate: 1.0 mL/min
Injection volume: 10 µL
System suitability
Samples: *Standard solution* and *Sample solution*
Suitability requirements
Resolution: NLT 1.5 between salidroside and tyrosol, *Sample solution*
Tailing factor: NMT 1.5 for salidroside, *Standard solution*
Relative standard deviation: NMT 2.0% for salidroside in repeated injections, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Using the chromatogram of the *Standard solution* and the relative retention times, identify the peaks corresponding to gallic acid, salidroside, and tyrosol in the *Sample solution*.
[NOTE—The approximate relative retention times for the peaks of gallic acid, salidroside, and tyrosol are 0.48, 1.00, and 1.05, respectively.]
Separately calculate the percentages of salidroside and tyrosol in the portion of *Rhodiola crenulata* Root and Rhizome Dry Extract taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of salidroside from the *Standard solution*
C_S = concentration of USP Salidroside RS in the *Standard solution* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of *Rhodiola crenulata* Root and Rhizome Dry Extract taken to prepare the *Sample solution* (mg)
F = conversion factors for the analytes: 1.00 for salidroside, 0.43 for tyrosol

Calculate the content of total phenylethanoids as the sum of the percentages of salidroside and tyrosol.
Calculate the percentage of the labeled amount of total phenylethanoids in the portion of *Rhodiola crenulata* Root and Rhizome Dry Extract taken:

$$\text{Result} = (P/L) \times 100$$

P = content of total phenylethanoids as determined above (%)
L = labeled amount of total phenylethanoids (%)

Acceptance criteria
Salidroside: NLT 2.0% on the dried basis
Total phenylethanoids: NLT 90.0% and NMT 110.0% on the dried basis

CONTAMINANTS
• **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩
Acceptance criteria
Arsenic: NMT 2.0 µg/g
Cadmium: NMT 0.5 µg/g
Lead: NMT 5.0 µg/g
Mercury: NMT 0.2 µg/g
• **BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*: Meets the requirements
• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^4 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g.
• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS
• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry at 105° for 5 h.
Acceptance criteria: NMT 5.0%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.
• **LABELING:** The label states the Latin binomial following the official name of the plant contained in the article. It meets other labeling requirements in *Botanical Extracts* ⟨565⟩.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Rosavin RS
USP Salidroside RS

Rhodiola crenulata Root and Rhizome Powder

DEFINITION
Rhodiola crenulata Root and Rhizome Powder consists of the dried roots and rhizomes of *Rhodiola crenulata* (Hook.f. & Thomson) H.Ohba (alt. name *Sedum crenulatum* Hook.f. & Thomson) (Fam. Crassulaceae), collected after the scape withers in autumn, reduced to a fine or very fine powder. It contains NLT 1.0% of total phenylethanoids calculated as the sum of salidroside ($C_{14}H_{20}O_7$) and tyrosol ($C_8H_{10}O_2$) on the dried basis, and NLT 0.6% of salidroside on the dried basis.

IDENTIFICATION
• **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩
Standard solution: 2.0 mg/mL of USP Salidroside RS and 1.0 mg/mL of USP Rosavin RS in methanol
Sample solution: 500 mg of *Rhodiola crenulata* Root and Rhizome Powder in 5 mL of methanol. Sonicate for 10 min, centrifuge, and use the supernatant.
Chromatographic system
Adsorbent: Chromatographic silica gel with an average particle size of about 5 µm
Application volume: 5 µL, as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
Developing solvent system: Ethyl acetate, methanol, water, and formic acid (77:13:10:2)
Developing distance: 6 cm
Derivatization reagent: Dissolve 1 g of diphenylamine in 40 mL of acetone, add 1 mL of aniline, and mix. Carefully add 7.5 mL of phosphoric acid, and mix.

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Analysis
Samples: *Standard solution* and *Sample solution*
Apply the *Samples* as bands and dry in air. Develop in a saturated chamber, remove the plate from the chamber, and dry in air. Treat with *Derivatization reagent*, heat at 120° for 5 min, and examine under white light.
System suitability: The *Standard solution* shows two bands in the lower half of the chromatogram. The band with a higher R_F is due to salidroside, and the band with a lower R_F is due to rosavin.
Acceptance criteria: The *Sample solution* exhibits a band corresponding in R_F and color to salidroside in the *Standard solution*. In the *Sample solution*, no band corresponding in R_F and color to rosavin is observed (a distinction from *Rhodiola rosea*). The *Sample solution* exhibits additional bands, including two bands below salidroside but above the position corresponding to rosavin, two yellow bands in the upper half of the chromatogram, and a couple of bands close to the starting position.

• **B. HPLC**
Analysis: Proceed as directed in the test for *Content of Phenylethanoids*.
Acceptance criteria: The *Sample solution* exhibits a peak with a retention time corresponding to salidroside in the *Standard solution*, a peak due to tyrosol with lower intensity than salidroside, and a peak due to gallic acid. The content ratio of salidroside to tyrosol is NLT 2.

COMPOSITION

• **CONTENT OF PHENYLETHANOIDS**
Solution A: 0.02% phosphoric acid in water
Solution B: Methanol and acetonitrile (9:1)
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	95	5
10	95	5
15	83	17
28	83	17
28.1	10	90
43	10	90
43.1	95	5
53	95	5

Solvent: Methanol and water (6:4)
Standard solution: 0.30 mg/mL of USP Salidroside RS in methanol
Sample solution: Accurately transfer about 500 mg of *Rhodiola crenulata* Root and Rhizome Powder to a suitable flask, accurately add 15.0 mL of *Solvent*, and tightly close. Weigh the filled flask with a precision of ±0.1 mg and sonicate for 30 min. Cool to room temperature and adjust to the initial weight by adding *Solvent* if needed. Before injection, pass through a suitable membrane filter of 0.45-µm or finer pore size and discard the first portion of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 275 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 25°
Flow rate: 1.0 mL/min
Injection volume: 10 µL
System suitability
Samples: *Standard solution* and *Sample solution*
Suitability requirements
Resolution: NLT 1.5 between salidroside and tyrosol, *Sample solution*
Tailing factor: NMT 1.5 for salidroside, *Standard solution*
Relative standard deviation: NMT 2.0% for salidroside in repeated injections, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Using the chromatogram of the *Standard solution* and the relative retention times, identify the peaks corresponding to gallic acid, salidroside, and tyrosol in the *Sample solution*.
[NOTE—The approximate relative retention times for the peaks of gallic acid, salidroside, and tyrosol are 0.48, 1.00, and 1.05, respectively.]
Separately calculate the percentages of salidroside and tyrosol in the portion of *Rhodiola crenulata* Root and Rhizome Powder taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of salidroside from the *Standard solution*
C_S = concentration of USP Salidroside RS in the *Standard solution* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of *Rhodiola crenulata* Root and Rhizome Powder taken to prepare the *Sample solution* (mg)
F = conversion factors for the analytes: 1.00 for salidroside, 0.43 for tyrosol

Calculate the content of total phenylethanoids as the sum of the percentages of salidroside and tyrosol.
Acceptance criteria
Salidroside: NLT 0.6% on the dried basis
Total phenylethanoids: NLT 1.0% on the dried basis

CONTAMINANTS

• **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Limits of Elemental Impurities*: Meets the requirements
• **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Pesticide Residue Analysis*: Meets the requirements
• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacterial count does not exceed 10^3 cfu/g.
• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures*, *Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS

• **BOTANICAL CHARACTERISTICS**
Macroscopic: Pinkish-brown or light reddish-brown powder
Microscopic: Cork cell fragments subrectangular or polygonal, containing yellowish-brown pigments, 10–150 µm in diameter. Spiral vessels are 6–58 µm in diameter. Fibers mostly in bundles, long fusiform with oblique or blunt-round ends and striations, 10–60 µm in diameter. Parenchymatous cells are square or irregular, 2–20 µm in diameter, containing calcium oxalate prism crystals. Abundant simple starch grains rounded with indistinct hilum and striation, 2–10 µm in diameter.

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- **LOSS ON DRYING** ⟨731⟩
  Analysis: Dry at 105° for 5 h.
  Acceptance criteria: NMT 12.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Alcohol-Soluble Extractives, Method 1*: NLT 25.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Total Ash*: NMT 6.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Acid-Insoluble Ash*: NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial following the official name of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Rosavin RS
  USP Salidroside RS

Rhodiola rosea

DEFINITION

Rhodiola rosea consists of the dried roots and rhizomes of *Rhodiola rosea* L. (Fam. Crassulaceae). It contains NLT 0.3% of phenylpropenoid glycosides calculated as the sum of rosarin, rosavin, and rosin; and NLT 0.08% salidroside; both calculated on the dried basis.

IDENTIFICATION

- **A.** *Rhodiola rosea* meets the requirements under *Specific Tests, Botanic Characteristics.*
- **B. THIN-LAYER CHROMATOGRAPHY**
  Standard solution A: 1.0 mg/mL of USP Rosavin RS in methanol
  Standard solution B: 50 mg/mL of USP *Rhodiola rosea* Root and Rhizome Dry Extract RS in methanol. Sonicate for 10 min, centrifuge, and use the supernatant.
  Sample solution: Sonicate for 10 min about 0.5 g of *Rhodiola rosea*, finely powdered, in 5 mL of methanol, centrifuge, and use the supernatant.
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography.*)
  Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plates)
  Application volume: 3 µL of *Standard solution A*, 5 µL each of *Standard solution B* and *Sample solution*; as 8-mm bands
  Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device
  Developing solvent system: A mixture of ethyl acetate, methanol, water, and formic acid (77:13:10:2)
  Developing distance: 6 cm
  Derivatization reagent: Dissolve 1 g of diphenylamine in 40 mL of acetone, add 1 mL of aniline, and mix. Carefully add 7.5 mL of phosphoric acid, and mix.
  Analysis
  Samples: *Standard solution A, Standard solution B,* and *Sample solution*
  Apply the samples as bands to a suitable high performance thin-layer chromatographic plate, and dry in air. Develop the chromatograms in a saturated chamber, remove the plate from the chamber, and dry in air. Derivatize the plate with *Derivatization reagent*, heat at 120° for 5 min, and examine under visible light.
  System suitability: The chromatogram of *Standard solution B* exhibits, in the lower half, three gray bands and two brownish bands, one above and the other below the gray bands; the most intense band in the chromatogram is the brownish band with an R_F below the gray bands; the most intense gray band is the lower band at an R_F corresponding to the band due to rosavin in the chromatogram of *Standard Solution A*; the upper gray band due to rosarin is less intense.
  Acceptance criteria: The chromatogram of the *Sample solution* exhibits a gray band corresponding to the band due to rosavin in the chromatogram of *Standard Solution A*, and the following bands corresponding to similar bands in the chromatogram of *Standard solution B*: two additional gray bands and two brownish bands, one above and the other below the gray bands; the most intense band in the chromatogram is the brownish band with an R_F below the gray bands; the most intense gray band is the lower band due to rosavin.
- **C. HPLC**
  Analysis: Proceed as directed in the test for *Content of Phenylpropenoid Glycosides and Salidroside.*
  Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks at the retention times corresponding to the peaks due to salidroside, tyrosol, rosarin, rosavin, rosin, and rosiridin in the chromatogram of *Standard solution C.* The ratio of the content of phenylpropenoid glycosides: rosarin, rosavin, and rosin, to the content of salidroside is about 3:1.

COMPOSITION

- **CONTENT OF PHENYLPROPENOID GLYCOSIDES AND SALIDROSIDE**
  Solution A: Water
  Solution B: Acetonitrile
  Mobile phase: See *Table 1.*

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	94	6
6	83	17
7	80.3	19.7
9	80.3	19.7
10	0	100
12	94	6
17	94	6

Solvent: 75% methanol in water
Standard solution A: 1.0 mg/mL of USP Rosavin RS in methanol
Standard solution B: 0.3 mg/mL of USP Salidroside RS in methanol
Standard solution C: 4.0 mg/mL of USP *Rhodiola rosea* Root and Rhizome Dry Extract RS in methanol. Sonicate to dissolve, if necessary. Before injection, pass through a membrane filter of 0.45-µm or finer pore size.
Sample solution: Transfer about 5.0 g of *Rhodiola rosea,* finely powdered and accurately weighed, to a 25-mL flask. Add 7 mL of *Solvent*, sonicate for 15 min, and filter into a 10-mL volumetric flask. Wash the residue on the filter paper twice, using 1 mL of *Solvent* each; add the washings to the volumetric flask; adjust the volume using *Solvent*; and mix. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability.*)

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Mode: LC
Detector: UV, 205 nm
Column: 3.0-mm × 10-cm; 2.5-µm packing L1
Column temperature: 40 ± 1°
Flow rate: 1.0 mL/min
Injection volume: 1 µL
System suitability
Samples: *Standard solution A* and *Standard solution C*
Suitability requirements
Chromatogram similarity: The chromatogram obtained from *Standard solution C* is similar to the reference chromatogram provided with the lot of USP *Rhodiola rosea* Root and Rhizome Dry Extract RS being used.
Resolution: NLT 1.5 between the rosarin and rosavin peaks, *Standard solution C*
Relative standard deviation: NMT 2% determined from the rosavin peak in repeated injections, *Standard solution A*
Analysis
Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*
Using the chromatograms of *Standard solution A*, *Standard solution B*, *Standard solution C*, and the reference chromatogram provided with the lot of USP *Rhodiola rosea* Root and Rhizome Dry Extract RS being used, identify the retention time of the peaks corresponding to salidroside, tyrosol, rosarin, rosavin, rosin, and rosiridin in the *Sample solution*.
Separately calculate the percentages of rosarin, rosavin, and rosin as rosavin in the portion of *Rhodiola rosea* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of the relevant analyte in the *Sample solution*
r_S = peak area of rosavin in *Standard solution A*
C_S = concentration of rosavin in *Standard solution A* (mg/mL)
V = volume of *Sample solution* (mL)
W = weight of *Rhodiola rosea* taken to prepare the *Sample solution* (mg)

Calculate the percentage of phenylpropenoid glycosides as the sum of the percentages of rosarin, rosavin, and rosin.
Calculate the percentage of salidroside in the portion of *Rhodiola rosea* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of salidroside from the *Sample solution*
r_S = peak area of salidroside from *Standard solution B*
C_S = concentration of salidroside in *Standard solution B* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of *Rhodiola rosea* taken to prepare the *Sample solution* (g)

Acceptance criteria: NLT 0.3% of phenylpropenoid glycosides and NLT 0.08% salidroside; both calculated on the dried basis.

CONTAMINANTS

- **Elemental Impurities—Procedures** ⟨233⟩

Acceptance criteria
Arsenic: NMT 2.0 µg/g
Cadmium: NMT 1.0 µg/g
Lead: NMT 5.0 µg/g
Mercury: NMT 1.0 µg/g

Change to read:

- **▲Articles of Botanical Origin,** *Pesticide Residue Analysis* ⟨561⟩▲ (CN 1-May-2019)**:** Meets the requirements
- **Microbial Enumeration Tests** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria count does not exceed 10^3 cfu/g.
- **Absence of Specified Microorganisms** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*
- **Articles of Botanical Origin,** *Test for Aflatoxins* ⟨561⟩**:** Meets the requirements

SPECIFIC TESTS

- **Botanic Characteristics**

Macroscopic: Underground parts consist of numerous rhizomes united at their base into a long taproot. Both the rhizome and root exhibit secondary growth. Pharmacopeial article consists of dry pieces of rhizomes and roots of various shapes. Pieces of rhizomes are thick, wrinkly, with remains of stems and scales, and pieces of roots branching off the rhizome. The surface of the rhizome and the roots is shiny, grayish-brown; after peeling off the cork, a golden-yellow layer is revealed; fracture, pinkish-brown or light brown.
Microscopic
Transverse section of rhizome: Cork, narrow to broad, depending on the sample; cork cells may be dark brown, greenish, or nearly colorless; cortex, large, loosely arranged parenchymatous cells, with slightly thickened walls; secondary phloem, parenchymatous, no sclereides; small narrow vascular bundles occur in a ring surrounding a broad parenchymatous pith; pith includes scattered vascular bundles; parenchyma of the rhizome is filled with starch granules, simple, round or oval-shaped, 5–20 µm in diameter; hilum, if present, appears as a small dot.
Transverse section of root: Cork, narrow to broad, depending on the sample; cortex, large, parenchymatous cells, may contain orange-brown tannin ducts; tannin ducts are also found embedded in the cork of old roots; secondary phloem, parenchymatous, no sclereides; small narrow vascular bundles occur in a ring surrounding a narrow parenchymatous pith; starch granules, simple, round or oval-shaped, 5–20 µm in diameter; hilum, if present, appears as a small dot.

- **Articles of Botanical Origin,** *Foreign Organic Matter* ⟨561⟩**:** NMT 2.0%
- **Loss on Drying** ⟨731⟩

Sample: 1.0 g of finely powdered *Rhodiola rosea*
Analysis: Dry at 105° for 2 h.
Acceptance criteria: NMT 12%

- **Articles of Botanical Origin,** *Total Ash* ⟨561⟩

Sample: 2 g of finely powdered *Rhodiola rosea*
Acceptance criteria: NMT 12%

- **Articles of Botanical Origin,** *Acid-Insoluble Ash* ⟨561⟩

Sample: 2–4 g of finely powdered *Rhodiola rosea*
Acceptance criteria: NMT 3%

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **Labeling:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP Reference Standards** ⟨11⟩

USP *Rhodiola rosea* Root and Rhizome Dry Extract RS

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USP Rosavin RS
USP Salidroside RS

Powdered *Rhodiola rosea*

DEFINITION

Powdered *Rhodiola rosea* is *Rhodiola rosea* L. reduced to a powder or fine powder. It contains NLT 0.3% of phenylpropanoid glycosides, calculated as the sum of rosarin, rosavin, and rosin, and NLT 0.08% of salidroside, both calculated on the dried basis.

IDENTIFICATION

• **A.** Powdered *Rhodiola rosea* meets the requirements under *Specific Tests, Botanic Characteristics.*

• **B. THIN-LAYER CHROMATOGRAPHY**

Standard solution A: 1.0 mg/mL of USP Rosavin RS in methanol

Standard solution B: 50 mg/mL of USP *Rhodiola rosea* Root and Rhizome Dry Extract RS in methanol. Sonicate for 10 min, centrifuge, and use the supernatant.

Sample solution: Sonicate for 10 min about 0.5 g of Powdered *Rhodiola rosea* in 5 mL of methanol, centrifuge, and use the supernatant.

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography.*)

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plates)

Application volume: 3 µL of *Standard solution A* and 5 µL each of *Standard solution B* and *Sample solution*; as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

Developing solvent system: A mixture of ethyl acetate, methanol, water, and formic acid (77:13:10:2)

Developing distance: 6 cm

Derivatization reagent: Dissolve 1 g of diphenylamine in 40 mL of acetone, add 1 mL of aniline, and mix. Carefully add 7.5 mL of phosphoric acid, and mix.

Analysis

Samples: *Standard solution A, Standard solution B,* and *Sample solution*

Apply the samples as bands to a suitable high performance thin-layer chromatographic plate, and dry in air. Develop the chromatograms in a saturated chamber, remove the plate from the chamber, and dry in air. Derivatize the plate with *Derivatization reagent,* heat at 120° for 5 min, and examine under visible light.

System suitability: The chromatogram of *Standard solution B* exhibits, in the lower half, three gray bands and two brownish bands, one above and the other below the gray bands; the most intense band in the chromatogram is the brownish band with an R_F below the gray bands; the most intense gray band is the lower band at an R_F corresponding to the band due to rosavin in the chromatogram of *Standard solution A*; the upper gray band due to rosarin is less intense.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits a gray band corresponding to the band due to rosavin in the chromatogram of *Standard solution A*, and the following bands corresponding to similar bands in the chromatogram of *Standard solution B*: two additional gray bands and two brownish bands, one above and the other below the gray bands; the most intense band in the chromatogram is the brownish band with an R_F below the gray bands; the most intense gray band is the lower band due to rosavin.

• **C. HPLC**

Analysis: Proceed as directed in the test for *Content of Phenylpropenoid Glycosides and Salidroside.*

Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks at the retention times corresponding to the peaks due to salidroside, tyrosol, rosarin, rosavin, rosin, and rosiridin in the chromatogram of *Standard solution C*. The ratio of the content of the phenylpropenoid glycosides, rosarin, rosavin, and rosin, to the content of salidroside is about 3:1.

COMPOSITION

• **CONTENT OF PHENYLPROPENOID GLYCOSIDES AND SALIDROSIDE**

Solution A: Water

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	94	6
6	83	17
7	80.3	19.7
9	80.3	19.7
10	0	100
12	94	6
17	94	6

Solvent: 75% methanol in water

Standard solution A: 1.0 mg/mL of USP Rosavin RS in methanol

Standard solution B: 0.3 mg/mL of USP Salidroside RS in methanol

Standard solution C: 4.0 mg/mL of USP *Rhodiola rosea* Root and Rhizome Dry Extract RS in methanol. Sonicate to dissolve, if necessary. Before injection, pass through a membrane filter of 0.45-µm or finer pore size.

Sample solution: Transfer about 5.0 g of Powdered *Rhodiola rosea*, accurately weighed, to a 25-mL flask. Add 7 mL of *Solvent*, sonicate for 15 min, and filter into a 10-mL volumetric flask. Wash the residue on the filter paper twice using 1 mL of *Solvent* each, add the washings to the volumetric flask, dilute with *Solvent* to volume, and mix. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability.*)

Mode: LC

Detector: UV 205 nm

Column: 3.0-mm × 10-cm; 2.5-µm packing L1

Column temperature: 40 ± 1°

Flow rate: 1.0 mL/min

Injection volume: 1 µL

System suitability

Samples: *Standard solution A* and *Standard solution C*

Suitability requirements

Chromatogram similarity: The chromatogram obtained from *Standard solution C* is similar to the reference chromatogram provided with the lot of USP *Rhodiola rosea* Root and Rhizome Dry Extract RS being used.

Resolution: NLT 1.5 between the rosarin and rosavin peaks, *Standard solution C*

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Relative standard deviation: NMT 2% determined from the rosavin peak in repeated injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Using the chromatograms of *Standard solution A*, *Standard solution B*, *Standard solution C*, and the reference chromatogram provided with the lot of USP *Rhodiola rosea* Root and Rhizome Dry Extract RS being used, identify the retention time of the peaks corresponding to salidroside, tyrosol, rosarin, rosavin, rosin, and rosiridin from the *Sample solution*.

Separately calculate the percentages of rosarin, rosavin, and rosin as rosavin in the portion of Powdered *Rhodiola rosea* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of rosavin from *Standard solution A*
C_S = concentration of rosavin in *Standard solution A* (mg/mL)
V = volume of *Sample solution* (mL)
W = weight of Powdered *Rhodiola rosea* taken to prepare the *Sample solution* (mg)

Calculate the percentage of phenylpropenoid glycosides as the sum of the percentages of rosarin, rosavin, and rosin.

Calculate the percentage of salidroside in the portion of Powdered *Rhodiola rosea* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of salidroside in the *Sample solution*
r_S = peak area of salidroside in *Standard solution B*
C_S = concentration of salidroside in *Standard solution B* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Powdered *Rhodiola rosea* taken to prepare the *Sample solution* (g)

Acceptance criteria: NLT 0.3% of phenylpropenoid glycosides and NLT 0.08% of salidroside, both calculated on the dried basis

CONTAMINANTS

- **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩

Acceptance criteria

Arsenic: NMT 2.0 µg/g
Cadmium: NMT 1.0 µg/g
Lead: NMT 5.0 µg/g
Mercury: NMT 1.0 µg/g

Change to read:

- **▲ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Pesticide Residue Analysis*:▲ (CN 1-May-2019) Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩: Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*
- **ARTICLES OF BOTANICAL ORIGIN,** *Test for Aflatoxins* ⟨561⟩: Meets the requirements

SPECIFIC TESTS

- **BOTANIC CHARACTERISTICS**

Macroscopic: Pinkish-brown or light brown in color

Microscopic It shows fragments of cork cells, subrectangular or polygonal, containing yellowish-brown pigments, 10–150 µm in diameter; parenchymatous cells of cortex, subsquare or polygonal, containing reddish-brown pigments; stone cells, subrounded, subtriangular, subrectangular or irregular shape, some elongated, mostly 14–70 µm in diameter, up to 270 µm in length; fibers mostly in bundles, long fusiform in shape, ends oblique-sharp or blunt-round, with oblique or criss-cross striations, 10–60 µm in diameter; and reticulate and pitted vessels, up to 120 µm in diameter.

- **LOSS ON DRYING** ⟨731⟩

Sample: 1.0 g of Powdered *Rhodiola rosea*
Analysis: Dry at 105° for 2 h.
Acceptance criteria: NMT 12%

- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩

Sample: 2 g of Powdered *Rhodiola rosea*
Acceptance criteria: NMT 12%

- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩

Sample: 2–4 g of Powdered *Rhodiola rosea*
Acceptance criteria: NMT 3%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant from which the article was obtained.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP *Rhodiola rosea* Root and Rhizome Dry Extract RS
USP Rosavin RS
USP Salidroside RS

Powdered *Rhodiola rosea* Extract

DEFINITION

Powdered *Rhodiola rosea* Extract is prepared from *Rhodiola rosea* by extraction with hydroalcoholic mixtures. The ratio of plant material to extract is between 1.5:1 and 5:1. It contains NLT 90.0% and NMT 110.0% of the labeled amount of phenylpropenoid glycosides calculated as the sum of rosarin, rosavin, and rosin, and NLT 90.0% and NMT 110.0% of the labeled amount of salidroside, both calculated on the dried basis. It may contain suitable added substances as carriers.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHY**

Standard solution A: 1.0 mg/mL of USP Rosavin RS in methanol
Standard solution B: 50 mg/mL of USP *Rhodiola rosea* Root and Rhizome Dry Extract RS in methanol. Sonicate for 10 min, centrifuge, and use the supernatant.
Sample solution: 50 mg/mL of Powdered *Rhodiola rosea* Extract in methanol. Sonicate for 10 min, centrifuge, and use the supernatant.

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plates)
Application volume: 3 µL of *Standard solution A* and 5 µL each of *Standard solution B* and the *Sample solution*; as 8-mm bands

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Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
Developing solvent system: A mixture of ethyl acetate, methanol, water, and formic acid (77:13:10:2)
Developing distance: 6 cm
Derivatization reagent: Dissolve 1 g of diphenylamine in 40 mL of acetone, add 1 mL of aniline, and mix. Carefully add 7.5 mL of phosphoric acid, and mix.

Analysis
Samples: *Standard solution A, Standard solution B,* and *Sample solution*
Apply the samples as bands to a suitable high performance thin-layer chromatographic plate, and dry in air. Develop the chromatograms in a saturated chamber, remove the plate from the chamber, and dry in air. Derivatize the plate with *Derivatization reagent,* heat at 120° for 5 min, and examine under visible light.

System suitability: The chromatogram of *Standard solution B* exhibits, in the lower half, three gray bands and two brownish bands, one above and the other below the gray bands; the most intense band in the chromatogram is the brownish band with an R_F below the gray bands; the most intense gray band is the lower band at an R_F corresponding to the band due to rosavin in the chromatogram of *Standard solution A*; the upper gray band due to rosarin is less intense.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits a gray band corresponding to the band due to rosavin in the chromatogram of *Standard solution A,* and the following bands corresponding to similar bands in the chromatogram of *Standard solution B*: two additional gray bands and two brownish bands, one above and the other below the gray bands; the most intense band in the chromatogram is the brownish band with an R_F below the gray bands; the most intense gray band is the lower band due to rosavin.

• **B. HPLC**

Analysis: Proceed as directed in the test for *Content of Phenylpropenoid Glycosides and Salidroside.*

Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks at the retention times corresponding to the peaks due to salidroside, tyrosol, rosarin, rosavin, rosin, and rosiridin in the chromatogram of *Standard solution C.* The ratio of the contents of rosarin, rosavin, and rosin is about 2.5:6.0:1.5, respectively.

COMPOSITION

• **CONTENT OF PHENYLPROPENOID GLYCOSIDES AND SALIDROSIDE**

Solution A: Water
Solution B: Acetonitrile
Mobile phase: See *Table 1.*

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	94	6
6	83	17
7	80.3	19.7
9	80.3	19.7
10	0	100
12	94	6
17	94	6

Standard solution A: 1.0 mg/mL of USP Rosavin RS in methanol
Standard solution B: 0.3 mg/mL of USP Salidroside RS in methanol
Standard solution C: 4.0 mg/mL of USP *Rhodiola rosea* Root and Rhizome Dry Extract RS in methanol. Sonicate to dissolve, if necessary. Before injection, pass through a membrane filter of 0.45-µm or finer pore size.
Sample solution: 4.0 mg/mL of Powdered *Rhodiola rosea* Extract in methanol. Sonicate to dissolve, if necessary. Before injection, pass through a membrane filter of 0.45-µm or finer pore size.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability.*)
Mode: LC
Detector: UV 205 nm
Column: 3.0-mm × 10-cm; 2.5-µm packing L1
Column temperature: 40 ± 1°
Flow rate: 1.0 mL/min
Injection volume: 1 µL
System suitability
Samples: *Standard solution A* and *Standard solution C*
Suitability requirements
Chromatogram similarity: The chromatogram obtained from *Standard solution C* is similar to the reference chromatogram provided with the lot of USP *Rhodiola rosea* Root and Rhizome Dry Extract RS being used.
Resolution: NLT 1.5 between the rosarin and rosavin peaks, *Standard solution C*
Relative standard deviation: NMT 2% determined from the rosavin peak in repeated injections, *Standard solution A*

Analysis
Samples: *Standard solution A, Standard solution B, Standard solution C,* and *Sample solution*
Using the chromatograms of *Standard solution A, Standard solution B, Standard solution C,* and the reference chromatogram provided with the lot of USP *Rhodiola rosea* Root and Rhizome Dry Extract RS being used, identify the retention time of the peaks corresponding to salidroside, tyrosol, rosarin, rosavin, rosin, and rosiridin from the *Sample solution.*
Separately calculate the percentage of rosarin, rosavin, and rosin as rosavin in the portion of Powdered *Rhodiola rosea* Extract taken:

$$P_1 = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of rosavin from *Standard solution A*
C_S = concentration of rosavin in *Standard solution A* (mg/mL)
C_U = concentration of Powdered *Rhodiola rosea* Extract in the *Sample solution* (mg/mL)

Calculate the percentage of phenylpropenoid glycosides as the sum of the percentages of rosarin, rosavin, and rosin.
Calculate the percentage of salidroside in the portion of Powdered *Rhodiola rosea* Extract taken:

$$P_2 = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of salidroside from the *Sample solution*
r_S = peak area of salidroside from *Standard solution B*
C_S = concentration of salidroside in *Standard solution B* (mg/mL)
C_U = concentration of Powdered *Rhodiola rosea* Extract in the *Sample solution* (mg/mL)

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Calculate the percentage of the labeled amount of phenylpropenoid glycosides in the portion of Powdered *Rhodiola rosea* Extract taken:

$$\text{Result} = (P_1/L) \times 100$$

P_1 = content of phenylpropenoid glycosides, as determined above (%)
L = labeled amount of phenylpropenoid glycosides (%)

Calculate the percentage of the labeled amount of salidroside in the portion of Powdered *Rhodiola rosea* Extract taken:

$$\text{Result} = (P_2/L) \times 100$$

P_2 = content of salidroside, as determined above (%)
L = labeled amount of salidroside (%)

Acceptance criteria: NLT 90.0% and NMT 110.0% of the labeled amount of phenylpropenoid glycosides, and NLT 90.0% and NMT 110.0% of the labeled amount of salidroside, both calculated on the dried basis.

CONTAMINANTS

• **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩
Acceptance criteria
Arsenic: NMT 2.0 µg/g
Cadmium: NMT 1.0 µg/g
Lead: NMT 5.0 µg/g
Mercury: NMT 1.0 µg/g

Change to read:

• **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020)
: Meets the requirements
• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*
• **ARTICLES OF BOTANICAL ORIGIN,** *Test for Aflatoxins* ⟨561⟩**:** Meets the requirements

SPECIFIC TESTS

• **LOSS ON DRYING** ⟨731⟩
Sample: 2.0 g of Powdered *Rhodiola rosea* Extract
Analysis: Dry at 105° for 2 h.
Acceptance criteria: NMT 5%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.
• **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived. It meets other labeling requirements under *Botanical Extracts* ⟨565⟩.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP *Rhodiola rosea* Root and Rhizome Dry Extract RS
USP Rosavin RS
USP Salidroside RS

Rhodiola rosea Tincture

DEFINITION

Rhodiola rosea Tincture is prepared as follows.

Rhodiola rosea	1 part (g)
A mixture of Alcohol and Water (40:60), a sufficient quantity to make	5 parts (mL)

Prepare the Tincture as directed for *Botanical Extracts* ⟨565⟩, *Tinctures, Maceration Process*. It contains NLT 0.06% (w/v) of phenylpropenoid glycosides calculated as the sum of rosarin, rosavin, and rosin; and NLT 0.016% of salidroside.

IDENTIFICATION

• **A. THIN-LAYER CHROMATOGRAPHY**
(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
Standard solution A: 1.0 mg/mL of USP Rosavin RS in methanol
Standard solution B: 50 mg/mL of USP *Rhodiola rosea* Root and Rhizome Dry Extract RS in methanol. Sonicate for 10 min, centrifuge, and use the supernatant.
Sample solution: Centrifuge a portion of Tincture, and use the supernatant.
Chromatographic system
Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plates)
Application volume: 3 µL of *Standard solution A*, 5 µL of *Standard solution B*, and 10 µL of *Sample solution*; as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
Developing solvent system: A mixture of ethyl acetate, methanol, water, and formic acid (77:13:10:2)
Developing distance: 6 cm
Derivatization reagent: Dissolve 1 g of diphenylamine in 40 mL of acetone, add 1 mL of aniline, and mix. Carefully add 7.5 mL of phosphoric acid, and mix.
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Apply the samples as bands to a suitable high performance thin-layer chromatographic plate, and dry in air. Develop the chromatograms in a saturated chamber, remove the plate from the chamber, and dry in air. Derivatize the plate with *Derivatization reagent*, heat at 120° for 5 min, and examine under visible light.
System suitability: The chromatogram of *Standard solution B* exhibits, in the lower half, three gray bands and two brownish bands, one above and the other below the gray bands; the most intense band in the chromatogram is the brownish band with an R_F below the gray bands; the most intense gray band is the lower band at an R_F corresponding to the band due to rosavin in the chromatogram of *Standard solution A*; the upper gray band due to rosarin is less intense.
Acceptance criteria: The chromatogram of the *Sample solution* exhibits a gray band corresponding to the band due to rosavin in the chromatogram of *Standard solution A*, and the following bands corresponding to similar bands in the chromatogram of *Standard solution B*: two additional gray bands and two brownish bands, one above and the other below the gray bands; the most intense band in the chromatogram is the brownish band with an R_F below the gray bands; the most intense gray band is the lower band due to rosavin.

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• **B. HPLC**

Analysis: Proceed as directed in the test for *Content of Phenylpropenoid Glycosides and Salidroside.*

Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks at the retention times corresponding to the peaks due to salidroside, tyrosol, rosarin, rosavin, rosin, and rosiridin in the chromatogram of *Standard solution C.*

The ratio of the contents of rosarin, rosavin, and rosin is about 2.5:6.0:1.5.

COMPOSITION

• **CONTENT OF PHENYLPROPENOID GLYCOSIDES AND SALIDROSIDE**

Solution A: Water

Solution B: Acetonitrile

Mobile phase: See *Table 1.*

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	94	6
6	83	17
7	80.3	19.7
9	80.3	19.7
10	0	100
12	94	6
17	94	6

Standard solution A: 1.0 mg/mL of USP Rosavin RS in methanol

Standard solution B: 0.3 mg/mL of USP Salidroside RS in methanol

Standard solution C: 4.0 mg/mL of USP *Rhodiola rosea* Root and Rhizome Dry Extract RS in methanol. Sonicate to dissolve, if necessary. Before injection, pass through a membrane filter of 0.45-µm or finer pore size.

Sample solution: Before injecting the Tincture, pass through a membrane filter of 0.45-µm or finer pore size. [NOTE—The sample can be weighed and converted to volume using the density of the Tincture.]

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability.*)

Mode: LC

Detector: UV 205 nm

Column: 3.0-mm × 10-cm; 2.5-µm packing L1

Column temperature: 40 ± 1°

Flow rate: 1.0 mL/min

Injection volume: 1 µL

System suitability

Samples: *Standard solution A* and *Standard solution C*

Suitability requirements

Chromatogram similarity: The chromatogram obtained from *Standard solution C* is similar to the reference chromatogram provided with the lot of USP *Rhodiola rosea* Root and Rhizome Dry Extract RS being used.

Resolution: NLT 1.5 between the rosarin and rosavin peaks, *Standard solution C*

Relative standard deviation: NMT 2% determined from the rosavin peak in repeated injections, *Standard solution A*

Analysis

Samples: *Standard solution A, Standard solution B, Standard solution C,* and *Sample solution*

Using the chromatograms of *Standard solution A, Standard solution B, Standard solution C,* and the reference chromatogram provided with the lot of USP *Rhodiola rosea* Root and Rhizome Dry Extract RS being used, identify the retention time of the peaks corresponding to salidroside, tyrosol, rosarin, rosavin, rosin, and rosiridin in the *Sample solution.*

Separately calculate the percentages of rosarin, rosavin, and rosin as rosavin in the portion of Tincture taken:

$$\text{Result} = (r_U/r_S) \times C_S \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*

r_S = peak area of rosavin from *Standard solution A*

C_S = concentration of rosavin in *Standard solution A* (g/mL)

Calculate the percentage of phenylpropenoid glycosides as the sum of the percentages of rosarin, rosavin, and rosin.

Calculate the percentage of salidroside in the portion of Tincture taken:

$$\text{Result} = (r_U/r_S) \times C_S \times 100$$

r_U = peak area of salidroside from the *Sample solution*

r_S = peak area of salidroside from *Standard solution B*

C_S = concentration of salidroside in *Standard solution B* (g/mL)

Acceptance criteria: NLT 0.06% (w/v) of phenylpropenoid glycosides; and NLT 0.016% of salidroside

OTHER COMPONENTS

Change to read:

• **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticides Residues*▲ (CN 1-May-2020)**:** NLT 90.0% and NMT 110.0% of the labeled amount of ethanol (C_{25}

CONTAMINANTS

• **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at room temperature.

• **LABELING:** The label states the official name of the article, the Latin binomial, and the part of the plant from which the article was prepared. Label it to indicate the content of phenylpropenoid glycosides and salidroside, the solvent mixture used for extraction, and the ratio of the starting crude plant material to Tincture.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP *Rhodiola rosea* Root and Rhizome Dry Extract RS

USP Rosavin RS

USP Salidroside RS

Rhodiola rosea Capsules

DEFINITION

Rhodiola rosea Capsules contain Powdered *Rhodiola rosea* Extract. They contain NLT 95.0% of the labeled amount of phenylpropenoid glycosides, calculated as the sum of rosarin ($C_{20}H_{28}O_{10}$), rosavin ($C_{20}H_{28}O_{10}$), and rosin

USP Monographs

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($C_{15}H_{20}O_6$); and NLT 95.0% of the labeled amount of salidroside ($C_{14}H_{20}O_7$).

IDENTIFICATION

Add the following:

• **▲A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 2.0 mg/mL of USP Salidroside RS and 1.0 mg/mL of USP Rosavin RS in absolute alcohol

Standard solution B: 50 mg/mL of USP *Rhodiola rosea* Root and Rhizome Dry Extract RS in absolute alcohol. Sonicate for 10 min, centrifuge, and use the supernatant.

Sample solution: Open 10 Capsules, combine their contents in an appropriate container, and mix well. Transfer a portion of the Capsule contents to a centrifuge tube and dilute with absolute alcohol to obtain a solution nominally equivalent to 50 mg/mL of extract. Sonicate for 10 min, centrifuge, and use the supernatant.

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plates)

Application volume: 5 µL, as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of 33%.

Temperature: Ambient, not to exceed 30°

Developing solvent system: Ethyl acetate, methanol, water, and formic acid (77:13:10:2)

Developing distance: 6 cm

Derivatization reagent: Dissolve 1 g of diphenylamine in 40 mL of acetone, add 1 mL of aniline, and mix. Carefully add 7.5 mL of phosphoric acid and mix.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands and dry in air. Develop in a saturated chamber and dry in air. Treat the plate with *Derivatization reagent*, heat at 120° for 5 min, and examine under white light.

System suitability: *Standard solution A* shows two bands in the lower half of the chromatogram. The band with a higher R_F is due to salidroside, and the band with a lower R_F is due to rosavin. *Standard solution B* exhibits, in the lower half of the chromatogram, two to four gray bands and several brownish bands, one above and the other below the gray bands; the most intense band is the brownish band with an R_F below the gray bands; the most intense gray band is the band at an R_F corresponding to rosavin in *Standard solution A*.

Acceptance criteria: The *Sample solution* exhibits an intense band corresponding in R_F and color to rosavin in *Standard solution A* and additional bands corresponding in R_F and color to similar bands in the chromatogram of *Standard solution B*; the most intense band is the brownish band with an R_F below the gray bands; the most intense gray band is that due to rosavin. Additional bands of varying coloration and intensity may be observed. ▲(USP 1-May-2019)

Change to read:

• **▲B.▲(USP 1-May-2019) HPLC**

Analysis: Proceed as directed in the test for *Content of Phenylpropenoid Glycosides and Salidroside, Method 1* or *Method 2*.

Acceptance criteria: The *Sample solution* exhibits peaks at retention times corresponding to the peaks due to salidroside, rosarin, rosavin, and rosin in *Standard solution B* in *Method 1* or *Standard solution C* in *Method 2*.

STRENGTH

[NOTE—The strength requirement may be met by following either one of the specified methods; the method used should be stated on the label only if *Method 1* is not used.]

• **CONTENT OF PHENYLPROPENOID GLYCOSIDES AND SALIDROSIDE,** *Method 1*

Extraction solvent: Methanol and water (2:8)

Solution A: 0.2% *o*-phosphoric acid in water

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	94	6
10	94	6
11	85	15
21	77	23
22	10	90
24	10	90
25	94	6
35	94	6

Standard solution A: 0.04 mg/mL of USP Rosavin RS and 0.02 mg/mL of USP Salidroside RS in *Extraction solvent*. Sonicate to dissolve, if necessary.

Standard solution B: 2.0 mg/mL of USP *Rhodiola rosea* Root and Rhizome Dry Extract RS in *Extraction solvent*. Sonicate to dissolve, if necessary. Before injection, pass through a PVDF membrane filter of 0.45-µm or finer pore size.

Sample solution: Determine the total weight of 20 Capsules. Open the Capsules and combine their contents in an appropriate container. Weigh the empty Capsule shells and calculate the average fill weight per Capsule. Transfer a portion of the Capsule contents, nominally equivalent to 3 mg of phenylpropenoid glycosides (sum of rosarin, rosavin, and rosin) into a 50-mL volumetric flask. Add 40 mL of *Extraction solvent* and sonicate for 30 min. Cool to room temperature, dilute with *Extraction solvent* to volume, mix well, and pass through a PVDF membrane filter of 0.45-µm pore size.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 221 nm for 0–15 min and UV 251 nm for 15–35 min

Column: 4.6-mm × 15-cm; 3-µm packing L1

Column temperature: 40°

Flow rate: 1.25 mL/min

Injection volume: 20 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram obtained with *Standard solution B* is similar to the reference chromatogram provided with the lot of USP *Rhodiola rosea* Root and Rhizome Dry Extract RS being used.

Resolution: NLT 1.5 between the rosarin and rosavin peaks, *Standard solution B*

Relative standard deviation: NMT 2.0% for the rosavin and salidroside peaks in replicate injections, *Standard solution A*

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Analysis
Samples: *Standard solution A, Standard solution B,* and *Sample solution*
Using the chromatograms of *Standard solution A, Standard solution B,* and the reference chromatogram provided with the lot of USP *Rhodiola rosea* Root and Rhizome Dry Extract RS being used, identify the peaks corresponding to salidroside, rosarin, rosavin, and rosin in the *Sample solution.*
Calculate the percentage of the labeled amount of phenylpropenoid glycosides calculated as the sum of rosarin, rosavin, and rosin in the portion of Capsules taken:

$$\text{Result} = (r_{sum}/r_S) \times (C_S \times V/W) \times (W_{av}/L) \times 100$$

r_{sum} = sum of the peak areas of rosarin, rosavin, and rosin from the *Sample solution*
r_S = peak area of rosavin from *Standard solution A*
C_S = concentration of rosavin in *Standard solution A* (mg/mL)
V = volume of the solvent taken for preparation of the *Sample solution* (mL)
W = weight of the sample taken for preparation of the *Sample solution* (mg)
W_{av} = average Capsule fill weight (mg)
L = labeled amount of phenylpropenoid glycosides (mg/Capsule)

Calculate the percentage of the labeled amount of salidroside in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S \times V/W) \times (W_{av}/L) \times 100$$

r_U = peak area of salidroside from the *Sample solution*
r_S = peak area of salidroside from *Standard solution A*
C_S = concentration of salidroside in *Standard solution A* (mg/mL)
V = volume of the solvent taken for preparation of the *Sample solution* (mL)
W = weight of the sample taken for preparation of the *Sample solution* (mg)
W_{av} = average Capsule fill weight (mg)
L = labeled amount of salidroside (mg/Capsule)

Acceptance criteria: NLT 95.0% of the labeled amount of phenylpropenoid glycosides as the sum of rosarin, rosavin, and rosin; and NLT 95.0% of the labeled amount of salidroside

• **CONTENT OF PHENYLPROPENOID GLYCOSIDES AND SALIDROSIDE,** *Method 2*
Solution A: Water
Solution B: Acetonitrile
Mobile phase: See *Table 2.*

Table 2

Time (min)	Solution A (%)	Solution B (%)
0	94	6
6	83	17
7	80.3	19.7
9	80.3	19.7
10	0	100
12	94	6
17	94	6

Standard solution A: 0.1 mg/mL of USP Rosavin RS in methanol
Standard solution B: 0.04 mg/mL of USP Salidroside RS in methanol
Standard solution C: 4.0 mg/mL of USP *Rhodiola rosea* Root and Rhizome Dry Extract RS in methanol. Sonicate to dissolve, if necessary. Before injection, pass through a PVDF membrane filter of 0.45-µm pore size.
Sample solution: Determine the total weight of 20 Capsules. Open the Capsules and combine their contents in an appropriate container. Weigh the empty Capsule shells and calculate the average fill weight per Capsule. Transfer a portion of the Capsule contents, equivalent to 6 mg of phenylpropenoid glycosides (sum of rosarin, rosavin, and rosin) into a 50-mL volumetric flask. Add 40 mL of methanol and sonicate for 30 min. Cool to room temperature, dilute with methanol to volume, mix well, and pass through a PVDF membrane filter of 0.45-µm pore size.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability.*)
Mode: LC
Detector: UV 205 nm
Column: 3.0-mm × 10-cm; 2.5-µm packing L1
Column temperature: 40°
Flow rate: 1.0 mL/min
Injection volume: 1 µL
System suitability
Samples: *Standard solution A, Standard solution B,* and *Standard solution C*
Suitability requirements
Chromatogram similarity: The chromatogram obtained with *Standard solution C* is similar to the reference chromatogram provided with the lot of USP *Rhodiola rosea* Root and Rhizome Dry Extract RS being used.
Resolution: NLT 1.5 between the rosarin and rosavin peaks, *Standard solution C*
Relative standard deviation: NMT 2.0% for the rosavin and salidroside peaks in replicate injections, *Standard solution A* and *Standard solution B*
Analysis
Samples: *Standard solution A, Standard solution B, Standard solution C,* and *Sample solution*
Using the chromatograms of *Standard solution A, Standard solution B, Standard solution C,* and the reference chromatogram provided with the lot of USP *Rhodiola rosea* Root and Rhizome Dry Extract RS being used, identify the peaks corresponding to salidroside, rosarin, rosavin, rosin, and rosiridin in the *Sample solution.*
Calculate the percentage of the labeled amount of phenylpropenoid glycosides calculated as the sum of rosarin, rosavin, and rosin in the portion of Capsules taken:

$$\text{Result} = (r_{sum}/r_S) \times (C_S \times V/W) \times (W_{av}/L) \times 100$$

r_{sum} = sum of the peak areas of rosarin, rosavin, and rosin from the *Sample solution*
r_S = peak area of rosavin from *Standard solution A*
C_S = concentration of rosavin in *Standard solution A* (mg/mL)
V = volume of the solvent taken for preparation of the *Sample solution* (mL)
W = weight of the sample taken for preparation of the *Sample solution* (mg)
W_{av} = average Capsule fill weight (mg)
L = labeled amount of phenylpropenoid glycosides (mg/Capsule)

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Calculate the percentage of the labeled amount of salidroside in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S \times V/W) \times (W_{av}/L) \times 100$$

r_U = peak area of salidroside from the *Sample solution*
r_S = peak area of salidroside from *Standard solution B*
C_S = concentration of salidroside in *Standard solution B* (mg/mL)
V = volume of the solvent taken for preparation of the *Sample solution* (mL)
W = weight of the sample taken for preparation of the *Sample solution* (mg)
W_{av} = average Capsule fill weight (mg)
L = labeled amount of salidroside (mg/Capsule)

Acceptance criteria: NLT 95.0% of the labeled amount of phenylpropenoid glycosides as the sum of rosarin, rosavin, and rosin; and NLT 95.0% of the labeled amount of salidroside

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩, *Disintegration*: Meet the requirements
- **WEIGHT VARIATION** ⟨2091⟩: Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩: Meet the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived. The label states the amount of phenylpropenoid glycosides, as the sum of rosarin, rosavin, and rosin; the amount of salidroside; and the amount of Powdered *Rhodiola rosea* Extract in mg/Capsule. It also states the assay method used if different from *Method 1*.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP *Rhodiola rosea* Root and Rhizome Dry Extract RS
  USP Rosavin RS
  USP Salidroside RS

Rhodiola rosea Tablets

DEFINITION

Rhodiola rosea Tablets contain Powdered *Rhodiola rosea* Extract. They contain NLT 95.0% of the labeled amount of phenylpropenoid glycosides, calculated as the sum of rosarin ($C_{20}H_{28}O_{10}$), rosavin ($C_{20}H_{28}O_{10}$), and rosin ($C_{15}H_{20}O_6$); and NLT 95.0% of the labeled amount of salidroside ($C_{14}H_{20}O_7$).

IDENTIFICATION

Add the following:

- **▲A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩
  Standard solution A: 2.0 mg/mL of USP Salidroside RS and 1.0 mg/mL of USP Rosavin RS in absolute alcohol
  Standard solution B: 50 mg/mL of USP *Rhodiola rosea* Root and Rhizome Dry Extract RS in absolute alcohol. Sonicate for 10 min, centrifuge, and use the supernatant.
  Sample solution: Grind 10 Tablets to a powder, transfer the powder to a suitable container, and homogenize. Transfer a portion of the homogenized powder to a centrifuge tube and dilute with absolute alcohol to obtain a solution nominally equivalent to 50 mg/mL of extract. Sonicate for 10 min, centrifuge, and use the supernatant.
  Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plates)
  Application volume: 5 µL, as 8-mm bands
  Relative humidity: Condition the plate to a relative humidity of 33%.
  Temperature: Ambient, not to exceed 30°
  Developing solvent system: Ethyl acetate, methanol, water, and formic acid (77:13:10:2)
  Developing distance: 6 cm
  Derivatization reagent: Dissolve 1 g of diphenylamine in 40 mL of acetone, add 1 mL of aniline, and mix. Carefully add 7.5 mL of phosphoric acid and mix.
  Analysis
  Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
  Apply the *Samples* as bands and dry in air. Develop in a saturated chamber and dry in air. Treat the plate with *Derivatization reagent*, heat at 120° for 5 min, and examine under white light.
  System suitability: *Standard solution A* shows two bands in the lower half of the chromatogram. The band with a higher R_F is due to salidroside, and the band with a lower R_F is due to rosavin. *Standard solution B* exhibits, in the lower half of the chromatogram, two to four gray bands and several brownish bands, one above and the other below the gray bands; the most intense band is the brownish band with an R_F below the gray bands; the most intense gray band is the band at an R_F corresponding to rosavin in *Standard solution A*.
  Acceptance criteria: The *Sample solution* exhibits an intense band corresponding in R_F and color to rosavin in *Standard solution A* and additional bands corresponding in R_F and color to similar bands in the chromatogram of *Standard solution B*; the most intense band is the brownish band with an R_F below the gray bands; the most intense gray band is that due to rosavin. Additional bands of varying coloration and intensity may be observed. ▲ (USP 1-May-2019)

Change to read:

- **▲B.▲ (USP 1-May-2019) HPLC**
  Analysis: Proceed as directed in the test for *Content of Phenylpropenoid Glycosides and Salidroside, Method 1* or *Method 2*.
  Acceptance criteria: The *Sample solution* exhibits peaks at retention times corresponding to the peaks due to salidroside, rosarin, rosavin, and rosin in *Standard solution B* in *Method 1* or *Standard solution C* in *Method 2*.

STRENGTH

[NOTE—The strength requirement may be met by following either one of the specified methods; the method used should be stated on the label only if *Method 1* is not used.]

- **CONTENT OF PHENYLPROPENOID GLYCOSIDES AND SALIDROSIDE,** *Method 1*
  Extraction solvent: Methanol and water (2:8)
  Solution A: 0.2% *o*-phosphoric acid in water
  Solution B: Acetonitrile

DS Monographs

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Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	94	6
10	94	6
11	85	15
21	77	23
22	10	90
24	10	90
25	94	6
35	94	6

Standard solution A: 0.04 mg/mL of USP Rosavin RS and 0.02 mg/mL of USP Salidroside RS in *Extraction solvent*. Sonicate to dissolve, if necessary.
Standard solution B: 2.0 mg/mL of USP *Rhodiola rosea* Root and Rhizome Dry Extract RS in *Extraction solvent*. Sonicate to dissolve, if necessary. Before injection, pass through a PVDF membrane filter of 0.45-µm or finer pore size.
Sample solution: Weigh NLT 20 Tablets, determine the average Tablet weight, and finely powder. Transfer a portion of finely powdered Tablets, nominally equivalent to 3 mg of phenylpropenoid glycosides (sum of rosarin, rosavin, and rosin) into a 50-mL volumetric flask. Add 40 mL of *Extraction solvent* and sonicate for 30 min. Cool to room temperature, dilute with *Extraction solvent* to volume, mix well, and pass through a PVDF membrane filter of 0.45-µm pore size.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 221 nm for 0–15 min and 251 nm for 15–35 min
Column: 4.6-mm × 15-cm; 3-µm packing L1
Column temperature: 40°
Flow rate: 1.25 mL/min
Injection volume: 20 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram obtained with *Standard solution B* is similar to the reference chromatogram provided with the lot of USP *Rhodiola rosea* Root and Rhizome Dry Extract RS being used.
Resolution: NLT 1.5 between the rosarin and rosavin peaks, *Standard solution B*
Relative standard deviation: NMT 2.0% for the rosavin and salidroside peaks in replicate injections, *Standard solution A*
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP *Rhodiola rosea* Root and Rhizome Dry Extract RS being used, identify the peaks corresponding to salidroside, rosarin, rosavin, and rosin in the *Sample solution*.
Calculate the percentage of the labeled amount of phenylpropenoid glycosides calculated as the sum of rosarin, rosavin, and rosin in the portion of Tablets taken:

$$\text{Result} = (r_{sum}/r_S) \times (C_S \times V/W) \times (W_{av}/L) \times 100$$

r_{sum} = sum of the peak areas of rosarin, rosavin, and rosin from the *Sample solution*
r_S = peak area of rosavin from *Standard solution A*
C_S = concentration of rosavin in *Standard solution A* (mg/mL)
V = volume of the solvent taken for preparation of the *Sample solution* (mL)
W = weight of the sample taken for preparation of the *Sample solution* (mg)
W_{av} = average Tablet weight (mg)
L = labeled amount of phenylpropenoid glycosides (mg/Tablet)

Calculate the percentage of the labeled amount of salidroside in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S \times V/W) \times (W_{av}/L) \times 100$$

r_U = peak area of salidroside from the *Sample solution*
r_S = peak area of salidroside from *Standard solution A*
C_S = concentration of salidroside in *Standard solution A* (mg/mL)
V = volume of the solvent taken for preparation of the *Sample solution* (mL)
W = weight of the sample taken for preparation of the *Sample solution* (mg)
W_{av} = average Tablet weight (mg)
L = labeled amount of salidroside (mg/Tablet)

Acceptance criteria: NLT 95.0% of the labeled amount of phenylpropenoid glycosides as the sum of rosarin, rosavin, and rosin; and NLT 95.0% of the labeled amount of salidroside

• **CONTENT OF PHENYLPROPENOID GLYCOSIDES AND SALIDROSIDE,** *Method 2*
Solution A: Water
Solution B: Acetonitrile
Mobile phase: See *Table 2*.

Table 2

Time (min)	Solution A (%)	Solution B (%)
0	94	6
6	83	17
7	80.3	19.7
9	80.3	19.7
10	0	100
12	94	6
17	94	6

Standard solution A: 0.1 mg/mL of USP Rosavin RS in methanol
Standard solution B: 0.04 mg/mL of USP Salidroside RS in methanol
Standard solution C: 4.0 mg/mL of USP *Rhodiola rosea* Root and Rhizome Dry Extract RS in methanol. Sonicate to dissolve, if necessary. Before injection, pass through a PVDF membrane filter of 0.45-µm pore size.
Sample solution: Weigh NLT 20 Tablets, determine the average Tablet weight, and finely powder. Transfer a portion of finely powdered Tablets, equivalent to 6 mg of phenylpropenoid glycosides (sum of rosarin, rosavin, and rosin) into a 50-mL volumetric flask. Add 40 mL of methanol

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and sonicate for 30 min. Cool to room temperature, dilute with methanol to volume, mix well, and pass through a PVDF membrane filter of 0.45-µm pore size.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 205 nm
Column: 3.0-mm × 10-cm; 2.5-µm packing L1
Column temperature: 40°
Flow rate: 1.0 mL/min
Injection volume: 1 µL

System suitability
Samples: *Standard solution A*, *Standard solution B*, and *Standard solution C*
Suitability requirements
Chromatogram similarity: The chromatogram obtained with *Standard solution C* is similar to the reference chromatogram provided with the lot of USP *Rhodiola rosea* Root and Rhizome Dry Extract RS being used.
Resolution: NLT 1.5 between the rosarin and rosavin peaks, *Standard solution C*
Relative standard deviation: NMT 2.0% for the rosavin and salidroside peaks in replicate injections, *Standard solution A* and *Standard solution B*

Analysis
Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*
Using the chromatograms of *Standard solution A*, *Standard solution B*, *Standard solution C*, and the reference chromatogram provided with the lot of USP *Rhodiola rosea* Root and Rhizome Dry Extract RS being used, identify the peaks corresponding to salidroside, rosarin, rosavin, rosin, and rosiridin in the *Sample solution*.
Calculate the percentage of the labeled amount of phenylpropenoid glycosides calculated as the sum of rosarin, rosavin, and rosin in the portion of Tablets taken:

$$\text{Result} = (r_{sum}/r_S) \times (C_S \times V/W) \times (W_{av}/L) \times 100$$

r_{sum} = sum of the peak areas of rosarin, rosavin, and rosin from the *Sample solution*
r_S = peak area of rosavin from *Standard solution A*
C_S = concentration of rosavin in *Standard solution A* (mg/mL)
V = volume of the solvent taken for preparation of the *Sample solution* (mL)
W = weight of the sample taken for preparation of the *Sample solution* (mg)
W_{av} = average Tablet weight (mg)
L = labeled amount of phenylpropenoid glycosides (mg/Tablet)

Calculate the percentage of the labeled amount of salidroside in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S \times V/W) \times (W_{av}/L) \times 100$$

r_U = peak area of salidroside from the *Sample solution*
r_S = peak area of salidroside from *Standard solution B*
C_S = concentration of salidroside in *Standard solution B* (mg/mL)
V = volume of the solvent taken for preparation of the *Sample solution* (mL)
W = weight of the sample taken for preparation of the *Sample solution* (mg)
W_{av} = average Tablet weight (mg)
L = labeled amount of salidroside (mg/Tablet)

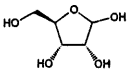
Acceptance criteria: NLT 95.0% of the labeled amount of phenylpropenoid glycosides as the sum of rosarin, rosavin, and rosin; and NLT 95.0% of the labeled amount of salidroside

PERFORMANCE TESTS
- **Disintegration and Dissolution** ⟨2040⟩, *Disintegration*: Meet the requirements
- **Weight Variation** ⟨2091⟩: Meet the requirements

CONTAMINANTS
- **Microbial Enumeration Tests** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **Absence of Specified Microorganisms** ⟨2022⟩: Meet the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS
- **Packaging and Storage:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **Labeling:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived. The label states the amount of phenylpropenoid glycosides, as the sum of rosarin, rosavin, and rosin; the amount of salidroside; and the amount of Powdered *Rhodiola rosea* Extract in mg/Tablet. It also states the assay method used if different from *Method 1*.
- **USP Reference Standards** ⟨11⟩
  USP *Rhodiola rosea* Root and Rhizome Dry Extract RS
  USP Rosavin RS
  USP Salidroside RS

Ribose



$C_5H_{10}O_5$ 150.13
(2*S*,3*R*,4*S*,5*R*)-5-(Hydroxymethyl)oxolane-2,3,4-triol; D-Ribose [50-69-1].

DEFINITION
Ribose contains NLT 98.0% and NMT 102.0% of D-ribose ($C_5H_{10}O_5$), calculated on the dried basis.

IDENTIFICATION

Change to read:

- **A.** ▲**Spectroscopic Identification Tests** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
- **B.** It meets the requirements in *Specific Tests* for *Optical Rotation* ⟨781S⟩, *Specific Rotation*.
- **C.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

ASSAY
- **Procedure**
  Mobile phase: Degassed water
  System suitability solution: 20 mg/mL of USP Ribose RS and 0.2 mg/mL of USP Arabinose RS in *Mobile phase*
  Standard solution: 20 mg/mL of USP Ribose RS in *Mobile phase*
  Sample solution: 20 mg/mL of Ribose in *Mobile phase*

DS Monographs

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Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Refractive index
Column: 8.0-mm × 30-cm; 6-µm packing L22
Temperatures
Detector: 40°
Column: 80°
Flow rate: 1.0 mL/min
Injection volume: 10 µL
System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The relative retention times for arabinose and ribose are 0.9 and 1.0, respectively.]
Suitability requirements
Resolution: NLT 1.2 between ribose and arabinose, *System suitability solution*
Tailing factor: NMT 1.5, *Standard solution*
Column efficiency: NLT 2500 theoretical plates for the ribose peak, *Standard solution*
Relative standard deviation: NMT 2.0%, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of D-ribose in the portion of Ribose taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP Ribose RS in the *Standard solution* (mg/mL)
C_U = concentration of Ribose in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0–102.0% on the dried basis

IMPURITIES
• **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 0.2%
• **CHLORIDE AND SULFATE,** *Chloride* ⟨221⟩
Standard: 0.10 mL of 0.020 N hydrochloric acid
Sample: 3.6 g of Ribose
Acceptance criteria: NMT 0.002%
• **CHLORIDE AND SULFATE,** *Sulfate* ⟨221⟩
Standard: 0.10 mL of 0.020 N sulfuric acid
Sample: 3.3 g of Ribose
Acceptance criteria: NMT 0.003%
• **RELATED COMPOUNDS**
Mobile phase, System suitability solution, Sample solution, Chromatographic system, and **System suitability:** Proceed as directed in the *Assay*.
Standard solution: 0.02 mg/mL of USP Arabinose RS in *Mobile phase*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of arabinose in the portion of Ribose taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of arabinose from the *Sample solution*
r_S = peak response of arabinose from the *Standard solution*
C_S = concentration of USP Arabinose RS in the *Standard solution* (mg/mL)
C_U = concentration of Ribose in the *Sample solution* (mg/mL)

Calculate the percentage of any unspecified impurity in the portion of Ribose taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of any unspecified impurity from the *Sample solution*
r_T = sum of all the peak responses from the *Sample solution*

Acceptance criteria
Arabinose: NMT 1.0%
Unspecified impurity: NMT 0.1%
Total unspecified impurities: NMT 1.0%

SPECIFIC TESTS
• **OPTICAL ROTATION,** *Specific Rotation* ⟨781S⟩
Sample solution: 20 mg/mL in water
Acceptance criteria: –18.0° to –22.0°
• **COLOR OF SOLUTION**
Sample solution: Dissolve 5.0 g of Ribose in 50 mL of water. Centrifuge or filter, if necessary, to obtain a clear solution.
Blank solution: Water
Analysis: Absorbance at 430 nm in a 1-cm cell
Acceptance criteria: NMT 0.2 AU
• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry at 105° for 2 h.
Acceptance criteria: NMT 0.5%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Arabinose RS
USP Ribose RS

Rosemary

DEFINITION
Rosemary consists of the dried leaf of *Rosmarinus officinalis* L. (Fam. Lamiaceae). It contains NLT 2.0% of phenolic diterpenes, calculated as the sum of carnosic acid and carnosol, and NLT 1.2% (v/w) of volatile oil, both calculated on the dried basis.

IDENTIFICATION
• **A.** Rosemary meets the requirements for *Specific Tests, Botanic Characteristics*.
• **B. THIN-LAYER CHROMATOGRAPHY**
Standard solution A: 0.5 mg/mL of USP Rosmarinic Acid RS in methanol
Standard solution B: 100 mg/mL of USP Powdered Rosemary Hydrophilic Extract RS in methanol
Sample solution: Sonicate for 10 min about 1 g of Rosemary, finely powdered, in 10 mL of methanol. Centrifuge, and use the supernatant.
Chromatographic system
(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)
Application volume: 2 µL of *Standard solution A*, 4 µL of *Standard solution B*, and 8 µL of the *Sample solution* as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
Developing solvent system: A mixture of ethyl acetate, formic acid, and water (15:1:1)
Developing distance: 6 cm

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Derivatization reagent A: 5-mg/mL solution of 2-aminoethyl diphenylborinate in ethyl acetate
Derivatization reagent B: 50-mg/mL solution of polyethylene glycol 400 in dichloromethane
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*. Apply the samples as bands to a suitable high performance thin-layer chromatographic plate. Develop the chromatograms in a saturated chamber, remove the plate from the chamber, heat for 3 min at 100°, derivatize the plate while still warm with *Derivatization reagent A*, dry in air, then derivatize with *Derivatization reagent B*, dry in air, and examine under UV light at 366 nm.
System suitability: The chromatogram of *Standard solution B* exhibits, in the upper-third section, a blue fluorescent band corresponding to the band due to rosmarinic acid in the chromatogram of *Standard solution A*, and a less intense band, right above the band due to rosmarinic acid, corresponding to caffeic acid. The bands due to rosmarinic acid and caffeic acid are clearly separated.
Acceptance criteria: The chromatogram of the *Sample solution* exhibits the following main bands similar in positions and colors to the corresponding bands in the chromatogram of *Standard solution B*: a blue fluorescent band at an R_F corresponding to the rosmarinic acid band in *Standard solution A*; a band at an R_F corresponding to the caffeic acid; a green band at about the middle of the chromatogram and an intense orange band in the lower third section of the chromatogram; below the green band, the *Sample solution* chromatogram exhibits a pattern of characteristically colored bands of low intensity (distinction from sage leaf, thyme leaf, holy basil leaf, basil leaf, and oregano leaf).
The *Sample solution* chromatogram does not show a pair of major orange bands in the lower-third section of the chromatogram (distinction from sage leaf and thyme leaf), a light blue band at an R_F of about one-third of the chromatogram (distinction from holy basil leaf), an orange band in the lower-third section of the chromatogram and a light blue band at about two-thirds of the chromatogram (distinction from basil leaf), or a red band right above the band due to caffeic acid in the upper-third section of the chromatogram (distinction from thyme leaf and oregano leaf). [NOTE—This red band is different from the red band, at the solvent front, present in Rosemary and other leaves due to chlorophyll.]

• **C. HPLC**
Analysis: Proceed as directed in the test for *Content of Phenolic Diterpenes*.
Acceptance criteria: The chromatogram of the *Sample solution* exhibits the major peak at the retention time corresponding to the carnosic acid peak in the chromatogram of *Standard solution A* and an additional peak corresponding to carnosol at a relative retention time of about 0.64 compared to that of the carnosic acid peak.

COMPOSITION

• **CONTENT OF PHENOLIC DITERPENES**
Mobile phase: A mixture of acetonitrile and a solution of 0.5% phosphoric acid in water (65:35)
[NOTE—Proceed under subdued light.]
Solvent: 0.5% phosphoric acid in methanol
Standard solution A: [NOTE—Use clear glassware.] 0.2 mg/mL of USP Carnosic Acid RS in *Solvent*. Sonicate to dissolve if necessary.
Sample solution: 1.0 g of Rosemary, finely powdered, in 50 mL of methanol. Sonicate for 3 h. Before injection, pass through a membrane filter of 0.45-µL or finer pore size, discarding the first few mL of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV, 230 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 25 ± 1°
Flow rate: 1.5 mL/min
Injection volume: 5 µL
System suitability
Sample: *Standard solution A*
Suitability requirements
Tailing factor: Between 0.90 and 1.30 for the carnosic acid peak, *Standard solution A*
Relative standard deviation: NMT 2% determined from the carnosic acid peak in repeated injections, *Standard solution A*
Analysis
Samples: *Standard solution A* and *Sample solution*
[NOTE—*Standard solution A* and the *Sample solution* are stable for 12 h at room temperature.]
Using the chromatograms of *Standard solution A*, identify the retention times of the peaks corresponding to carnosic acid and carnosol in the *Sample solution* chromatogram. [NOTE—The approximate relative retention times for the carnosol and carnosic acid peaks are 0.64 and 1.00, respectively.]
Calculate the percentages of carnosic acid and carnosol in the portion of Rosemary taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte in the *Sample solution* chromatogram
r_S = peak area for carnosic acid in the *Standard solution A* chromatogram
C_S = concentration of USP Carnosic Acid RS in *Standard solution A* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Rosemary taken to prepare the *Sample solution* (mg)
F = conversion factor (1.00 for carnosic acid and 0.92 for carnosol)

Add the percentages of carnosic acid and carnosol.
Acceptance criteria: NLT 2.0% on the dried basis

• **ARTICLES OF BOTANICAL ORIGIN,** *Volatile Oil Determination* ⟨561⟩**:** Use 25.0 g of coarsely powdered Rosemary. Distill for 3 h at a rate of 2–3 mL/min.
Acceptance criteria: NLT 1.2% v/w of volatile oil on the dried basis

CONTAMINANTS

• **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩
Acceptance criteria
Arsenic: NMT 1.0 µg/g
Cadmium: NMT 0.5 µg/g
Lead: NMT 5.0 µg/g
Mercury: NMT 1.0 µg/g

Change to read:

• **▲ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Pesticide Residue Analysis*▲ (CN 1-May-2019)**:** Meets the requirements
• **MICROBIAL ENUMERATION TESTS—NUTRITIONAL AND DIETARY SUPPLEMENTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the

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bile-tolerant Gram-negative bacteria do not exceed 10^3 cfu/g.

- **Microbiological Procedures for Absence of Specified Microorganisms—Nutritional and Dietary Supplements** ⟨2022⟩: Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

- **Botanic Characteristics**

Macroscopic: The leaves are sessile, linear to linear lanceolate, needle-like, 1–4 cm long and 2–4 mm wide, with strongly revolute margins; apex obtuse, base tapering. The upper surface is dark green, somewhat pubescent when young, glabrous in older leaves, the lower surface is grayish-green and densely tomentose with a prominent midrib. Pharmacopeial article consists of dry, grayish-green to yellowish-green, brittle, leaves.

Microscopic

Transverse section: Upper epidermis, cells are tabular with slightly thickened walls and occasional pits, rare conical covering trichomes, stomata are absent; an underlying hypodermis composed of large, irregularly rounded to ovoid cells with thickened anticlinal walls, one to several layers of cell extending across the lamina at intervals, separating the one or two-layered palisade into large, crescent-shaped areas, each with a group of spongy mesophyll; lower epidermis, numerous stomata, masses of multicellular and characteristic, thin-walled, uniseriate, extensively branched covering trichomes, up to 300 µm in length, forming dense felted masses, with each branch arising from a slightly swollen joint and terminating in a single tapering cell; glandular trichomes of two types are present on both epidermis, more on the lower epidermis, the majority with a short, unicellular stalk and a radiate head composed of eight cells, less abundant with a unicellular stalk and a spherical, unicellular or bicellular head.

- **Articles of Botanical Origin,** *Foreign Organic Matter* ⟨561⟩: NMT 5% of stems; NMT 2.0% of other foreign matter
- **Loss on Drying** ⟨731⟩

Sample: 1.0 g of finely powdered Rosemary
Analysis: Dry at 105° for 2 h.
Acceptance criteria: NMT 10%

- **Articles of Botanical Origin,** *Total Ash* ⟨561⟩

Sample: 2–4 g of finely powdered Rosemary
Acceptance criteria: NMT 9.0%

- **Articles of Botanical Origin,** *Acid-Insoluble Ash* ⟨561⟩

Sample: 2–4 g of finely powdered Rosemary
Acceptance criteria: NMT 1.5%

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **Labeling:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP Reference Standards** ⟨11⟩

USP Carnosic Acid RS
USP Powdered Rosemary Hydrophilic Extract RS
USP Rosmarinic Acid RS

Powdered Rosemary

DEFINITION

Powdered Rosemary is Rosemary reduced to powder or very fine powder. It contains NLT 2.0% of phenolic diterpenes, calculated as the sum of carnosic acid and carnosol, on the dried basis.

IDENTIFICATION

- **A.** Powdered Rosemary meets the requirements for *Specific Tests, Botanic Characteristics.*
- **B. Thin-Layer Chromatography**

Standard solution A: 0.5 mg/mL of USP Rosmarinic Acid RS in methanol
Standard solution B: 100 mg/mL of USP Powdered Rosemary Hydrophilic Extract RS in methanol
Sample solution: Sonicate for 10 min about 1 g of Powdered Rosemary in 10 mL of methanol. Centrifuge, and use supernatant.
Chromatographic system
(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography.*)
Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)
Application volume: 2 µL of *Standard solution A,* 4 µL of *Standard solution B,* and 8 µL of the *Sample solution* as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
Developing solvent system: A mixture of ethyl acetate, formic acid, and water (15:1:1)
Developing distance: 6 cm
Derivatization reagent A: 5-mg/mL solution of 2-aminoethyl diphenylborinate in ethyl acetate
Derivatization reagent B: 50-mg/mL solution of polyethylene glycol 400 in dichloromethane
Analysis
Samples: *Standard solution A, Standard solution B,* and *Sample solution.* Apply the samples as bands to a suitable high performance thin-layer chromatographic plate (see *Chromatography* ⟨621⟩), and dry in air. Develop the chromatograms in a saturated chamber, remove the plate from the chamber, heat at 100° for 3 min, derivatize the plate while still warm with *Derivatization reagent A,* dry in air, then derivatize with *Derivatization reagent B,* dry in air, and examine under UV light at 366 nm.
System suitability: The chromatogram of *Standard solution B* exhibits, in the upper-third section, a blue fluorescent band corresponding to the band due to rosmarinic acid in the chromatogram of *Standard solution A,* and a less intense band, right above the band due to rosmarinic acid, corresponding to caffeic acid. The bands due to rosmarinic acid and caffeic acid are clearly separated.
Acceptance criteria: The chromatogram of the *Sample solution* exhibits the following main bands similar in positions and colors to the corresponding bands in the chromatogram of *Standard solution B.* A blue fluorescent band at an R_F corresponding to the rosmarinic acid band in *Standard solution A;* a band at an R_F corresponding to the caffeic acid; a green band at about the middle of the chromatogram and an intense orange band in the lower third section of the chromatogram; below the green band, the *Sample solution* chromatogram exhibits a pattern of characteristically colored bands of low intensity (distinction from sage leaf, thyme leaf, holy basil leaf, basil leaf, and oregano leaf).
The *Sample solution* chromatogram does not show a pair of major orange bands in the lower-third section of the chromatogram (distinction from sage leaf and thyme leaf), a light blue band at an R_F of about one-third of the

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chromatogram (distinction from holy basil leaf), an orange band in the lower-third section of the chromatogram and a light blue band at about two-thirds of the chromatogram (distinction from basil leaf), or a red band right above the band due to caffeic acid in the upper-third section of the chromatogram (distinction from thyme leaf and oregano leaf.) [NOTE—This red band is different from the red band, at the solvent front, present in Rosemary and other leaves due to chlorophyll.]

- **C. HPLC**

Analysis: Proceed as directed in the test for *Content of Phenolic Diterpenes*.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits the major peak at the retention time corresponding to carnosic acid in the chromatogram of *Standard solution A* and an additional peak corresponding to carnosol at a relative retention time of about 0.64 compared to that of the carnosic acid peak.

COMPOSITION

- **CONTENT OF PHENOLIC DITERPENES**

Mobile phase: A mixture of acetonitrile and a solution of 0.5% phosphoric acid in water (65:35) [NOTE—Proceed under subdued light.]

Solvent: 0.5% phosphoric acid in methanol

Standard solution A: [NOTE—Use clear glassware.] 0.2 mg/mL of USP Carnosic Acid RS in *Solvent*. Sonicate to dissolve if necessary.

Sample solution: 1.0 g of Powdered Rosemary in 50 mL of methanol. Sonicate for 3 h. Before injection, pass through a membrane filter of 0.45-µL or finer pore size, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV, 230 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 25 ± 1°

Flow rate: 1.5 mL/min

Injection volume: 5 µL

System suitability

Sample: *Standard solution A*

Suitability requirements

Tailing factor: Between 0.90 and 1.30 for the carnosic acid peak, *Standard solution A*

Relative standard deviation: NMT 2% determined from the carnosic acid peak in repeated injections, *Standard solution A*

Analysis

Samples: *Standard solution A* and *Sample solution* [NOTE—*Standard solution A* and *Sample solution* are stable for 12 h at room temperature.]

Using the chromatograms of *Standard solution A*, identify the retention time of the peaks corresponding to carnosic acid and carnosol in the *Sample solution* chromatogram. [NOTE—The approximate relative retention times for the carnosol and carnosic acid peaks are 0.64 and 1.00, respectively.]

Calculate the percentages of carnosic acid and carnosol in the portion of Powdered Rosemary taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte in the *Sample solution* chromatogram

r_S = peak area for carnosic acid in the *Standard solution A* chromatogram

C_S = concentration of carnosic acid in *Standard solution A* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Powdered Rosemary taken to prepare the *Sample solution* (mg)

F = conversion factor (1.00 for carnosic acid and 0.92 for carnosol)

Add the percentages of carnosic acid and carnosol.

Acceptance criteria: NLT 2.0% on the dried basis

CONTAMINANTS

- **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩

Acceptance criteria

Arsenic: NMT 1.0 µg/g

Cadmium: NMT 0.5 µg/g

Lead: NMT 5.0 µg/g

Mercury: NMT 1.0 µg/g

Change to read:

- **▲ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Pesticide Residue Analysis:*▲ (CN 1-May-2019) Meets the requirements
- **MICROBIAL ENUMERATION TESTS—NUTRITIONAL AND DIETARY SUPPLEMENTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria do not exceed 10^3 cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS—NUTRITIONAL AND DIETARY SUPPLEMENTS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

- **BOTANIC CHARACTERISTICS**

Macroscopic: Grayish-green to yellowish-green powder

Microscopic: It shows fragments of upper epidermal cell, polygonal to irregular, with slightly thickened walls and occasional pits, stomata absent; lower epidermal cells, with straight or slightly sinuous walls, numerous diacytic stomata, masses of multicellular and characteristic, thin-walled, uniseriate, extensively branched covering trichomes, up to 300 µm in length, with each branch arising from a slightly swollen joint and terminating in a single tapering cell; glandular trichomes of two types, the majority with a short, unicellular stalk and a radiate head composed of eight cells, less abundant with a unicellular stalk and a spherical, unicellular or bicellular head; glandular trichomes in surface view appear as a disk with eight cells; hypodermis cells with distinctly beaded walls; fibers; vascular tissue.

- **LOSS ON DRYING** ⟨731⟩

Sample: 1.0 g of Powdered Rosemary

Analysis: Dry at 105° for 2 h.

Acceptance criteria: NMT 10%

- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩

Sample: 2–4 g of Powdered Rosemary

Acceptance criteria: NMT 9.0%

- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩

Sample: 2–4 g of Powdered Rosemary

Acceptance criteria: NMT 1.5%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was obtained.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Carnosic Acid RS

USP Powdered Rosemary Hydrophilic Extract RS

USP Rosmarinic Acid RS

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Rosemary Leaf Dry Aqueous Extract

DEFINITION

Rosemary Leaf Dry Aqueous Extract is prepared from Rosemary by extraction with water. It contains NLT 90% and NMT 110% of the labeled amount of rosmarinic acid, on the dried basis. It may contain suitable added substances as carriers.

IDENTIFICATION

• **A. Thin-Layer Chromatography**

Standard solution A: 0.5 mg/mL of USP Rosmarinic Acid RS in methanol

Standard solution B: 100 mg/mL of USP Powdered Rosemary Hydrophilic Extract RS in methanol

Sample solution: 100 mg/mL of Rosemary Leaf Dry Aqueous Extract in methanol

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)

Application volume: 2 µL of the *Standard solution A*, and 4 µL of the *Standard solution B* and *Sample solution* as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

Developing solvent system: A mixture of ethyl acetate, formic acid, and water (15:1:1)

Developing distance: 6 cm

Derivatization reagent A: 5-mg/mL solution of 2-aminoethyl diphenylborinate in ethyl acetate

Derivatization reagent B: 50-mg/mL solution of polyethylene glycol 400 in dichloromethane

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*. Apply the samples as bands to a suitable high performance thin-layer chromatographic plate, and dry in air. Develop the chromatograms in a saturated chamber, remove the plate from the chamber, heat at 100° for 3 min, derivatize the plate while still warm with *Derivatization reagent A*, dry in air, then derivatize with *Derivatization reagent B*, dry in air, and examine under UV light at 366 nm.

System suitability: The chromatogram of *Standard solution B* shows, in the upper-third section, a blue fluorescent band corresponding to the band due to rosmarinic acid in the chromatogram of *Standard solution A*, and a less intense band, right above the band due to rosmarinic acid, corresponding to caffeic acid. The bands due to rosmarinic acid and caffeic acid are clearly separated.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits the following main bands similar in positions and colors to the corresponding bands in the chromatogram of *Standard solution B*. A blue fluorescent band at an R_F corresponding to the rosmarinic acid band in *Standard solution A*; a band at an R_F corresponding to the caffeic acid; a green band at about the middle of the chromatogram and an intense orange band in the lower third section of the chromatogram; below the green band, the *Sample solution* chromatogram exhibits a pattern of characteristically colored bands of low intensity (distinction from sage leaf, thyme leaf, holy basil leaf, basil leaf, and oregano leaf).

The *Sample solution* chromatogram does not show a pair of major orange bands in the lower-third section of the chromatogram (distinction from sage leaf and thyme leaf), a light blue band at an R_F of about one-third of the chromatogram (distinction from holy basil leaf), an orange band in the lower-third section of the chromatogram and a light blue band at about two-thirds of the chromatogram (distinction from basil leaf), or a red band right above the band due to caffeic acid in the upper-third section of the chromatogram (distinction from thyme leaf and oregano leaf). [Note—This red band is different from the red band, at the solvent front, present in Rosemary and other leaves due to chlorophyll.]

• **B. HPLC**

Analysis: Proceed as directed in the test for *Content of Rosmarinic Acid*.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits the major peak at the retention time corresponding to the rosmarinic acid peak in the chromatogram of *Standard solution A*. It also shows a less intense peak corresponding to the luteolin-3-*O*-glucuronide peak in the chromatogram of *Standard solution B* and at a relative retention time of about 0.8 compared to the rosmarinic acid peak.

COMPOSITION

• **Content of Rosmarinic Acid**

Mobile phase: A mixture of acetonitrile and a solution of 0.1% trifluoroacetic acid in water (32:68)

Solvent: Methanol and water (1:1)

Standard solution A: 0.3 mg/mL of USP Rosmarinic Acid RS in *Solvent*. Sonicate to dissolve if necessary.

Standard solution B: 10 mg/mL of USP Powdered Rosemary Hydrophilic Extract RS in *Solvent*. Sonicate to dissolve if necessary. Before injection, pass through a membrane filter of 0.45-µL or finer pore size, discarding the first few mL of the filtrate.

Sample solution: Sonicate for 5 min an amount of Rosemary Leaf Dry Aqueous Extract equivalent to 15 mg of rosmarinic acid in 50 mL of *Solvent*. Before injection, pass through a membrane filter of 0.45-µL or finer pore size, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV, 328 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 20 ± 1°

Flow rate: 1.0 mL/min

Injection volume: 5 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Rosemary Hydrophilic Extract RS being used.

Tailing factor: Between 0.90 and 1.30 for the rosmarinic acid peak, *Standard solution A*

Relative standard deviation: NMT 2% determined from the rosmarinic acid peak in repeated injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Rosemary Hydrophilic Extract RS being used, identify the retention time of the peak corresponding to rosmarinic acid in the *Sample solution* chromatogram.

Calculate the percentage of rosmarinic acid in the portion of Rosemary Leaf Dry Aqueous Extract taken:

$$P = (r_U/r_S) \times (C_S/C_U) \times 100$$

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r_U = peak area for rosmarinic acid in the *Sample solution* chromatogram
r_S = peak area for rosmarinic acid in *Standard solution A* chromatogram
C_S = concentration of rosmarinic acid in *Standard solution A* (mg/mL)
C_U = concentration of Dry Aqueous Extract in the *Sample solution* (mg/mL)

Calculate the percentage of the labeled amount of rosmarinic acid in the portion of Rosemary Leaf Dry Aqueous Extract taken:

$$\text{Result} = (P/L) \times 100$$

P = percentage of rosmarinic acid as determined above (%)
L = labeled amount of rosmarinic acid (%)

Acceptance criteria: 90%–110% on the dried basis

CONTAMINANTS

- **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩
  Acceptance criteria
  Arsenic: NMT 1.0 µg/g
  Cadmium: NMT 0.5 µg/g
  Lead: NMT 5.0 µg/g
  Mercury: NMT 1.0 µg/g

Change to read:

- **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (Official 1-May-2020)**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS—NUTRITIONAL AND DIETARY SUPPLEMENTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^4 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS—NUTRITIONAL AND DIETARY SUPPLEMENTS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

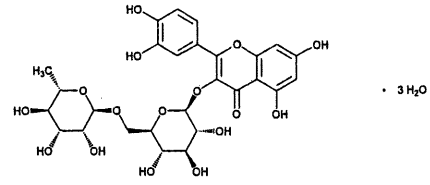
- **LOSS ON DRYING** ⟨731⟩
  Sample: 1.0 g of Rosemary Leaf Dry Aqueous Extract
  Analysis: Dry at 105° for 2 h.
  Acceptance criteria: NMT 8%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light, moisture, and oxygen; store at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived. It meets other labeling requirements in *Botanical Extracts* ⟨565⟩.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Powdered Rosemary Hydrophilic Extract RS
  USP Rosmarinic Acid RS

Rutin

Change to read:



$C_{27}H_{30}O_{16} \cdot 3H_2O$ 664.56

3-Rhamnoglucoside of 5,7,3′,4′-tetrahydroxyflavonol ▲trihydrate;▲ (ERR 1-Apr-2019)
2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4*H*-chromen-4-one-3-yl 6-*O*-α-L-rhamnopyranosyl-β-D-glucoside ▲trihydrate▲ (ERR 1-Apr-2019) [250249-75-3].

DEFINITION

Rutin contains NLT 95.0% and NMT 101.0% of rutin ($C_{27}H_{30}O_{16}$), calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

Change to read:

- **B. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Ultraviolet-Visible Spectroscopy*: **197U**▲ (CN 1-May-2020)
  Analytical wavelength: 210–450 nm
  Sample solution: 20 µg/mL of Rutin in methanol. Filter if necessary.
  Acceptance criteria: The spectrum exhibits two absorption maxima at 257 nm and 358 nm. The absorptivity at the maximum at 358 nm is 30.5–33.0, calculated on the anhydrous basis.
- **C.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Quercetin and Other Related Compounds*.

ASSAY

- **PROCEDURE**
  Sample: 200 mg of Rutin
  Blank: 20 mL of dimethylformamide
  Titrimetric system
  (See *Titrimetry* ⟨541⟩.)
  Mode: Direct titration
  Titrant: Tetrabutylammonium hydroxide
  Endpoint detection: Potentiometric
  Analysis: Dissolve the *Sample* in 20 mL of dimethylformamide, and titrate with *Titrant*. Perform the *Blank* determination and make corrections if necessary.
  Calculate the percentage of rutin ($C_{27}H_{30}O_{16}$) in the portion of the *Sample* taken:

$$\text{Result} = \{[(V_S - V_B) \times M \times F]/W\} \times 100$$

V_S = *Titrant* volume consumed by the *Sample* (mL)
V_B = *Titrant* volume consumed by the *Blank* (mL)
M = actual *Titrant* molarity (mmol/mL)
F = equivalency factor, 305.3 mg/mmol
W = *Sample* weight (mg)

DS Monographs

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Acceptance criteria: 95.0%–101.0% on the anhydrous basis

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%
- **LIGHT ABSORBING SUBSTANCES**

Analytical wavelength: 450–800 nm
Sample solution: Dissolve 0.200 g of Rutin in 40 mL of 2-propanol. Stir for 15 min, dilute with 2-propanol to 50.0 mL, and filter.
Acceptance criteria: The absorbance of any substance is NMT 0.10.

- **METHANOL INSOLUBLE SUBSTANCES**

Sample: 2.5 g
Analysis: Shake the *Sample* for 15 min in 50 mL of methanol at 20°–25°. Filter under reduced pressure through a sintered-glass filter previously dried for 15 min at 100°–105°, cooled in a desiccator, and tared. Wash the filter three times with 20 mL of methanol. Dry the filter for 30 min at 105°. Allow to cool, and weigh.
Acceptance criteria: The residue weighs NMT 75 mg (3%).

- **QUERCETIN AND OTHER RELATED COMPOUNDS**

Solution A: Mix 50 mL of tetrahydrofuran with 950 mL of a 15.6 mg/mL solution of monobasic sodium phosphate. Adjust with phosphoric acid to a pH of 3.0.
Solution B: Mix 400 mL of tetrahydrofuran with 600 mL of a 15.6 mg/mL solution of monobasic sodium phosphate. Adjust with phosphoric acid to a pH of 3.0.
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	50	50
10	0	100
20	0	100
21	50	50
25	50	50

Standard solution A: Transfer 10 mg of USP Rutin RS into a 10-mL volumetric flask, add 2 mL of methanol, and mix to dissolve. Dilute with *Solution B* to volume.
Standard solution B: 20 µg/mL of USP Rutin RS in *Solution B*, prepared from the dilution of *Standard solution A* with *Solution B*
Standard solution C: 20 µg/mL of USP Quercetin RS in *Solution B*
Sample solution: Transfer 100 mg of Rutin into a 100-mL volumetric flask, add 20 mL of methanol, and mix to dissolve. Dilute with *Solution B* to volume.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 280 nm
Column: 4-mm × 25-cm; 5-µm packing L7
Column temperature: 30°
Flow rate: 1.0 mL/min
Injection volume: 20 µL
System suitability
Sample: *Standard solution A*
[NOTE—The relative retention times for rutin, kaempferol 3-rutinoside, isoquercitroside, and quercetin are 1.0, 1.1, 1.2, and 2.5, respectively.]
Suitability requirements
Chromatographic similarity: The chromatogram from *Standard solution A* is similar to the reference chromatogram provided with the USP Rutin RS being used.
Resolution: NLT 1.5, between the rutin and kaempferol 3-rutinoside peaks
Analysis
Samples: *Standard solution B*, *Standard solution C*, and *Sample solution*
Calculate the percentage of quercetin[1] in the portion of Rutin taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of quercetin from the *Sample solution*
r_S = peak response of quercetin from *Standard solution C*
C_S = concentration of USP Quercetin RS in *Standard solution C* (mg/mL)
C_U = concentration of Rutin in the *Sample solution* (mg/mL)

Calculate the percentage of each impurity in the portion of Rutin taken: [NOTE—Disregard any impurity less than 0.1%.]

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak response of each impurity from the *Sample solution*
r_S = peak response of rutin from *Standard solution B*
C_S = concentration of USP Rutin RS in *Standard solution B* (mg/mL)
C_U = concentration of Rutin in the *Sample solution* (mg/mL)
F = correction factor for each individual impurity (see *Table 2*)

Acceptance criteria: See *Table 2*.

Table 2

Name	Relative Retention Time	Correction Factor (*F*)	Acceptance Criteria, NMT (%)
Kaempferol 3-rutinoside[a]	1.1	1	2.0
Isoquercitroside[b]	1.2	0.8	2.0
Quercetin	—	—	2.0
Any other impurity	—	1	1.0
Total impurities	—	—	4.0

[a] 3-[[6-*O*-(6-Deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-5,7-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one.
[b] 2-(3,4-Dihydroxyphenyl)-3-(β-D-glucofuranosyloxy)-5,7-dihydroxy-4*H*-1-benzopyran-4-one.

SPECIFIC TESTS

- **WATER DETERMINATION** ⟨921⟩, *Method I, Method Ia*

Sample: 100 mg
Acceptance criteria: 7.5%–9.5%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed, tight, light-resistant containers.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Quercetin RS
USP Rutin RS

[1] 2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-1-benzopyran-4-one.

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St. John's Wort Flowering Top

DEFINITION

St. John's Wort Flowering Top consists of the dried flowering tops of *Hypericum perforatum* L. (Fam. Hypericaceae), gathered shortly before or during flowering. It contains NLT 0.6% of hyperforin ($C_{35}H_{52}O_4$) and NLT 0.04% of the combined total of hypericin ($C_{30}H_{16}O_8$) and pseudohypericin ($C_{30}H_{16}O_9$), on the dried basis.

IDENTIFICATION

- **A.** It meets the requirements in *Specific Tests, Botanical Characteristics*.
- **B. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 0.5 mg/mL of USP Rutin RS in methanol

Standard solution B: 0.5 mg/mL of USP Hyperoside RS in methanol

Standard solution C: 50 mg/mL of USP Powdered St. John's Wort Extract RS in methanol. Sonicate for 20 min, centrifuge, and use the clear supernatant.

Sample solution: Finely powder 50 g of St. John's Wort Flowering Top. Sonicate 1 g in 10 mL of methanol for about 20 min, centrifuge, and use the clear supernatant.

Chromatographic system

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)

Application volume: 2 µL, as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of 33%.

Temperature: Ambient, not to exceed 30°

Developing solvent system: Ethyl acetate, glacial acetic acid, formic acid, water, and methylene chloride (10: 1.0: 1.0: 1.1: 2.5)

Developing distance: 6 cm

Derivatization reagent A: 5-mg/mL solution of 2-aminoethyl diphenylborinate in ethyl acetate

Derivatization reagent B: 10-mg/mL solution of polyethylene glycol 400 in methylene chloride

Analysis

Samples: *Standard solution A, Standard solution B, Standard solution C,* and *Sample solution*

Apply the *Samples* as bands, and dry in air. Develop in a saturated chamber, remove the plate from the chamber, and dry in air. Heat the plate at 105° for 3 min, treat while still warm with *Derivatization reagent A,* and dry in air. Then treat with *Derivatization reagent B,* dry in air, and examine under UV light at 365 nm.

System suitability: *Standard solution C* exhibits, in the lower third section, two yellowish-orange fluorescent bands corresponding to rutin and hyperoside in *Standard solution A* and *Standard solution B,* respectively; a blue fluorescent band directly below the hyperoside band corresponding to chlorogenic acid; and two red fluorescent bands due to pseudohypericin (lower R_F) and hypericin in the upper third section. The bands due to pseudohypericin and hypericin are clearly separated.

Acceptance criteria: The *Sample solution* exhibits the following: two yellowish-orange fluorescent bands at R_F corresponding to rutin and hyperoside in *Standard solution A, Standard solution B,* and *Standard solution C;* a blue fluorescent band directly below the hyperoside band corresponding to the chlorogenic acid in *Standard solution C;* two red fluorescent bands at R_F corresponding to pseudohypericin and hypericin in *Standard solution C;* and two to three yellowish-orange fluorescent bands in the middle third section corresponding to similar bands in *Standard solution C.*

COMPOSITION

- **CONTENT OF HYPERICIN AND PSEUDOHYPERICIN**

[NOTE—Conduct all sample preparations with minimal exposure to subdued light, and use low-actinic glassware.]

Solvent: Methanol and acetone (1:1)

Solution A: Phosphoric acid and water (3:997)

Solution B: Acetonitrile

Solution C: Methanol

Mobile phase: See *Table 1.*

Table 1

Time (min)	Solution A (%)	Solution B (%)	Solution C (%)
0	100	0	0
10	85	15	0
30	70	20	10
40	10	75	15
55	5	80	15
56	100	0	0
66	100	0	0

Standard solution A: 2.5 µg/mL of USP Oxybenzone RS in *Solvent*

Standard solution B: 1 mg/mL of USP Powdered St. John's Wort Extract RS in *Solvent*

Sample solution: Pulverize 10 g of St. John's Wort Flowering Top. Accurately weigh and transfer about 1 g to a round-bottom flask equipped with a condenser and protected from light, add 50 mL of *Solvent* and a magnetic stirring bar, and heat at 60° for 2 h while stirring. Cool to room temperature, and pass through a filter paper into a 50-mL volumetric flask. Wash the flask and the residue on the filter with *Solvent,* and dilute with the washings to volume. Pass the solution through a PTFE membrane filter of 0.45-µm or finer pore size, and use the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability.*)

Mode: LC

Detector: UV 270 nm and Vis 588 nm

Columns

Guard: Packing L1

Analytical: 4.6-mm × 25-cm; packing L1

Column temperature: 30°

Flow rate: 1 mL/min

Injection volume: 20 µL

System suitability

Samples: *Standard solution A* (record the peak responses at 270 nm) and *Standard solution B* (record the peak responses at 270 and 588 nm)

Suitability requirements

Chromatogram similarity: The chromatograms of *Standard solution B* are similar to the respective reference chromatograms provided with the lot of USP Powdered St. John's Wort Extract RS being used.

Column efficiency: NLT 100,000 theoretical plates for oxybenzone, *Standard solution A*

Tailing factor: NMT 1.5 for oxybenzone, *Standard solution A*

Relative standard deviation: NMT 2.0% in replicate injections, *Standard solution A*

Analysis

Samples: *Standard solution A* and *Sample solution*

Measure the areas of the relevant peaks in the *Sample solution* at 270 nm.

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Calculate the combined total of hypericin ($C_{30}H_{16}O_8$) and pseudohypericin ($C_{30}H_{16}O_9$) percentages in the portion of St. John's Wort Flowering Top taken:

$$\text{Result} = (C_S/r_S) \times \Sigma(r_{UI}/F_I) \times (V/W) \times 100$$

C_S = concentration of USP Oxybenzone RS in *Standard solution A* (mg/mL)
r_S = peak area of oxybenzone in *Standard solution A*
r_{UI}/F_I = peak area of hypericin or pseudohypericin in the *Sample solution* divided by their respective response factors relative to oxybenzone; 1.30 for hypericin and 1.24 for pseudohypericin
V = volume of the *Sample solution* (mL)
W = weight of St. John's Wort Flowering Top taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 0.04% on the dried basis

- **CONTENT OF HYPERFORIN**

Analysis: Using the chromatograms obtained in the test for *Content of Hypericin and Pseudohypericin*, calculate the percentage of hyperforin ($C_{35}H_{52}O_4$) in the portion of St. John's Wort Flowering Top taken:

$$\text{Result} = C_S \times (r_U/r_S) \times (V/W) \times (1/F) \times 100$$

C_S = concentration of USP Oxybenzone RS in *Standard solution A* (mg/mL)
r_U = peak area of hyperforin in the *Sample solution*
r_S = peak area of oxybenzone in *Standard solution A*
V = volume of the *Sample solution* (mL)
W = weight of St. John's Wort Flowering Top taken to prepare the *Sample solution* (mg)
F = relative response factor for hyperforin relative to oxybenzone, 0.46

Acceptance criteria: NLT 0.6% on the dried basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Limits of Elemental Impurities*: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Pesticide Residue Analysis*: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: The two-edged stem is greenish-yellow, rounded, and has two ribs running longitudinally on opposite sides. The plant is adversifoliate, its leaves are sessile, ovoid or elongated, up to 3.5 cm in length, smooth-edged, and hairless with translucent perforations. The very numerous yellow, short-stemmed, pentamerous flowers form false umbels shaped like grape clusters. The five lanceolate and black-dotted sepals are about one-half the length of the dark yellow petals, which are shaped like slanted ovals and whose edges are set with dark red glands. The numerous stamens are joined in three to six bundles (usually three). The ovary is surmounted by three styles. Some ovaries are already developed into greenish, elongated, oval triovular capsules with various degrees of maturity. When chopped, the crude plant material is distinguished by numerous yellow to yellowish-brown flower buds and individual petals with dark red glands at the edges. The light green to brown-green leaf fragments, characterized by plicate marcescence, appear stippled when held up to the light. The greenish-yellow or reddish-brown hollow stem fragments are distinguished by two longitudinal edges.

Microscopic: The stems have elongated epidermal cells with straight beaded, anticlinal walls; cuticle smooth; frequent paracytic stomata with two small adjacent epidermal cells; cortex of five to six rows of collenchyma; stele with secondary growth consisting of a compacted ring of phloem, with a wide area of lignified xylem and small areas of intraxylary phloem; parenchymatous pith, lignified and pitted in older stems; oil glands may occur in the cortex and phloem.

The upper surface of the leaf has polygonal cells with sinuous, slightly beaded, anticlinal walls; cells of lower surface smaller, with anticlinal walls more wavy, with frequent paracytic, sometimes anomocytic, stomata; smooth cuticle, thicker on upper surface, straight-walled, elongated epidermal cells of veins, occasionally beaded. Dorsiventral, single palisade lamina; large oil glands equal to depth of spongy mesophyll. Midrib containing single, collateral bundle with a small area of lignified xylem. Trichomes and calcium oxalate are absent.

The sepal of the flower has characteristics resembling those of the leaf. Petal, narrow, elongated, thin-walled; epidermal cells with straight anticlinal walls on the outer surface and wavy on the inner surface. Stamen, lignified fibrous layer of anther wall; elongated, thin-walled cells of filament with striated cuticle; subprolate pollen grains, about 20 µm in diameter with three pores and a smooth exine. Ovary, small polygonal cells with underlying oil glands; seed testa, brown, thick-walled hexagonal cells.

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Foreign Organic Matter*: NMT 2.0%
- **LOSS ON DRYING** ⟨731⟩

Sample: 1.0 g of finely powdered St. John's Wort Flowering Top
Analysis: Dry the *Sample* at 105° for 2 h.
Acceptance criteria: NMT 10%

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Total Ash*: NMT 5.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Hyperoside RS
USP Oxybenzone RS
USP Rutin RS
USP Powdered St. John's Wort Extract RS

St. John's Wort Flowering Top Powder

DEFINITION

St. John's Wort Flowering Top Powder consists of the dried flowering tops of *Hypericum perforatum* L. (Fam. Hypericaceae), gathered shortly before or during flowering, and reduced to a fine or a very fine powder. It contains NLT 0.6% of hyperforin ($C_{35}H_{52}O_4$) and NLT 0.04% of the combined total of hypericin ($C_{30}H_{16}O_8$) and pseudohypericin ($C_{30}H_{16}O_9$), on the dried basis.

Supplements

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IDENTIFICATION

• **A.** It meets the requirements in *Specific Tests, Botanical Characteristics.*

• **B. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 0.5 mg/mL of USP Rutin RS in methanol

Standard solution B: 0.5 mg/mL of USP Hyperoside RS in methanol

Standard solution C: 50 mg/mL of USP Powdered St. John's Wort Extract RS in methanol. Sonicate for 20 min, centrifuge, and use the clear supernatant.

Sample solution: Sonicate 1 g of St. John's Wort Flowering Top Powder in 10 mL of methanol for about 20 min, centrifuge, and use the clear supernatant.

Chromatographic system

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)

Application volume: 2 µL, as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of 33%.

Temperature: Ambient, not to exceed 30°

Developing solvent system: Ethyl acetate, glacial acetic acid, formic acid, water, and methylene chloride (10: 1.0: 1.0: 1.1: 2.5)

Developing distance: 6 cm

Derivatization reagent A: 5-mg/mL solution of 2-aminoethyl diphenylborinate in ethyl acetate

Derivatization reagent B: 10-mg/mL solution of polyethylene glycol 400 in methylene chloride

Analysis

Samples: *Standard solution A, Standard solution B, Standard solution C,* and *Sample solution*

Apply the *Samples* as bands, and dry in air. Develop in a saturated chamber, remove the plate from the chamber, and dry in air. Heat the plate at 105° for 3 min, treat while still warm with *Derivatization reagent A,* and dry in air. Then treat with *Derivatization reagent B,* dry in air, and examine under UV light at 365 nm.

System suitability: *Standard solution C* exhibits, in the lower third section, two yellowish-orange fluorescent bands corresponding to rutin and hyperoside in *Standard solution A* and *Standard solution B,* respectively; a blue fluorescent band directly below the hyperoside band corresponding to chlorogenic acid; and two red fluorescent bands due to pseudohypericin (lower R_F) and hypericin in the upper third section. The bands due to pseudohypericin and hypericin are clearly separated.

Acceptance criteria: The *Sample solution* exhibits the following: two yellowish-orange fluorescent bands at R_F corresponding to rutin and hyperoside in *Standard solution A, Standard solution B,* and *Standard solution C;* a blue fluorescent band directly below the hyperoside band corresponding to the chlorogenic acid in *Standard solution C;* two red fluorescent bands at R_F corresponding to pseudohypericin and hypericin in *Standard solution C;* and two to three yellowish-orange fluorescent bands in the middle third section corresponding to similar bands in *Standard solution C.*

COMPOSITION

• **CONTENT OF HYPERICIN AND PSEUDOHYPERICIN**

[NOTE—Conduct all sample preparations with minimal exposure to subdued light, and use low-actinic glassware.]

Solvent: Methanol and acetone (1:1)

Solution A: Phosphoric acid and water (3:997)

Solution B: Acetonitrile

Solution C: Methanol

Mobile phase: See *Table 1.*

Table 1

Time (min)	Solution A (%)	Solution B (%)	Solution C (%)
0	100	0	0
10	85	15	0
30	70	20	10
40	10	75	15
55	5	80	15
56	100	0	0
66	100	0	0

Standard solution A: 2.5 µg/mL of USP Oxybenzone RS in *Solvent*

Standard solution B: 1 mg/mL of USP Powdered St. John's Wort Extract RS in *Solvent*

Sample solution: Accurately weigh about 1 g of St. John's Wort Flowering Top Powder into a round-bottom flask equipped with a condenser and protected from light, add 50 mL of *Solvent* and a magnetic stirring bar, and heat at 60° for 2 h while stirring. Cool to room temperature, and pass through a filter paper into a 50-mL volumetric flask. Wash the flask and the residue on the filter with *Solvent,* and dilute with the washings to volume. Pass the solution through a PTFE membrane filter of 0.45-µm or finer pore size, and use the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability.*)

Mode: LC

Detector: UV 270 nm and Vis 588 nm

Columns

Guard: Packing L1

Analytical: 4.6-mm × 25-cm; packing L1

Column temperature: 30°

Flow rate: 1 mL/min

Injection volume: 20 µL

System suitability

Samples: *Standard solution A* (record the peak responses at 270 nm) and *Standard solution B* (record the peak responses at 270 and 588 nm)

Suitability requirements

Chromatogram similarity: The chromatograms of *Standard solution B* are similar to the respective reference chromatograms provided with the lot of USP Powdered St. John's Wort Extract RS being used.

Column efficiency: NLT 100,000 theoretical plates for oxybenzone, *Standard solution A*

Tailing factor: NMT 1.5 for oxybenzone, *Standard solution A*

Relative standard deviation: NMT 2.0% in replicate injections, *Standard solution A*

Analysis

Samples: *Standard solution A* and *Sample solution*

Measure the areas of the relevant peaks in the *Sample solution* at 270 nm.

Calculate the combined total of hypericin ($C_{30}H_{16}O_8$) and pseudohypericin ($C_{30}H_{16}O_9$) percentages in the portion of St. John's Wort Flowering Top Powder taken:

$$\text{Result} = (C_S/r_S) \times \Sigma(r_{Ui}/F_i) \times (V/W) \times 100$$

C_S = concentration of USP Oxybenzone RS in *Standard solution A* (mg/mL)

r_S = peak area of oxybenzone in *Standard solution A*

r_{Ui}/F_i = peak area of hypericin or pseudohypericin in the *Sample solution* divided by their respective

DS Monographs

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response factors relative to oxybenzone; 1.30 for hypericin and 1.24 for pseudohypericin
V = volume of the *Sample solution* (mL)
W = weight of St. John's Wort Flowering Top Powder taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 0.04% on the dried basis

• **CONTENT OF HYPERFORIN**

Analysis: Using the chromatograms obtained in the test for *Content of Hypericin and Pseudohypericin*, calculate the percentage of hyperforin ($C_{35}H_{52}O_4$) in the portion of St. John's Wort Flowering Top Powder taken:

$$\text{Result} = C_S \times (r_U/r_S) \times (V/W) \times (1/F) \times 100$$

C_S = concentration of USP Oxybenzone RS in *Standard solution A* (mg/mL)
r_U = peak area of hyperforin in the *Sample solution*
r_S = peak area of oxybenzone in *Standard solution A*
V = volume of the *Sample solution* (mL)
W = weight of St. John's Wort Flowering Top Powder taken to prepare the *Sample solution* (mg)
F = relative response factor for hyperforin relative to oxybenzone, 0.46

Acceptance criteria: NLT 0.6% on the dried basis

CONTAMINANTS

• **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Limits of Elemental Impurities*: Meets the requirements
• **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Pesticide Residue Analysis*: Meets the requirements
• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.
• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS

• **BOTANICAL CHARACTERISTICS**

Macroscopic: Buff to greenish-brown powder with an aromatic and balsamic odor

Microscopic: Elongated and polygonal epidermal cells with thickened and beaded anticlinal walls, some accompanied by paracytic (occasionally anomocytic) stomata; fragments of leaf and sepal with schizogenous oil and pigment glands; narrow, thin-walled, elongated epidermal cells with straight and wavy anticlinal walls from petal; narrow, lignified vessels with annular or reticulate thickening; tracheids and tracheidal vessels with lignified, pitted thickening; thick-walled lignified fibers with tapering apices and occasional oblique pits; rectangular, lignified, pitted parenchyma; fibrous layer of antheral wall; pollen grains, 20–25 µm in diameter, with smooth exine.

• **LOSS ON DRYING** ⟨731⟩

Sample: 1.0 g of St. John's Wort Flowering Top Powder
Analysis: Dry the *Sample* at 105° for 2 h.
Acceptance criteria: NMT 10%

• **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Total Ash*: NMT 5.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture.
• **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant source from which the article was derived.
• **USP REFERENCE STANDARDS** ⟨11⟩

USP Hyperoside RS
USP Oxybenzone RS
USP Rutin RS
USP Powdered St. John's Wort Extract RS

St. John's Wort Flowering Top Dry Extract

DEFINITION

St. John's Wort Flowering Top Dry Extract is prepared from comminuted dried flowering tops of *Hypericum perforatum* L. (Fam. Hypericaceae), gathered shortly before or during flowering, and extracted with 80% methanol or other suitable solvents. It contains NLT 90.0% and NMT 110.0% of the labeled combined total of hypericin ($C_{30}H_{16}O_8$) and pseudohypericin ($C_{30}H_{16}O_9$) and NLT 90.0% and NMT 110.0% of hyperforin ($C_{35}H_{52}O_4$), on the anhydrous basis. It may contain suitable added substances.

IDENTIFICATION

• **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 0.5 mg/mL of USP Rutin RS in methanol
Standard solution B: 0.5 mg/mL of USP Hyperoside RS in methanol
Standard solution C: 50 mg/mL of USP Powdered St. John's Wort Extract RS in methanol. Sonicate for 20 min, centrifuge, and use the clear supernatant.
Sample solution: 50 mg/mL of St. John's Wort Flowering Top Dry Extract in methanol, sonicate for 20 min, centrifuge, and use the clear supernatant.
Chromatographic system
Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)
Application volume: 2 µL, as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of 33%.
Temperature: Ambient, not to exceed 30°
Developing solvent system: Ethyl acetate, glacial acetic acid, formic acid, water, and methylene chloride (10: 1.0: 1.0: 1.1: 2.5)
Developing distance: 6 cm
Derivatization reagent A: 5 mg/mL of 2-aminoethyl diphenylborinate in ethyl acetate
Derivatization reagent B: 10 mg/mL of polyethylene glycol 400 in methylene chloride
Analysis
Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Apply the *Samples* as bands, and dry in air. Develop in a saturated chamber, remove the plate from the chamber, and dry in air. Heat the plate at 105° for 3 min, treat while still warm with *Derivatization reagent A*, and dry in air. Then treat with *Derivatization reagent B*, dry in air, and examine under UV light at 365 nm.

System suitability: *Standard solution C* exhibits, in the lower third section, two yellowish-orange fluorescent bands corresponding to rutin and hyperoside in *Standard solution A* and *Standard solution B*, respectively; a blue fluorescent band directly below the hyperoside band corresponding to chlorogenic acid; and two red fluorescent bands due to pseudohypericin (lower R_F) and hypericin in the upper third section. The bands due to pseudohypericin and hypericin are clearly separated.

Acceptance criteria: The *Sample solution* exhibits the following: two yellowish-orange fluorescent bands at R_F corresponding to rutin and hyperoside in *Standard solution*

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A, *Standard solution B*, and *Standard solution C*; a blue fluorescent band directly below the hyperoside band corresponding to chlorogenic acid in *Standard solution C*; two red fluorescent bands at R_F corresponding to pseudohypericin and hypericin in *Standard solution C*; and two to three yellowish-orange fluorescent bands in the middle third section corresponding to similar bands in *Standard solution C*.

• **B. THIN-LAYER CHROMATOGRAPHY**

Presence of hyperforin

Standard solution: 50 mg/mL of USP Powdered St. John's Wort Extract RS in methanol. Sonicate for 20 min, and use the clear supernatant.

Sample solution: 50 mg/mL of St. John's Wort Flowering Top Dry Extract in methanol. Sonicate for 20 min, and use the clear supernatant.

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Adsorbent: 0.50-mm layer of chromatographic silica gel mixture

Application volume: 10 µL

Developing solvent system: Solvent hexane and ethyl acetate (4:1)

Derivatization reagent: Prepare a solution containing 0.38 g of ceric ammonium sulfate and 3.8 g of ammonium molybdate in 100 mL of 2 N sulfuric acid.

Analysis

Samples: *Standard solution* and *Sample solution*

Develop in a saturated chamber until the solvent front has moved NLT 18 cm, and dry the plate in a current of air. Treat the plate with *Derivatization reagent*, heat at 140° for 15 min, and examine under white light.

Acceptance criteria: The blue zone due to hyperforin at an R_F value of about 0.54 in the *Sample solution* corresponds in color and position to that in the *Standard solution*.

COMPOSITION

• **CONTENT OF HYPERICIN AND PSEUDOHYPERICIN**

[NOTE—Conduct all sample preparations with minimal exposure to subdued light, and use low-actinic glassware.]

Solvent: Methanol and acetone (1:1)

Solution A: Phosphoric acid and water (3:997)

Solution B: Acetonitrile

Solution C: Methanol

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)	Solution C (%)
0	100	0	0
10	85	15	0
30	70	20	10
40	10	75	15
55	5	80	15
56	100	0	0
66	100	0	0

Standard solution A: 2.5 µg/mL of USP Oxybenzone RS in *Solvent*

Standard solution B: 1 mg/mL of USP Powdered St. John's Wort Extract RS in *Solvent*

Sample solution: 1 mg/mL of St. John's Wort Flowering Top Dry Extract in a mixture of methanol and water (9:1). Sonicate to dissolve, pass through a PTFE filter of 0.45-µm or finer pore size, and use the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 270 nm and Vis 588 nm

Columns

Guard: Packing L1

Analytical: 4.6-mm × 25-cm; packing L1

Column temperature: 30°

Flow rate: 1 mL/min

Injection volume: 20 µL

System suitability

Samples: *Standard solution A* (record the peak responses at 270 nm) and *Standard solution B* (record the peak responses at 270 and 588 nm)

Suitability requirements

Chromatogram similarity: The chromatograms of *Standard solution B* are similar to the respective reference chromatograms provided with the lot of USP Powdered St. John's Wort Extract RS being used.

Column efficiency: NLT 100,000 theoretical plates for oxybenzone, *Standard solution A*

Tailing factor: NMT 1.5 for oxybenzone, *Standard solution A*

Relative standard deviation: NMT 2.0% in replicate injections, *Standard solution A*

Analysis

Samples: *Standard solution A* and *Sample solution*

Measure the areas of the relevant peaks in the *Sample solution* at 270 nm.

Calculate the percentage of the combined total of hypericin ($C_{30}H_{16}O_8$) and pseudohypericin ($C_{30}H_{16}O_9$) in the portion of St. John's Wort Flowering Top Dry Extract taken:

$$\text{Result} = (C_S/r_S) \times \Sigma(r_{Ui}/F_i) \times (V/W) \times 100$$

C_S = concentration of USP Oxybenzone RS in *Standard solution A* (mg/mL)

r_S = peak area of oxybenzone in the *Standard solution A*

r_{Ui}/F_i = peak area of hypericin or pseudohypericin in the *Sample solution* divided by their respective response factors relative to oxybenzone; 1.30 for hypericin and 1.24 for pseudohypericin

V = volume of the *Sample solution* (mL)

W = weight of St. John's Wort Flowering Top Dry Extract taken to prepare the *Sample solution* (mg)

Calculate the percentage of the labeled amount of hypericin ($C_{30}H_{16}O_8$) and pseudohypericin ($C_{30}H_{16}O_9$) in the portion of St. John's Wort Flowering Top Dry Extract taken:

$$\text{Result} = (P/L) \times 100$$

P = combined total percentage of hypericin and pseudohypericin, as determined above (%)

L = labeled percentage of hypericin and pseudohypericin in St. John's Wort Flowering Top Dry Extract (%)

Acceptance criteria: 90.0%–110.0% on the anhydrous basis

• **CONTENT OF HYPERFORIN**

Analysis: Using the chromatograms obtained in the test for *Content of Hypericin and Pseudohypericin*, calculate the percentage of hyperforin ($C_{35}H_{52}O_4$) in the portion of St. John's Wort Flowering Top Dry Extract taken:

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$$\text{Result} = C_S \times (r_U/r_S) \times (V/W) \times (1/F) \times 100$$

C_S = concentration of USP Oxybenzone RS in *Standard solution A* (mg/mL)
r_U = peak area of hyperforin in the *Sample solution*
r_S = peak area of oxybenzone in the *Standard solution A*
V = volume of the *Sample solution* (mL)
W = weight of St. John's Wort Flowering Top Dry Extract taken to prepare the *Sample solution* (mg)
F = relative response factor for hyperforin relative to that of oxybenzone, 0.46

Calculate the percentage of the labeled amount of hyperforin ($C_{35}H_{52}O_4$) in the portion of St. John's Wort Flowering Top Dry Extract taken:

$$\text{Result} = (P/L) \times 100$$

P = percentage of hyperforin, as determined above (%)
L = labeled percentage of hyperforin in St. John's Wort Flowering Top Dry Extract (%)

Acceptance criteria: 90.0%–110.0% on the anhydrous basis

CONTAMINANTS

Change to read:

- **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*:▲ (CN 1-May-2020) Meets the requirements
- **BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Residual Solvents*: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS

- **WATER DETERMINATION** ⟨921⟩, *Method I*: NMT 5%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Total Ash*: NMT 7.0%
- **BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Residue on Evaporation*: Meets the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. The label also indicates the content of hypericin, pseudohypericin, and hyperforin; the extracting solvent or solvent mixture used for preparation; and the ratio of the starting crude plant material to Dry Extract. The label bears a statement indicating that "Rare cases of allergic reactions and photosensitivity have been reported with the use of St. John's Wort. St. John's Wort interacts with numerous medications. Check with your healthcare provider before using."
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Hyperoside RS
  USP Oxybenzone RS
  USP Rutin RS
  USP Powdered St. John's Wort Extract RS

St. John's Wort Flowering Top Dry Extract Capsules

DEFINITION

St. John's Wort Flowering Top Dry Extract Capsules contain St. John's Wort Flowering Top Dry Extract. They contain NLT 90% and NMT 110% of the labeled amount of hypericins, calculated as the sum of hypericin ($C_{30}H_{16}O_8$) and pseudohypericin ($C_{30}H_{16}O_9$) and NLT 90.0% and NMT 110.0% of the labeled amount of hyperforin ($C_{35}H_{52}O_4$).

IDENTIFICATION

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩
  Standard solution A: 0.5 mg/mL of USP Rutin RS in methanol
  Standard solution B: 0.5 mg/mL of USP Hyperoside RS in methanol
  Standard solution C: 50 mg/mL of USP Powdered St. John's Wort Extract RS in methanol. Sonicate for 20 min, centrifuge, and use the clear supernatant.
  Sample solution: Transfer a portion of the contents of the Capsules, equivalent to 500 mg of St. John's Wort Flowering Top Dry Extract, to a conical flask, add 10 mL of methanol, mix and sonicate for 20 min, centrifuge, and use the supernatant.
  Chromatographic system
  Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)
  Application volume: 2 µL, as 8-mm bands
  Relative humidity: Condition the plate to a relative humidity of 33%.
  Temperature: Ambient, not to exceed 30°
  Developing solvent system: Ethyl acetate, glacial acetic acid, formic acid, water, and methylene chloride (10: 1.0: 1.0: 1.1: 2.5)
  Developing distance: 6 cm
  Derivatization reagent A: 5 mg/mL of 2-aminoethyl diphenylborinate in ethyl acetate
  Derivatization reagent B: 10 mg/mL of polyethylene glycol 400 in methylene chloride
  Analysis
  Samples: *Standard solution A, Standard solution B, Standard solution C,* and *Sample solution*
  Apply the *Samples* as bands, and dry in air. Develop in a saturated chamber, remove the plate from the chamber, and dry in air. Heat the plate at 105° for 3 min, treat while still warm with *Derivatization reagent A,* and dry in air. Then treat with *Derivatization reagent B,* dry in air, and examine under UV light at 366 nm.
  System suitability: *Standard solution C* exhibits, in the lower third section, two yellowish-orange fluorescent bands corresponding to rutin and hyperoside in *Standard solution A* and *Standard solution B,* respectively; a blue fluorescent band directly below the hyperoside band corresponding to chlorogenic acid; and two red fluorescent bands due to pseudohypericin (lower R_F) and hypericin in the upper third section. The bands due to pseudohypericin and hypericin are clearly separated.
  Acceptance criteria: The *Sample solution* exhibits the following: two yellowish-orange fluorescent bands at R_F corresponding to rutin and hyperoside in *Standard solution A, Standard solution B,* and *Standard solution C;* a blue fluorescent band directly below the hyperoside band

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corresponding to chlorogenic acid in *Standard solution C*; two red fluorescent bands at R_F corresponding to pseudohypericin and hypericin in *Standard solution C*; and two to three yellowish-orange fluorescent bands in the middle third section corresponding to similar bands in *Standard solution C*.

• **B. LC**

Analysis: Proceed as directed in the *Content of Hypericin, Pseudohypericin, and Hyperforin.*

Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks at the retention times corresponding to the peaks due to hypericin, pseudohypericin, and hyperforin in the chromatogram of *Standard solution B.*

STRENGTH

• **CONTENT OF HYPERICIN, PSEUDOHYPERICIN, AND HYPERFORIN**

[NOTE—Conduct all sample preparations with minimal exposure to subdued light, and use low-actinic glassware.]

Solvent: Methanol and acetone (1:1)

Solution A: Phosphoric acid and water (3:997)

Solution B: Acetonitrile

Solution C: Methanol

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)	Solution C (%)
0	100	0	0
10	85	15	0
30	70	20	10
40	10	75	15
55	5	80	15
56	100	0	0
66	100	0	0

Standard solution A: 2.5 µg/mL of USP Oxybenzone RS in *Solvent*

Standard solution B: 1 mg/mL of USP Powdered St. John's Wort Extract RS in *Solvent*

Sample solution: Determine the total weight of 20 Capsules. Open the Capsules and combine their contents in an appropriate container. Weigh the empty Capsule shells and calculate the average fill weight per Capsule. Transfer a portion of the Capsule contents, equivalent to 200 mg of St. John's Wort Flowering Top Dry Extract into a 200-mL volumetric flask. Add 150 mL of *Solvent* and sonicate for 30 min with occasional shaking. Cool to room temperature, dilute to volume with *Solvent*, mix well, centrifuge. Before injection, pass through a polytetrafluoroethylene (PTFE) membrane filter of 0.45-µm pore size and use the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability.*)

Mode: LC

Detector: UV 270 nm and Vis 588 nm

Columns

Guard: Packing L1

Analytical: 4.6-mm × 25-cm; packing L1

Column temperature: 30°

Flow rate: 1 mL/min

Injection volume: 20 µL

System suitability

Samples: *Standard solution A* (record the peak responses at 270 nm) and *Standard solution B* (record the peak responses at 270 and 588 nm)

Suitability requirements

Column efficiency: NLT 100,000 theoretical plates for oxybenzone, *Standard solution A*

Tailing factor: NMT 1.5 for oxybenzone, *Standard solution A*

Relative standard deviation: NMT 2.0% in replicate injections, *Standard solution A*

Chromatogram similarity: The chromatograms of *Standard solution B* are similar to the respective reference chromatograms provided with the lot of USP Powdered St. John's Wort Extract RS being used.

Analysis

Samples: *Standard solution A*, *Standard solution B* and *Sample solution*

Record the peak responses at 270 nm. Using the chromatograms of *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered St. John's Wort Extract RS being used, identify the peaks corresponding to hypericin, pseudohypericin, and hyperforin in the *Sample solution*. Measure the areas of the relevant peaks in the *Sample solution* chromatogram.

Calculate the percentage of the labeled amount of hypericin ($C_{30}H_{16}O_8$) and pseudohypericin ($C_{30}H_{16}O_9$) in the portion of Capsules taken:

$$\text{Result} = \Sigma(r_{Ui}/F_i) \times (1/r_S) \times (C_S/C_U) \times 100$$

$\Sigma(r_{Ui}/F_i)$ = sum of the peak areas of hypericin and pseudohypericin from the *Sample solution* divided by their respective response factors relative to oxybenzone; 1.30 for hypericin and 1.24 for pseudohypericin

r_S = peak area of oxybenzone from *Standard solution A*

C_S = concentration of USP Oxybenzone RS in *Standard solution A* (mg/mL)

C_U = nominal concentration of the total hypericins content in the *Sample solution* (mg/mL)

Calculate the percentage of the labeled amount of hyperforin ($C_{35}H_{52}O_4$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 1/F \times 100$$

r_U = peak area of hyperforin from the *Sample solution*

r_S = peak area of oxybenzone from *Standard solution A*

C_S = concentration of USP Oxybenzone RS in *Standard solution A* (mg/mL)

C_U = nominal concentration of hyperforin in the *Sample solution* (mg/mL)

F = relative response factor for hyperforin relative to oxybenzone, 0.46

Acceptance criteria: 90.0%–110.0% of the combined total of hypericin and pseudohypericin and 90.0%–110.0% of hyperforin

PERFORMANCE TESTS

• **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩, *Disintegration*: Meet the requirements

• **WEIGHT VARIATION** ⟨2091⟩: Meet the requirements

CONTAMINANTS

• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.

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- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meet the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and the official name. The label states the amount of hypericins (as sum of hypericin and pseudohypericin) and amount of hyperforin in mg/Capsule. The label also bears a statement that rare cases of allergic reactions and photosensitivity have been reported with the use of St. John's Wort Flowering Top Dry Extract, that St. John's Wort Flowering Top Dry Extract interacts with numerous medications, and that use of the product should be discussed with a healthcare provider prior to use.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Hyperoside RS
  USP Oxybenzone RS
  USP Rutin RS
  USP Powdered St. John's Wort Extract RS

St. John's Wort Flowering Top Dry Extract Tablets

DEFINITION

St. John's Wort Flowering Top Dry Extract Tablets contain St. John's Wort Flowering Top Dry Extract. They contain NLT 90% and NMT 110% of the labeled amount of hypericins, calculated as the sum of hypericin ($C_{30}H_{16}O_8$) and pseudohypericin ($C_{30}H_{16}O_9$) and NLT 90.0% and NMT 110.0% of the labeled amount of hyperforin ($C_{35}H_{52}O_4$).

IDENTIFICATION

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 0.5 mg/mL of USP Rutin RS in methanol

Standard solution B: 0.5 mg/mL of USP Hyperoside RS in methanol

Standard solution C: 50 mg/mL of USP Powdered St. John's Wort Extract RS in methanol. Sonicate for 20 min, centrifuge, and use the clear supernatant.

Sample solution: Transfer a portion of the powdered Tablets, equivalent to 500 mg of St. John's Wort Flowering Top Dry Extract, to a conical flask, add 10 mL of methanol, mix and sonicate for 20 min, centrifuge, and use the supernatant.

Chromatographic system

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)

Application volume: 2 µL, as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of 33%.

Temperature: Ambient, not to exceed 30°

Developing solvent system: Ethyl acetate, glacial acetic acid, formic acid, water, and methylene chloride (10: 1.0: 1.0: 1.1: 2.5)

Developing distance: 6 cm

Derivatization reagent A: 5 mg/mL of 2-aminoethyl diphenylborinate in ethyl acetate

Derivatization reagent B: 10 mg/mL of polyethylene glycol 400 in methylene chloride

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Apply the *Samples* as bands, and dry in air. Develop in a saturated chamber, remove the plate from the chamber, and dry in air. Heat the plate at 105° for 3 min, treat while still warm with *Derivatization reagent A*, and dry in air. Then treat with *Derivatization reagent B*, dry in air, and examine under UV light at 366 nm.

System suitability: *Standard solution C* exhibits, in the lower third section, two yellowish-orange fluorescent bands corresponding to rutin and hyperoside in *Standard solution A* and *Standard solution B*, respectively; a blue fluorescent band directly below the hyperoside band corresponding to chlorogenic acid; and two red fluorescent bands due to pseudohypericin (lower R_F) and hypericin in the upper third section. The bands due to pseudohypericin and hypericin are clearly separated.

Acceptance criteria: The *Sample solution* exhibits the following: two yellowish-orange fluorescent bands at R_F corresponding to rutin and hyperoside in *Standard solution A*, *Standard solution B*, and *Standard solution C*; a blue fluorescent band directly below the hyperoside band corresponding to chlorogenic acid in *Standard solution C*; two red fluorescent bands at R_F corresponding to pseudohypericin and hypericin in *Standard solution C*; and two to three yellowish-orange fluorescent bands in the middle third section corresponding to similar bands in *Standard solution C*.

- **B. LC**

Analysis: Proceed as directed in the *Content of Hypericin, Pseudohypericin, and Hyperforin*.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks at the retention times corresponding to the peaks due to hypericin, pseudohypericin, and hyperforin in the chromatogram of *Standard solution B*.

STRENGTH

- **CONTENT OF HYPERICIN, PSEUDOHYPERICIN, AND HYPERFORIN**

[NOTE—Conduct all sample preparations with minimal exposure to subdued light, and use low-actinic glassware.]

Solvent: Methanol and acetone (1:1)

Solution A: Phosphoric acid and water (3:997)

Solution B: Acetonitrile

Solution C: Methanol

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)	Solution C (%)
0	100	0	0
10	85	15	0
30	70	20	10
40	10	75	15
55	5	80	15
56	100	0	0
66	100	0	0

Standard solution A: 2.5 µg/mL of USP Oxybenzone RS in *Solvent*

Standard solution B: 1 mg/mL of USP Powdered St. John's Wort Extract RS in *Solvent*

Sample solution: Weigh NLT 20 Tablets, determine the average Tablet weight, and finely powder. Transfer a portion of finely powdered Tablets, nominally equivalent to 200 mg of St. John's Wort Flowering Top Dry Extract into a 200-mL volumetric flask. Add 150 mL of *Solvent* and

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sonicate for 30 min with occasional shaking. Cool to room temperature, dilute to volume with *Solvent*, mix well, and centrifuge. Before injection, pass through a polytetrafluoroethylene (PTFE) membrane filter of 0.45-µm pore size and use the filtrate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 270 nm and Vis 588 nm
Columns
Guard: Packing L1
Analytical: 4.6-mm × 25-cm; packing L1
Column temperature: 30°
Flow rate: 1 mL/min
Injection volume: 20 µL

System suitability
Samples: *Standard solution A* (record the peak responses at 270 nm) and *Standard solution B* (record the peak responses at 270 and 588 nm)
Suitability requirements
Column efficiency: NLT 100,000 theoretical plates for oxybenzone, *Standard solution A*
Tailing factor: NMT 1.5 for oxybenzone, *Standard solution A*
Relative standard deviation: NMT 2.0% in replicate injections, *Standard solution A*
Chromatogram similarity: The chromatograms of *Standard solution B* are similar to the respective reference chromatograms provided with the lot of USP Powdered St. John's Wort Extract RS being used.

Analysis
Samples: *Standard solution A*, *Standard solution B* and *Sample solution*
Record the peak responses at 270 nm. Using the chromatograms of *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered St. John's Wort Extract RS being used, identify the peaks corresponding to hypericin, pseudohypericin, and hyperforin in the *Sample solution* chromatogram. Measure the areas of the relevant peaks in the *Sample solution*.
Calculate the percentage of the labeled amount of hypericin ($C_{30}H_{16}O_8$) and pseudohypericin ($C_{30}H_{16}O_9$) in the portion of Tablets taken:

$$\text{Result} = \Sigma(r_{Ui}/F_i) \times (1/r_S) \times (C_S/C_U) \times 100$$

$\Sigma(r_{Ui}/F_i)$ = sum of the peak areas of hypericin and pseudohypericin from the *Sample solution* divided by their respective response factors relative to oxybenzone; 1.30 for hypericin and 1.24 for pseudohypericin
r_S = peak area of oxybenzone from *Standard solution A*
C_S = concentration of USP Oxybenzone RS in *Standard solution A* (mg/mL)
C_U = nominal concentration of the total hypericins content in the *Sample solution* (mg/mL)

Calculate the percentage of the labeled amount of hyperforin ($C_{35}H_{52}O_4$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 1/F \times 100$$

r_U = peak area of hyperforin from the *Sample solution*
r_S = peak area of oxybenzone from *Standard solution A*
C_S = concentration of USP Oxybenzone RS in *Standard solution A* (mg/mL)
C_U = nominal concentration of hyperforin in the *Sample solution* (mg/mL)
F = relative response factor for hyperforin relative to oxybenzone, 0.46

Acceptance criteria: 90.0%–110.0% of the combined total of hypericin and pseudohypericin and 90.0%–110.0% of hyperforin

PERFORMANCE TESTS
- **Disintegration and Dissolution** ⟨2040⟩, *Disintegration*: Meet the requirements
- **Weight Variation** ⟨2091⟩: Meet the requirements

CONTAMINANTS
- **Microbial Enumeration Tests** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **Absence of Specified Microorganisms** ⟨2022⟩, *Test Procedures*, *Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meet the requirements

ADDITIONAL REQUIREMENTS
- **Packaging and Storage:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **Labeling:** The label states the Latin binomial and the official name. The label states the amount of hypericins (as sum of hypericin and pseudohypericin) and amount of hyperforin in mg/Tablet. The label also bears a statement that rare cases of allergic reactions and photosensitivity have been reported with the use of St. John's Wort Flowering Top Dry Extract, that St. John's Wort Flowering Top Dry Extract interacts with numerous medications, and that use of the product should be discussed with a healthcare provider prior to use.
- **USP Reference Standards** ⟨11⟩
USP Hyperoside RS
USP Oxybenzone RS
USP Rutin RS
USP Powdered St. John's Wort Extract RS

Change to read:

Salix Species Bark

▲▲ (ERR 1-Feb-2019)

DEFINITION
Salix Species Bark is prepared from the whole or fragmented dried bark of the young branches, or whole dried pieces of the current-year twigs, obtained from *Salix* species (Fam. Salicaceae). Common in pharmacopeial use are *S. alba* L., *S. babylonica* L., *S. daphnoides* Vill., *S. fragilis* L., *S. chilensis* Molina, *S. pentandra* L., *S. purpurea* L., and a number of other complying willow species and their hybrids. It contains NLT 1.50% of total salicylate derivatives, calculated as salicin ($C_{13}H_{18}O_7$) on the dried basis.

IDENTIFICATION
- **A. HPTLC for Articles of Botanical Origin** ⟨203⟩
Standard solution A: 1.50 mg/mL of USP Salicin RS in methanol
Standard solution B: 30 mg/mL of USP Salix Species Bark Dry Extract RS in methanol. Sonicate for 10 min, centrifuge, and use the supernatant.
Sample solution A: Suspend 1000 mg of *Salix* Species Bark, finely powdered, in 10.0 mL of methanol. Sonicate for 10 min, centrifuge, and use the supernatant.

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Sample solution B: Combine 5.0 mL of *Sample solution A* with 1.0 mL of 50-mg/mL anhydrous sodium carbonate. Cap tightly and incubate at 60° for 10 min. Centrifuge and use the supernatant.

Chromatographic system

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plate)[1]

Application volume: 5.0 µL each of *Standard solution A*, *Standard solution B*, and *Sample solution A* and 6.0 µL of *Sample solution B* as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of 33%.

Temperature: Ambient, not to exceed 30°

Developing solvent system: Ethyl acetate, methanol, and water (77:13:10)

Developing distance: 6 cm

Derivatization reagent: Sulfuric acid and methanol (1:9). Slowly add sulfuric acid to ice-cold methanol.

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Sample solution A*, and *Sample solution B*

Apply the *Samples* as bands and dry in air. Develop in a saturated chamber, and dry in a current of air for 5 min. Treat with *Derivatization reagent*, heat at 100° for 5 min, and examine under white light.

System suitability: Under white light, the derivatized chromatogram of *Standard solution B* displays, in the middle third of the plate, three brown bands: the lower corresponds to the salicin band in *Standard solution A*; the one above it is due to salicortin; the top band is due to tremulacin. A faint band, which may appear between the salicortin and tremulacin bands, is due to tremuloidin. Two darker brown bands are seen in the lower third of the plate: one proximate to the application line; another, more intense, above it.

Acceptance criteria: Under white light, the derivatized chromatogram of *Sample solution A* shows one or several dark bands due to different salicin esters, whose position and intensity are contingent on the *Salix* species being used. The salicylate bands of interest are predominantly located in the middle third of the plate, demarcated by the salicin and tremulacin bands of *Standard solution B*. The salicin band in *Sample solution A*, corresponding to that in *Standard solution A*, may be faint or not visible. Additional bands may be seen in *Sample solution A* and *Sample solution B*. In *Sample solution B*, the bands due to salicin esters are not present, while the salicin band corresponding to that in *Standard solution A* is the principal band observed. The salicin band in *Sample solution A* is of lower intensity than the corresponding band in *Standard solution A*. The salicin band in *Sample solution B* is of comparable or higher intensity than the corresponding band in *Standard solution A*.

• **B. HPLC**

Analysis: Proceed as directed in the test for *Salicylates Profile*.

Acceptance criteria: The *Sample solution* exhibits peaks at retention times corresponding to those of salicin and salicin derivatives in the *Standard solution*.

COMPOSITION

• **CONTENT OF SALICIN**

Diluent: Methanol and water (1:1)

Solution A: 0.01% trifluoroacetic acid

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	90	10
10	85	15
30	50	50
32	10	90
35	10	90
37	90	10
45	90	10

Standard solution: 0.50 mg/mL of USP Salicin RS in *Diluent*

Sample solution: Reduce to a fine powder and accurately weigh about 1.3 g of *Salix* Species Bark, transfer to a 200-mL round-bottom flask, and add 40 mL of methanol and 3 mL of 1 N sodium hydroxide. Attach the condenser and heat under reflux for 2 h, with intermittent shaking. Allow to cool, neutralize with 3 mL of 1 N hydrochloric acid, and pass through a paper filter into a 100-mL volumetric flask. Wash the round-bottom flask twice with 5-mL aliquots of methanol and filter into the same 100-mL volumetric flask. Adjust with water to volume, mix well, allow to equilibrate to room temperature, and readjust with water. Pass through a PTFE filter of 0.45-µm pore size, discarding the initial 3 mL of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 270 nm

Column: 4.6-mm × 25-cm; 5-µm base-deactivated packing L1

Column temperature: 30°

Flow rate: 1.0 mL/min

Injection volume: 10 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Tailing factor: 0.8–2.0 for the salicin peak

Relative standard deviation: NMT 2.0% for the salicin peak in replicate injections

Analysis

Samples: *Standard solution* and *Sample solution*

Using the chromatogram of the *Standard solution*, identify the salicin peak in the *Sample solution* chromatogram.

Calculate the percentage of salicin hydrolytically derived from constituent salicylates in the portion of *Salix* Species Bark taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of salicin from the *Sample solution*

r_S = peak area of salicin from the *Standard solution*

C_S = concentration of USP Salicin RS in the *Standard solution* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of *Salix* Species Bark taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 1.50% of salicin on the dried basis

CONTAMINANTS

• **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Limits of Elemental Impurities*: Meets the requirements

• **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Pesticide Residue Analysis*: Meets the requirements

[1] A suitable commercially available plate is HPTLC Silica Gel 60 F_{254} from EMD Millipore (e.g., Part No. 1.05642.0001).

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- **Microbial Enumeration Tests** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined yeasts and molds count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria count does not exceed 10^3 cfu/g.
- **Absence of Specified Microorganisms** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS

- **Salicylates Profile**

Diluent, Mobile phase, and Chromatographic system: Proceed as directed in the test for *Content of Salicin*.

Standard solution: 5 mg/mL of USP Salix Species Bark Dry Extract RS in *Diluent*. Sonicate for 5 min, mix well, and pass through a PTFE filter of 0.45-µm pore size, discarding the initial 3 mL of the filtrate.

Sample solution: Reduce to fine powder and accurately weigh about 650 mg of *Salix* Species Bark, transfer to a 50-mL volumetric flask, add 25 mL of methanol, and sonicate for 30 min. Adjust with water to volume, mix well, allow to equilibrate to room temperature, and readjust with water. Pass through a PTFE filter of 0.45-µm pore size, discarding the initial 3 mL of the filtrate.

System suitability

Suitability requirements: The chromatogram of the *Standard solution* is similar to the reference chromatogram provided with the lot of USP Salix Species Bark Dry Extract RS being used.

Analysis

Samples: *Standard solution* and *Sample solution*

Using the chromatogram of the *Standard solution* and the reference chromatogram provided with the lot of USP Salix Species Bark Dry Extract RS being used, identify salicin esters present in the *Sample solution* chromatogram. The approximate relative retention times, with respect to salicin, are provided in *Table 2*.

Table 2

Analyte	Relative Retention Time
Salicin	1.0
Salicortin	3.0
Tremuloidin	3.6
Tremulacin	4.6

Acceptance criteria: The peak area of salicin is NMT 50% of the combined peak areas of all identified constituent salicylates.

- **Botanical Characteristics**

Macroscopic: The bark is 1–2 cm wide and 1–2 mm thick, and occurs in flexible, elongated, quilled or curved pieces. The outer surface is glossy, smooth or slightly wrinkled longitudinally; greenish yellow in the younger bark to brownish grey in the older bark. The inner surface is smooth or finely striated longitudinally and white, pale yellow or reddish brown, depending on the species. The fracture is short in the outer part and coarsely fibrous in the inner region, and is easily split longitudinally. The diameter of current year twigs is NMT 10 mm. The xylem of young twigs is white or pale yellow.

Microscopic: Two or three rows of poorly developed cork cells with thickened outer walls; cortex of collenchymatous and parenchymatous cells. The latter contains cluster crystals of calcium oxalate, 20–25 µm in diameter, and occasionally tannin. Phloem is characterized by tangential groups of lignified fibers associated with a crystal sheath containing prismatic crystals of calcium oxalate. Simple, rounded starch granules 6–8 µm in diameter in the parenchymatous cells of the phloem and medullary rays.

- **Articles of Botanical Origin** ⟨561⟩, *Methods of Analysis, Foreign Organic Matter*: NMT 3.0% of twigs with a diameter greater than 10 mm, and NMT 2.0% of other foreign matter
- **Loss on Drying** ⟨731⟩

Sample: 1.0 g of *Salix* Species Bark, finely powdered
Analysis: Dry the *Sample* at 105° for 2 h.
Acceptance criteria: NMT 10.0%

- **Articles of Botanical Origin** ⟨561⟩, *Methods of Analysis, Total Ash*

Sample: 2.0 g of *Salix* Species Bark, finely powdered
Acceptance criteria: NMT 10.0%

- **Articles of Botanical Origin** ⟨561⟩, *Methods of Analysis, Acid-Insoluble Ash*

Sample: 2.0 g of *Salix* Species Bark, finely powdered
Acceptance criteria: NMT 3.0%

- **Articles of Botanical Origin** ⟨561⟩, *Methods of Analysis, Water-Soluble Extractives*

Sample: 2.0 g of *Salix* Species Bark, finely powdered
Acceptance criteria: NLT 10.0%

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.

Change to read:

- **Labeling:** The label states the Latin binomial(s) of one or several *Salix* species included in the article. ▲Dosage forms prepared with this article should bear the following statement: Not for use in children, women who are pregnant or nursing, or by persons with known sensitivity to aspirin.▲ (ERR 1-Feb-2019)

Change to read:

- **USP Reference Standards** ⟨11⟩

USP Salicin RS
USP Salix Species Bark Dry Extract RS

▲▲ (ERR 1-Feb-2019)

Salix Species Bark Dry Extract

DEFINITION

Salix Species Bark Dry Extract is prepared from *Salix* Species Bark by extraction with hydroalcoholic, aqueous, or other suitable solvents. It contains NLT 90.0% and NMT 110.0% of the labeled amount of salicylates, calculated as salicin ($C_{13}H_{18}O_7$) on the anhydrous basis. The ratio of starting plant material to extract is between 5:1 and 20:1. It may contain suitable added substances.

IDENTIFICATION

- **A. HPTLC for Articles of Botanical Origin** ⟨203⟩

Standard solution A: 1.50 mg/mL of USP Salicin RS in methanol

Standard solution B: 30 mg/mL of USP Salix Species Bark Dry Extract RS in methanol. Sonicate for 10 min, centrifuge, and use the supernatant.

Sample solution A: Suspend the amount of *Salix* Species Bark Dry Extract calculated to contain 15 mg of salicin (post-hydrolysis) in 10.0 mL of methanol. Sonicate for 10 min, centrifuge, and use the supernatant.

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Sample solution B: Combine 5.0 mL of *Sample solution A* with 1.0 mL of 50-mg/mL anhydrous sodium carbonate. Cap tightly and incubate at 60° for 10 min. Centrifuge and use the supernatant.

Chromatographic system

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plate)[1]

Application volume: 5.0 µL each of *Standard solution A*, *Standard solution B*, and *Sample solution A* and 6.0 µL of *Sample solution B* as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of 33%.

Temperature: Ambient, not to exceed 30°

Developing solvent system: Ethyl acetate, methanol, and water (77:13:10)

Developing distance: 6 cm

Derivatization reagent: Sulfuric acid and methanol (1:9). Slowly add sulfuric acid to ice-cold methanol.

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Sample solution A*, and *Sample solution B*

Apply the *Samples* as bands and dry in air. Develop in a saturated chamber, and dry in a current of air for 5 min. Treat with *Derivatization reagent*, heat at 100° for 5 min, and examine under white light.

System suitability: Under white light, the derivatized chromatogram of *Standard solution B* displays, in the middle third of the plate, three brown bands: the lower corresponds to the salicin band in *Standard solution A*; the one above it is due to salicortin; the top band is due to tremulacin. A faint band, which may appear between the salicortin and tremulacin bands, is due to tremuloidin. Two darker brown bands are seen in the lower third of the plate: one proximate to the application line; another, more intense, above it.

Acceptance criteria: Under white light, the derivatized chromatogram of *Sample solution A* shows one or several dark bands due to different salicin esters, whose position and intensity are contingent on the *Salix* species being used. The salicylate bands of interest are predominantly located in the middle third of the plate, demarcated by the salicin and tremulacin bands of *Standard solution B*. The salicin band in *Sample solution A*, corresponding to that in *Standard solution A*, may be faint or not visible. Additional bands may be seen in *Sample solution A* and *Sample solution B*. In *Sample solution B*, the bands due to salicin esters are not present, while the salicin band corresponding to that in *Standard solution A* is the principal band observed. The salicin band in *Sample solution A* is of lower intensity than the corresponding band in *Standard solution A*. The salicin band in *Sample solution B* is of comparable or higher intensity than the corresponding band in *Standard solution A*.

• B. HPLC

Analysis: Proceed as directed in the test for *Salicylates Profile and Limit of Free Salicin*.

Acceptance criteria: The *Sample solution* exhibits peaks at retention times corresponding to those of salicin and salicin derivatives in the *Standard solution*.

COMPOSITION

• CONTENT OF SALICIN

Diluent: Methanol and water (1:1)

Solution A: 0.01% trifluoroacetic acid

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	90	10
10	85	15
30	50	50
32	10	90
35	10	90
37	90	10
45	90	10

Standard solution: 0.50 mg/mL of USP Salicin RS in *Diluent*

Sample solution: Accurately weigh the amount of *Salix* Species Bark Dry Extract calculated to contain about 30 mg of salicin, transfer to a 200-mL round-bottom flask, and add 40 mL of methanol and 3 mL of 1 N sodium hydroxide. Attach the condenser and heat under reflux for 2 h, with intermittent shaking. Allow to cool, neutralize with 3 mL of 1 N hydrochloric acid, and pass through a paper filter into a 100-mL volumetric flask. Wash the round-bottom flask twice with 5-mL aliquots of methanol and filter into the same 100-mL volumetric flask. Adjust with water to volume, mix well, allow to equilibrate to room temperature, and readjust with water. Pass through a PTFE filter of 0.45-µm pore size, discarding the initial 3 mL of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 270 nm

Column: 4.6-mm × 25-cm; 5-µm base-deactivated packing L1

Column temperature: 30°

Flow rate: 1.0 mL/min

Injection volume: 10 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Tailing factor: 0.8–2.0 for the salicin peak

Relative standard deviation: NMT 2.0% for the salicin peak in replicate injections

Analysis

Samples: *Standard solution* and *Sample solution*

Using the chromatogram of the *Standard solution*, identify the salicin peak in the *Sample solution* chromatogram.

Calculate the percentage of salicin hydrolytically derived from constituent salicylates in the portion of *Salix* Species Bark Dry Extract taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of salicin from the *Sample solution*

r_S = peak area of salicin from the *Standard solution*

C_S = concentration of USP Salicin RS in the *Standard solution* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of *Salix* Species Bark Dry Extract taken to prepare the *Sample solution* (mg)

Calculate the percentage of the labeled content of salicin in the portion of *Salix* Species Bark Dry Extract taken:

$$\text{Result} = (P/L) \times 100$$

P = percentage of salicylates as determined above

L = labeled content of salicin

[1] A suitable commercially available plate is HPTLC Silica Gel 60 F_{254} from EMD Millipore (e.g., Part No. 1.05642.0001).

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Acceptance criteria: 90.0%–110.0% of the labeled amount of salicylates calculated as salicin on the anhydrous basis

CONTAMINANTS

Change to read:

- **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues:*▲ (CN 1-May-2020) Meets the requirements
- **BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Residual Solvents:* Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^4 cfu/g, and total combined yeasts and molds count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli:* Meets the requirements

SPECIFIC TESTS

- **SALICYLATES PROFILE AND LIMIT OF FREE SALICIN**[2]

Diluent, Mobile phase, and Chromatographic system: Proceed as directed in the test for *Content of Salicin.*

Standard solution: 5 mg/mL of USP Salix Species Bark Dry Extract RS in *Diluent.* Sonicate for 5 min, mix well, and pass through a PTFE filter of 0.45-µm pore size, discarding the initial 3 mL of the filtrate.

Sample solution: Weigh the amount of *Salix* Species Bark Dry Extract calculated to contain about 15 mg of salicin, transfer to a 50-mL volumetric flask, add 25 mL of methanol, and sonicate for 5 min. Adjust with water to volume, mix well, allow to equilibrate to room temperature, and readjust with water. Pass through a PTFE filter of 0.45-µm pore size, discarding the initial 3 mL of the filtrate.

System suitability

Suitability requirements: The chromatogram of the *Standard solution* is similar to the reference chromatogram provided with the lot of USP Salix Species Bark Dry Extract RS being used.

Analysis

Samples: *Standard solution* and *Sample solution*

Using the chromatogram of the *Standard solution* and the reference chromatogram provided with the lot of USP Salix Species Bark Dry Extract RS being used, identify salicin esters present in the *Sample solution* chromatogram. The approximate relative retention times, with respect to salicin, are provided in *Table 2.*

Table 2

Analyte	Relative Retention Time
Salicin	1.0
Salicortin	3.0
Tremuloidin	3.6
Tremulacin	4.6

Acceptance criteria: The peak area of salicin is NMT 50% of the combined peak areas of all identified constituent salicylates.

- **WATER DETERMINATION** ⟨921⟩, *Method I, Method Ia:* NMT 5.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Total Ash*

Sample: 2.0 g of *Salix* Species Bark Dry Extract

Acceptance criteria: NMT 5.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial(s) of one or several *Salix* species from which the article was prepared. The label also indicates the content of salicin, the solvent used in extract preparation, and the ratio of the starting crude plant material to dry extract. It meets the labeling requirements of *Botanical Extracts* ⟨565⟩. Dosage forms prepared with this article should bear the following statement: Not for use in children, women who are pregnant or nursing, or by persons with known sensitivity to aspirin.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Salicin RS

USP Salix Species Bark Dry Extract RS

Change to read:

Salix Species Bark Powder

▲▲ (ERR 1-Feb-2019)

DEFINITION

Salix Species Bark Powder consists of *Salix* Species Bark reduced to fine or very fine powder. It contains NLT 1.50% of total salicylate derivatives, calculated as salicin ($C_{13}H_{18}O_7$) on the dried basis. *Salix* Species Bark Powder contains NMT 1.50% of free salicin, calculated on the dried basis.

IDENTIFICATION

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 1.50 mg/mL of USP Salicin RS in methanol

Standard solution B: 30 mg/mL of USP Salix Species Bark Dry Extract RS in methanol. Sonicate for 10 min, centrifuge, and use the supernatant.

Sample solution A: Suspend 1000 mg of *Salix* Species Bark Powder in 10.0 mL of methanol. Sonicate for 10 min, centrifuge, and use the supernatant.

Sample solution B: Combine 5.0 mL of *Sample solution A* with 1.0 mL of 50-mg/mL anhydrous sodium carbonate. Cap tightly and incubate at 60° for 10 min. Centrifuge and use the supernatant.

Chromatographic system

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plate)[1]

Application volume: 5.0 µL each of *Standard solution A, Standard solution B,* and *Sample solution A* and 6.0 µL of *Sample solution B* as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of 33%.

Temperature: Ambient, not to exceed 30°

Developing solvent system: Ethyl acetate, methanol, and water (77:13:10)

Developing distance: 6 cm

Derivatization reagent: Sulfuric acid and methanol (1:9). Slowly add sulfuric acid to ice-cold methanol.

[2] Elevated free salicin content may indicate prehydrolysis or fortification with extraneous salicin.

[1] A suitable commercially available plate is HPTLC Silica Gel 60 F_{254} from EMD Millipore (e.g., Part No. 1.05642.0001).

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Analysis
Samples: *Standard solution A*, *Standard solution B*, *Sample solution A*, and *Sample solution B*
Apply the *Samples* as bands and dry in air. Develop in a saturated chamber, and dry in a current of air for 5 min. Treat with *Derivatization reagent*, heat at 100° for 5 min, and examine under white light.
System suitability: Under white light, the derivatized chromatogram of *Standard solution B* displays, in the middle third of the plate, three brown bands: the lower corresponds to the salicin band in *Standard solution A*; the one above it is due to salicortin; the top band is due to tremulacin. A faint band, which may appear between the salicortin and tremulacin bands, is due to tremuloidin. Two darker brown bands are seen in the lower third of the plate: one proximate to the application line; another, more intense, above it.
Acceptance criteria: Under white light, the derivatized chromatogram of *Sample solution A* shows one or several dark bands due to different salicin esters, whose position and intensity are contingent on the *Salix* species being used. The salicylate bands of interest are predominantly located in the middle third of the plate, demarcated by the salicin and tremulacin bands of *Standard solution B*. The salicin band in *Sample solution A*, corresponding to that in *Standard solution A*, may be faint or not visible. Additional bands may be seen in *Sample solution A* and *Sample solution B*. In *Sample solution B*, the bands due to salicin esters are not present, while the salicin band corresponding to that in *Standard solution A* is the principal band observed. The salicin band in *Sample solution A* is of lower intensity than the corresponding band in *Standard solution A*. The salicin band in *Sample solution B* is of comparable or higher intensity than the corresponding band in *Standard solution A*.

• **B. HPLC**
Analysis: Proceed as directed in the test for *Salicylates Profile and Limit of Free Salicin*.
Acceptance criteria: The *Sample solution* exhibits peaks at retention times corresponding to those of salicin and salicin derivatives in the Standard solutions.

COMPOSITION

• **CONTENT OF SALICIN**
Diluent: Methanol and water (1:1)
Solution A: 0.01% trifluoroacetic acid
Solution B: Acetonitrile
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	90	10
10	85	15
30	50	50
32	10	90
35	10	90
37	90	10
45	90	10

Standard solution A: 0.50 mg/mL of USP Salicin RS in *Diluent*
Sample solution: Accurately weigh about 1.3 g of *Salix* Species Bark Powder, transfer to a 200-mL round-bottom flask, and add 40 mL of methanol and 3 mL of 1 N sodium hydroxide. Attach the condenser and heat under reflux for 2 h, with intermittent shaking. Allow to cool, neutralize with 3 mL of 1 N hydrochloric acid, and pass through a paper filter into a 100-mL volumetric flask. Wash the round-bottom flask twice with 5-mL aliquots of methanol and filter into the same 100-mL volumetric flask. Adjust with water to volume, mix well, allow to equilibrate to room temperature, and readjust with water. Pass through a PTFE filter of 0.45-µm pore size, discarding the initial 3 mL of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 270 nm
Column: 4.6-mm × 25-cm; 5-µm base-deactivated packing L1
Column temperature: 30°
Flow rate: 1.0 mL/min
Injection volume: 10 µL
System suitability
Sample: *Standard solution A*
Suitability requirements
Tailing factor: 0.8–2.0 for the salicin peak
Relative standard deviation: NMT 2.0% for the salicin peak in replicate injections
Analysis
Samples: *Standard solution A* and *Sample solution*
Using the chromatogram of *Standard solution A*, identify the salicin peak in the *Sample solution* chromatogram.
Calculate the percentage of salicin hydrolytically derived from constituent salicylates in the portion of *Salix* Species Bark Powder taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of salicin from the *Sample solution*
r_S = peak area of salicin from *Standard solution A*
C_S = concentration of USP Salicin RS in *Standard solution A* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of *Salix* Species Bark Powder taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 1.50% of salicin on the dried basis

CONTAMINANTS

• **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Limits of Elemental Impurities*: Meets the requirements
• **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Pesticide Residue Analysis*: Meets the requirements
• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined yeasts and molds count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria count does not exceed 10^3 cfu/g.
• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures*, *Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS

• **SALICYLATES PROFILE AND LIMIT OF FREE SALICIN**[2]
Diluent, Mobile phase, Standard solution A, and Chromatographic system: Proceed as directed in the test for *Content of Salicin*.
Standard solution B: 5 mg/mL of USP Salix Species Bark Dry Extract RS in *Diluent*. Sonicate for 5 min, mix well, and pass through a PTFE filter of 0.45-µm pore size, discarding the initial 3 mL of the filtrate.

[2] Elevated free salicin content may indicate prehydrolysis or fortification with extraneous salicin.

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Sample solution: Accurately weigh about 650 mg of *Salix* Species Bark Powder, transfer to a 50-mL volumetric flask, add 25 mL of methanol, and sonicate for 30 min. Adjust with water to volume, mix well, allow to equilibrate to room temperature, and readjust with water. Pass through a PTFE filter of 0.45-µm pore size, discarding the initial 3 mL of the filtrate.

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Tailing factor: 0.8–2.0 for the salicin peak, *Standard solution A*

Relative standard deviation: NMT 2.0% determined for the salicin peak in replicate injections, *Standard solution A*

Chromatogram similarity: The chromatogram is similar to the reference chromatogram provided with the lot of USP Salix Species Bark Dry Extract RS being used, *Standard solution B.*

Analysis

Samples: *Standard solution A, Standard solution B,* and *Sample solution*

Using the chromatograms of *Standard solution A, Standard solution B,* and the reference chromatogram provided with the lot of USP Salix Species Bark Dry Extract RS being used, identify salicin esters present in the *Sample solution* chromatogram. The approximate relative retention times, with respect to salicin, are provided in *Table 2.*

Table 2

Analyte	Relative Retention Time
Salicin	1.0
Salicortin	3.0
Tremuloidin	3.6
Tremulacin	4.6

Calculate the percentage of salicin in the portion of *Salix* Species Bark Powder taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of salicin from the *Sample solution*
r_S = peak area of salicin from *Standard solution A*
C_S = concentration of USP Salicin RS in *Standard solution A* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of *Salix* Species Bark Powder taken to prepare the *Sample solution* (mg)

Acceptance criteria: NMT 1.50% of salicin on the dried basis. The peak area of salicin is NMT 50% of the combined peak areas of all identified constituent salicylates.

• **BOTANICAL CHARACTERISTICS**

Macroscopic: Pale yellow, greenish-yellow, or light brown powder

Microscopic: Bundles of narrow fibers, up to about 600 µm long, with very thick walls, lignified, and surrounded by a crystal sheath containing prism crystals of calcium oxalate; parenchyma of the cortex with thick, pitted and deeply beaded walls, and containing large cluster crystals of calcium oxalate; uniseriate medullary rays; thickened and suberized cork cells. Groups of brownish collenchyma from the bud may be present. Twigs show fragments of lignified fibers and vessels from the xylem.

• **LOSS ON DRYING** ⟨731⟩

Sample: 1.0 g of *Salix* Species Bark Powder

Analysis: Dry the *Sample* at 105° for 2 h.

Acceptance criteria: NMT 10.0%

• **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Total Ash*

Sample: 2.0 g of *Salix* Species Bark Powder

Acceptance criteria: NMT 10.0%

• **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Acid-Insoluble Ash*

Sample: 2.0 g of *Salix* Species Bark Powder

Acceptance criteria: NMT 3.0%

• **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Water-Soluble Extractives*

Sample: 2.0 g of *Salix* Species Bark Powder

Acceptance criteria: NLT 10.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.

Change to read:

• **LABELING:** The label states the Latin binomial(s) of one or several *Salix* species included in the article. ▲Dosage forms prepared with this article should bear the following statement: Not for use in children, women who are pregnant or nursing, or by persons with known sensitivity to aspirin.▲ (ERR 1-Feb-2019)

Change to read:

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Salicin RS

USP Salix Species Bark Dry Extract RS

▲▲ (ERR 1-Feb-2019)

SAMe—*see S-adenosyl-L-methionine Disulfate Tosylate*

Saw Palmetto

DEFINITION

Saw Palmetto consists of partially dried, ripe fruit of *Serenoa repens* (W. Bartram) Small (Fam. Arecaceae) [*Serenoa serrulatum* Schult.; *Sabal serrulata* (Michx.) Nutt. ex Schult. & Schult. f.]. It contains NLT 2% (v/w) of volatile oil, NLT 7% of lipophilic extract, and NLT 9.0% of total fatty acids, determined on the dried basis.

IDENTIFICATION

• **A. THIN-LAYER CHROMATOGRAPHY**

[NOTE—Perform the following test under subdued light.]

Reagent solution: 3.7 mg/mL of 4-bromomethyl-7-methoxycoumarin in acetone. Store this solution in a dark place.

Standard solution: 5.0 g of magnesium stearate in a 100-mL round-bottom flask fitted with a reflux condenser. Add 50 mL of ether, 20 mL of 12.5% nitric acid, and 20 mL of water, and heat until dissolution is complete. Cool, transfer the contents of the flask to a separatory funnel, withdraw, and retain the lower aqueous phase. Extract the ether phase twice, each time using 4 mL of water, separating the aqueous phases. Extract the combined aqueous phases with 15 mL of ether, combining the ether extracts. Evaporate to dryness, and dry the residue at 105°.

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Transfer 1 mg of the residue to an amber glass vial fitted with a metal-clamped rubber cap. Add 10 mg of lithium carbonate, 3 µL of tris-[2-(2-methoxyethoxy)ethyl]amine, and 1.0 mg of the *Reagent solution*. Seal the metal-clamped rubber cap, incubate at 105° for 2 h, and cool.

Sample solution: 10.0 g of finely powdered Saw Palmetto in a 250-mL round-bottom flask fitted with a reflux condenser. Add 150 mL of alcohol, and reflux for 1 h. Cool, filter, wash the residue with alcohol, and dilute the combined washings and filtrate with alcohol to 200.0 mL. Transfer 0.6 mL of this solution to a suitable flask, and evaporate to dryness. To the residue add 1.0 mL of the *Reagent solution*. Transfer this solution with the aid of a pipet to an amber glass vial fitted with a metal-clamped rubber cap. Add 3 µL of tris-[2-(2-methoxyethoxy)ethyl] amine and 10 mg of lithium carbonate to the vial. Seal the metal-clamped rubber cap, incubate at 105° for 2 h, and cool.

Blank solution: To 10 mg of lithium carbonate in an amber glass vial fitted with a metal-clamped rubber cap, add 3 µL of tris-[2-(2-methoxyethoxy)ethyl]amine and 1.0 mL of the *Reagent solution*. Seal the metal-clamped rubber cap, incubate at 105° for 2 h, and cool.

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture, typically 20 cm in length (TLC plates)

Application volume: 2 µL

Developing solvent system: Cyclohexane, ethyl acetate, and acetic acid (70:30:1)

Analysis

Samples: *Standard solution*, *Sample solution*, and *Blank solution*

Develop the chromatograms in the solvent system until the solvent front has moved three-fourths of the length of the plate. Remove the plate from the chromatographic chamber, mark the solvent front, and allow the plate to air-dry. Examine the plate under long-wave UV light (365 nm).

Acceptance criteria: The chromatogram of the *Sample solution* exhibits at least two blue fluorescent zones corresponding to similar zones in the *Standard solution*. The blue fluorescent zones in the *Sample solution* appear above the blue fluorescent zones in the *Blank solution*.

COMPOSITION

- **ARTICLES OF BOTANICAL ORIGIN,** *Volatile Oil Determination* ⟨561⟩: NLT 2% (v/w) of oil that solidifies to a white solid at room temperature
- **CONTENT OF LIPOPHILIC EXTRACT**

Analysis: Transfer 10 g of pulverized Saw Palmetto to a 250-mL round-bottom flask fitted with a reflux condenser, and add 150 mL of alcohol. Maintain under reflux for 1 h. Cool, filter, and wash the residue with small portions of alcohol. Combine the filtrate and washings in a 200-mL volumetric flask, and dilute with alcohol to volume. Evaporate 100.0 mL of this solution to dryness in a rotary evaporator under vacuum. Add 40 mL of *n*-hexane to the residue, stir for 5 min, filter, and collect the filtrate in a round-bottom flask. Repeat the above operation of washing with *n*-hexane two more times, and combine all of the filtrates in the same flask. Using a rotary evaporator, evaporate to dryness. Dry the residue at 105° for 2 h.

Acceptance criteria: The weight of the residue is NLT 0.35 g (NLT 7%).

- **CONTENT OF FATTY ACIDS**

Internal standard solution: 12 mg/mL of nonadecane in hexanes

Standard stock solution: Dissolve quantities of USP Methyl Laurate RS, USP Methyl Oleate RS, USP Methyl Myristate RS, USP Methyl Palmitate RS, USP Methyl Linoleate RS, USP Methyl Caproate RS, USP Methyl Caprylate RS, USP Methyl Caprate RS, USP Methyl Palmitoleate RS, USP Methyl Stearate RS, and USP Methyl Linolenate RS in hexanes to obtain concentrations of each methyl ester as given in *Table 1*.

Table 1

Methyl Ester	Concentration (mg/mL)
Methyl laurate	5
Methyl oleate	5
Methyl myristate	2
Methyl palmitate	2
Methyl linoleate	1
Methyl caproate	0.4
Methyl caprylate	0.4
Methyl caprate	0.4
Methyl palmitoleate	0.4
Methyl stearate	0.4
Methyl linolenate	0.4

Standard solution: Combine 1.0 mL of *Internal standard solution* with 5.0 mL of the *Standard stock solution*.

Sample solution: Pulverize 50 g of dried Saw Palmetto to a moderately coarse powder. Transfer 1 g of powder, accurately weighed, to a 100-mL round-bottom flask fitted with a reflux condenser and a magnetic bar. Add 10 mL of 0.5 N methanolic sodium hydroxide solution (20 mg/mL of sodium hydroxide in methanol), and reflux, while stirring, for 15 min. Add 5 mL of a 140 mg/mL solution of boron trifluoride in methanol through the condenser into the flask, and reflux for 2 more min. Add 5.0 mL of hexanes through the condenser, and reflux for an additional 1 min. Cool the flask, remove the condenser, add 15 mL of saturated sodium chloride solution, and 1.0 mL of the *Internal standard solution*. While the solution is still tepid, stopper the flask and shake vigorously. Pipet 1.0 mL of the upper hexanes layer into a glass-stoppered test tube containing a small quantity of anhydrous sodium sulfate. Filter the solution, and, if necessary, dilute a volume of the filtrate with hexanes to obtain a known volume. [NOTE—Store this solution in a refrigerator until just before use.]

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.25-mm × 30-m fused silica capillary; 0.25-µm film of phase G16 coating

Temperatures

Injector: 250°

Detector: 300°

Column: See *Table 2*.

Table 2

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
120	0	120	3
120	50	220	12

Carrier gas: Helium

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Flow rate: 1 mL/min
Injection size: 1 µL
System suitability
Sample: *Standard solution*
[NOTE—See *Table 3* for the relative retention times.]

Table 3

Methyl Ester	Relative Retention Time
Methyl caproate	0.39
Methyl caprylate	0.56
Methyl caprate	0.76
Methyl laurate	0.94
Nonadecane (internal standard)	1.0
Methyl myristate	1.1
Methyl palmitate	1.3
Methyl palmitoleate	1.35
Methyl stearate	1.65
Methyl oleate	1.7
Methyl linoleate	1.8
Methyl linolenate	2.0

Suitability requirements
Resolution: NLT 1.5 between methyl stearate and methyl oleate
Tailing factor: NMT 2.0 for each of the methyl ester peaks
Relative standard deviation: NMT 5.0% for each of the methyl ester peaks
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of each fatty acid in the portion of the Saw Palmetto taken:

$$\text{Result} = (R_U/R_S) \times (C_S \times V) \times (1/W) \times (M_{r1}/M_{r2}) \times 100$$

R_U = peak response ratio of the relevant methyl ester to the internal standard obtained from the *Sample solution*
R_S = peak response ratio of the relevant methyl ester to the internal standard obtained from the *Standard solution*
C_S = concentration of the respective methyl ester in the *Standard stock solution* (mg/mL)
V = volume of the *Standard stock solution* used to prepare the *Standard solution* (mL)
W = weight of Saw Palmetto taken to prepare the *Sample solution* (mg)
M_{r1} = molecular weight of the relevant fatty acid
M_{r2} = molecular weight of the methyl ester of the relevant fatty acid

Acceptance criteria: NLT 9.0% for the sum of the percentages of all the fatty acids, on the dried basis. See *Table 4* for individual fatty acids.

Table 4

Individual Fatty Acids	Percentages (%)
Oleic acid	NLT 3.0
Lauric acid	NLT 2.0
Myristic acid	NLT 1.2
Palmitic acid	NLT 1.0
Linoleic acid	NLT 0.4
Caproic acid	NLT 0.2
Caprylic acid	NLT 0.2
Capric acid	NLT 0.2
Stearic acid	NLT 0.1
Linolenic acid	NLT 0.05
Palmitoleic acid	NLT 0.01

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩: It meets the requirements of the test for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: Subspherical to ovoid drupes, about 2–3 cm in length and about 1.5 cm thick, dark brown to black with a smooth, dull surface and with large, irregular depressions and ridges caused by shrinkage on drying; remains of style at the summit; base bearing a small depression with the scar of the stalk; epicarp and underlying sarcocarp forming a fragile layer that partially peels off, revealing the hard, pale brown layer of endocarp surrounding the seed. Seed irregularly spherical to ovoid, up to about 1.2 cm in length and 1 cm in width, hard, surface finely pitted and reddish brown with a paler, raised and membranous area over the raphe and micropyle; when cut transversely, it shows a thin testa, a narrow perisperm, and a large area of dense, horny, grayish-white endosperm.
Microscopic: The pulp is covered by a small-celled, thin-walled epidermis and consists chiefly of a very large-celled, mostly thin-walled parenchyma. The outermost layers contain brown substances; farther inside, single cells only with brown contents are scattered in the tissue; occasional large, thick-walled, rather punctate stone cells with a wide lumen are also found. The vascular bundles are accompanied by fibers with cover cells (stigmata) containing siliceous solids attached. The innermost layers of the pulp wall consist of almost completely thickened, rather punctate, quite irregularly shaped stone cells (astrosclereids). The outer layers of the seed coat are large-celled, the cells are coarse-walled; the middle layers are thin-walled, the cells are smaller; the innermost layers are small-celled, flattened. All cells are filled with a brown substance. On the outside, the endosperm exhibits radial elongated, nonpunctate, coarse-walled cells; in the deeper layers, the cells are larger, thick-walled, rather coarsely punctate. The middle lamella is fairly recognizable. Aleurone with protein crystalloids is present in the cell contents.

- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩: NMT 2.0%

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- **LOSS ON DRYING** ⟨731⟩
  Sample: 1.0 g of Saw Palmetto, finely powdered
  Analysis: Dry the *Sample* at 105° for 2 h.
  Acceptance criteria: NMT 12.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 5.0%, determined on 1.0 g of finely powdered Saw Palmetto
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Methyl Caprate RS
  USP Methyl Caproate RS
  USP Methyl Caprylate RS
  USP Methyl Laurate RS
  USP Methyl Linoleate RS
  USP Methyl Linolenate RS
  USP Methyl Myristate RS
  USP Methyl Oleate RS
  USP Methyl Palmitate RS
  USP Methyl Palmitoleate RS
  USP Methyl Stearate RS

Powdered Saw Palmetto

DEFINITION

Powdered Saw Palmetto is Saw Palmetto reduced to a fine or a very fine powder. It contains NLT 2% (v/w) of volatile oil, NLT 7% of lipophilic extract, and NLT 9.0% of total fatty acids, determined on the dried basis.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHY**
  [NOTE—Perform the following test under subdued light.]
  Reagent solution: 3.7 mg/mL of 4-bromomethyl-7-methoxycoumarin in acetone. Store this solution in a dark place.
  Standard solution: 5.0 g of magnesium stearate in a 100-mL round-bottom flask fitted with a reflux condenser. Add 50 mL of ether, 20 mL of 12.5% nitric acid, and 20 mL of water, and heat until dissolution is complete. Cool, transfer the contents of the flask to a separatory funnel, withdraw, and retain the lower aqueous phase. Extract the ether phase twice, each time using 4 mL of water, separating the aqueous phases. Extract the combined aqueous phases with 15 mL of ether, combining the ether extracts. Evaporate to dryness, and dry the residue at 105°. Transfer 1 mg of the residue to an amber glass vial fitted with a metal-clamped rubber cap. Add 10 mg of lithium carbonate, 3 µL of tris-[2-(2-methoxyethoxy)ethyl]amine, and 1.0 mg of the *Reagent solution*. Seal the metal-clamped rubber cap, incubate at 105° for 2 h, and cool.
  Sample solution: 10.0 g of Powdered Saw Palmetto in a 250-mL round-bottom flask fitted with a reflux condenser. Add 150 mL of alcohol, and reflux for 1 h. Cool, filter, wash the residue with alcohol, and dilute the combined washings and filtrate with alcohol to 200.0 mL. Transfer 0.6 mL of this solution to a suitable flask, and evaporate to dryness. To the residue add 1.0 mL of the *Reagent solution*. Transfer this solution with the aid of a pipet to an amber glass vial fitted with a metal-clamped rubber cap. Add 3 µL of tris-[2-(2-methoxyethoxy)ethyl]amine and 10 mg of lithium carbonate to the vial. Seal the metal-clamped rubber cap, incubate at 105° for 2 h, and cool. Use the cooled solution.
  Blank solution: To 10 mg of lithium carbonate in an amber glass vial fitted with a metal-clamped rubber cap, add 3 µL of tris-[2-(2-methoxyethoxy)ethyl]amine and 1.0 mL of the *Reagent solution*. Seal the metal-clamped rubber cap, incubate at 105° for 2 h, and cool.
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
  Adsorbent: 0.25-mm layer of chromatographic silica gel mixture, typically 20 cm in length (TLC plates)
  Application volume: 2 µL
  Developing solvent system: Cyclohexane, ethyl acetate, and acetic acid (70:30:1)
  Analysis
  Samples: *Standard solution*, *Sample solution*, and *Blank solution*
  Develop the chromatograms in the solvent system until the solvent front has moved three-fourths of the length of the plate. Remove the plate from the chromatographic chamber, mark the solvent front, and allow the plate to air-dry. Examine the plate under long-wave UV light (365 nm).
  Acceptance criteria: The chromatogram of the *Sample solution* exhibits at least two blue fluorescent zones of corresponding to similar zones in the *Standard solution*. The blue fluorescent zones in the *Sample solution* appear above the blue fluorescent zones in the *Blank solution*.

COMPOSITION

- **ARTICLES OF BOTANICAL ORIGIN,** *Volatile Oil Determination* ⟨561⟩**:** NLT 2 mL/100 g of oil that solidifies to a white solid at room temperature
- **CONTENT OF LIPOPHILIC EXTRACT**
  Analysis: Transfer 10 g of Powdered Saw Palmetto to a 250-mL round-bottom flask fitted with a reflux condenser, and add 150 mL of alcohol. Maintain under reflux for 1 h. Cool, filter, and wash the residue with small portions of alcohol. Combine the filtrate and washings in a 200-mL volumetric flask, and dilute with alcohol to volume. Evaporate 100.0 mL of this solution to dryness in a rotary evaporator under vacuum. Add 40 mL of *n*-hexane to the residue, stir for 5 min, filter, and collect the filtrate in a round-bottom flask. Repeat the above operation of washing with *n*-hexane two more times, and combine all of the filtrates in the same flask. Using a rotary evaporator, evaporate to dryness. Dry the residue at 105° for 2 h.
  Acceptance criteria: The weight of the residue is NLT 0.35 g (NLT 7%).
- **CONTENT OF FATTY ACIDS**
  Internal standard solution: 12 mg/mL of nonadecane in hexanes.
  Standard stock solution: Dissolve quantities of USP Methyl Laurate RS, USP Methyl Oleate RS, USP Methyl Myristate RS, USP Methyl Palmitate RS, USP Methyl Linoleate RS, USP Methyl Caproate RS, USP Methyl Caprylate RS, USP Methyl Caprate RS, USP Methyl Palmitoleate RS, USP Methyl Stearate RS, and USP Methyl Linolenate RS in hexanes to obtain concentrations of each methyl ester as given in *Table 1*.

Table 1

Methyl Ester	Concentration (mg/mL)
Methyl laurate	5
Methyl oleate	5

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Table 1 *(continued)*

Methyl Ester	Concentration (mg/mL)
Methyl myristate	2
Methyl palmitate	2
Methyl linoleate	1
Methyl caproate	0.4
Methyl caprylate	0.4
Methyl caprate	0.4
Methyl palmitoleate	0.4
Methyl stearate	0.4
Methyl linolenate	0.4

Standard solution: Combine 1.0 mL of *Internal standard solution* with 5.0 mL of *Standard stock solution.*

Sample solution: Transfer 1 g of Powdered Saw Palmetto, accurately weighed, to a 100-mL round-bottom flask fitted with a reflux condenser and a magnetic bar. Add 10 mL of 0.5 N methanolic sodium hydroxide solution (20 mg/mL of sodium hydroxide in methanol), and reflux while stirring for 15 min. Add 5 mL of a 140 mg/mL solution of boron trifluoride in methanol through the condenser into the flask, and reflux for 2 more min. Add 5.0 mL of hexanes through the condenser, and reflux for an additional 1 min. Cool the flask, remove the condenser, add 15 mL of saturated sodium chloride solution, and 1.0 mL of the *Internal standard solution.* While the solution is still tepid, stopper the flask, and shake vigorously. Pipet 1.0 mL of the upper hexanes layer into a glass-stoppered test tube containing a small quantity of anhydrous sodium sulfate. Filter the solution, and, if necessary, dilute a volume of the filtrate with hexanes to obtain a known volume. [NOTE—Store this solution in a refrigerator until just before use.]

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability.*)

Mode: GC

Detector: Flame ionization

Column: 0.25-mm × 30-m fused silica capillary; 0.25-µm film of phase G16 coating

Temperatures

Injector: 250°

Detector: 300°

Column: See *Table 2.*

Table 2

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
120	0	120	3
120	50	220	12

Carrier gas: Helium

Flow rate: 1 mL/min

Injection size: 1 µL

System suitability

Sample: *Standard solution*

[NOTE—See *Table 3* for the relative retention times.]

Table 3

Methyl Ester	Relative Retention Time
Methyl caproate	0.39
Methyl caprylate	0.56
Methyl caprate	0.76
Methyl laurate	0.94
Nonadecane (internal standard)	1.0
Methyl myristate	1.1
Methyl palmitate	1.3
Methyl palmitoleate	1.35
Methyl stearate	1.65
Methyl oleate	1.7
Methyl linoleate	1.8
Methyl linolenate	2.0

Suitability requirements

Resolution: NLT 1.5 between methyl stearate and methyl oleate

Tailing factor: NMT 2.0 for each of the methyl ester peaks

Relative standard deviation: NMT 5.0% for each of the methyl ester peaks

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of each fatty acid in the portion of the Powdered Saw Palmetto taken:

$$\text{Result} = (R_U/R_S) \times (C_S \times V) \times (1/W) \times (M_{r1}/M_{r2}) \times 100$$

R_U = peak response ratio of the relevant methyl ester to the internal standard obtained from the *Sample solution*

R_S = peak response ratio of the relevant methyl ester to the internal standard obtained from the *Standard solution*

C_S = concentration of the respective methyl ester in the *Standard stock solution* (mg/mL)

V = volume of the *Standard stock solution* used to prepare the *Standard solution* (mL)

W = weight of Powdered Saw Palmetto taken to prepare the *Sample solution* (mg)

M_{r1} = molecular weight of the relevant fatty acid

M_{r2} = molecular weight of the methyl ester of the relevant fatty acid

Acceptance criteria: NLT 9.0% for the sum of the percentages of all the fatty acids on the dried basis. See *Table 4* for individual fatty acids.

Table 4

Individual Fatty Acids	Percentages (%)
Oleic acid	NLT 3.0
Lauric acid	NLT 2.0
Myristic acid	NLT 1.2
Palmitic acid	NLT 1.0
Linoleic acid	NLT 0.4
Caproic acid	NLT 0.2

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Table 4 *(continued)*

Individual Fatty Acids	Percentages (%)
Caprylic acid	NLT 0.2
Capric acid	NLT 0.2
Stearic acid	NLT 0.1
Linolenic acid	NLT 0.05
Palmitoleic acid	NLT 0.01

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN,** *Limits of Elemental Impurities* ⟨561⟩: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩: It meets the requirements of the test for absence of *Salmonella* species and *Escherichia coli.*

SPECIFIC TESTS

- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩: NMT 2%
- **LOSS ON DRYING** ⟨731⟩
  Sample: 1.0 g of Powdered Saw Palmetto
  Analysis: Dry the *Sample* at 105° for 2 h.
  Acceptance criteria: NMT 12.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩
  Sample: 1.0 g of Powdered Saw Palmetto
  Acceptance criteria: NMT 5.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩: NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant source from which the article was derived.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Methyl Caprate RS
  USP Methyl Caproate RS
  USP Methyl Caprylate RS
  USP Methyl Laurate RS
  USP Methyl Linoleate RS
  USP Methyl Linolenate RS
  USP Methyl Myristate RS
  USP Methyl Oleate RS
  USP Methyl Palmitate RS
  USP Methyl Palmitoleate RS
  USP Methyl Stearate RS

Saw Palmetto Extract

DEFINITION

Saw Palmetto Extract is obtained from comminuted Saw Palmetto by extraction with hydroalcoholic mixtures or solvent hexane, or by supercritical extraction with carbon dioxide. The ratio of starting crude plant material to Extract is from 8.0:1 to 14.3:1. The Extract contains NLT 80.0% of fatty acids, NLT 0.2% of sterols, and NLT 0.1% of β-sitosterol, all on the anhydrous basis. The lipophilic Extract contains NLT 0.15% and NMT 0.35% of long-chain alcohols. The hydroalcoholic Extract contains NLT 0.01% and NMT 0.15% of long-chain alcohols. It contains no added substances.

IDENTIFICATION

- **A. GAS CHROMATOGRAPHY**
  Analysis: Proceed as directed in *Content of Fatty Acids.*
  Acceptance criteria: The retention times of the 11 major peaks of the *Sample solution* correspond to those in the chromatogram of the *Standard solution.* The ranges for ratios of the concentration of lauric acid to the concentration of the respective fatty acid in Extract prepared using hydroalcoholic mixtures or solvent hexane are in *Table 1.* The ranges for ratios of the concentration of lauric acid to the concentration of the respective fatty acid in Extract prepared by supercritical extraction with carbon dioxide are in *Table 2.*

Table 1

Fatty Acid	Minimum Ratio	Maximum Ratio
Capric	9.0	16
Caproic	8.5	24
Caprylic	8.5	17.5
Linoleic	5.0	16
Linolenic	31.5	55
Myristic	2.2	2.8
Oleic	0.60	1.15
Palmitic	2.8	3.9
Stearic	14	26

Table 2

Fatty Acid	Minimum Ratio	Maximum Ratio
Capric	9.0	16
Caproic	9.0	40
Caprylic	8.5	17.5
Linoleic	4.0	8.0
Linolenic	35	60
Myristic	2.2	2.8
Oleic	0.60	1.15
Palmitic	2.8	3.9
Stearic	13	20

COMPOSITION

- **CONTENT OF FATTY ACIDS**
  Internal standard solution: 12 mg/mL of nonadecane in hexanes
  Standard stock solution: Dissolve quantities of USP Methyl Laurate RS, USP Methyl Oleate RS, USP Methyl Myristate RS, USP Methyl Palmitate RS, USP Methyl Linoleate RS, USP Methyl Caproate RS, USP Methyl Caprylate RS, USP Methyl Caprate RS, USP Methyl Palmitoleate RS, USP Methyl Stearate RS, and USP Methyl Linolenate RS in hexanes to obtain concentrations of each methyl ester as given in *Table 3.*

Table 3

Methyl Ester	Concentration (mg/mL)
Methyl laurate	5

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Table 3 *(continued)*

Methyl Ester	Concentration (mg/mL)
Methyl oleate	5
Methyl myristate	2
Methyl palmitate	2
Methyl linoleate	1
Methyl caproate	0.4
Methyl caprylate	0.4
Methyl caprate	0.4
Methyl palmitoleate	0.4
Methyl stearate	0.4
Methyl linolenate	0.4

Standard solution: Transfer 1.0 mL of the *Internal standard solution* to 5.0 mL of the *Standard stock solution*.
Sample solution: Transfer 100 mg of Extract to a pressure-proof, screw-capped vial, and add 3.0 mL of a solution of sulfuric acid in methanol (5 in 100). Heat at 100° in an oil bath for 2 h, shaking from time to time. Allow to cool, and add 1.0 mL of the *Internal standard solution*, 10.0 mL of water, 1 g of sodium chloride, and 5 mL of hexanes. Shake well, allow the layers to separate completely, and use the hexanes layer. [NOTE—Store in a refrigerator until ready to use.]
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.25-mm × 30-m fused silica capillary; 0.25-µm film of phase G16 coating
Temperatures
Injector: 250°
Detector: 300°
Column: See *Table 4*.

Table 4

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
120	0	120	3
120	50	220	12

Carrier gas: Helium
Flow rate: 1 mL/min
Injection volume: 1 µL
System suitability
Sample: *Standard solution*
[NOTE—See *Table 5* for the relative retention times.]

Table 5

Methyl Ester	Relative Retention Time
Methyl caproate	0.39
Methyl caprylate	0.56
Methyl caprate	0.76
Methyl laurate	0.94
Nonadecane (internal standard)	1.0

Table 5 *(continued)*

Methyl Ester	Relative Retention Time
Methyl myristate	1.1
Methyl palmitate	1.3
Methyl palmitoleate	1.35
Methyl stearate	1.65
Methyl oleate	1.7
Methyl linoleate	1.8
Methyl linolenate	2.0

Suitability requirements
Resolution: NLT 1.5 between the methyl stearate and methyl oleate peaks
Tailing factor: NMT 2.0 for each of the methyl ester peaks
Relative standard deviation: NMT 5.0% for each of the methyl ester peaks
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of each fatty acid in the portion of Extract taken:

$$\text{Result} = (R_U/R_S) \times (C_S \times V) \times (1/W) \times (M_{r1}/M_{r2}) \times 100$$

R_U = peak response ratio of the relevant methyl ester to the internal standard from the *Sample solution*
R_S = peak response ratio of the relevant methyl ester to the internal standard from the *Standard solution*
C_S = concentration of the respective methyl ester in the *Standard stock solution* (mg/mL)
V = volume of the *Standard stock solution* used to prepare the *Standard solution* (mL)
W = weight of Extract taken to prepare the *Sample solution* (mg)
M_{r1} = molecular weight of the relevant fatty acid
M_{r2} = molecular weight of the methyl ester of the relevant fatty acid

Acceptance criteria: NLT 80.0% for the sum of the percentages of all the fatty acids, on the anhydrous basis

Change to read:

- **CONTENT OF LONG-CHAIN ALCOHOLS AND STEROLS**

Derivatizing solution A: Bis(trimethylsilyl)acetamide, trimethylsilylimidazole, and trimethylchlorosilane (3:3:2)
Derivatizing solution B: *Derivatizing solution A*, bis(trimethylsilyl)trifluoroacetamide, and pyridine (1:1:1)
Internal standard solution: 10 mg/mL of eicosanol and 5 mg/mL of cholesterol in chloroform
System suitability stock solution A: 2 mg/mL each of tetracosanol, octacosanol, USP Hexacosanol RS, and triacontanol in chloroform
System suitability solution A: Mix 5.0 mL of *System suitability stock solution A* with 1.0 mL of the *Internal standard solution*. Evaporate 0.75 mL of this solution to dryness using a stream of nitrogen. Dissolve the residue in 1.0 mL of *Derivatizing solution B*, and allow to stand for NLT 15 min at room temperature.
System suitability stock solution B: ▲0.37 mg/mL of stigmastanol and 2 mg/mL each of campesterol, stigmasterol, and USP β-Sitosterol RS in chloroform▲ (ERR 1-Jul-2019)

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System suitability solution B: Mix 5.0 mL of *System suitability stock solution B* with 1.0 mL of the *Internal standard solution*. Evaporate 0.75 mL of this solution to dryness using a stream of nitrogen. Dissolve the residue in 1.0 mL of *Derivatizing solution B*, and allow to stand for NLT 15 min at room temperature.

Standard stock solution: 0.75 mg/mL of USP Hexacosanol RS and 1.4 mg/mL of USP β-Sitosterol RS in chloroform

Standard solution: Mix 5.0 mL of the *Standard stock solution* with 1.0 mL of the *Internal standard solution*. Evaporate 0.75 mL of this solution to dryness using a stream of nitrogen. Dissolve the residue in 1.0 mL of *Derivatizing solution B*, and allow to stand for NLT 15 min at room temperature.

Sample solution: Transfer 3.35 g of Extract into a 50-mL round-bottomed flask. Add 1.0 mL of the *Internal standard solution*, and evaporate under vacuum at a temperature not exceeding 50°. Add 20 mL of a solution prepared by dissolving 130 g of potassium hydroxide in 200 mL of water in a 1000-mL volumetric flask, and dilute with methanol to volume. Attach a condenser, and reflux in a bath at 100° for 2 h. Quantitatively transfer this solution to a 25-mL volumetric flask, and dilute with water to volume. Transfer a 3-mL portion to a cartridge[1] containing diatomaceous earth capable of holding 3 mL of aqueous phase. Absorb the solution into the column under vacuum for 20 min until the column is not cold. Elute the analytes from the column with 90 mL of methylene chloride, and evaporate the eluate to dryness. Dissolve the residue in 1.0 mL of *Derivatizing solution B*, and allow to stand for NLT 15 min at room temperature.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.2-mm × 25-m capillary; 0.33-µm thickness of phase G1 coating

Temperatures

Injector: 325°

Detector: 325°

Column: See *Table 6*.

Table 6

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
200	0	200	3
200	10	300	35

Carrier gas: Helium

Flow rate: 0.5 mL/min

Make up gas flow: 25 mL/min

Injection volume: 1 µL

Injection type: Split ratio, 1:40

System suitability

Samples: *System suitability solution A* and *System suitability solution B*

[NOTE—The relative retention times for tetracosanol, hexacosanol, octacosanol, and triacontanol are 0.89, 1.00, 1.15, and 1.36, respectively, *System suitability solution A*. The relative retention times for cholesterol, campesterol, stigmasterol, β-sitosterol, and stigmastanol are 0.85, 0.92, 0.95, 1.00, and 1.01, respectively, *System suitability solution B*.]

[1] A suitable cartridge is Extrelut NT3, or an equivalent cartridge.

Suitability requirements

Resolution: NLT 2 between the β-sistosterol and stigmastanol peaks, *System suitability solution B*

Column efficiency: NLT 200,000 theoretical plates for the eicosanol peak, *System suitability solution A*; and NLT 150,000 theoretical plates for the cholesterol peak, *System suitability solution B*

Tailing factor: NMT 2.0 for each relevant peak, *System suitability solution A*; and NMT 2.0 for each relevant peak, *System suitability solution B*

Analysis

Samples: *Standard solution* and *Sample solution*

Identify the signals corresponding to the relevant analytes by comparing the chromatograms obtained with *System suitability solution A* and *System suitability solution B*.

Separately calculate the percentages of tetracosanol, hexacosanol, octacosanol, and triacontanol, respectively, in the portion of Extract taken:

$$\text{Result} = (R_U/R_S) \times (C_S \times V) \times (1/W) \times 100$$

R_U = peak response ratio of the relevant long-chain alcohol to the internal standard from the *Sample solution*

R_S = peak response ratio of hexacosanol to the internal standard from the *Standard solution*

C_S = concentration of hexacosanol in the *Standard stock solution* (mg/mL)

V = volume of the *Standard stock solution* used to prepare the *Standard solution* (mL)

W = weight of the Extract taken to prepare the *Sample solution* (mg)

Calculate the total content of long-chain alcohols as a percentage by adding the individual percentages.

Separately calculate the percentages of campesterol, stigmasterol, β-sitosterol, and stigmastanol, respectively, in the portion of Extract taken:

$$\text{Result} = (R_U/R_S) \times (C_S \times V) \times (1/W) \times 100$$

R_U = peak response ratio of the relevant sterol to the internal standard from the *Sample solution*

R_S = peak response ratio of β-sitosterol to the internal standard from the *Standard solution*

C_S = concentration of β-sitosterol in the *Standard stock solution* (mg/mL)

V = volume of the *Standard stock solution* used to prepare the *Standard solution* (mL)

W = weight of the Extract taken to prepare the *Sample solution* (mg)

Calculate the total content of sterols as a percentage by adding the individual percentages.

Acceptance criteria: NLT 0.2% for the sum of the percentages of all the sterols and NLT 0.1% of β-sitosterol, both on the anhydrous basis. The lipophilic Extract contains 0.15%–0.35% of long-chain alcohols, and the hydroalcoholic Extract contains 0.01%–0.15% of long-chain alcohols on the anhydrous basis.

CONTAMINANTS

- **BOTANICAL EXTRACTS,** *Pesticide Residues* ⟨565⟩**:** Meets the requirements

SPECIFIC TESTS

- **ALCOHOL DETERMINATION,** *Method II* ⟨611⟩ (if present)**:** NMT 1%
- **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩**:** 40–50
- **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩**:** 210–250

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- **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 3% is found in the hydroalcoholic Extract.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Meets the requirements in *Botanical Extracts* ⟨565⟩, *Packaging and Storage*
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. The label also indicates the content of fatty acids and sterols and the ratio of the starting crude plant material to Extract. It meets the requirements in *Botanical Extracts* ⟨565⟩, *Labeling*.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Hexacosanol RS
  USP Methyl Caprate RS
  USP Methyl Caproate RS
  USP Methyl Caprylate RS
  USP Methyl Laurate RS
  USP Methyl Linoleate RS
  USP Methyl Linolenate RS
  USP Methyl Myristate RS
  USP Methyl Oleate RS
  USP Methyl Palmitate RS
  USP Methyl Palmitoleate RS
  USP Methyl Stearate RS
  USP β-Sitosterol RS

Saw Palmetto Capsules

DEFINITION

Saw Palmetto Capsules contain Saw Palmetto Extract. Capsules contain NLT 22.0% and NMT 34.0% of lauric acid in the labeled amount of Saw Palmetto Extract. The ratio of the concentrations of lauric acid to caprylic acid is NLT 8.5 and NMT 17.5. The ratio of the concentrations of lauric acid to myristic acid is NLT 2.2 and NMT 2.8.

IDENTIFICATION

- **A.** The retention times of the peaks for methyl caprate, methyl caproate, methyl caprylate, methyl laurate, methyl linoleate, methyl linolenate, methyl myristate, methyl oleate, methyl palmitate, methyl palmitoleate, and methyl stearate of the *Sample solution* correspond to those of the *Standard solution*, as obtained in the test for *Content of Lauric Acid and the Ratios of the Concentrations of Lauric Acid to Caprylic Acid and Lauric Acid to Myristic Acid*.

Change to read:

- **B. PRESENCE OF STEROLS**

Derivatizing stock solution: *N,O*-bis(trimethylsilyl)-acetamide, trimethylsilylimidazole, and trimethylchlorosilane (3:3:2)

Derivatizing solution: *Derivatizing stock solution*, bis(trimethylsilyl)trifluoroacetamide, and pyridine (1:1:1)

Internal standard solution: 10 mg/mL of eicosanol and 5 mg/mL of cholesterol in chloroform

Standard stock solution: 0.75 mg/mL of USP Hexacosanol RS and 1.4 mg/mL of USP β-Sitosterol RS in chloroform

Standard solution: Mix 5.0 mL of *Standard stock solution* with 1.0 mL of the *Internal standard solution*. Evaporate 0.75 mL of this solution to dryness using a stream of nitrogen. Dissolve the residue in 1.0 mL of *Derivatizing solution*, and allow to stand for NLT 15 min at room temperature.

System suitability stock solution A: 2 mg/mL each of tetracosanol, octacosanol, USP Hexacosanol RS, and triacontanol in chloroform

System suitability solution A: Mix 5.0 mL of *System suitability stock solution A* with 1.0 mL of *Internal standard solution*. Evaporate 0.75 mL of this solution to dryness using a stream of nitrogen. Dissolve the residue in 1.0 mL of *Derivatizing solution*, and allow to stand for NLT 15 min at room temperature.

System suitability stock solution B: ▲0.37 mg/mL of stigmastanol and 2 mg/mL each of campesterol, stigmasterol, and USP β-Sitosterol RS in chloroform▲ (ERR 1-Jul-2019)

System suitability solution B: Mix 5.0 mL of *System suitability stock solution B* with 1.0 mL of *Internal standard solution*. Evaporate 0.75 mL of this solution to dryness using a stream of nitrogen. Dissolve the residue in 1.0 mL of *Derivatizing solution*, and allow to stand for NLT 15 min at room temperature.

β-Cholestanol solution: β-cholestanol in chloroform (1 in 100)

Sample solution

Sample: A number of Capsules, equivalent to 10 g of Saw Palmetto Extract

Open the Capsules, and transfer the shells and contents to a suitable container.

Transfer 5 g of the *Sample* to a 250-mL round-bottom flask, and evaporate in vacuum at a temperature of NMT 50°. Add 50 mL of a solution prepared by dissolving 130 mg/mL of potassium hydroxide in methanol and water (4:1). Attach a condenser, and reflux in a bath at 100° until a clear solution is obtained. Reflux for an additional 10 min, and cool by adding 50 mL of water through the condenser. Transfer to a separation funnel, rinsing the flask with a total of 50 mL of water in small portions. Extract with 80 mL of ether, shaking for 30 s, and repeat twice. [NOTE—If an emulsion forms, it can be eliminated by adding small quantities of methanol.] Transfer the combined ether layers to a separation funnel, and wash with successive portions of 50 mL of water until a neutral washing is obtained. [NOTE—If an emulsion forms, it can be eliminated by adding small quantities of methanol.] Pass the ether extract through filter paper containing anhydrous sodium sulfate, wash the filter with 30 mL of ether, and evaporate to dryness in vacuum. Dissolve the residue in 2.0 mL of chloroform. Extract the sterols using the following chromatographic system.

Chromatographic extraction system

Mode: TLC

Absorbent: Chromatographic plate coated with 0.25-mm silica gel having an application zone that was previously dipped under 3 cm of a solution prepared by dissolving 13 mg/mL of potassium hydroxide in methanol and water (49:1)

Developing solvent system: Hexanes and ether (7:3)

Application volumes: 200 µL of chloroform solution containing Capsule residue and 20 µL of *β-Cholestanol solution*

After the spots have been applied, allow the plate to dry, and heat it to 100° for 1 h before use. The plate can be stored in a desiccator containing calcium chloride until the time of use. Develop the plates until the solvent front has moved 17–19 cm. Keep the chamber temperature between 15° and 20°. Dry the plate in a current of warm air, then spray with an alkaline solution of 2,7-dichlorofluorescein in alcohol (0.2 in 100). Observe the plate under 366-nm wavelength light, and identify the bands corresponding to the sterols by

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referring to the β-cholestanol spot. Scrape off these bands and transfer them to a test tube. Add 10 mL of warm chloroform, and shake for 2 min with the aid of several glass beads. Filter the chloroform solution, wash the filter with chloroform, and evaporate the combined filtrate and washings to dryness in vacuum. Dissolve the residue with some drops of anhydrous acetone, and evaporate in vacuum. Dry the residue in an oven at 105° for 15 min. Dissolve the residue in 0.2 mL of *Derivatizing solution*. Use this resulting solution as the *Sample solution* for GC analysis.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.2-mm × 25-m capillary, coated with a 0.33-µm thickness of phase G1
Temperature
Detector: 325°
Injector: 325°
Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
200	0	200	3
200	10	300	35

Carrier gas: Helium
Flow rate: 0.5 mL/min
Make up gas flow: 25 mL/min
Split ratio: 1:40
Injection size: 1 µL
Injection type: Split injection system

System suitability
Samples: *System suitability solution A* and *System suitability solution B*
[NOTE—The relative retention times for tetracosanol, octacosanol, hexacosanol, and triacontanol are 0.89, 1.00, 1.15, and 1.36, respectively, *System suitability solution A*; and the relative retention times for cholesterol, campesterol, stigmasterol, β-sitosterol, and stigmastanol are 0.85, 0.92, 0.95, 1.00, and 1.01, respectively, *System suitability solution B*.]
Suitability requirements
Resolution: NLT 2 between β-sistosterol and stigmastanol, *System suitability solution B*
Column efficiency: NLT 200,000 theoretical plates for the eicosanol peak, *System suitability solution A*; and NLT 150,000 theoretical plates for the cholesterol peak, *System suitability solution B*
Tailing factor: NMT 2.0 for each relevant peak, *System suitability solution A*; and NMT 2.0 for each relevant peak, *System suitability solution B*

Analysis
Samples: *Standard solution* and *Sample solution*
Identify the signals corresponding to the relevant analytes by comparison with the chromatograms obtained with *System suitability solutions A* and *B*.

Acceptance criteria: The *Sample solution* exhibits peaks for campesterol, β-sitosterol, and stigmasterol, identified by their retention times relative to the β-sitosterol peak in the *Standard solution*.

STRENGTH

• **CONTENT OF LAURIC ACID AND THE RATIOS OF THE CONCENTRATIONS OF LAURIC ACID TO CAPRYLIC ACID AND LAURIC ACID TO MYRISTIC ACID**

Internal standard solution: 12 mg/mL of nonadecane in hexanes
Standard stock solution: Dissolve quantities of USP Methyl Laurate RS, USP Methyl Oleate RS, USP Methyl Myristate RS, USP Methyl Palmitate RS, USP Methyl Linoleate RS, USP Methyl Caproate RS, USP Methyl Caprylate RS, USP Methyl Caprate RS, USP Methyl Palmitoleate RS, USP Methyl Stearate RS, and USP Methyl Linolenate RS in hexanes to obtain concentration of each methyl ester as given in the table below.

Methyl Ester	Concentration (mg/mL)
Methyl laurate	5
Methyl oleate	5
Methyl myristate	2
Methyl palmitate	2
Methyl linoleate	1
Methyl caproate	0.4
Methyl caprylate	0.4
Methyl caprate	0.4
Methyl palmitoleate	0.4
Methyl stearate	0.4
Methyl linolenate	0.4

Standard solution: Add 1.0 mL of *Internal standard solution* to 5.0 mL of the *Standard stock solution*.
Sample solution: Take a number of Capsules, equivalent to 10 g of Extract, open the Capsules, and transfer the shells and contents to a suitable container. Transfer 100 mg to a pressure-proof screw-capped vial, and add 3.0 mL of a solution of sulfuric acid in methanol (5 in 100). Heat in an oil bath at 100° for 2 h, shaking from time to time. Allow to cool, and add 1.0 mL of *Internal standard solution*, 10.0 mL of water, 1 g of sodium chloride, and 5 mL of hexanes. Shake well, and allow the layers to separate completely. Use the hexanes layer. [NOTE—Store this solution in a refrigerator until use.]
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitabiity*.)
Mode: GC
Detector: Flame ionization
Column: 0.25-mm × 30-m fused silica capillary, coated with a 0.25-µm film of phase G16
Temperature
Detector: 300°
Injector: 250°
Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
120	0	120	3
120	50	220	12

Carrier gas: Helium
Flow rate: 1 mL/min
Injection size: 1 µL

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System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for methyl caproate, methyl caprylate, methyl caprate, methyl laurate, nonadecane (internal standard), methyl myristate, methyl palmitate, methyl palmitoleate, methyl stearate, methyl oleate, methyl linoleate, and methyl linolenate are about 0.39, 0.56, 0.76, 0.94, 1.0, 1.1, 1.3, 1.35, 1.65, 1.7, 1.8, and 2.0, respectively.]
Suitability requirements
Resolution: NLT 1.5 between the methyl stearate and methyl oleate peaks
Tailing factor: NMT 2.0 for each of the methyl ester peaks
Relative standard deviation: NMT 5.0% for each of the methyl ester peaks
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentages of lauric acid, myristic acid, and caprylic acid in the labeled amount of Saw Palmetto Extract in the portion of Capsules taken:

$$\text{Result} = (R_U/R_S) \times (C_S \times V/W) \times (M_{r1}/M_{r2}) \times A_W/L_E \times 100$$

R_U = ratio of the response of the relevant methyl ester peak and the internal standard peak from the *Sample solution*
R_S = ratio of the response of the relevant methyl ester peak and the internal standard peak from the *Standard solution*
C_S = concentration of the respective methyl ester in the *Standard stock solution* (mg/mL)
V = volume of the *Standard stock solution* taken to prepare the *Standard solution* (5.0 mL)
W = weight of sample used to prepare the *Sample solution* (mg)
M_{r1} = molecular weight of the relevant fatty acid
M_{r2} = molecular weight of the methyl ester of the relevant fatty acid
A_W = average weight of the Capsule contents (mg/Capsule)
L_E = labeled amount of Saw Palmetto Extract per Capsule (mg/Capsule)

Using these percentages, calculate the individual ratios of the concentration of lauric acid to caprylic acid and of lauric acid to myristic acid in the portion of Capsules taken.
Acceptance criteria: 22.0%–34.0% of lauric acid in the labeled amount of Saw Palmetto Extract. The ratio of lauric acid to caprylic acid is 8.5–17.5. The ratio of lauric acid to myristic acid is 2.2–2.8.

PERFORMANCE TESTS
- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** ⟨2040⟩**:** Meet the requirements for *Rupture Test for Soft Shell Capsules*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩**:** Meet the requirements

CONTAMINANTS
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total bacterial count does not exceed 10^4 cfu/g, the total combined molds and yeasts count does not exceed 1000 cfu/g, the coliform count does not exceed 100 cfu/g, and the count for enterobacteria does not exceed 100 cfu/g.
- **MICROBIAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Capsules meet the requirements of the tests for absence of *Salmonella* species, *Escherichia coli*, and *Staphylococcus aureus*.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** The label states the Latin binomial and, following the official name, the name of article from which the Capsules were prepared. Label it to indicate the amount of Extract in mg/Capsule.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Hexacosanol RS
USP Methyl Caprate RS
USP Methyl Caproate RS
USP Methyl Caprylate RS
USP Methyl Laurate RS
USP Methyl Linoleate RS
USP Methyl Linolenate RS
USP Methyl Myristate RS
USP Methyl Oleate RS
USP Methyl Palmitate RS
USP Methyl Palmitoleate RS
USP Methyl Stearate RS
USP β-Sitosterol RS

Northern Schisandra Fruit

DEFINITION
Northern Schisandra Fruit consists of the dried ripe fruits of *Schisandra chinensis* (Turcz.) Baill. (Fam. Schisandraceae) collected in the fall. It contains NLT 0.40% of schisandrin (schisandrol A) on the dried basis; NLT 0.95% of lignans, calculated as the sum of schisandrin, schisandrol B, deoxyschisandrin (schisandrin A), and γ-schisandrin (schisandrin B) on the dried basis.

IDENTIFICATION
- **A. BOTANIC CHARACTERISTICS:** Meets the requirements in *Specific Tests* for *Botanic Characteristics*
- **B. THIN-LAYER CHROMATOGRAPHY**
Standard solution A: 1.0 mg/mL of USP Schisandrin RS in ethanol
Standard solution B: Sonicate 50 mg/mL of USP *Schisandra chinensis* Fruit Dry Extract RS in alcohol for 10 min. Centrifuge, and use the supernatant.
Sample solution: Sonicate 2.5 g of Northern Schisandra Fruit, finely powdered, in 10 mL of alcohol for 10 min. Centrifuge, and use the supernatant.
Chromatographic system
(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)
Application volume: 3 µL, as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
Developing solvent system: Toluene, ethyl acetate, and glacial acetic acid (23:6:1)
Developing distance: 6 cm
Derivatization reagent: 10% Sulfuric acid in ethanol. [NOTE—Slowly add sulfuric acid to ice-cold ethanol.]
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Apply the *Samples* as bands to a suitable HPTLC plate, and dry in air. Develop the chromatograms in a saturated chamber, remove the plate from the chamber, dry, and examine under UV light at 254 nm. Then treat the plate

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with *Derivatization reagent*, heat at 120° for 7 min, and examine under UV light at 366 nm.

System suitability: Under UV light at 254 nm, the chromatogram of *Standard solution B* exhibits an intense band corresponding in R_F to the band due to schisandrin in the chromatogram of *Standard solution A*. *Standard solution B* also exhibits a band due to deoxyschisandrin in the middle of the chromatogram, four or five bands between the positions of the bands of schisandrin and deoxyschisandrin. In the upper-half section, *Standard solution B* exhibits an intense band corresponding to γ-schisandrin.

Acceptance criteria: Under UV light at 254 nm, the *Sample solution* chromatogram exhibits an intense band at an R_F corresponding to the band due to schisandrin in the chromatogram of *Standard solution A*. The *Sample solution* exhibits additional bands corresponding to similar bands in the chromatogram of *Standard solution B*. These include one or two bands below the position of schisandrin; a band due to deoxyschisandrin in the middle of the chromatogram; four or five bands between the positions of the bands of schisandrin and deoxyschisandrin; two or three bands in the upper-half section, the most intense band at an R_F corresponding to the band of γ-schisandrin. Under UV light at 366 nm after derivatization, the chromatogram of the *Sample solution* does not exhibit an intense blue fluorescent band in the upper-third of the chromatogram.

• **C. HPLC**

Analysis: Proceed as directed in *Content of Lignans*.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits the most intense peak with a retention time corresponding to schisandrin in *Standard solution A*, and the peaks due to schisandrol B, deoxyschisandrin, and γ-schisandrin corresponding to the retention times for the same lignans in *Standard solution B*. There is no principal peak due to deoxyschisandrin at a relative retention time of about 2.1 relative to schisandrin (distinction from Southern *Schisandra* fruit).

COMPOSITION

• **CONTENT OF LIGNANS**

Solution A: Water

Solution B: Acetonitrile and methanol (1:1) (v/v)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	47	53
30	20	80

Standard solution A: 0.06 mg/mL of USP Schisandrin RS in methanol

Standard solution B: 10 mg/mL of USP *Schisandra chinensis* Fruit Dry Extract RS in methanol. Before injection, pass through a polytetrafluoroethylene filter of 0.2-µm pore size, and discard the first portion of the filtrate.

Sample solution: Transfer about 250 mg of Northern Schisandra Fruit, moderately powdered and accurately weighed, to a 50-mL centrifuge tube. Add 10.0 mL of methanol, and sonicate for 10 min (140 W, 42 kHz). Centrifuge, and transfer this solution to a 25-mL volumetric flask. Repeat this extraction one more time. Combine the extracts in the 25-mL volumetric flask. Adjust with methanol to volume, and mix. Before injection, pass through a polytetrafluoroethylene filter of 0.2-µm pore size, and discard the first portion of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: HPLC

Detector: UV 251 nm

Column: 2.1-mm × 15-cm; 1.8-µm packing L1

Column temperature: 35°

Flow rate: 0.3 mL/min

Injection volume: 3 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

[NOTE—The relative retention times of the peaks for schisandrin, schisandrol B, deoxyschisandrin, and γ-schisandrin are 1.00, 1.29, 2.90, and 3.31, respectively.]

Suitability requirements

Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP *Schisandra chinensis* Fruit Dry Extract RS being used.

Tailing factor: NMT 2.0 for the schisandrin peak, *Standard solution A*

Relative standard deviation: NMT 2.0%, determined from the schisandrin peak in repeated injections, *Standard solution A*

Resolution: NLT 1.5 between the schisandrol B peak and its following peak, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatogram of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP *Schisandra chinensis* Fruit Dry Extract RS being used, identify the retention times of the peaks corresponding to different lignans in the *Sample solution* chromatogram.

Calculate the percentages of schisandrin, schisandrol B, deoxyschisandrin, and γ-schisandrin in the portion of Northern Schisandra Fruit taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*

r_S = peak area of schisandrin from *Standard solution A*

C_S = concentration of USP Schisandrin RS in *Standard solution A* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Northern Schisandra Fruit taken to prepare the *Sample solution* (mg)

F = conversion factor for analytes (1.00 for schisandrin, 1.21 for schisandrol B, 1.00 for deoxyschisandrin, and 1.23 for γ-schisandrin)

Calculate the content of lignans as the sum of the percentages of schisandrin, schisandrol B, deoxyschisandrin, and γ-schisandrin.

Acceptance criteria

Schisandrin: NLT 0.40% on the dried basis

Lignans: NLT 0.95% on the dried basis

CONTAMINANTS

• **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩

Acceptance criteria

Arsenic: NMT 2.0 µg/g

Cadmium: NMT 0.3 µg/g

Lead: NMT 5.0 µg/g

Mercury: NMT 0.2 µg/g

• **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements

Supplements

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- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩: Meets the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli*
- **ARTICLES OF BOTANICAL ORIGIN,** *Test for Aflatoxins* ⟨561⟩: Meets the requirements

SPECIFIC TESTS

- **BOTANIC CHARACTERISTICS**

Macroscopic: Irregularly spheroidal or compressed-spheroidal, 5–8 mm in diameter; externally red, purplish-red or dull red, shrunken, oily, with soft pulp, sometimes externally blackish-red or covered with "white frost". One or two seeds: reniform, externally brownish-yellow, shiny; testa, thin and fragile. Pulp: odor, slight; taste, sour. Seeds: odor, aromatic on crushing; taste, pungent and slightly bitter.

Microscopic

Transverse section: Epidermal cells of pericarp: polygonal in surface view, oil cells presented scattered; mesocarp consisting of 10 or more layers of parenchymatous cells containing starch granules, small collateral vascular bundles present and scattered; endocarp consisting of one layer of square parenchymatous cells; most outer layer of testa consisting of radially elongated stone cells, thick-walled, with fine and close pit canals; beneath showing several layers of stone cells, subrounded, triangular or polygonal with larger pits; a few layers of parenchymatous cells, inner layer of testa consisting of small, slightly thick-wall cells; endosperm cells containing oil droplets and aleurone grains. Raphe has vascular bundles; oil cell layer consists of one layer of rectangular oil cells containing yellowish-brown oil, with 3–5 layers of small cells lying below.

- **LOSS ON DRYING** ⟨731⟩

Sample: 2 g of Northern Schisandra Fruit, finely powdered
Analysis: Dry the *Sample* at 105° for 5 h.
Acceptance criteria: NMT 16%

- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩

Analysis: 2 g of Northern Schisandra Fruit, finely powdered
Acceptance criteria: NMT 7%

- **ARTICLES OF BOTANICAL ORIGIN,** *Water-Soluble Extractives* ⟨561⟩

Analysis: Cold extraction method
Acceptance criteria: NMT 35%

- **ARTICLES OF BOTANICAL ORIGIN,** *Alcohol-Soluble Extractives* ⟨561⟩

Analysis: Cold extraction method
Acceptance criteria: NMT 40%

- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩: NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial following the official name.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP *Schisandra chinensis* Fruit Dry Extract RS
USP Schisandrin RS

Northern Schisandra Fruit Dry Extract

DEFINITION

Northern Schisandra Fruit Dry Extract is prepared from the dried ripe fruits of *Schisandra chinensis* (Turcz.) Baill. (Fam. Schisandraceae) collected in the fall, by extraction with hydroalcoholic mixtures. It contains NLT 90.0% and NMT 110.0% of the labeled amount of schisandrin on the dried basis; NLT 90.0% and NMT 110.0% of the labeled amount of total lignans, calculated as the sum of schisandrin, schisandrol B, schisandrin A (deoxyschisandrin), and schisandrin B (γ-schisandrin) on the dried basis.

IDENTIFICATION

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 0.5 mg/mL of USP Schisandrin RS in methanol
Standard solution B: Sonicate 100 mg/mL of USP *Schisandra chinensis* Fruit Dry Extract RS in methanol for 10 min. Centrifuge and use the supernatant.
Sample solution: Sonicate about 250 mg (adjust the amount properly, if necessary) of Northern Schisandra Fruit Dry Extract in 5 mL of methanol for 10 min. Centrifuge, and use the supernatant.
Chromatographic system
Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)
Application volume: 3 µL, as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
Developing solvent system: Toluene, ethyl acetate, and glacial acetic acid (23:6:1)
Developing distance: 6 cm
Derivatization reagent: 10% Sulfuric acid in ethanol. [NOTE—Slowly add sulfuric acid to ice-cold ethanol.]
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Apply the *Samples* as bands to a suitable HPTLC plate, and dry in air. Develop the chromatograms in a saturated chamber, remove the plate from the chamber, air-dry, and examine under UV light at 254 nm. Then treat the plate with *Derivatization reagent*, heat at 120° for 7 min, and examine under UV light at 366 nm.
System suitability: Under UV light at 254 nm, *Standard solution B* exhibits an intense band corresponding in R_F to the band of schisandrin in *Standard solution A*. *Standard solution B* also exhibits a band due to schisandrin A in the middle of the chromatogram, and four or five bands between the positions of the bands of schisandrin and schisandrin A. In the upper-half section, *Standard solution B* exhibits an intense band due to schisandrin B.
Acceptance criteria: Under UV light at 254 nm, the *Sample solution* exhibits an intense band at an R_F corresponding to the band due to schisandrin [distinction from southern schisandra (*Schisandra sphenanthera*) fruit] in *Standard solution A*. The *Sample solution* exhibits additional bands corresponding in R_F to similar bands in *Standard solution B*, including a band due to schisandrin A in the middle of the chromatogram; four or five bands between the positions of the bands of schisandrin A and schisandrin; and two or three bands in the upper-half section, the most intense band due to schisandrin B. Under UV light at 366 nm after derivatization, the chromatogram of the *Sample solution* does not exhibit an intense blue fluorescent band (distinction from *Schisandra sphenanthera* fruit) in the upper-third of the chromatogram.

- **B. HPLC**

Analysis: Proceed as directed in *Content of Lignans*.

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Acceptance criteria: The chromatogram of the *Sample solution* exhibits the most intense peak with a retention time corresponding to schisandrin in *Standard solution A*, and the peaks for schisandrol B, schisandrin A, and schisandrin B correspond to the retention times for the same lignans in *Standard solution B*. There is no principal peak due to schisantherin A at a relative retention time of about 2.1 relative to schisandrin (distinction from *Schisandra sphenanthera* fruit).

COMPOSITION

• **CONTENT OF LIGNANS**

Solution A: Water

Solution B: Acetonitrile and methanol (1:1) (v/v)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	47	53
30	20	80

Standard solution A: 0.06 mg/mL of USP Schisandrin RS in methanol

Standard solution B: 10 mg/mL of USP *Schisandra chinensis* Fruit Dry Extract RS in methanol. Sonicate and pass through a polytetrafluoroethylene filter of 0.2-µm pore size.

Sample solution: Accurately transfer an amount, equivalent to 4 mg of total lignans according to the labeled content, of Northern Schisandra Fruit Dry Extract to a 50-mL round-bottom centrifuge tube. Add 10 mL of methanol, and sonicate for 10 min (140 W, 42 kHz). Centrifuge, and transfer the supernatant to a 25-mL volumetric flask. Repeat the extraction one more time. Combine the extracts in the 25-mL volumetric flask and dilute with methanol to volume. Mix, pass through a polytetrafluoroethylene filter of 0.2-µm pore size before injection, and discard the first portion of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: UPLC

Detector: UV 251 nm

Column: 2.1-mm × 15-cm; 1.8-µm packing L1

Column temperature: 35°

Flow rate: 0.3 mL/min

Injection volume: 3 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP *Schisandra chinensis* Fruit Dry Extract RS being used.

Resolution: NLT 1.5 between the schisandrol B peak and its following peak, *Standard solution B*

Tailing factor: NMT 2.0 for the schisandrin peak, *Standard solution A*

Relative standard deviation: NMT 2.0% for the schisandrin peak, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatogram of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP *Schisandra chinensis* Fruit Dry Extract RS being used, identify the retention times of the peaks corresponding to schisandrin, schisandrol B, schisandrin A, and schisandrin B in the *Sample solution*.

[NOTE—The approximate relative retention times of the analytes are provided in *Table 2*.]

Table 2

Analyte	Approximate Relative Retention Time	Conversion Factor
Schisandrin	1.00	1.00
Schisandrol B	1.29	1.21
Schisandrin A	2.90	1.00
Schisandrin B	3.31	1.23

Separately calculate the percentages of schisandrin, schisandrol B, schisandrin A, and schisandrin B in the portion of Northern Schisandra Fruit Dry Extract taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*

r_S = peak area of schisandrin from *Standard solution A*

C_S = concentration of USP Schisandrin RS in *Standard solution A* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Northern Schisandra Fruit Dry Extract taken to prepare the *Sample solution* (mg)

F = conversion factor for analytes (see *Table 2*)

Calculate the percentage of the labeled amount of schisandrin in the portion of Northern Schisandra Fruit Dry Extract taken:

$$\text{Result} = (P/L) \times 100$$

P = content of schisandrin as determined above (%)

L = labeled amount of schisandrin (%)

Acceptance criteria: 90.0%–110.0% on the dried basis

Calculate the percentage of the labeled amount of total lignans as the sum of schisandrin, schisandrol B, schisandrin A, and schisandrin B in the portion of Northern Schisandra Fruit Dry Extract taken:

$$\text{Result} = (P/L) \times 100$$

P = content of total lignans as determined above (%)

L = labeled amount of total lignans (%)

Acceptance criteria: 90.0%–110.0% on the dried basis

CONTAMINANTS

Change to read:

• **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020)**:** Meets the requirements

• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^4 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria do not exceed 10^3 cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures*, *Test for Absence of Salmonella Species* and *Test*

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Procedures, Test for Absence of Escherichia coli: Meets the requirements

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Test for Aflatoxins*: Meets the requirements

SPECIFIC TESTS

- **LOSS ON DRYING** ⟨731⟩
  Sample: 2 g of Northern Schisandra Fruit Dry Extract
  Analysis: Dry the *Sample* at 105° for 5 h.
  Acceptance criteria: NMT 8%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Total Ash*
  Sample: 2 g of Northern Schisandra Fruit Dry Extract
  Acceptance criteria: NMT 5%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial following the official name of the plant from which the article was derived. It meets other labeling requirements in *Botanical Extracts* ⟨565⟩.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP *Schisandra chinensis* Fruit Dry Extract RS
  USP Schisandrin RS

Northern Schisandra Fruit Powder

DEFINITION

Northern Schisandra Fruit Powder consists of dried ripe fruits of *Schisandra chinensis* (Turcz.) Baill. (Fam. Schisandraceae) reduced to a powder or very fine powder. It contains NLT 0.40% of schisandrin (schisandrol A) on the dried basis; NLT 0.95% of lignans, calculated as the sum of schisandrin, schisandrol B, deoxyschisandrin (schisandrin A), and γ-schisandrin (schisandrin B) on the dried basis.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHY**
  Standard solution A: 1.0 mg/mL of USP Schisandrin RS in ethanol
  Standard solution B: Sonicate 50 mg/mL of USP *Schisandra chinensis* Fruit Dry Extract RS in alcohol for 10 min. Centrifuge, and use the supernatant.
  Sample solution: Sonicate 2.5 g of Northern Schisandra Fruit Powder in 10 mL of alcohol for 10 min. Centrifuge, and use the supernatant.
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
  Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)
  Application volume: 3 µL, as 8-mm bands
  Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
  Developing solvent system: Toluene, ethyl acetate, and glacial acetic acid (23:6:1)
  Developing distance: 6 cm
  Derivatization reagent: 10% Sulfuric acid in ethanol. [NOTE—Slowly add sulfuric acid to ice-cold ethanol.]
  Analysis
  Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
  Apply the *Samples* as bands to a suitable HPTLC plate, and dry in air. Develop the chromatograms in a saturated chamber, remove the plate from the chamber, dry, and examine under UV light at 254 nm. Then treat the plate with *Derivatization reagent*, heat at 120° for 7 min, and examine under UV light at 366 nm.
  System suitability: Under UV light at 254 nm, the chromatogram of *Standard solution B* exhibits an intense band corresponding in R_F to the band due to schisandrin in the chromatogram of *Standard solution A*. *Standard solution B* also exhibits a band due to deoxyschisandrin in the middle of the chromatogram and four or five bands between the positions of the bands of schisandrin and deoxyschisandrin. In the upper-half section *Standard solution B* exhibits an intense band corresponding to γ-schisandrin.
  Acceptance criteria: Under UV light at 254 nm, the *Sample solution* chromatogram exhibits an intense band at an R_F corresponding to the band due to schisandrin in the chromatogram of *Standard solution A*. The *Sample solution* exhibits additional bands corresponding to similar bands in the chromatogram of *Standard solution B*. These include one or two bands below the position of schisandrin; a band due to deoxyschisandrin in the middle of the chromatogram; four or five bands between the positions of the bands of schisandrin and deoxyschisandrin; two or three bands in the upper-half section, the most intense band at an R_F corresponding to the band of γ-schisandrin. Under UV light at 366 nm after derivatization, the chromatogram of the *Sample solution* does not exhibit an intense blue fluorescent band in the upper-third of the chromatogram.
- **B. HPLC**
  Analysis: Proceed as directed in *Content of Lignans*.
  Acceptance criteria: The chromatogram of the *Sample solution* exhibits the most intense peak with a retention time corresponding to schisandrin in *Standard solution A*, and the peaks due to schisandrol B, deoxyschisandrin, and γ-schisandrin corresponding to the retention times for the same lignans in *Standard solution B*. There is no principal peak due to schisandrin A at a relative retention time of about 2.1 relative to schisandrin (distinction from *Northern Schisandra Fruit*).

COMPOSITION

- **CONTENT OF LIGNANS**
  Solution A: Water
  Solution B: Acetonitrile and methanol (1:1) (v/v)
  Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	47	53
30	20	80

Standard solution A: 0.06 mg/mL of USP Schisandrin RS in methanol
Standard solution B: 10 mg/mL of USP *Schisandra chinensis* Fruit Dry Extract RS in methanol. Before injection, pass through a polytetrafluoroethylene filter of 0.2-µm pore size, and discard the first portion of the filtrate.
Sample solution: Transfer about 250 mg of Northern Schisandra Fruit Powder, accurately weighed, to a 50-mL centrifuge tube. Add 10.0 mL of methanol, and sonicate for 10 min (140 W, 42 kHz). Centrifuge, and transfer this solution to a 25-mL volumetric flask. Repeat this extraction one more time. Combine the extracts in the 25-mL volumetric flask. Adjust with methanol to volume, and mix. Before injection, pass through a polytetrafluoroethylene filter of 0.2-µm pore size, and discard the first portion of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)

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Mode: UPLC
Detector: UV 251 nm
Column: 2.1-mm × 15-cm; 1.8-µm packing L1
Column temperature: 35°
Flow rate: 0.3 mL/min
Injection volume: 3 µL

System suitability
Samples: *Standard solution A* and *Standard solution B*
[NOTE—The relative retention times of the peaks for schisandrin, schisandrol B, deoxyschisandrin, and γ-schisandrin are about 1.00, 1.29, 2.90, 3.31, respectively.]
Suitability requirements
Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP *Schisandra chinensis* Fruit Dry Extract RS being used.
Tailing factor: NMT 2.0 for the schisandrin peak, *Standard solution A*
Relative standard deviation: NMT 2.0%, determined from the schisandrin peak in repeated injections, *Standard solution A*
Resolution: NLT 1.5 between the schisandrol B peak and its following peak, *Standard solution B*

Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Using the chromatogram of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP *Schisandra chinensis* Fruit Dry Extract RS being used, identify the retention times of the peaks corresponding to different lignans in the *Sample solution* chromatogram.
Calculate the percentages of schisandrin, schisandrol B, deoxyschisandrin, and γ-schisandrin in the portion of Powder taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of schisandrin from *Standard solution A*
C_S = concentration of USP Schisandrin RS in *Standard solution A* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Powder taken to prepare the *Sample solution* (mg)
F = conversion factor for analytes (1.00 for schisandrin, 1.21 for schisandrol B, 1.00 for deoxyschisandrin, and 1.23 for γ-schisandrin)

Calculate the content of lignans as the sum of the percentages of schisandrin, schisandrol B, deoxyschisandrin, and γ-schisandrin.

Acceptance criteria
Schisandrin: NLT 0.40% on the dried basis
Lignans: NLT 0.95% on the dried basis

CONTAMINANTS

- **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩
  Acceptance criteria
  Arsenic: NMT 2.0 µg/g
  Cadmium: NMT 0.3 µg/g
  Lead: NMT 5.0 µg/g
  Mercury: NMT 0.2 µg/g
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli*
- **ARTICLES OF BOTANICAL ORIGIN,** *Test for Aflatoxins* ⟨561⟩**:** Meets the requirements

SPECIFIC TESTS

- **BOTANIC CHARACTERISTICS**
  Macroscopic: Dark purple in color
  Microscopic: Stone cells of epidermis of testa polygonal or elongated-polygonal in surface view, 18–50 µm in diameter, walls thickened with very fine and close pit canals, lumina containing dark brown contents. Stone cells of the inner layers of testa polygonal, subrounded or irregular, NMT 83 µm in diameter, walls slightly thickened, with relatively large pits. Epidermal cells of pericarp: polygonal in surface view, anticlinal walls slightly beaded, with cuticle striations (unlike *Schisandra sphenanthera*, where anticlinal walls are not beaded) scattered with oil cells. Cells of the mesocarp are shriveled, containing dark brown contents and starch granules.
- **LOSS ON DRYING** ⟨731⟩
  Sample: 2 g of Powder, finely powdered
  Analysis: Dry the *Sample* at 105° for 5 h.
  Acceptance criteria: NMT 16%
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩
  Analysis: 2 g of Powder, finely powdered
  Acceptance criteria: NMT 7%
- **ARTICLES OF BOTANICAL ORIGIN,** *Water-Soluble Extractives* ⟨561⟩
  Analysis: Cold extraction method
  Acceptance criteria: NMT 35%
- **ARTICLES OF BOTANICAL ORIGIN,** *Alcohol-Soluble Extractives* ⟨561⟩
  Analysis: Cold extraction method
  Acceptance criteria: NMT 40%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial following the official name.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP *Schisandra chinensis* Fruit Dry Extract RS
  USP Schisandrin RS

Schizochytrium Oil

DEFINITION

Schizochytrium Oil is obtained by fermentation and extraction of algae of the genus *Schizochytrium* and contains docosahexaenoic acid (DHA; $C_{22}H_{32}O_2$) (C22:6 n–3) as the major polyunsaturated fatty acid component. It may contain eicosapentaenoic acid (EPA; $C_{20}H_{30}O_2$) (C20:5 n–3), docosapentaenoic acid (DPA; $C_{22}H_{34}O_2$) (C22:5 n–6), arachidonic acid ($C_{20}H_{32}O_2$) (C20:4 n–6), and dihomo gamma linolenic acid ($C_{20}H_{34}O_2$) (C20:3 n–6) as the minor polyunsaturated fatty acid components. DHA content may be standardized with other appropriate oils. Suitable antioxidants in appropriate concentrations may be added.

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IDENTIFICATION

• **A. Long Chain Unsaturated Fatty Acid Profile:**
Proceed as directed in *Fats and Fixed Oils* ⟨401⟩, *Omega-3 Fatty Acids Determination and Profile.*

Analysis

Samples: *Standard Solution 2a*, *Standard Solution 2b*, and *Test Solution 1*

Calculate the area percentage for dihomo gamma linolenic acid, arachidonic acid, and docosapentaenoic acid as methyl ester in *Test Solution 1*:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of each individual fatty acid as methyl ester

r_T = sum of the responses of all the peaks, except the solvent and butylated hydroxytoluene peaks

Acceptance criteria: The retention times of the peaks of the docosahexaenoic acid methyl ester and the eicosapentaenoic acid methyl ester of *Test Solution 1* correspond to those of *Standard Solution 2a* and *Standard Solution 2b*, respectively, as obtained in the test for *Content of DHA and EPA*. The area percentages for the methyl esters of dihomo gamma linolenic acid, arachidonic acid, and docosapentaenoic acid from the chromatogram of *Test Solution 1* meet *Acceptance Criteria I*, *II*, *III*, or *IV* in *Table 1*.

COMPOSITION

• **Content of DHA and EPA**

Analysis: Proceed as directed in *Fats and Fixed Oils* ⟨401⟩, *Omega-3 Fatty Acids Determination and Profile.* Use *Analysis* (for triglycerides).

Acceptance criteria: Meets the acceptance criteria for DHA and EPA in *Table 1*

IMPURITIES

• **Limit of Arsenic**

[Note—For the preparation of all aqueous solutions and for the rinsing of glass, polytef, and plastic vessels before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin before use. Select all reagents to have as low a content of arsenic as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytef, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

Solution A: Transfer 1 g of ultrapure palladium metal to a Teflon beaker. Add 20 mL of water and 10 mL of nitric acid, and warm on a hot plate to dissolve. Allow the solution to cool to room temperature, transfer it to a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution B: Transfer 1 g of ultrapure magnesium nitrate to a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it to a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution C: *Solution A*, *Solution B*, and 2% nitric acid (3:2:5). A volume of 5 µL provides 0.015 mg of palladium and 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (1:19)

Standard stock solution: Transfer 10.0 mL of *Standard Arsenic Solution*, prepared as directed in *Arsenic* ⟨211⟩, to a 100-mL volumetric flask. Add 40 mL of water and 5 mL of nitric acid, and dilute with water to volume. This solution contains 0.10 µg/mL of arsenic.

Standard solutions: Dilute the *Standard stock solution* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 µg/mL of arsenic.

Sample solution: For preparation of the *Sample solution*, use a microwave oven with a magnetron frequency of 2455 MHz and a selectable output power of 0–950 W in 1% increments, equipped with advanced composite vessels with 100-mL polytef liners. Use rupture membranes to vent vessels should the pressure exceed 125 psi. The vessels fit into a turntable, and each vessel can be vented into an overflow container. Equip the microwave oven with an exhaust tube to ventilate fumes. [**Caution**—Wear proper eye protection and protective clothing and gloves.] Transfer approximately 500 mg of Schizochytrium Oil, weighed to the nearest 0.1 mg, to a Teflon digestion vessel liner. Prepare samples in duplicate. Add 15 mL of nitric acid, and swirl gently. Cover the vessels with lids, leaving the vent fitting off. Predigest overnight under a hood. Place the rupture membrane in the vent fitting, and tighten the lid. Place all vessels on the microwave oven turntable. Connect the vent tubes to the vent trap, and connect the pressure-sensing line to the appropriate vessel. Initiate a two-stage digestion procedure by heating the microwave at 15% power for 15 min, followed by 25% power for 45 min. Remove the turntable of vessels from the oven, and allow the vessels to cool to room temperature. [Note—A cool water bath may be used to speed the cooling process.] Vent the vessels when they reach room temperature. Remove the lids, and slowly add 2 mL of hydrogen peroxide to each. Allow the reactions to subside, and seal the vessels. Return the vessels on the turntable to the microwave oven, and heat for an additional 15 min at 30% power. Remove the vessels from the oven, and allow them to cool to room temperature. Transfer the cooled digests to 25-mL volumetric flasks, and dilute with water to volume.

Analysis: Program the graphite furnace as follows. Dry at 115°, using a 1-s ramp, a 65-s hold, and an argon flow of

Table 1 (Schizochytrium Oil)

Fatty Acid	Relative Retention Time	Shorthand Notation	Acceptance Criteria I Limit (area %)	Acceptance Criteria II (for articles labeled as Type IA) Limit (area %)	Acceptance Criteria III (for articles labeled as Type II) Limit (area %)	Acceptance Criteria IV (for articles labeled as Type III) Limit (area %)
Dihomo gammalinolenic acid	0.71	20:3 n–6	NMT 2.8	NMT 1.0	NMT 1.0	NMT 1.0
Arachidonic acid	0.73	20:4 n–6	NMT 1.3	NMT 3.5	NMT 3.5	NMT 3.5
Eicosapentaenoic acid (EPA)	0.79	20:5 n–3	NMT 3.9[a]	NMT 3.5[a]	NLT 10.0[a]	NMT 20.0[a]
Docosapentaenoic acid (DPA)	0.94	22:5 n–6	NMT 16.5	NMT 25.0	NMT 3.5	NMT 6.0
Docosahexaenoic acid (DHA)	1.00	22:6 n–3	NLT 30.0[a]	NLT 35.0[a]	NLT 20.0[a]	NLT 35.0[a]

[a] Limit is expressed as w/w%.

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300 mL/min; char the sample at 1000°, using a 1-s ramp, a 20-s hold, and an airflow of 300 mL/min; cool down, and purge the air from the furnace for 10 s, using a 20° set temperature and an argon flow of 300 mL/min; atomize at 2400°, using a 0-s ramp and a 5-s hold with the argon flow stopped; and clean out at 2600° with a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 µL) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by an injection of 5 µL of *Solution C* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for arsenic. Determine the peak area at the arsenic emission line at 193.7 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of arsenic, in µg/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in µg/mL, of arsenic in each mL of the *Sample solution* by interpolation from the regression line.

Calculate the content of arsenic in the portion of Schizochytrium Oil taken:

$$\text{Result} = (C/W) \times 25$$

C = concentration as obtained above
W = weight of Schizochytrium Oil taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g

• **LIMIT OF LEAD**

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytef, and plastic vessels before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin before use. Select all reagents to have as low a content of lead as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytef, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

Solution A: 10 g of ultrapure monobasic ammonium phosphate in 1 mL of nitric acid and 40 mL of water to dissolve the phosphate. Dilute with deionized water to 100 mL.

Solution B: Transfer 1 g of ultrapure magnesium nitrate to a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it to a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution C: *Solution A*, *Solution B*, and 2% nitric acid (2:1:2). A volume of 5 µL provides 0.2 mg of phosphate plus 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (1:19)

Standard stock solution: Transfer 10.0 mL of lead nitrate stock solution TS to a 100-mL volumetric flask, add 40 mL of water and 5 mL of nitric acid, and dilute with water to volume. Transfer 1.0 mL of this solution to a second 100-mL volumetric flask, add 50 mL of water and 1 mL of nitric acid, and dilute with water to volume. This solution contains 0.10 µg/mL of lead.

Standard solutions: Dilute the *Standard stock solution* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 µg/mL of lead.

Sample solution: Prepare as directed for *Sample solution* in the test for *Limit of Arsenic*.

Analysis: Program the graphite furnace as follows. Dry at 120°, using a 1-s ramp, a 55-s hold, and an argon flow of 300 mL/min; char the sample at 850°, using a 1-s ramp, a 30-s hold, and an airflow of 300 mL/min; cool down, and purge the air from the furnace for 10 s, using a 20° set temperature and an argon flow of 300 mL/min; atomize at 2100°, using a 0-s ramp and a 5-s hold with the argon flow stopped; and clean out at 2600° with a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 µL) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by an injection of 5 µL of *Solution C* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for lead. Determine the peak area at the lead emission line at 283.3 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of lead, in µg/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in µg/mL, of lead in each mL of the *Sample solution* by interpolation from the regression line.

Calculate the content of lead in the portion of Schizochytrium Oil taken:

$$\text{Result} = (C/W) \times 25$$

C = concentration, as obtained above
W = weight of Schizochytrium Oil taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g

• **LIMIT OF CADMIUM**

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytef, and plastic vessels before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin before use. Select all reagents to have as low a content of cadmium as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytef, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

Solution A: 10 g of ultrapure monobasic ammonium phosphate in 40 mL of water and 1 mL of nitric acid to dissolve the phosphate. Dilute with deionized water to 100 mL.

Solution B: Transfer 1 g of ultrapure magnesium nitrate to a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it to a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution C: *Solution A*, *Solution B*, and 2% nitric acid to volume (2:1:2). A volume of 5 µL provides 0.2 mg of phosphate and 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (1:19)

Standard stock solution A: 0.1372 mg/mL of cadmium nitrate

Standard stock solution B: *Standard stock solution A*, nitric acid, and water (2:1:97). This solution contains 0.10 µg/mL of cadmium. [NOTE—Before makeup to final volume, dissolve in a portion of water and nitric acid.]

Standard solutions: Dilute *Standard stock solution B* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 µg/mL of cadmium.

Sample solution: Prepare as directed for *Sample solution* in the test for *Limit of Arsenic*.

Analysis: Program the graphite furnace as follows. Dry at 120°, using a 1-s ramp, a 55-s hold, and an argon flow of 300 mL/min; char the sample at 850°, using a 1-s ramp, a 30-s hold, and an airflow of 300 mL/min; cool down, and purge the air from the furnace for 10 s, using a 20° set temperature and an argon flow of 300 mL/min; atomize at 2400°, using a 0-s ramp and a 5-s hold with the argon flow

Supplements

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stopped; and clean out at 2600° with a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 µL) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by an injection of 5 µL of *Solution C* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for cadmium. Determine the peak area at the cadmium emission line at 228.8 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of cadmium, in µg/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in µg/mL, of cadmium in each mL of the *Sample solution* by interpolation from the regression line.

Calculate the content of cadmium in the portion of Schizochytrium Oil taken:

$$\text{Result} = (C/W) \times 25$$

C = concentration, as obtained above
W = weight of Schizochytrium Oil taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g

- **LIMIT OF MERCURY**

Sample solution: Prepare as directed for the *Sample solution* in the test for *Limit of Arsenic*, combining the two duplicate cooled digests into 1.0 mL of *Potassium Permanganate Solution* (see *Mercury* ⟨261⟩, *Method IIa and Method IIb, Reagents*).

Analysis: Proceed as directed for *Mercury* ⟨261⟩, *Method IIa and Method IIb*, except use a *Standard Mercury Solution* with the equivalent of 0.1 µg/mL of mercury.

Acceptance criteria: NMT 0.1 µg/g

SPECIFIC TESTS

- **FATS AND FIXED OILS** ⟨401⟩, *Anisidine Value*: NMT 20.0
- **FATS AND FIXED OILS** ⟨401⟩, *Acid Value*: The free fatty acids in 10 g require NMT 1.42 mL of 0.1 N sodium hydroxide for neutralization.
- **FATS AND FIXED OILS** ⟨401⟩, *Peroxide Value*: NMT 5.0
- **FATS AND FIXED OILS** ⟨401⟩, *Total Oxidation Value*: NMT 26, calculated:

$$\text{Result} = (2 \times PV) + AV$$

PV = peroxide value
AV = anisidine value

- **FATS AND FIXED OILS** ⟨401⟩, *Unsaponifiable Matter*: NMT 4.5%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and avoid exposure to excessive heat.
- **LABELING:** The label states the content of docosahexaenoic acid in mg/g and eicosapentaenoic acid in mg/g if its content is NLT 10%. It also states the name and concentration of any added antioxidant. Articles intended to meet Acceptance Criteria II, III, or IV are labeled as Type 1A, Type II, or Type III, respectively.

Schizochytrium Oil Capsules

DEFINITION

Schizochytrium Oil Capsules are prepared from Schizochytrium Oil and contain NLT 95.0% and NMT 105.0% of the labeled amount of docosahexaenoic acid (DHA; $C_{22}H_{32}O_2$) (C22:6 n–3).

IDENTIFICATION

- **LONG CHAIN UNSATURATED FATTY ACID PROFILE:** Proceed as directed under *Strength, Content of DHA*.

Analysis

Samples: *Standard Solution 2a*, *Standard Solution 2b*, and *Test solution 1*

Calculate the area percentage for each fatty acid as methyl ester in *Test solution 1*:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of each individual fatty acid as methyl ester
r_T = sum of the responses of all the peaks, except the solvent and butylated hydroxytoluene peaks

Acceptance criteria: The retention times of the peaks of the docosahexaenoic acid methyl ester and the eicosapentanoic acid methyl ester of *Test solution 1* correspond to those of the docosahexaenoic acid methyl ester and eicosapentaenoic acid methyl ester peaks from *Standard Solution 2a* and *Standard Solution 2b*, respectively, as obtained in the test for *Fats and Fixed Oils* ⟨401⟩, *Omega-3 Fatty Acids Determination and Profile, Content of EPA and DHA*. The area percentage for the methyl esters of the fatty acids from the chromatogram of *Test solution 1* in the test for *Content of EPA and DHA* meet the requirements for each fatty acid shown in the table below.

Fatty Acid	Relative Retention Time	Shorthand Notation	Lower Limit (Area %)	Upper Limit (Area %)
Dihomo-gamma-linolenic acid	0.71	20:3 n–6	1.7	2.8
Arachidonic acid	0.73	20:4 n–6	0.6	1.3
Eicosapentaenoic acid (EPA)	0.79	20:5 n–3	1.3	3.9
Docosapentaenoic acid (DPAn–6)	0.94	22:5 n–6	10.5	16.5
Docosahexaenoic acid (DHA)	1.00	22:6 n–3	30.0	40.0

STRENGTH

- **CONTENT OF DHA**

Test solution 1 and **Test solution 2:** Weigh NLT 10 Capsules in a tared weighing bottle. With a sharp blade or other appropriate means, carefully open the Capsules, without loss of the shell material, and transfer the combined Capsule contents to a 100-mL beaker. Remove any adhering substance from the emptied Capsules by washing with several small portions of isooctane. Discard the washings, and allow the empty Capsules to dry in a current of dry air until the isooctane is completely evaporated. Weigh the empty Capsules in the original tared weighing bottle, and calculate the average fill weight (AFW) of schizochytrium oil per Capsule. Proceed with the content of Capsules as directed in the *Analysis*.

Analysis: Proceed as directed in *Fats and Fixed Oils* ⟨401⟩, *Omega-3 Fatty Acids Determination and Profile, Content of EPA and DHA*.

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Calculate the percentage of the labeled amount of docosahexaenoic acid (DHA) in the Capsules taken:

$$\text{Result} = R \times AFW/L$$

R = percentage of DHA in the portion of oil taken from the Capsules
AFW = average fill weight of the Capsules taken (mg)
L = the labeled amount of DHA (mg/Capsule)

Acceptance criteria: NLT 95.0% and NMT 105.0% of the labeled amount of DHA

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS ⟨2040⟩:** Meet the requirements of the *Rupture Test for Soft Shell Capsules*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS ⟨2091⟩:** Meet the requirements

IMPURITIES

- **LIMIT OF ARSENIC**

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytef, and plastic vessels before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin before use. Select all reagents to have as low a content of arsenic as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytef, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

Solution A: Transfer 1 g of ultrapure palladium metal into a Teflon beaker. Add 20 mL of water and 10 mL of nitric acid, and warm on a hot plate to dissolve. Allow the solution to cool to room temperature, transfer it into a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution B: Transfer 1 g of ultrapure magnesium nitrate into a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it into a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution C: *Solution A*, *Solution B*, and 2% nitric acid (3:2:5). A volume of 5 µL provides 0.015 mg of palladium and 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (1:19)

Standard stock solution: Transfer 10.0 mL of *Standard Arsenic Solution*, prepared as directed in *Arsenic* ⟨211⟩, to a 100-mL volumetric flask. Add 40 mL of water and 5 mL of nitric acid, and dilute with water to volume. This solution contains 0.10 µg/mL of arsenic.

Standard solutions: Dilute the *Standard stock solution* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 µg/mL of arsenic.

Sample solution: For preparation of the *Sample solution*, use a microwave oven with a magnetron frequency of 2455 MHz and a selectable output power of 0–950 watts in 1% increments, equipped with advanced composite vessels with 100-mL polytef liners. Use rupture membranes to vent vessels should the pressure exceed 125 psi. The vessels fit into a turntable, and each vessel can be vented into an overflow container. Equip the microwave oven with an exhaust tube to ventilate fumes. [**CAUTION**—Wear proper eye protection and protective clothing and gloves.] Transfer approximately 500 mg of schizochytrium oil from Capsules, weighed to the nearest 0.1 mg, into a Teflon digestion vessel liner. Prepare samples in duplicate. Add 15 mL of nitric acid, and swirl gently. Cover the vessels with lids, leaving the vent fitting off. Predigest overnight under a hood. Place the rupture membrane in the vent fitting, and tighten the lid. Place all vessels on the microwave oven turntable. Connect the vent tubes to the vent trap, and connect the pressure-sensing line to the appropriate vessel. Initiate a two-stage digestion procedure by heating the microwave at 15% power for 15 min, followed by 25% power for 45 min. Remove the turntable of vessels from the oven, and allow the vessels to cool to room temperature. [NOTE—A cool water bath may be used to speed the cooling process.] Vent the vessels when they reach room temperature. Remove the lids, and slowly add 2 mL of 30% hydrogen peroxide to each. Allow the reactions to subside, and seal the vessels. Return the vessels on the turntable to the microwave oven, and heat for an additional 15 min at 30% power. Remove the vessels from the oven, and allow them to cool to room temperature. Transfer the cooled digests into 25-mL volumetric flasks, and dilute with water to volume.

Analysis: Program the graphite furnace as follows. Dry at 115°, using a 1-s ramp, a 65-s hold, and an argon flow of 300 mL/min; char the sample at 1000°, using a 1-s ramp, a 20-s hold, and an airflow of 300 mL/min; cool down, and purge the air from the furnace for 10 s, using a 20° set temperature and an argon flow of 300 mL/min; atomize at 2400°, using a 0-s ramp and a 5-s hold with the argon flow stopped; and clean out at 2600° with a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 µL) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by an injection of 5 µL of *Solution C* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for arsenic. Determine the peak area at the arsenic emission line at 193.7 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of arsenic, in µg/mL, and calculate the regression line best fitting the points. Determine the concentration, C, in µg/mL, of arsenic in each mL of the *Sample solution* by interpolation from the regression line.

Calculate the content of arsenic in the portion of Capsules taken:

$$\text{Result} = (C/W) \times 25$$

C = concentration as obtained above
W = weight of Capsule content taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g

- **LIMIT OF LEAD**

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytef, and plastic vessels before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin before use. Select all reagents to have as low a content of lead as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytef, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

Solution A: 10 g of ultrapure monobasic ammonium phosphate in 1 mL of nitric acid and 40 mL of water to dissolve the phosphate. Dilute with deionized water to 100 mL.

Solution B: 1 g of ultrapure magnesium nitrate in a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it to a 100-mL volumetric flask, and dilute with deionized water to volume.

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Solution C: *Solution A*, *Solution B*, and 2% nitric acid (2:1:2). A volume of 5 µL provides 0.2 mg of phosphate plus 0.01 mg of magnesium nitrate.
Blank: Nitric acid and water (1:19)
Standard stock solution: Transfer 10.0 mL of lead nitrate stock solution TS to a 100-mL volumetric flask. Add 40 mL of water and 5 mL of nitric acid, and dilute with water to volume. Transfer 1.0 mL of this solution to a second 100-mL volumetric flask, add 50 mL of water and 1 mL of nitric acid, and dilute with water to volume. This solution contains 0.10 µg/mL of lead.
Standard solutions: Dilute the *Standard stock solution* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 µg/mL of lead.
Sample solution: Prepare as directed for *Sample solution* in the test for *Limit of Arsenic*.
Analysis: Program the graphite furnace as follows. Dry at 120°, using a 1-s ramp, a 55-s hold, and an argon flow of 300 mL/min; char the sample at 850°, using a 1-s ramp, a 30-s hold, and an airflow of 300 mL/min; cool down, and purge the air from the furnace for 10 s, using a 20° set temperature and an argon flow of 300 mL/min; atomize at 2100°, using a 0-s ramp and a 5-s hold with the argon flow stopped; and clean out at 2600° with a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 µL) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by an injection of 5 µL of *Solution C* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for lead. Determine the peak area at the lead emission line at 283.3 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of lead, in µg/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in µg/mL, of lead in each mL of the *Sample solution* by interpolation from the regression line.
Calculate the content of lead in the portion of Capsules taken:

$$\text{Result} = (C/W) \times 25$$

C = concentration, as obtained above
W = weight of Capsule content taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g

- **LIMIT OF CADMIUM**

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytef, and plastic vessels before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin before use. Select all reagents to have as low a content of cadmium as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytef, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]
Solution A: 10 g of ultrapure monobasic ammonium phosphate in 40 mL of water and 1 mL of nitric acid to dissolve the phosphate. Dilute with deionized water to 100 mL.
Solution B: Transfer 1 g of ultrapure magnesium nitrate to a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it to a 100-mL volumetric flask, and dilute with deionized water to volume.
Solution C: *Solution A*, *Solution B*, and 2% nitric acid to volume (2:1:2). A volume of 5 µL provides 0.2 mg of phosphate and 0.01 mg of magnesium nitrate.
Blank: Nitric acid and water (1:19)
Standard stock solution A: 0.1372 mg/mL of cadmium nitrate
Standard stock solution B: *Standard stock solution A*, nitric acid, and water (2:1:97). This solution contains 0.10 µg/mL of cadmium. [NOTE—Before makeup to final volume, dissolve in a portion of water and nitric acid.]
Standard solutions: Dilute *Standard stock solution B* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 µg/mL of cadmium.
Sample solution: Prepare as directed for *Sample solution* in the test for *Limit of Arsenic*.
Analysis: Program the graphite furnace as follows. Dry at 120°, using a 1-s ramp, a 55-s hold, and an argon flow of 300 mL/min; char the sample at 850°, using a 1-s ramp, a 30-s hold, and an airflow of 300 mL/min; cool down, and purge the air from the furnace for 10 s, using a 20° set temperature and an argon flow of 300 mL/min; atomize at 2400°, using a 0-s ramp and a 5-s hold with the argon flow stopped; and clean out at 2600° with a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 µL) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by an injection of 5 µL of *Solution C* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for cadmium. Determine the peak area at the cadmium emission line at 228.8 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of cadmium, in µg/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in µg/mL, of cadmium in each mL of the *Sample solution* by interpolation from the regression line.
Calculate the content of cadmium in the Capsules taken:

$$\text{Result} = (C/W) \times 25$$

C = concentration, as obtained above
W = weight of Capsule content taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g

- **LIMIT OF MERCURY**

Proceed as directed for *Mercury* ⟨261⟩, *Method IIa and Method IIb*, except use a *Standard Mercury Solution* having the equivalent of 0.1 µg/mL of mercury.
Sample solution: Prepare as directed for the *Sample solution* in the test for *Limit of Arsenic*, combining the two duplicate cooled digests into 1.0 mL of *Potassium Permanganate Solution*.
Acceptance criteria: NMT 0.1 µg/g

SPECIFIC TESTS

- **FATS AND FIXED OILS,** *Anisidine Value* ⟨401⟩**:** NMT 20.0, determined on the contents of the Capsules
- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩**:** The free fatty acids in 10 g require for neutralization NMT 1.42 mL of 0.1 N sodium hydroxide.
- **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩**:** NMT 5.0, determined on the contents of the Capsules
- **FATS AND FIXED OILS,** *Total Oxidation Value (TOTOX)* ⟨401⟩**:** NMT 26 (determined on the contents of the Capsules), calculated as:

$$\text{Result} = (2 \times PV) + AV$$

PV = peroxide value

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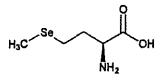
AV = anisidine value

- **FATS AND FIXED OILS,** *Unsaponifiable Matter* ⟨401⟩: NMT 4.5%, determined on the contents of the Capsules

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and avoid exposure to excessive heat.
- **LABELING:** The label states the content of docosahexaenoic acid in mg/Capsule. It also states the name and concentration of any added antioxidant.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Docosahexaenoic Acid Ethyl Ester RS
  USP Eicosapentaenoic Acid Ethyl Ester RS
  USP Methyl Tricosanoate RS

Selenomethionine



$C_5H_{11}NO_2Se$ 196.11

Butanoic acid, 2-amino-4-(methylseleno)-, (*S*)-;
(*S*)-2-Amino-4-(methylselenyl)butyric acid [3211-76-5].

DEFINITION

Selenomethionine contains NLT 97.0% and NMT 103.0% of selenomethionine ($C_5H_{11}NO_2Se$) and contains NLT 39.0% and NMT 41.0% of selenium (Se), calculated on the as-is basis.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

ASSAY

- **PROCEDURE**

Mobile phase: 6.8 g/L of monobasic potassium phosphate in water. Adjust with phosphoric acid to a pH of 2.75 ± 0.25.

System suitability solution: 0.16 mg/mL of USP Selenomethionine RS and 0.8 mg/mL of USP L-Methionine RS in *Mobile phase*

Standard solution: 0.16 mg/mL of USP Selenomethionine RS in *Mobile phase*

Sample solution: 0.16 mg/mL of Selenomethionine in *Mobile phase* with sonication. Pass through a membrane filter of 0.45-µm pore size.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 220 nm
Column: 4.6-mm × 25-cm; packing L1 with polar end-capping
Flow rate: 1 mL/min
Injection size: 20 µL

System suitability
Sample: *System suitability solution*
[NOTE—The relative retention times for methionine and selenomethionine are 0.8 and 1.0, respectively.]
Suitability requirements
Resolution: NLT 3.0 between methionine and selenomethionine
Tailing factor: NMT 2.0 for the selenomethionine peak
Relative standard deviation: NMT 2.0% for the selenomethionine peak

Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of selenomethionine ($C_5H_{11}NO_2Se$) in the portion of Selenomethionine taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP Selenomethionine RS in the *Standard solution* (mg/mL)
C_U = concentration of Selenomethionine in the *Sample solution* (mg/mL)

Acceptance criteria: 97.0%–103.0% on the as-is basis

IMPURITIES

- **LIMIT OF SODIUM**

Standard stock solution: 10 µg/mL of sodium from sodium chloride, previously dried at 105° for 2 h
Standard solutions: 0.2, 0.5, and 1.0 µg/mL of sodium. Pipet 2.0, 5.0, and 10.0 mL of *Standard stock solution* into separate 100-mL volumetric flasks. To each flask add 2.0 mL of potassium chloride solution (1 in 5) and 1.0 mL of hydrochloric acid, and dilute with water to volume.
Sample solution: Transfer 100 mg of Selenomethionine to a 100-mL volumetric flask, add 2.0 mL of potassium chloride solution (1 in 5) and 1.0 mL of hydrochloric acid, and dilute with water to volume.
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Analytical wavelength: 589 nm
Lamp: Sodium hollow-cathode
Flame: Air–acetylene
Analysis
Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions. Plot the absorbances of the *Standard solutions* versus their concentrations (µg/mL of sodium), and draw the straight line best fitting the plotted points. From the graph so obtained, determine the concentration of sodium, C_{Na} (µg/mL), in the *Sample solution*.
Calculate the percentage of sodium in the portion of Selenomethionine taken:

$$\text{Result} = (C_{Na}/C_{SA}) \times F \times 100$$

C_{Na} = concentration of sodium in the *Sample solution* (µg/mL), determined from the regression line
C_{SA} = concentration of Selenomethionine in the *Sample solution* (mg/mL)
F = conversion factor, 0.001 mg/µg

Acceptance criteria: NMT 0.1%

- **CHROMATOGRAPHIC PURITY**

Standard solution A: Transfer 50 mg of USP Selenomethionine RS to a 10-mL volumetric flask, add 2 mL of water, warm to dissolve if necessary, and dilute with methanol to volume.
Standard solution B: Transfer 1.0 mL of *Standard solution A* to a 100-mL volumetric flask, and dilute with methanol to volume.
Sample solution: Transfer 50 mg of Selenomethionine to a 10-mL volumetric flask, add 2 mL of water, warm to dissolve if necessary, and dilute with methanol to volume.

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Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: TLC
Absorbent: 0.25-mm layer of chromatographic silica gel mixture
Application volume: 10 µL
Developing solvent: Butanol, glacial acetic acid, and water (4:1:1)
Spray reagent: 2 mg/mL of ninhydrin in alcohol
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Proceed as directed for *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*. Allow the spots to dry, and develop the chromatogram in the *Developing solvent* until the solvent front has moved three-fourths of the length of the plate. Remove the plate from the developing chamber, mark the solvent front, and allow the solvent to evaporate. Locate the spots on the plate by spraying with *Spray reagent* and drying it at 110° for 10 min.
Acceptance criteria: NMT 1.0%. The R_F value of the principal spot of the *Sample solution* corresponds to that of *Standard solution A*; and no spot, other than the principal spot of the *Sample solution*, is larger or more intense than the principal spot of *Standard solution B*.

SPECIFIC TESTS
• **OPTICAL ROTATION,** *Specific Rotation* ⟨781S⟩
Sample solution: 10 mg/mL in 1 N hydrochloric acid
Acceptance criteria: +17.0° to +19.5°
• **CONTENT OF SELENIUM**
[**CAUTION**—Selenium is toxic; handle it with care.]
Standard stock solution: Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness, add 2 mL of water, and evaporate to dryness. Repeat the addition of water and evaporation to dryness three times. Dissolve the residue in 3 N hydrochloric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume. This solution contains 1000 µg/mL of selenium.
Standard solutions: 20, 50, and 100 µg/mL of selenium. Pipet 2.0, 5.0, and 10.0 mL of *Standard stock solution* into separate 100-mL volumetric flasks. Dilute the contents of each flask with water to volume.
Sample solution: 0.125 mg/mL of Selenomethionine in water
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Analytical wavelength: 196 nm
Lamp: Selenium hollow-cathode
Flame: Air–acetylene
Blank: Water
Analysis
Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions. Plot the absorbances of the *Standard solutions* versus their concentrations (µg/mL of selenium), and draw the straight line best fitting the plotted points. From the graph so obtained, determine the concentration of selenium, C_{Se} (µg/mL), in the *Sample solution*.
Calculate the percentage of selenium in the portion of Selenomethionine taken:

$$\text{Result} = (C_{Se}/C_{SA}) \times F \times 100$$

C_{Se} = concentration of selenium in the *Sample solution* (µg/mL), determined from the regression line
C_{SA} = concentration of Selenomethionine in the *Sample solution* (mg/mL)
F = conversion factor, 0.001 mg/µg

Acceptance criteria: 39.0%–41.0% on the as-is basis

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP L-Methionine RS
USP Selenomethionine RS

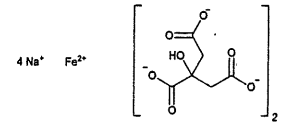
Serine—*see Serine General Monographs*

Ginseng, Siberian—*see Eleuthero*

Slippery Elm—*see Elm General Monographs*

Sodium Ascorbate
—see Sodium Ascorbate General Monographs

Sodium Ferrous Citrate



$C_{12}H_{10}FeNa_4O_{14}$ 526.00
Tetrasodium biscitrato iron (II) [43160-25-4].

DEFINITION
Sodium Ferrous Citrate contains NLT 10.0% and NMT 11.0% of iron (Fe), calculated on the as-is basis.

IDENTIFICATION
• **A. SODIUM**
Sample: Ignite 3 g of Sodium Ferrous Citrate at 500°–600° for 3 h.
Acceptance criteria: The residue obtained from the ignition imparts an intense yellow color to a nonluminous flame.
• **B. IDENTIFICATION TESTS—GENERAL** ⟨191⟩, *Chemical Identification Tests, Iron, A., Ferrous salts*: A solution of 10 mg/mL of Sodium Ferrous Citrate in 5% hydrochloric acid meets the requirements.
• **C. CITRATE**
Sample: 0.5 g of Sodium Ferrous Citrate
Analysis: Transfer the *Sample* to an appropriate container. Add 5 mL of water and 10 mL of 4% potassium hydroxide solution. Heat the solution in a water bath for 10 min while stirring well. Allow the solution to cool to room temperature and filter. Neutralize a portion of the filtrate with 50% acetic acid, add an excessive amount of 7.5% calcium chloride solution and boil. A white crystalline precipitate is formed. Collect the precipitate.
Acceptance criteria: The precipitate formed does not dissolve upon addition of 4% sodium hydroxide solution;

DS Monographs

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the precipitate does dissolve in 25% hydrochloric acid solution.

• **D. Complex Salt of Ferrous Iron and Citric Acids**
Sample solution: 10 mg/mL of Sodium Ferrous Citrate
Analysis: Add 2 mL of stronger ammonia water to 5 mL of the *Sample solution*.
Acceptance criteria: A red-brown color develops with no precipitation.

ASSAY

• **Content of Iron**
Sample: 1 g of Sodium Ferrous Citrate
Blank: Proceed as directed in the *Analysis* without the *Sample*.
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.1 N sodium thiosulfate VS
Endpoint detection: Visual
Analysis: Transfer the *Sample* to a flask with a ground-glass stopper. Carefully add 25 mL of 5% sulfuric acid and 2 mL of nitric acid to the flask, and boil the mixture for 10 min. Allow the mixture to cool to room temperature. Add 20 mL of water and 4 g of potassium iodide, and immediately stopper the flask tightly. Allow the mixture to stand in the dark for 15 min, then add 100 mL of water. Titrate the liberated iodine with *Titrant* (indicator: starch TS). Perform a blank determination.
Calculate the percentage of iron (Fe) in the portion of the *Sample* taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

V_S = *Titrant* volume consumed by the *Sample* (mL)
V_B = *Titrant* volume consumed by the *Blank* (mL)
N = actual *Titrant* normality (mEq/mL)
F = equivalency factor, 55.85 mg/mEq
W = *Sample* weight (mg)

Acceptance criteria: 10.0%–11.0% on the as-is basis

IMPURITIES

• **Chloride and Sulfate** ⟨221⟩, *Chloride*
Standard solution: 0.10 mL of 0.020 N hydrochloric acid
Sample: 73 mg of Sodium Ferrous Citrate
Acceptance criteria: NMT 0.1%

• **Chloride and Sulfate** ⟨221⟩, *Sulfate*
Standard solution: 1.0 mL of 0.20 N sulfuric acid
Sample: 200 mg of Sodium Ferrous Citrate
Acceptance criteria: NMT 0.5%

• **Ferric Iron**
Sample: 2.0 g of Sodium Ferrous Citrate
Analysis: Transfer the *Sample* to an appropriate flask with a ground-glass stopper, dissolve in 5 mL of hydrochloric acid, and dilute with water to 30 mL. Add 4 g of potassium iodide and close the flask with the stopper. Allow the mixture to stand in the dark for 15 min. Add 2 mL of starch TS, and mix well.
Acceptance criteria: A color develops and disappears upon addition of 1.0 mL of 0.1 N sodium thiosulfate VS to the solution.

• **Elemental Impurities—Procedures** ⟨233⟩
Acceptance criteria
Arsenic: NMT 4.0 µg/g
Lead: NMT 10 µg/g
Mercury: NMT 3.0 µg/g

SPECIFIC TESTS

• **Tartrate**
Sample: 1.0 g of Sodium Ferrous Citrate
Analysis: Transfer the *Sample* into an appropriate container. Carefully add 5 mL of water and 10 mL of 6.7% (w/v) potassium hydroxide solution. Heat the mixture in a water bath for 10 min while stirring well. Allow the mixture to cool to room temperature and filter. To 5 mL of the filtrate, add 25% acetic acid solution to make the mixture weakly acidic, and 2 mL of acetic acid. Allow the solution to stand for 24 h.
Acceptance criteria: No white, crystalline precipitate is formed.

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Preserve in well-closed containers.
• **Labeling:** Label it to indicate that it is not to be used if it is coated with brownish yellow basic ferric sulfate.

Powdered Soy Isoflavones Extract

DEFINITION

Powdered Soy Isoflavones Extract is prepared from the seeds of *Glycine max* Merr. (Fam. Fabaceae) by extraction with water or hydroalcoholic mixtures. It contains NLT 90.0% and NMT 110.0% of the labeled amount of isoflavones, calculated on the dried basis as the sum of daidzin, glycitin, genistin, and one or more of the following isoflavones: malonyl daidzin, malonyl glycitin, malonyl genistin, acetyl daidzin, acetyl glycitin, acetyl genistin, daidzein, glycitein, and genistein.

IDENTIFICATION

• **A. HPLC Identification Test**
Analysis: Proceed as directed in the test for *Content of Isoflavones*.
Acceptance criteria: The retention times of the daidzin, glycitin, and genistin peaks from the *Sample solution* correspond to those of *Standard solutions A–E*.

COMPOSITION

• **Content of Isoflavones**
Diluent: Acetonitrile and water (2:3)
Internal standard solution: 2.0 mg/mL USP Apigenin RS in dimethyl sulfoxide. [Note—This solution is stable for 6 months when stored in a tightly closed, light-resistant glass container at room temperature.]
System suitability solution 1: Transfer 1 g of USP Defatted Powdered Soy RS to a centrifuge tube, fitted with PTFE or polyethylene-lined screw caps. Add the following in exact volumes: 0.5 mL of *Internal standard solution*, 10 mL of acetonitrile (swirl to disperse), and 6.0 mL of water. Cap, shake on an orbital or wrist-action shaker for 60 min, add 8.5 mL of water, and centrifuge. Pass a portion of the supernatant through a hydrophilic propylene or PVDF membrane having a 0.45-µm or finer pore size, discarding the first 5 mL of filtrate.
System suitability solution 2: Heat 1 g of USP Defatted Powdered Soy RS in a shallow porcelain dish at 120° for 120 min, and transfer to a centrifuge tube, fitted with PTFE or polyethylene-lined screw caps. Add the following in exact volumes: 0.5 mL of *Internal standard solution*, 10 mL of acetonitrile (swirl to disperse), and 6.0 mL of water. Cap, shake on an orbital or wrist-action shaker for 60 min, add 8.5 mL of water, and centrifuge. Pass a portion of the supernatant through a hydrophilic propylene or PVDF membrane having a 0.45-µm or finer pore size, discarding the first 5 mL of filtrate.
[Note—*Standard stock solution* and *Standard solutions A–E* are stable for 2 months when stored in a tightly

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closed, light-resistant glass container at room temperature.]

Standard stock solution: Contains the following in dimethyl sulfoxide: 2.0 mg/mL of USP Daidzin RS, 0.5 mg/mL of USP Glycitin RS, 2.0 mg/mL of USP Genistin RS, 0.2 mg/mL of USP Daidzein RS, 0.2 mg/mL of USP Glycitein RS, and 0.2 mg/mL of USP Genistein RS

Standard solution A: Add 0.5 mL of *Standard stock solution* and 0.5 mL of *Internal standard solution* to a 25-mL volumetric flask, and dilute with *Diluent* to volume.

Standard solution B: Add 1.0 mL of *Standard stock solution* and 0.5 mL of *Internal standard solution* to a 25-mL volumetric flask, and dilute with *Diluent* to volume.

Standard solution C: Add 1.5 mL of *Standard stock solution* and 0.5 mL of *Internal standard solution* to a 25-mL volumetric flask, and dilute with *Diluent* to volume.

Standard solution D: Add 2.0 mL of *Standard stock solution* and 0.5 mL of *Internal standard solution* to a 25-mL volumetric flask, and dilute with *Diluent* to volume.

Standard solution E: Add 2.5 mL of *Standard stock solution* and 0.5 mL of *Internal standard solution* to a 25-mL volumetric flask, and dilute with *Diluent* to volume.

Sample solution: Transfer a quantity of Powdered Soy Isoflavones Extract, equivalent to NMT 5 mg of isoflavones, to a 30-mL glass centrifuge tube, fitted with a PTFE or polyethylene-lined screw cap. Add the following in exact volumes: 0.5 mL of *Internal standard solution*, 10 mL of acetonitrile (swirl to disperse), and 6.0 mL of water. Cap, shake on an orbital or wrist-action shaker for 60 min, add 8.5 mL of water, and centrifuge. Pass a portion of the supernatant through a hydrophilic propylene or PVDF membrane having a 0.45-µm or finer pore size, discarding the first 5 mL of filtrate. [NOTE—Do not use nylon filters. Analyze samples containing significant amounts of acetyl and/or malonyl isoflavones within 4 h of preparation.]

Solution A: 0.05% phosphoric acid in water

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	90	10
60	70	30
60.5	10	90
63.5	10	90
64	90	10
74	90	10

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 260 nm
Column: 3.0-mm × 25-cm; 5-µm packing L1
Column temperature: 40°
Flow rate: 0.65 mL/min
Injection size: 5 µL
[NOTE—*System suitability 1* and *System suitability 2* must both be met.]

System suitability 1
Sample: *Standard solution C*
Suitability requirements
Chromatogram similarity: The chromatogram from *Standard solution C* is similar to the refence chromatogram provided with the lot of USP reference standard being used for the isoflavones mentioned.
Tailing factor: NLT 0.8 and NMT 1.2 for the daidzin peak
Relative standard deviation: NMT 2.0% for the genistin peak

System suitability 2
Samples: *System suitability solution 1* and *System suitability solution 2*
Suitability requirements
Chromatogram similarity: The chromatograms from *System suitability solution 1* and *System suitability solution 2* are similar to those provided with the lot of USP Defatted Powdered Soy RS being used.
Resolution: NLT 1.0 between the acetyl glycitin and malonyl genistin peaks, and NLT 2.0 between any other consecutive pair of isoflavone peaks

Analysis
Samples: *Standard solutions A–E* and *Sample solution*
Measure the peak areas of the analytes and the internal standard. Determine the ratio of the peak areas of each analyte to the internal standard peak area. Plot the ratios of the relevant peak responses versus the concentrations, in mg/mL, of each analyte obtained from the *Standard solutions A–E*, and determine the regression line by least-squares analysis. The correlation coefficient for each of the regression lines is NLT 0.999. From the graphs so obtained, determine the concentration, *C*, in mg/mL, of the relevant analyte in the *Sample solution*.
Separately calculate the percentages of daidzin, glycitin, and genistin, and of daidzein, glycitein, and genistein, if present, in the portion of Powdered Soy Isoflavones Extract taken:

$$\text{Result} = (C/W) \times V \times 100$$

C = concentration of each isoflavone as determined above, for the *Sample solution* (mg/mL)
W = weight of Powdered Soy Isoflavones Extract taken to prepare the *Sample solution* (mg)
V = final solution volume of the *Sample solution* (mL)

Identify the peaks of malonyl daidzin, malonyl glycitin, acetyl daidzin, acetyl glycitin, malonyl genistin, and acetyl genistin by comparison with the reference chromatograms provided with USP Defatted Powdered Soy RS and by the difference in peak abundance between *System suitability solution 1* and *System suitability solution 2*. [NOTE—Malonyl derivates are converted into acetyl derivatives by heat. Malonyl derivatives are more abundant in *System suitability solution 1* and acteyl derivatives are more abundant in *System suitability solution 2*.] From the graphs obtained for daidzin, glycitin, and genistin, determine the corresponding concentration, C, in mg/mL, of the malonyl and acetyl derivatives, if present, in the *Sample solution*.
Separately calculate the percentages of malonyl daidzin, acetyl daidzin, malonyl glycitin, acetyl glycitin, malonyl genistin, and acetyl genistin in the portion of Powdered Soy Isoflavones Extract taken:

$$\text{Result} = (C/W) \times V \times F \times 100$$

C = concentration of each isoflavone as determined above, for the *Sample solution* (mg/mL)
W = weight of Powdered Soy Isoflavones Extract taken to prepare the *Sample solution* (mg)
V = final solution volume of the *Sample solution* (mL)
F = conversion factor: malonyl daidzin, 1.207; acetyl daidzin, 1.101; malonyl glycitin, 1.193; acetyl

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glycitin, 1.094; malonyl genistin, 1.199; and acetyl genistin, 1.097

Calculate the percentage of isoflavones in the portion of the Powdered Soy Isoflavones Extract taken (*P*) by adding the percentages calculated for all analytes present.
Calculate the percentage of the labeled amount of isoflavones in the portion of the Powdered Soy Isoflavones Extract taken:

$$\text{Result} = (P/L) \times 100$$

P = sum of the individual percentages of each isoflavone in the Powdered Soy Isoflavones Extract taken, as calculated above (%)
L = labeled amount of isoflavones in the Powdered Soy Isoflavones Extract (%)

Acceptance criteria: 90.0%–110.0% on the dried basis

CONTAMINANTS
- **ARTICLES OF BOTANICAL ORIGIN,** *Test for Aflatoxins* ⟨561⟩**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count is NMT 10^4 cfu/g, and the total combined molds and yeasts count is NMT 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli.*

SPECIFIC TESTS
- **LOSS ON DRYING** ⟨731⟩**:** Dry 1 g at 130° for 2 h: it loses NMT 7.0% of its weight.
- **OTHER REQUIREMENTS:** It meets the requirements for *Residual Solvents* and *Pesticide Residues* in *Botanical Extracts* ⟨565⟩.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. The label also indicates the content of isoflavones. It meets other labeling requirements in *Botanical Extracts* ⟨565⟩.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Apigenin RS
  USP Daidzein RS
  USP Daidzin RS
  USP Defatted Powdered Soy RS
  USP Genistein RS
  USP Genistin RS
  USP Glycitein RS
  USP Glycitin RS

Soy Isoflavones Capsules

DEFINITION
Soy Isoflavones Capsules contain Powdered Soy Isoflavones Extract. Capsules contain NLT 90.0% and NMT 110.0% of the labeled amount of Extract, represented by the sum of the content of the isoflavones daidzin, glycitin, genistin, and one or more of the following isoflavones: malonyl daidzin, malonyl glycitin, malonyl genistin, acetyl daidzin, acetyl glycitin, acetyl genistin, daidzein, glycitein, and genistein.

IDENTIFICATION
- The retention times of the daidzin, glycitin, and genistin peaks in the chromatogram of the *Sample solution* correspond to those of *Standard solutions A–E*, as obtained in the test for *Content of Isoflavones.*

STRENGTH
- **CONTENT OF ISOFLAVONES**

Diluent: Acetonitrile and water (2:3)
Internal standard solution: 2.0 mg/mL of USP Apigenin RS in dimethyl sulfoxide. [NOTE—This solution is stable for 6 months when stored in a tightly closed, light-resistant glass container at room temperature.]
Malonyl/Acetyl isoflavones retention times check solution 1: Heat 1 g of USP Defatted Powdered Soy RS in a shallow porcelain dish at 120° for 120 min, and transfer to a centrifuge tube, fitted with PTFE or polyethylene-lined screw cap. Add the following in exact volumes: 0.5 mL of *Internal standard solution*, 10 mL of acetonitrile (swirl to disperse), and 6.0 mL of water. Cap, shake on an orbital or wrist-action shaker for 60 min, add 8.5 mL of water, mix, and centrifuge. Pass a portion of the supernatant through a hydrophilic propylene or PVDF membrane of 0.45-µm or finer pore size, discarding the first 5 mL of filtrate.
Malonyl/Acetyl isoflavones retention times check solution 2: Transfer 1 g of USP Defatted Powdered Soy RS to a centrifuge tube, fitted with PTFE or polyethylene-lined screw cap. Add the following in exact volumes: 0.5 mL of *Internal standard solution*, 10 mL of acetonitrile (swirl to disperse), and 6.0 mL of water. Cap, shake on an orbital or wrist-action shaker for 60 min, add 8.5 mL of water, mix, and centrifuge. Pass a portion of the supernatant through a hydrophilic propylene or PVDF membrane of 0.45-µm or finer pore size, discarding the first 5 mL of filtrate.
[NOTE—All *Standard solutions* and *Standard stock solutions* are stable for 2 months when stored in a tightly closed, light-resistant glass container at room temperature.]
Standard stock solution: Contains the following in dimethyl sulfoxide: 2.0 mg/mL of USP Daidzin RS, 0.5 mg/mL of USP Glycitin RS, and 2.0 mg/mL of USP Genistin RS, 0.2 mg/mL of USP Daidzein RS, 0.2 mg/mL of USP Glycitein RS, and 0.2 mg/mL of USP Genistein RS
Standard solution A: Add 0.5 mL of *Standard stock solution* and 0.5 mL of *Internal standard solution* to a 25-mL volumetric flask, and dilute with *Diluent* to volume.
Standard solution B: Add 1.0 mL of *Standard stock solution* and 0.5 mL of *Internal standard solution* to a 25-mL volumetric flask, and dilute with *Diluent* to volume.
Standard solution C: Add 1.5 mL of *Standard stock solution* and 0.5 mL of *Internal standard solution* to a 25-mL volumetric flask, and dilute with *Diluent* to volume.
Standard solution D: Add 2.0 mL of *Standard stock solution* and 0.5 mL of *Internal standard solution* to a 25-mL volumetric flask, and dilute with *Diluent* to volume.
Standard solution E: Add 2.5 mL of *Standard stock solution* and 0.5 mL of *Internal standard solution* to a 25-mL volumetric flask, and dilute with *Diluent* to volume.
Sample solution
Hard-gelatin Capules: Weigh and finely powder the content of NLT 20 Capsules. Transfer an accurately weighed quantity of the powder, equivalent to NMT 5 mg of isoflavones, to a suitable glass centrifuge tube, fitted with PTFE or polyethylene-lined screw cap.
Soft-gelatin Capsules: Weigh and homogenize NLT 20 Capsules. Transfer an accurately weighed quantity of the mixture, equivalent to NMT 5 mg of isoflavones, to a suitable glass centrifuge tube, fitted with PTFE or polyethylene-lined screw cap.
Add the following in exact volumes to the centrifuge tube: 0.5 mL of *Internal standard solution*, 10 mL of acetonitrile (swirl to disperse), and 6.0 mL of water. Cap, shake on an orbital or wrist-action shaker for 60 min, add 8.5 mL of water, mix, and centrifuge. Pass a portion of the

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supernatant through a hydrophilic propylene or PVDF membrane of 0.45-µm or finer pore size, discarding the first 5 mL of filtrate. [NOTE—Do not use nylon filters. Analyze samples containing significant amounts of acetyl and/or malonyl isoflavones within 4 h of preparation.]

Solution A: 0.05% phosphoric acid in water

Solution B: Acetonitrile

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	90	10
60	70	30
60.5	10	90
63.5	10	90
64	90	10
74	90	10

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 260 nm

Column: 3.0-mm × 25-cm; 5-µm packing L1

Column temperature: 40°

Flow rate: 0.65 mL/min

Injection size: 5 µL

System suitability

Samples: *Standard solution C, Malonyl/Acetyl isoflavones retention times check solution 1,* and *Malonyl/Acetyl isoflavones retention times check solution 2*

Suitability requirements

Chromatogram similarity: The chromatograms obtained from *Standard solution C, Malonyl/Acetyl isoflavones retention times check solution 1,* and *Malonyl/Acetyl isoflavones retention times check solution 2* are similar to the Reference chromatograms provided with the USP Defatted Powdered Soy RS.

Tailing factor: NLT 0.8 and NMT 1.2 for the daidzin peak, *Standard solution C*

Relative standard deviation: NMT 2.0%, for the genistin peak, in repeated injections, *Standard solution C*

Resolution: NLT 1.0 between acetyl glycitin and malonyl genistin, and NLT 2.0 between any other consecutive pair of isoflavone peaks, *Malonyl/Acetyl isoflavones retention times check solution 2*

Analysis

Samples: *Standard solution A, Standard solution B, Standard solution C, Standard solution D, Standard solution E, Malonyl/Acetyl isoflavones retention times check solution 1, Malonyl/Acetyl isoflavones retention times check solution 2,* and *Sample solution*

Identify the peaks of daidzin, glycitin, genistin, daidzein, glycitein, and genistein in the chromatograms of *Standard solutions A–E* by comparison with the Reference Standard Chromatogram provided with the appropriate USP Reference Standard used. Measure the peak areas of the analytes and the internal standard peaks. Determine the ratio of the peak areas of each analyte to the internal standard peak areas. Plot the ratios of the relevant peak responses versus the concentrations, in mg/mL, of each analyte obtained from *Standard solutions A–E,* and determine the regression line by least-squares analysis. The correlation coefficient for each of the regression lines is NLT 0.999. From the graphs obtained, determine the concentration, C, in mg/mL, of daidzin, glycitin, and genistin, in addition to daidzein, glycitein, and genistein, if present, in the *Sample solution.* Identify the peaks of malonyl daidzin, malonyl glycitin, acetyl daidzin, acetyl glycitin, malonyl genistin, and acetyl genistin in the chromatograms of the *Malonyl/Acetyl isoflavones retention times check solutions* by comparison with the Reference Chromatograms provided with the USP Defatted Powdered Soy RS. From the graphs obtained for daidzin, glycitin and genistin, determine the concentration, C, in mg/mL, of the corresponding malonyl and acetyl derivatives, if present, in the *Sample solution*:

$$\text{Result} = C_o \times F$$

C_o = concentration obtained from the relevant graph (mg/mL)

F = conversion factor for each analyte (1.207 for malonyl daidzin, 1.101 for acetyl daidzin, 1.193 for malonyl glycitin, 1.094 for acetyl glycitin, 1.199 for malonyl genistin, and 1.097 for acetyl genistin)

Calculate the quantity, T, in mg, of total isoflavones in the portion of Capsules contents taken:

$$\text{Result} = V \times C_T$$

V = final volume of the *Sample solution* (mL)

C_T = sum of concentrations (C) (mg/mL) of all relevant isoflavones

Calculate the percentage of Powdered Soy Isoflavones Extract with respect to the label claim:

$$\text{Result} = T \times (A_{WT}/W) \times (100/L_E) \times (100/L)$$

T = content of total isoflavones in the portion of Capsules contents taken (mg)

A_{WT} = average weight of Capsules contents (mg/Capsule)

W = weight of the portion of Capsules contents taken (mg)

L_E = content of total isoflavones, mg, in 100 mg of the Extract used to prepare the Capsules

L = amount of Extract per Capsule according to label claim (mg/Capsule)

Acceptance criteria: NLT 90.0% and NMT 110.0% of the labeled amount of Extract, represented by the sum of the content of the isoflavones daidzin, glycitin, genistin and one or more of the following isoflavones: malonyl daidzin, malonyl glycitin, malonyl genistin, acetyl daidzin, acetyl glycitin, acetyl genistin, daidzein, glycitein, and genistein

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** ⟨2040⟩**:** Meet the requirements for *Disintegration*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩**:** Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count is NMT 10^4 cfu/g; and the total combined molds and yeasts count is NMT 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Capsules meet the requirements of the tests for absence of *Salmonella* species and *Escherichia coli.*

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ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the article from which the Capsules were prepared. Label it to indicate the amount of Extract, in mg, per Capsule. Label it to indicate the percentage of isoflavones in the Extract used to prepare the Capsules.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Apigenin RS
  USP Daidzein RS
  USP Daidzin RS
  USP Defatted Powdered Soy RS
  USP Genistein RS
  USP Genistin RS
  USP Glycitein RS
  USP Glycitin RS

Soy Isoflavones Tablets

DEFINITION

Soy Isoflavones Tablets contain Powdered Soy Isoflavones Extract. Tablets contain NLT 90.0% and NMT 110.0% of the labeled amount of Extract, represented by the sum of the content of the isoflavones daidzin, glycitin, genistin and one or more of the following isoflavones: malonyl daidzin, malonyl glycitin, malonyl genistin, acetyl daidzin, acetyl glycitin, acetyl genistin, daidzein, glycitein, and genistein.

IDENTIFICATION

- The retention times of the daidzin, glycitin, and genistin peaks in the chromatogram of the *Sample solution* correspond to those of *Standard solutions A–E*, as obtained in the test for *Content of Isoflavones*.

STRENGTH

- **CONTENT OF ISOFLAVONES**

Diluent: Acetonitrile and water (2:3)

Internal standard solution: 2.0 mg/mL of USP Apigenin RS in dimethyl sulfoxide. [NOTE—This solution is stable for 6 months when stored in a tightly closed, light-resistant glass container at room temperature.]

Malonyl/Acetyl isoflavones retention times check solution 1: Heat 1 g of USP Defatted Powdered Soy RS in a shallow porcelain dish at 120° for 120 min, and transfer to a centrifuge tube, fitted with PTFE or polyethylene-lined screw cap. Add the following in exact volumes: 0.5 mL of *Internal standard solution*, 10 mL of acetonitrile (swirl to disperse), and 6.0 mL of water. Cap, shake on an orbital or wrist-action shaker for 60 min, add 8.5 mL of water, mix, and centrifuge. Pass a portion of the supernatant through a hydrophilic propylene or PVDF membrane of 0.45-µm or finer pore size, discarding the first 5 mL of filtrate.

Malonyl/Acetyl isoflavones retention times check solution 2: Transfer 1 g of USP Defatted Powdered Soy RS to a centrifuge tube, fitted with PTFE or polyethylene-lined screw cap. Add the following in exact volumes: 0.5 mL of *Internal standard solution*, 10 mL of acetonitrile (swirl to disperse), and 6.0 mL of water. Cap, shake on an orbital or wrist-action shaker for 60 min, add 8.5 mL of water, mix, and centrifuge. Pass a portion of the supernatant through a hydrophilic propylene or PVDF membrane of 0.45-µm or finer pore size, discarding the first 5 mL of filtrate.

[NOTE—All *Standard solutions* and *Standard stock solutions* are stable for 2 months when stored in a tightly closed, light-resistant glass container at room temperature.]

Standard stock solution: Contains the following in dimethyl sulfoxide: 2.0 mg/mL of USP Daidzin RS, 0.5 mg/mL of USP Glycitin RS, 2.0 mg/mL of USP Genistin RS, 0.2 mg/mL of USP Daidzein RS, 0.2 mg/mL of USP Glycitein RS, and 0.2 mg/mL of USP Genistein RS

Standard solution A: Add 0.5 mL of *Standard stock solution* and 0.5 mL of *Internal standard solution* to a 25-mL volumetric flask, and dilute with *Diluent* to volume.

Standard solution B: Add 1.0 mL of *Standard stock solution* and 0.5 mL of *Internal standard solution* to a 25-mL volumetric flask, and dilute with *Diluent* to volume.

Standard solution C: Add 1.5 mL of *Standard stock solution* and 0.5 mL of *Internal standard solution* to a 25-mL volumetric flask, and dilute with *Diluent* to volume.

Standard solution D: Add 2.0 mL of *Standard stock solution* and 0.5 mL of *Internal standard solution* to a 25-mL volumetric flask, and dilute with *Diluent* to volume.

Standard solution E: Add 2.5 mL of *Standard stock solution* and 0.5 mL of *Internal standard solution* to a 25-mL volumetric flask, and dilute with *Diluent* to volume.

Sample solution: Accurately weigh and finely powder NLT 20 Tablets. Transfer an accurately weighed quantity of the powder, equivalent to NMT 5 mg of isoflavones, to a 30-mL glass centrifuge tube, fitted with a PTFE or polyethylene-lined screw cap. Add the following in exact volumes: 0.5 mL of *Internal standard solution*, 10 mL of acetonitrile (swirl to disperse), and 6.0 mL of water. Cap, shake on an orbital or wrist-action shaker for 60 min, add 8.5 mL of water, mix, and centrifuge. Pass a portion of the supernatant through a hydrophilic propylene or PVDF membrane of 0.45-µm or finer pore size, discarding the first 5 mL of filtrate. [NOTE—Do not use nylon filters. Analyze samples containing significant amounts of acetyl and/or malonyl isoflavones within 4 h of preparation.]

Solution A: 0.05% phosphoric acid in water

Solution B: Acetonitrile

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	90	10
60	70	30
60.5	10	90
63.5	10	90
64	90	10
74	90	10

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 260 nm
Column: 3.0-mm × 25-cm; 5-µm packing L1
Column temperature: 40°
Flow rate: 0.65 mL/min
Injection size: 5 µL

System suitability
Samples: *Standard solution C, Malonyl/Acetyl isoflavones retention times check solution 1*, and *Malonyl/Acetyl isoflavones retention times check solution 2*
Suitability requirements
Chromatogram similarity: The chromatograms obtained from *Standard solution C, Malonyl/Acetyl isoflavones retention times check solution 1*, and *Malonyl/Acetyl isoflavones retention times check solution 2* are similar to the Reference Chromatograms provided with the USP Defatted Powdered Soy RS.

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Tailing factor: NLT 0.8 and NMT 1.2 for the daidzin peak, *Standard solution C*

Relative standard deviation: NMT 2.0%, for the genistin peak, in repeated injections, *Standard solution C*

Resolution: NLT 1.0 between acetyl glycitin and malonyl genistin, and NLT 2.0 between any other consecutive pair of isoflavone peaks, *Malonyl/Acetyl isoflavones retention times check solution 2*

Analysis

Samples: *Standard solution A, Standard solution B, Standard solution C, Standard solution D, Standard solution E, Malonyl/Acetyl isoflavones retention times check solution 1, Malonyl/Acetyl isoflavones retention times check solution 2,* and *Sample solution*

Identify the peaks of daidzin, glycitin, genistin, daidzein, glycitein, and genistein in the chromatograms of *Standard solutions A–E* by comparison with the Reference Standard Chromatogram provided with the appropriate USP Reference Standard used. Measure the peak areas of the analytes and the internal standard peaks. Determine the ratio of the peak areas of each analyte to the internal standard peak areas. Plot the ratios of the relevant peak responses versus the concentrations, in mg/mL, of each analyte obtained from *Standard solutions A–E,* and determine the regression line by least-squares analysis. The correlation coefficient for each of the regression lines is NLT 0.999. From the graphs obtained, determine the concentration, C, in mg/mL, of daidzin, glycitin, and genistin, in addition to daidzein, glycitein, and genistein, if present, in the *Sample solution.* Identify the peaks of malonyl daidzin, malonyl glycitin, acetyl daidzin, acetyl glycitin, malonyl genistin, and acetyl genistin in the chromatograms of the *Malonyl/Acetyl isoflavones retention times check solutions* by comparison with the Reference Chromatograms provided with the USP Defatted Powdered Soy RS. From the graphs obtained for daidzin, glycitin, and genistin, determine the concentration, C, in mg/mL, of the corresponding malonyl and acetyl derivatives, if present, in the *Sample solution*:

$$\text{Result} = C_o \times F$$

C_o = concentration obtained from the relevant graph (mg/mL)

F = conversion factor for each analyte (1.207 for malonyl daidzin, 1.101 for acetyl daidzin, 1.193 for malonyl glycitin, 1.094 for acetyl glycitin, 1.199 for malonyl genistin, and 1.097 for acetyl genistin)

Calculate the quantity, T, in mg, of total isoflavones in the portion of Tablets taken:

$$\text{Result} = V \times C_T$$

V = final volume of the *Sample solution* (mL)

C_T = sum of concentrations (C) (mg/mL) of all relevant isoflavones

Calculate the percentage of Powdered Soy Isoflavones Extract with respect to the label claim:

$$\text{Result} = T \times (A_{WT}/W) \times (100/L_E) \times (100/L)$$

T = content of total isoflavones in the portion of Tablets taken (mg)

A_{WT} = average weight of Tablets (mg/Tablet)

W = weight of the portion of Tablets taken (mg)

L_E = content of total isoflavones, mg, in 100 mg of the Extract used to prepare the Tablets

L = amount of Extract per Tablet according to label claim (mg/Tablet)

Acceptance criteria: NLT 90.0% and NMT 110.0% of the labeled amount of Extract, represented by the sum of the content of the isoflavones daidzin, glycitin, genistin and one or more of the following isoflavones: malonyl daidzin, malonyl glycitin, malonyl genistin, acetyl daidzin, acetyl glycitin, acetyl genistin, daidzein, glycitein, and genistein

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** ⟨2040⟩**:** Meet the requirements for *Disintegration*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩**:** Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count is NMT 10^4 cfu/g; and the total combined molds and yeasts count is NMT 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Tablets meet the requirements of the tests for absence of *Salmonella* species and *Escherichia coli.*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the article from which the Tablets were prepared. Label it to indicate the amount of Extract, in mg, per Tablet. Label it to indicate the content, in percentage, of isoflavones in the Extract used to prepare the Tablets.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Apigenin RS
  USP Daidzein RS
  USP Daidzin RS
  USP Defatted Powdered Soy RS
  USP Genistein RS
  USP Genistin RS
  USP Glycitein RS
  USP Glycitin RS

Spirulina

DEFINITION

Spirulina consists of the dried, whole, blue-green microalgae *Arthrospira platensis* (Nordstedt) Gomont, synonym *Spirulina platensis* (Nordstedt) Geitler; *Arthrospira maxima* Setchell & Gardner, synonym *Spirulina maxima* (Setchell & Gardner) Geitler in Rabenhorst (illegitimate); or *Arthrospira fusiformis* (Voronichin) J. Komarek & J.W.G. Lund, synonym *Spirulina fusiformis* Voronichin.

IDENTIFICATION

- **A.** Spirulina meets the requirements in *Specific Tests, Description* for *Macroscopic* and *Microscopic characteristics.*
- **B. FATTY ACIDS PROFILE**

Methyl oleate solution: 1 mg/mL of USP Methyl Oleate RS in *n*-heptane

Standard solution: Dissolve quantities of USP Methyl Palmitate RS, USP Methyl Palmitoleate RS, USP Methyl Stearate RS, USP Methyl Oleate RS, USP Methyl Linoleate RS, USP Methyl Linolenate RS (methyl alpha linolenate), and methyl gamma linolenate (obtained

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commercially) in *n*-heptane to obtain concentrations of each methyl ester as given in *Table 1*.

Table 1

Methyl Ester	Relative Retention Time	Concentration (mg/mL)
Methyl palmitate	0.76	0.8
Methyl palmitoleate	0.79	0.8
Methyl stearate	0.97	0.8
Methyl oleate	1.00	0.8
Methyl linoleate	1.06	0.8
Methyl gamma linolenate	1.12	0.8
Methyl alpha linolenate	1.18	0.8

Sample solution: Transfer about 100 mg of Spirulina to a 50-mL round-bottom flask, fitted with a water-cooled reflux condenser and a magnetic stir bar. Add 100 mg of pyrogallic acid, 10 mL of 0.5 N methanolic sodium hydroxide solution (20 mg/mL of sodium hydroxide in methanol), and heat the flask while stirring under reflux for 15 min. Add 5 mL of 14% boron trifluoride–methanol through the reflux condenser into the flask, and continue boiling for 2 more min. Add 5.0 mL of *n*-heptane through the condenser, and continue boiling for an additional 1 min. Cool the flask, remove the condenser, and add 15 mL of saturated sodium chloride solution. While the solution is still tepid, insert a stopper into the flask, shake vigorously, and allow the layers to separate. Add sufficient saturated sodium chloride solution to bring the upper *n*-heptane layer up into the neck of the flask. Transfer the *n*-heptane layer into a suitable container, add a small quantity of anhydrous sodium sulfate (previously washed with *n*-heptane), and shake. [NOTE—Fatty acids methyl esters should be analyzed as soon as possible. They may be kept in an atmosphere of nitrogen at 2° for 24 h.]

Blank: Use *n*-heptane.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.25-mm × 30-m fused silica capillary; 0.25-µm film of phase G16 coating

Temperatures

Injector: 220°

Detector: 260°

Column: See *Table 2*.

Table 2

Initial Temperature (°)	Hold Time at 170° (min)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
170	2	5	240	5

Carrier gas: Helium

Flow rate: 1 mL/min

Split ratio: 100:1. [NOTE—If necessary, adjust the *Split ratio* and/or sample dilution to obtain a tailing factor of 0.8–1.5 for the fatty acid methyl ester peaks.]

Injection volume: 1 µL

System suitability

Samples: *Methyl oleate solution* and *Standard solution*

Suitability requirements

Resolution: NLT 1.5 between methyl stearate and methyl oleate peaks

Relative standard deviation: NMT 6.0% for the peak responses of each of the methyl palmitate and methyl stearate peaks for replicate injections; NMT 1.0% for the peak response ratio of the methyl palmitate to methyl stearate peaks for replicate injections

Analysis

Samples: *Methyl oleate solution*, *Standard solution*, *Sample solution*, and *Blank*

Identify the fatty acid ester peaks in the chromatogram of the *Sample solution* based on the relative retention times given in *Table 1* and by comparison to the chromatograms of the *Methyl oleate solution* and the *Standard solution*. Measure the peak response for all of the fatty acid ester peaks in the chromatogram from the *Sample solution*.

Calculate the percentage of each fatty acid component in the portion of Spirulina taken:

$$\text{Result} = (A/B) \times 100$$

A = peak response of individual fatty acid ester from the *Sample solution*

B = sum of responses of all peaks, excluding the solvent, from the *Sample solution*

Acceptance criteria: See *Table 3* for individual fatty acids.

Table 3

Fatty Acids	Percentage of Total (%)
Palmitic acid	35–60
Palmitoelic acid	2–8
Stearic acid	1–5
Oleic acid	1–7
Linoleic acid	13–25
Gamma linolenic acid	13–27
Alpha linolenic acid	<0.5

• **C. HPLC FOR CHLOROPHYLL A**

Analysis: Proceed as directed in the test for *Content of Beta Carotene and Total Carotenoids*, except analyze the *Sample solution* and *Standard solution* at 432 nm.

Acceptance criteria: The major peak in the *Sample solution* chromatogram is due to chlorophyll A, at a relative retention time of about 0.8 to the peak of all-*trans*-beta carotene.

• **D. HPLC FOR CAROTENOIDS**

Analysis: Proceed as directed in the test for *Content of Beta Carotene and Total Carotenoids*.

Acceptance criteria: The major peak from the *Sample solution* corresponds to that of all-*trans*-beta carotene from the *Standard solution*. The *Sample solution* chromatogram exhibits additional peaks due to oscillol rhamnoside, myxol rhamnoside, zeaxanthin, alloxanthin, echinenone, beta cryptoxanthin, and *cis*-isomers of beta carotene as shown in *Table 5*. [NOTE—The *cis*-beta carotene peaks may partially be resolved from the all-*trans*-beta carotene peak.]

COMPOSITION

• **CONTENT OF BETA CAROTENE AND TOTAL CAROTENOIDS**

[NOTE—It is important to have all glassware ready before starting the test. Minimize exposure of the

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Standard solution and *Sample solution* to light. Proceed under subdued light using low-actinic glassware at a room temperature not to exceed 25°. The *Standard solution* and *Sample solution* are stable for 12 h.]

Solution A: A mixture of a solution of 0.05% butylated hydroxytoluene in methanol and water (9:1). Allow the mixture to equilibrate to room temperature before adjusting to final volume with the solution of 0.05% butylated hydroxytoluene in methanol.

Solution B: A mixture of a solution of 0.05% butylated hydroxytoluene in methanol and methylene chloride (88:12). Allow the mixture to equilibrate to room temperature before adjusting to final volume with the solution of 0.05% butylated hydroxytoluene in methanol.

Mobile phase: See *Table 4*.

Table 4

Time (min)	Solution A (%)	Solution B (%)
0	95	5
1.0	95	5
2.5	0	100
10.75	0	100
11.00	95	5
16	95	5

Diluent: 50 µg/mL of butylated hydroxytoluene in methanol

Standard stock solution: 500 µg/mL of USP Beta Carotene RS in methylene chloride. [NOTE—Prepare fresh at the time of use, and minimize the time before it is used to prepare the *Standard solution*.]

Standard solution: 2.5 µg/mL of USP Beta Carotene RS in *Diluent* from *Standard stock solution*.

Determine the concentration of the *Standard solution* as follows.

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Mode: Visible

Analytical wavelength: 450 nm

Cell path: 1 cm

Blank: *Diluent*

Take three measurements using three different portions of the *Standard solution*.

Calculate the concentration of beta carotene (µg/mL) in the *Standard solution*:

$$C_S = (A/F) \times (P/100)$$

A = average absorbance of the three portions of *Standard solution*

F = one thousandth of the absorptivity value of beta carotene in *Diluent*, 0.25 mL · µg^{-1} · cm^{-1}

P = percentage of beta carotene relative to total carotenoids in the *Standard solution* as determined below in the *Analysis*

Sample solution: [NOTE—It may be necessary to grind Spirulina into a fine powder using a pestle and mortar for the extraction to be exhaustive.]

Transfer about 30 mg of Spirulina, accurately weighed, into a centrifuge tube, add 3 mL of dimethyl sulfoxide, and a few glass beads. Mix on a vortex mixer to disperse the sample. For 15 min, repeat the process of immersing in a water bath maintained at a temperature of 45° and mixing on a vortex mixer. Remove the tube from the water bath, and centrifuge at about 4000 rpm for 3 min. Transfer the supernatant to a 25-mL volumetric flask. To the residue, add 3 mL of methanol, mix on a vortex mixer, centrifuge at about 4000 rpm for 3 min, and transfer the supernatant to the volumetric flask. Repeat until the last methanol extract is colorless. Dilute with methanol to volume, and mix. If particles are still present in the final extract, centrifuge a portion at about 4000 rpm for 3 min, and use the supernatant.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 476 nm

Columns

Guard: 2.0-mm × 4-cm; 5-µm, 300 Å, packing L1

Analytical: 2.0-mm × 15-cm; 5-µm, 300 Å, packing L1

Column temperature: 25°

Flow rate: 0.5 mL/min

Injection volume: 50 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Inject the *Standard solution* repeatedly (about 2–3 times) until a consistent retention time, within ± 0.2 min, is obtained.

Relative standard deviation: NMT 2.0% for beta carotene peak

Analysis

Samples: *Standard solution* and *Sample solution*

Using the chromatogram of the *Standard solution*, identify the peak corresponding to all-*trans*-beta carotene in the *Sample solution* chromatogram. Identify the peaks corresponding to oscillol rhamnoside, myxol rhamnoside, zeaxanthin, alloxanthin, echinenone, beta cryptoxanthin, and *cis*-isomers of beta carotene in the *Sample solution* using the relative retention times provided in *Table 5*.

Table 5

Carotenoids	Relative Retention Time
Oscillol rhamnoside	0.15
Myxol rhamnoside	0.19
Zeaxanthin	0.29
Alloxanthin	0.53
Echinenone	0.60
Beta cryptoxanthin	0.64
All-*trans*-beta carotene	1.00
9-*cis*-Beta carotene	1.02
13-*cis*-Beta carotene	1.04

The *cis*-beta carotene peaks may partially be resolved from the all-*trans*-beta carotene peak. In this case, peak integration should be done as a single peak that includes all the isomer peaks.

Calculate the percentage (P) of beta carotene (sum of all the beta carotene isomers) relative to total carotenoids in the *Standard solution*:

$$P = (r_U/r_T) \times 100$$

r_U = peak response of beta carotene from the *Standard solution*

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r_T = sum of peak responses of all carotenoids detected from the *Standard solution*, including that of beta carotene

Calculate the percentage of beta carotene in the portion of Spirulina taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak response of beta carotene from the *Sample solution*
r_S = peak response of beta carotene from the *Standard solution*
C_S = concentration of beta carotene in the *Standard solution*, as determined above under *Standard solution* (µg/mL)
V = final volume of *Sample solution* (mL)
W = weight of Spirulina used to prepare the *Sample solution* (µg)

Calculate the percentage of total carotenoids in the portion of Spirulina taken:

$$\text{Result} = (\Sigma r_U/r_S) \times C_S \times (V/W) \times 100$$

Σr_U = sum of peak responses for all carotenoids from the *Sample solution*
r_S = peak response of beta carotene from the *Standard solution*
C_S = concentration of beta carotene in the *Standard solution*, as determined above under *Standard solution* (µg/mL)
V = final volume of *Sample solution* (mL)
W = weight of Spirulina used to prepare the *Sample solution* (µg)

Acceptance criteria: NLT 0.15% of beta carotene and NLT 0.35% of total carotenoids, both calculated on the dried basis

• **CONTENT OF C-PHYCOCYANIN**

Buffer solution: Prepare a solution having a pH of 7, containing 13.9 g/L of monobasic sodium phosphate, 26.81 g/L of dibasic sodium phosphate, and 0.05% of sodium azide.

Sample solution: Transfer about 100 mg of Spirulina, accurately weighed, into a centrifuge tube, and add 25.0 mL of *Buffer solution*. Cap the tube, mix well on a vortex mixer for 1 min, cover to protect from light, and store in a refrigerator overnight (16–24 h). After overnight refrigerator storage, mix the tube on a vortex mixer for 1 min, and centrifuge at 3500 rpm for 4 min in a refrigerated centrifuge (10°). Mix again on a vortex mixer for 1 min, and centrifuge at 3500 rpm for 6 min in a refrigerated centrifuge. Use the supernatant.

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Mode: Visible

Analytical wavelengths: 620 nm and 650 nm

Cell path: 1 cm

Blank: *Buffer solution*

Analysis: Measure the absorbance of the *Sample solution* at 620 nm and 650 nm using *Buffer solution* as *Blank*.

Calculate the percentage of C-phycocyanin in the portion of Spirulina taken:

$$\text{Result} = (A_{620nm} \times 0.1757 - A_{650nm} \times 0.1185) \times (V/W) \times 100$$

A_{620nm} = absorbance at 620 nm
A_{650nm} = absorbance at 650 nm
V = volume of *Sample solution* (mL)
W = weight of Spirulina taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 6.0%, calculated on the dried basis

• **CONTENT OF PROTEIN**

Sample: 100 mg of Spirulina

Analysis: Proceed as directed in *Nitrogen Determination* ⟨461⟩, and multiply the nitrogen content by 6.25.

Acceptance criteria: NLT 50.0%, calculated on the dried basis

CONTAMINANTS

• **LIMIT OF MICROCYSTINS**

Use a commercial ELISA kit with cross reactivity for microcystin LR and other microcystins, suitable to detect microcystin LR at a concentration of 0.5 ng/mL. Kits typically consist of antibody-coated test tubes or microplates, microcystin-horseradish peroxidase conjugate solution, a buffer diluent, and a substrate for peroxidase color development.[1]

Solvent: A mixture of methanol and water (75:25)

Standard solution A: A 3-ng/mL solution of microcystin-LR

Standard solution B: A 0.5-ng/mL solution of microcystin-LR

Blank: Water

Sample solution: Homogenize for 3 min 3.0 g of Spirulina, accurately weighed, in 20.0 mL of *Solvent*. Transfer the mixture to a Teflon centrifuge tube, and centrifuge at 4500 rpm for 10 min. Transfer the supernatant into a glass flask. Add 10.0 mL of *Solvent* to the homogenizer, and homogenize the residue for 30 s. Transfer the solution to the centrifuge tube, mix, and centrifuge at 4500 rpm for 10 min. Combine the supernatants, and mix. Dilute with water (1 in 100).

System suitability

Samples: *Standard solution A*, *Standard solution B*, and *Blank*

System suitability requirement

Perform the ELISA determination according to the directions of the commercial kit manufacturer.

Sensitivity: Color is developed with both the *Blank* and *Standard solution B*. The color developed with the *Blank* is darker than the color developed with *Standard solution B*, and the color developed with *Standard solution A* is lighter than the color developed with *Standard solution B*.

Analysis

Samples: *Standard solution B* and *Sample solution*

Acceptance criteria: NMT 0.5 µg/g as microcystin LR, indicated by a darker color developed for the *Sample solution* compared to that developed for *Standard solution B*.

• **ELEMENTAL CONTAMINANTS**

Analysis: Proceed as directed in *Elemental Impurities—Procedures* ⟨233⟩.

Acceptance criteria

Arsenic: NMT 0.5 µg/g

Cadmium: NMT 0.2 µg/g

Lead: NMT 0.2 µg/g

Mercury: NMT 0.025 µg/g

• **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meets the requirements

• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria does not

[1] Suitable kits are available from EnviroLogix, 500 Riverside Industrial Parkway, Portland, Maine, 04103, USA (www.envirologix.com).

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exceed 10^3 cfu/g. [NOTE—When the total combined molds and yeasts count is expected to exceed the acceptance criteria due to bacteria growth, *Sabouraud Dextrose Agar* containing antibiotics may be used.]

- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩: Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

- **DESCRIPTION**

Macroscopic characteristics: Spirulina occurs as a fine, uniform powder, blue-green to green in color.

Microscopic characteristics: Spirulina is a microscopic, filamentous, multicellular blue-green microalgae. When fresh, filaments may appear as tightly coiled, relaxed helical coils. Straight filaments of Spirulina may also be observed in cultures that have been under continuous cultivation for more than 1 year. Under high magnification, filaments show a typical prokaryotic structure without the presence of intracellular organelles other than gas vesicles. Filaments have a diameter of 6–10 µm and a length of 100–400 µm and may reach 1 mm if grown undisturbed. Spirulina filaments are broken when Spirulina is harvested and dried into roughly uniform particles, 6–10 µm in diameter, and 20–60 µm in length.

- **LOSS ON DRYING** ⟨731⟩

Sample: 2 g of Spirulina
Analysis: Dry the *Sample* at 105° for 3 h.
Acceptance criteria: NMT 10%

- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩: NMT 15%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, under nitrogen, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and the official name.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Beta Carotene RS
USP Methyl Linoleate RS
USP Methyl Linolenate RS
USP Methyl Oleate RS
USP Methyl Palmitate RS
USP Methyl Palmitoleate RS
USP Methyl Stearate RS

Spirulina Tablets

DEFINITION

Spirulina Tablets are prepared from Spirulina. It contains NLT 100% of the labeled amount of spirulina, represented by a content of beta carotene of NLT 0.15% of the labeled amount of spirulina, a content of total carotenoids of NLT 0.35% of the labeled amount of spirulina, and a content of C-phycocyanin of NLT 6.0% of the labeled amount of spirulina.

IDENTIFICATION

- **A. FATTY ACIDS PROFILE**

Methyl oleate solution: 1 mg/mL of USP Methyl Oleate RS in *n*-heptane

Standard solution: Dissolve quantities of USP Methyl Palmitate RS, USP Methyl Palmitoleate RS, USP Methyl Stearate RS, USP Methyl Oleate RS, USP Methyl Linoleate RS, USP Methyl Linolenate RS (methyl alpha linolenate), and methyl gamma linolenate (obtained commercially) in *n*-heptane to obtain concentrations of each methyl ester as given in *Table 1*.

Table 1

Methyl Ester	Relative Retention Time	Concentration (mg/mL)
Methyl palmitate	0.76	0.8
Methyl palmitoleate	0.79	0.8
Methyl stearate	0.97	0.8
Methyl oleate	1.00	0.8
Methyl linoleate	1.06	0.8
Methyl gamma linolenate	1.12	0.8
Methyl alpha linolenate	1.18	0.8

Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer an amount of the powder, equivalent to about 100 mg of spirulina, to a 50-mL round-bottom flask, fitted with a water-cooled reflux condenser and a magnetic stir bar. Add 100 mg of pyrogallic acid, 10 mL of 0.5 N methanolic sodium hydroxide solution (20 mg/mL of sodium hydroxide in methanol), and heat the flask while stirring and under reflux for 15 min. Add 5 mL of a solution of 14% boron trifluoride–methanol through the reflux condenser into the flask, and continue boiling for 2 more min. Add 5.0 mL of *n*-heptane through the condenser, and continue boiling for an additional 1 min. Cool the flask, remove the condenser, and add 15 mL of saturated sodium chloride solution. While the solution is still tepid, insert a stopper into the flask, shake vigorously, and allow the layers to separate. Add sufficient saturated sodium chloride solution to bring the upper *n*-heptane layer up into the neck of the flask. Transfer the *n*-heptane layer into a suitable container, add a small quantity of anhydrous sodium sulfate (previously washed with *n*-heptane), and shake. [NOTE—Fatty acids methyl esters should be analyzed as soon as possible. They may be kept in an atmosphere of nitrogen at 2° for 24 h.]

Blank: Use *n*-heptane.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.25-mm × 30-m fused silica capillary; 0.25-µm film of phase G16 coating
Temperatures
Injector: 220°
Detector: 260°
Column: See *Table 2*.

Table 2

Initial Temperature (°)	Hold Time at 170° (min)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
170	2	5	240	5

Carrier gas: Helium
Flow rate: 1 mL/min
Split ratio: 100:1. [NOTE—If necessary, adjust the *Split ratio* and/or sample dilution to obtain a tailing factor of 0.8–1.5 for the fatty acid methyl ester peaks.]
Injection volume: 1 µL

System suitability

Samples: *Methyl oleate solution* and *Standard solution*
Suitability requirements
Resolution: NLT 1.5 between methyl stearate and methyl oleate peaks

DS Monographs

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Relative standard deviation: NMT 6.0% for the peak responses of each of the methyl palmitate and methyl stearate peaks for replicate injections; NMT 1.0% for the peak response ratio of the methyl palmitate to methyl stearate peaks in replicate injections

Analysis

Samples: *Methyl oleate solution*, *Standard solution*, *Sample solution*, and *Blank*

Identify the fatty acid ester peaks in the chromatogram of the *Sample solution* based on the relative retention times provided in *Table 1* and by comparison to the chromatograms of the *Methyl oleate solution* and the *Standard solution*. Measure the peak response for all of the fatty acid ester peaks from the *Sample solution*.

Calculate the percentage of each fatty acid component in the portion of Tablets taken:

$$\text{Result} = (A/B) \times 100$$

A = peak response of individual fatty acid ester from the *Sample solution*

B = sum of responses of all peaks, excluding the solvent, from the *Sample solution*

Acceptance criteria: See *Table 3* for individual fatty acids.

Table 3

Fatty Acids	Percentage of Total (%)
Palmitic acid	35–60
Palmitoelic acid	2–8
Stearic acid	1–5
Oleic acid	1–7
Linoleic acid	13–25
Gamma linolenic acid	13–27
Alpha linolenic acid	<0.5

• B. HPLC FOR CHLOROPHYLL A

Analysis: Proceed as directed in the test for *Content of Beta Carotene and Total Carotenoids*, except analyze the *Sample solution* and *Standard solution* at 432 nm.

Acceptance criteria: The major peak in the *Sample solution* chromatogram is due to chlorophyll A, at a relative retention time of about 0.8 to the peak of all-*trans*-beta carotene.

• C. HPLC FOR CAROTENOIDS

Analysis: Proceed as directed in the test for *Content of Beta Carotene and Total Carotenoids*.

Acceptance criteria: The major peak from the *Sample solution* corresponds to that of all-*trans*-beta carotene from the *Standard solution*. The *Sample solution* chromatogram exhibits additional peaks due to oscillol rhamnoside, myxol rhamnoside, zeaxanthin, alloxanthin, echinenone, beta cryptoxanthin, and *cis*-isomers of beta carotene as shown in *Table 5*. [NOTE—The *cis*-beta carotene peaks may partially be resolved from the all-*trans*-beta carotene peak.]

STRENGTH

• CONTENT OF BETA CAROTENE AND TOTAL CAROTENOIDS

[NOTE—It is important to have all glassware ready before starting the test. Minimize exposure of the *Standard solution* and *Sample solution* to light. Proceed under subdued light using low-actinic glassware at a room temperature not to exceed 25°. The *Standard solution* and *Sample solution* are stable for 12 h.]

Solution A: A mixture of a solution of 0.05% butylated hydroxytoluene in methanol and water (9:1). Allow the mixture to equilibrate to room temperature before adjusting to final volume with the solution of 0.05% butylated hydroxytoluene in methanol.

Solution B: A mixture of a solution of 0.05% butylated hydroxytoluene in methanol and methylene chloride (88:12). Allow the mixture to equilibrate to room temperature before adjusting to final volume with the solution of 0.05% butylated hydroxytoluene in methanol.

Mobile phase: See *Table 4*.

Table 4

Time (min)	Solution A (%)	Solution B (%)
0	95	5
1.0	95	5
2.5	0	100
10.75	0	100
11.00	95	5
16	95	5

Diluent: 50 µg/mL of butylated hydroxytoluene in methanol

Standard stock solution: 500 µg/mL of USP Beta Carotene RS in methylene chloride. [NOTE—Prepare fresh at the time of use, and minimize the time before it is used to prepare the *Standard solution*.]

Standard solution: 2.5 µg/mL of USP Beta Carotene RS in *Diluent* from *Standard stock solution*.

Determine the concentration of the *Standard solution* as follows.

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Mode: Visible

Analytical wavelength: 450 nm

Cell path: 1 cm

Blank: *Diluent*

Take three measurements using three different portions of the *Standard solution*.

Calculate the concentration of beta carotene (µg/mL) in the *Standard solution*.

$$C_S = (A/F) \times (P/100)$$

A = average absorbance of the three portions of *Standard solution*

F = one thousandth of the absorptivity value of beta carotene in *Diluent*, 0.25 $mL \cdot \mu g^{-1} \cdot cm^{-1}$

P = percentage of beta carotene relative to total carotenoids in the *Standard solution* as determined below in the *Analysis*

Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer an accurately weighed amount of the powder, equivalent to about 30 mg of spirulina, into a centrifuge tube, add 3 mL of dimethyl sulfoxide, and a few glass beads. Mix on a vortex mixer to disperse the sample. For 15 min, repeat the process of immersing in a water bath maintained at a temperature of 45° and mixing on a vortex mixer. Remove the tube from the water bath, and centrifuge at about 4000 rpm for 3 min. Transfer the supernatant to a 25-mL volumetric flask. To the residue add 3 mL of methanol, mix on a vortex mixer, centrifuge at about

Supplement 2

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4000 rpm for 3 min, and transfer the supernatant to the volumetric flask. Repeat until the last methanol extract is colorless. Dilute with methanol to volume, and mix. If particles are still present in the final extract, centrifuge a portion at about 4000 rpm for 3 min, and use the supernatant.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 476 nm
Columns
Guard: 2.0-mm × 4-cm; 5-µm, 300 Å, packing L1
Analytical: 2.0-mm × 15-cm; 5-µm, 300 Å, packing L1
Column temperature: 25°
Flow rate: 0.5 mL/min
Injection volume: 50 µL

System suitability
Sample: *Standard solution*
Suitability requirements
Inject the *Standard solution* repeatedly (about 2–3 times) until a consistent retention time, within ± 0.2 min, is obtained.
Relative standard deviation: NMT 2.0% for beta carotene peak

Analysis
Samples: *Standard solution* and *Sample solution*
Using the chromatogram of the *Standard solution*, identify the peak corresponding to all-*trans*-beta carotene in the *Sample solution* chromatogram. Identify the peaks corresponding to oscillol rhamnoside, myxol rhamnoside, zeaxanthin, alloxanthin, echinenone, beta cryptoxanthin, and *cis*-isomers of beta carotene from the *Sample solution* using the relative retention times provided in *Table 5*.

Table 5

Carotenoids	Relative Retention Time
Oscillol rhamnoside	0.15
Myxol rhamnoside	0.19
Zeaxanthin	0.29
Alloxanthin	0.53
Echinenone	0.60
Beta cryptoxanthin	0.64
All-*trans*-beta carotene	1.00
9-*cis*-Beta carotene	1.02
13-*cis*-Beta carotene	1.04

The *cis*-beta carotene peaks may partially be resolved from the all-*trans*-beta carotene peak. In this case, peak integration should be done as a single peak that includes all the isomer peaks.
Calculate the percentage (P) of beta carotene (sum of all the beta carotene isomers) relative to total carotenoids in the *Standard solution*:

$$P = (r_U/r_T) \times 100$$

r_U = peak response of beta carotene from the *Standard solution*
r_T = sum of peak responses of all carotenoids detected from the *Standard solution*, including that of beta carotene

Calculate the percentage of beta carotene in spirulina present in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak response of beta carotene from the *Sample solution*
r_S = peak response of beta carotene from the *Standard solution*
C_S = concentration of beta carotene in the *Standard solution*, as determined above under *Standard solution* (µg/mL)
V = final volume of *Sample solution* (mL)
W = labeled amount of spirulina in the portion of Tablets taken to prepare the *Sample solution* (µg)

Calculate the percentage of total carotenoids in the labeled amount of spirulina in the portion of Tablets taken:

$$\text{Result} = (\Sigma r_U/r_S) \times C_S \times (V/W) \times 100$$

Σr_U = sum of peak responses for all carotenoids from the *Sample solution*
r_S = peak response for beta carotene from the *Standard solution*
C_S = concentration of beta carotene in the *Standard solution*, as determined above under *Standard solution* (µg/mL)
V = final volume of *Sample solution* (mL)
W = labeled amount of spirulina present in the portion of Tablets taken to prepare the *Sample solution* (µg)

Acceptance criteria: NLT 0.15% of beta carotene and NLT 0.35% of total carotenoids, of the labeled amount of spirulina

• **CONTENT OF C-PHYCOCYANIN**
Buffer solution: Prepare a solution having a pH of 7, containing 13.9 g/L of monobasic sodium phosphate, 26.81 g/L of dibasic sodium phosphate, and 0.05% of sodium azide.
Sample solution: Transfer a portion of powdered Tablets, equivalent to 100 mg of spirulina, accurately weighed, into a centrifuge tube, and add 25.0 mL of *Buffer solution*. Cap the tube, mix well on a vortex mixer for 1 min, cover to protect from light, and store in a refrigerator overnight (16–24 h). After overnight refrigerator storage, mix the tube on a vortex mixer for 1 min, and centrifuge at 3500 rpm for 4 min in a refrigerated centrifuge (10°). Mix again on a vortex mixer for 1 min, and centrifuge at 3500 rpm for 6 min in a refrigerated centrifuge. Use the supernatant.
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: Visible
Analytical wavelengths: 620 nm and 650 nm
Cell path: 1 cm
Blank: *Buffer solution*
Analysis: Measure the absorbance of the *Sample solution* at 620 nm and 650 nm using *Buffer solution* as the *Blank*.
Calculate the percentage of C-phycocyanin in spirulina present in the portion of Tablets taken:

$$\text{Result} = (A_{620\text{nm}} \times 0.1757 - A_{650\text{nm}} \times 0.1185) \times (V/W) \times 100$$

$A_{620\text{nm}}$ = absorbance at 620 nm
$A_{650\text{nm}}$ = absorbance at 650 nm
V = volume of *Sample solution* (mL)

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W = labeled amount of spirulina in the portion of Tablets taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 6.0% of the labeled amount of spirulina

• **CONTENT OF PROTEIN**

Sample: A portion of powdered Tablets equivalent to 100 mg of spirulina

Analysis: Proceed as directed in *Nitrogen Determination* ⟨461⟩, and multiply the nitrogen content by 6.25.

Acceptance criteria: NLT 50.0% of the labeled amount of spirulina

PERFORMANCE TESTS

• **WEIGHT VARIATION** ⟨2091⟩**:** Meet the requirements

CONTAMINANTS

• **LIMIT OF MICROCYSTINS**

Use a commercial ELISA kit with cross reactivity for microcystin LR and other microcystins, suitable to detect microcystin LR at a concentration of 0.5 ng/mL. Kits typically consist of antibody-coated test tubes or microplates, microcystin-horseradish peroxidase conjugate solution, a buffer diluent, and a substrate for peroxidase color development.[1]

Solvent: A mixture of methanol and water (75:25)

Standard solution A: A 3-ng/mL solution of microcystin-LR

Standard solution B: A 0.5-ng/mL solution of microcystin-LR

Blank: Water

Sample solution: Homogenize for 3 min a portion of powdered Tablets, equivalent to 3.0 g of the labeled amount of spirulina, accurately weighed, in 20.0 mL of *Solvent*. Transfer the mixture to a Teflon centrifuge tube, and centrifuge at 4500 rpm for 10 min. Transfer the supernatant into a glass flask. Add 10.0 mL of *Solvent* to the homogenizer, and homogenize the residue for 30 s. Transfer the solution to the centrifuge tube, mix, and centrifuge at 4500 rpm for 10 min. Combine the supernatants, and mix. Dilute with water (1 in 100).

System suitability

Samples: *Standard solution A*, *Standard solution B*, and *Blank*

System suitability requirement

Perform the ELISA determination according to the directions of the commercial kit manufacturer.

Sensitivity: Color is developed with both the *Blank* and *Standard solution B*. The color developed with the *Blank* is darker than the color developed with *Standard solution B*, and the color developed with *Standard solution A* is lighter than the color developed with *Standard solution B*.

Analysis

Samples: *Standard solution B* and *Sample solution*

Acceptance criteria: NMT 0.5 µg/g as microcystin LR, indicated by a darker color developed for the *Sample solution* compared to that developed for the *Standard solution B*.

• **ELEMENTAL CONTAMINANTS IN DIETARY SUPPLEMENTS** ⟨2232⟩**:** Meet the requirements

• **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩**:** Meet the requirements

• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 5×10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g. [NOTE—When the total combined molds and yeasts count is expected to exceed the acceptance criteria due to bacteria growth, *Sabouraud Dextrose Agar* containing antibiotics may be used.]

• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meet the requirements of the tests for absence of *Salmonella* species and *Escherichia coli* in 10 g

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.

• **LABELING:** The label states the Latin binomial and, following the official name, the article from which the Tablets were prepared. The label also indicates the amount, in mg/Tablet, of spirulina. Label Tablets to indicate the content, in percentage, of beta carotene, total carotenoids, and C-phycocyanin in the spirulina used to prepare the Tablets.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Beta Carotene RS
USP Methyl Linoleate RS
USP Methyl Linolenate RS
USP Methyl Oleate RS
USP Methyl Palmitate RS
USP Methyl Palmitoleate RS
USP Methyl Stearate RS

Stinging Nettle

DEFINITION

Stinging Nettle consists of dried roots and rhizomes of *Urtica dioica* L. subsp. *dioica* (Fam. Urticaceae), and may contain *Urtica urens* L., known in commerce as dwarf nettle, as a minor component. It contains NLT 0.8% of total amino acids, NLT 0.05% of β-sitosterol ($C_{29}H_{50}O$), and NLT 3 µg/g of scopoletin ($C_{10}H_8O_4$), calculated on the dried basis.

IDENTIFICATION

• **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution: 0.05 mg/mL of USP Scopoletin RS and 0.5 mg/mL of USP β-Sitosterol RS in methanol

Sample solution: Extract 1 g of powder by refluxing with 10 mL of a solution containing toluene, ethyl acetate, and methanol (7:2:1) for 15 min, cool, and filter. Evaporate the filtrate to dryness under reduced pressure at less than 40°, and dissolve the residue in 2 mL of the mixture containing toluene, ethyl acetate, and methanol.

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Adsorbent: 0.50-mm layer of chromatographic silica gel mixture

Application volume: 20 µL for the *Sample solution*; 10 µL for the *Standard solution*

Developing solvent system: Diethyl ether and methanol (9:1)

Spray reagent: 85% phosphoric acid, 10% vanillin in 96% ethanol, and water (4.5: 1: 4.5)

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the plate and examine under UV light at 365 nm. Spray the plate with *Spray reagent*, heat between 100° and 105° for 10 min, and examine under daylight.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits a violet-red zone corresponding to β-sitosterol at the same R_F value as β-sitosterol in the *Standard solution*, weakly visible zones above and below β-sitosterol, and a violet-red zone corresponding to β-sitosterolglucoside.

[1] Suitable kits are available from EnviroLogix, 500 Riverside Industrial Parkway, Portland, Maine, 04103, USA (www.envirologix.com).

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COMPOSITION

• **CONTENT OF β-SITOSTEROL**

Derivatizing reagent: A solution containing equal volumes (1:1:1) of BSTFA [*N,O*-bis(trimethylsilyl)tri fluoroacetamide], anhydrous pyridine, and a mixture of BSA [*N,O*-(trimethylsilyl)acetamide], TMSI (*N*-trimethylsilylimidazole), and TMCS (trimethylchlorosilane) (3:3:2)

Internal standard solution: 10 mg/mL of cholesterol in chloroform

Standard solution: Dissolve 50.0 mg of USP β-sitosterol RS in 2 mL of chloroform, add 1.0 mL of *Internal standard solution*, and dilute with chloroform to 5 mL. Dry 0.5 mL under reduced pressure, and add 1 mL of *Derivatizing reagent*.

Sample solution: Transfer 50.0 g of finely powdered Stinging Nettle to a Soxhlet apparatus, add chloroform, and extract for 6 h. The volume of chloroform used is at least twice the volume of the thimble with an appropriately sized flask. Dry the solvent under reduced pressure, add 1.0 mL of *Internal standard solution*, and dilute with chloroform to 10 mL. Transfer 0.5 mL of this solution to a 10-mL round-bottom flask, dry the solvent under reduced pressure, and add 0.5 mL of *Derivatizing reagent*.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.20-mm × 25-m fused-silica capillary; 0.35-µm film of phase G2 coating
Temperature
Injector: 325°
Detector: 325°
Column: 300°, for 60 min
Carrier gas: Helium
Flow rate: 0.5 mL/min
Injection size: 1 µL

System suitability
Sample: *Standard solution*
Suitability requirements
Tailing factor: NMT 2.0 for each sterol peak
Relative standard deviation: NMT 5.0% determined from each sterol peak

Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of β-sitosterol in the portion of Stinging Nettle taken:

$$\text{Result} = (R_U/R_S) \times (W_S/W_U) \times 100$$

R_U = peak response ratio of β-sitosterol to the internal standard from the *Sample solution*
R_S = peak response ratio of β-sitosterol to the internal standard from the *Standard solution*
W_S = weight of USP β-sitosterol RS used to prepare the *Standard solution* (mg)
W_U = weight of Stinging Nettle taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 0.05% on the dried basis

• **CONTENT OF SCOPOLETIN**

Solution A: Water
Solution B: Methanol
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	75	25
2	60	40
8	60	40
10	0	100
15	0	100
20	75	25
30	75	25

Standard solution: 0.02 µg/mL of USP Scopoletin RS in methanol
Sample stock solution: Extract 160 mg of finely powdered Stinging Nettle in each mL of methanol. Place in an ultrasonic bath for 25 min, and centrifuge.
Sample solution: *Sample stock solution* diluted with methanol 1 in 20
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Fluorescence detector; set at an excitation wavelength of 366 nm and an emission wavelength of 420 nm
Column: 4.6-mm × 25-cm; packing L1
Flow rate: 1 mL/min
Injection size: 10 µL

System suitability
Sample: *Standard solution*
Suitability requirements
Capacity factor (k'): NLT 5 determined from the scopoletin peak
Tailing factor: NMT 2.0 for the scopoletin peak
Relative standard deviation: NMT 5.0%

Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak responses for scopoletin.
Calculate the content of scopoletin ($C_{10}H_8O_4$), in µg/g, in the portion of Stinging Nettle taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D$$

r_U = peak area of scopoletin from the *Sample solution*
r_S = peak area of scopoletin from the *Standard solution*
C_S = concentration of USP Scopoletin RS in the *Standard solution* (µg/mL)
V = volume of the *Sample stock solution* (mL)
W = weight of Stinging Nettle taken to prepare the *Sample solution* (g)
D = dilution factor to prepare the *Sample solution* from the *Sample stock solution*

Acceptance criteria: NLT 3 µg/g on the dried basis

• **CONTENT OF TOTAL AMINO ACIDS**

Buffer: Mix 5.40 g of anhydrous sodium acetate, 0.3 mL of glacial acetic acid, and water to a final volume of 100 mL. Adjust to pH 5.5.
Reagent solution: Solution containing 1.00 g of ninhydrin, 150 mg of hydrindantin, and 37.5 mL of propylene glycol. Adjust with *Buffer* to 50.0 mL. [NOTE—Prepare the *Reagent solution* daily.]
Standard solution: 20 µg/mL each of USP Glutamic Acid RS and USP Aspartic Acid RS in water
Sample solution: Finely powder Stinging Nettle, and transfer 1.0 g of it to 80 mL of water. Place in an ultrasonic bath for 25 min, and centrifuge. Transfer the supernatant

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to a 100-mL volumetric flask, dilute with water to volume, and filter.
Blank: Water
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: Visible
Analytical wavelength: 570 nm
Cell: 1 cm
Analysis
Samples: *Standard solution*, *Sample solution*, and *Blank*
Transfer 5.0 mL of the *Standard solution*, *Sample solution*, and *Blank* to separate 50-mL volumetric flasks. Add 5.0 mL of *Reagent solution* to each flask. Heat in a boiling water bath for 30 min, cool, and adjust with a mixture of ethanol and water (1:1) to volume.
Calculate the percentage of total amino acids in the portion of Stinging Nettle taken:

$$\text{Result} = (A_U/A_S) \times C_S \times (V/W) \times F \times 100$$

A_U = absorbance of the *Sample solution*
A_S = absorbance of the *Standard solution*
C_S = sum of the concentration of USP Glutamic Acid RS and USP Aspartic Acid RS in the *Standard solution* (µg/mL)
V = volume of the *Sample solution*, 100 mL
W = amount of Stinging Nettle taken to prepare the *Sample solution* (mg)
F = unit conversion factor, 0.001 mg/µg

Acceptance criteria: NLT 0.8% on the dried basis

CONTAMINANTS
- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residues* ⟨561⟩**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^6 cfu/g, the total combined molds and yeasts count does not exceed 10^4 cfu/g, and the bile-tolerant Gram-negative bacteria count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS
- **BOTANIC CHARACTERISTICS**

Macroscopic: The rhizome is irregularly bent, about 3–10 mm thick, and light gray-brown on the outside; thin roots spring from the knotty bulges of a lengthwise furrow. A transverse cut of the rhizome shows it is fibrous, light yellowish white, and usually has a small medulla cave. The roots are often very long, usually 0.5–2 mm thick, light yellow-brown on the outside, and contain some deep longitudinal furrows; a transverse cut shows a pale and almost pure-white color.

Microscopic: The transverse section of the rhizome and root shows the following characteristics. The rhizome has a narrow cork composed of brown, thin-walled cells, a few rows of tangentially elongated cortical parenchyma, and a pericyclic region with numerous fibers occurring singly or, more frequently, in small groups. Fibers are much elongated with very thick and lignified walls. Some cells of the pericycle and outer part of secondary phloem contain large globular compound crystals of calcium oxalate. The vascular cambial region is distinct and continuous with narrow radial groups of vascular tissue separated by wide medullary rays. The secondary phloem is mainly parenchymatous with groups of thin-walled sieve tissue. The xylem is dense and completely lignified, containing scattered vessels, isolated or in small groups, associated with moderately thickened xylem parenchyma cells and numerous thicker-walled xylem fibers with slit-shaped pits. Individual vessels have fairly large, closely arranged, bordered pits, while the adjacent parenchyma has simple or bordered pits. Medullary rays indicate alternating areas of lignified and unlignified cells, appearing as tangential bands between the vascular bundles, each composed of 5 or 6 layers of cells; the lignified cells have moderately thickened walls with simple pits. The pith is composed of rounded, unlignified parenchyma, collapsed in the central part to form a cavity. Mature roots show a thin cork, narrow phelloderm, and secondary phloem and xylem with alternating areas of lignified and unlignified parenchyma in the wide medullary rays, similar to that found in the rhizome.

- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩**:** NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 10%
- **LOSS ON DRYING** ⟨731⟩ Dry 1.0 g of Stinging Nettle, finely powdered, at 105° for 2 h: it loses NMT 12.0% of its weight.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light. Store at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Aspartic Acid RS
  USP Glutamic Acid RS
  USP Scopoletin RS
  USP β-Sitosterol RS

Powdered Stinging Nettle

DEFINITION
Powdered Stinging Nettle is Stinging Nettle reduced to a fine or very fine powder. It contains NLT 0.8% of total amino acids, NLT 0.05% of β-sitosterol ($C_{29}H_{50}O$), and NLT 3 µg/g of scopoletin ($C_{10}H_8O_4$), calculated on the dried basis.

IDENTIFICATION
- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution: 0.05 mg/mL of USP Scopoletin RS and 0.5 mg/mL of USP β-Sitosterol RS in methanol
Sample solution: Extract 1 g of Powdered Stinging Nettle by refluxing with 10 mL of a solution containing toluene, ethyl acetate, and methanol (7:2:1) for 15 min, cool, and filter. Evaporate the filtrate to dryness under reduced pressure at less than 40°, and dissolve the residue in 2 mL of the mixture containing toluene, ethyl acetate, and methanol.
Chromatographic system
(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
Adsorbent: 0.50-mm layer of chromatographic silica gel mixture
Application volume: 20 µL for the *Sample solution*; 10 µL for the *Standard solution*
Developing solvent system: Diethyl ether and methanol (9:1)
Spray reagent: 85% phosphoric acid, 10% vanillin in 96% ethanol, and water (4.5:1:4.5)
Analysis
Samples: *Standard solution* and *Sample solution*
Develop the plate and examine under UV light at 365 nm. Spray the plate with *Spray reagent*, heat between 100° and 105° for 10 min, and examine under daylight.

Supplements

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Acceptance criteria: The chromatogram of the *Sample solution* exhibits a violet-red zone corresponding to β-sitosterol at the same R_F value as β-sitosterol of the *Standard solution*, weakly visible zones above and below β-sitosterol, and a violet-red zone corresponding to β-sitosterolglucoside.

COMPOSITION

• **CONTENT OF β-SITOSTEROL**

Derivatizing reagent: A solution containing equal volumes (1:1:1) of BSTFA [*N,O*-bis(trimethylsilyl)tri fluoroacetamide], anhydrous pyridine, and a mixture of BSA [*N,O*-(trimethylsilyl)acetamide], TMSI (*N*-trimethylsilylimidazole), and TMCS (trimethylchlorosilane) (3:3:2)

Internal standard solution: 10 mg/mL of cholesterol in chloroform

Standard solution: Dissolve 50.0 mg of USP β-sitosterol RS in 2 mL of chloroform, add 1.0 mL of *Internal standard solution*, and dilute to 5 mL with chloroform. Dry 0.5 mL under reduced pressure, and add 1 mL of *Derivatizing reagent*.

Sample solution: Transfer 50.0 g of Powdered Stinging Nettle to a Soxhlet apparatus, add chloroform, and extract for 6 h. The volume of chloroform used is at least twice the volume of the thimble with an appropriately sized flask. Dry the solvent under reduced pressure, add 1.0 mL of *Internal standard solution*, and dilute with chloroform to 10 mL. Transfer 0.5 mL of this solution to a 10-mL round-bottom flask, dry the solvent under reduced pressure, and add 0.5 mL of *Derivatizing reagent*.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.20-mm × 25-m fused-silica capillary; 0.35-µm film of phase G2 coating

Temperature

Injector: 325°

Detector: 325°

Column: 300°, for 60 min

Carrier gas: Helium

Flow rate: 0.5 mL/min

Injection size: 1 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Tailing factor: NMT 2.0 for each sterol peak

Relative standard deviation: NMT 5.0% determined from each sterol peak

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of β-sitosterol in the portion of Powdered Stinging Nettle taken:

$$\text{Result} = (R_U/R_S) \times (W_S/W_U) \times 100$$

R_U = peak response ratio of β-sitosterol to the internal standard from the *Sample solution*

R_S = peak response ratio of β-sitosterol to the internal standard from the *Standard solution*

W_S = weight of USP β-sitosterol RS used to prepare the *Standard solution* (mg)

W_U = weight of Powdered Stinging Nettle taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 0.05% on the dried basis

• **CONTENT OF SCOPOLETIN**

Solution A: Water

Solution B: Methanol

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	75	25
2	60	40
8	60	40
10	0	100
15	0	100
20	75	25
30	75	25

Standard solution: 0.02 µg/mL of USP Scopoletin RS in methanol

Sample stock solution: 160 mg of Powdered Stinging Nettle in each mL of methanol. Place in an ultrasonic bath for 25 min, and centrifuge.

Sample solution: *Sample stock solution* diluted with methanol 1 in 20

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Fluorescence detector; set at an excitation wavelength of 366 nm and an emission wavelength of 420 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection size: 10 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Capacity factor (k′): NLT 5 determined from the scopoletin peak

Tailing factor: NMT 2.0 for the scopoletin peak

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak responses for scopoletin.

Calculate the content of scopoletin ($C_{10}H_8O_4$), in µg/g, in the portion of Powdered Stinging Nettle taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D$$

r_U = peak area of scopoletin from the *Sample solution*

r_S = peak area of scopoletin from the *Standard solution*

C_S = concentration of USP Scopoletin RS in the *Standard solution* (µg/mL)

V = volume of the *Sample stock solution* (mL)

W = weight of Powdered Stinging Nettle taken to prepare the *Sample stock solution* (g)

D = dilution factor to prepare the *Sample solution* from the *Sample stock solution*

Acceptance criteria: NLT 3 µg/g on the dried basis

• **CONTENT OF TOTAL AMINO ACIDS**

Buffer: Mix 5.40 g of anhydrous sodium acetate, 0.3 mL of glacial acetic acid, and water to a final volume of 100 mL. Adjust to pH 5.5.

Reagent solution: Solution containing 1.00 g of ninhydrin, 150 mg of hydrindantin, and 37.5 mL of propylene glycol. Adjust with *Buffer* to 50.0 mL. [NOTE—Prepare the *Reagent solution* daily.]

Standard solution: 20 µg/mL each of USP Glutamic Acid RS and USP Aspartic Acid RS, in water

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Sample solution: Transfer 1.0 g of Powdered Stinging Nettle to 80 mL of water. Place in an ultrasonic bath for 25 min, and centrifuge. Transfer the supernatant to a 100-mL volumetric flask, dilute with water to volume, and filter.
Blank: Water
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: Visible
Analytical wavelength: 570 nm
Cell: 1 cm
Analysis
Samples: *Standard solution*, *Sample solution*, and *Blank*
Transfer 5.0 mL of the *Standard solution*, *Sample solution*, and *Blank* to separate 50-mL volumetric flasks. Add 5.0 mL of *Reagent solution* to each flask. Heat in a boiling water bath for 30 min, cool, and adjust with a mixture of ethanol and water (1:1) to volume.
Calculate the percentage of total amino acids in the portion of Powdered Stinging Nettle taken:

$$\text{Result} = (A_U/A_S) \times C_S \times (V/W) \times F \times 100$$

A_U = absorbance from the *Sample solution*
A_S = absorbance from the *Standard solution*
C_S = sum of the concentration of USP Glutamic Acid RS and USP Aspartic Acid RS in the *Standard solution* (µg/mL)
V = volume of the *Sample solution*, 100 mL
W = amount of Powdered Stinging Nettle taken to prepare the *Sample solution* (mg)
F = unit conversion factor, 0.001 mg/µg

Acceptance criteria: NLT 0.8% on the dried basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residues* ⟨561⟩**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^6 cfu/g, the total combined molds and yeasts count does not exceed 10^4 cfu/g, and the bile-tolerant Gram-negative bacteria count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

- **BOTANIC CHARACTERISTICS**

Macroscopic: The powder is yellowish to brownish gray.
Microscopic: Under a microscope using chloral hydrate solution, it shows several fragments of net and pitted vessels with a strongly varying diameter, usually 50–150 µm. Few, if any, pitted bast fibers are present. Clearly pitted wood fibers, 200–800 µm in length, and thin-walled parenchyma cells, sometimes containing compounded calcium oxalate crystals and occasionally with single crystals and fragments of cork consisting of flat-shaped, thin-walled cells, are also present.

- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩**:** NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 10%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light and excessive heat.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Aspartic Acid RS
USP Glutamic Acid RS
USP Scopoletin RS
USP β-Sitosterol RS

Powdered Stinging Nettle Extract

DEFINITION

Powdered Stinging Nettle Extract is prepared from comminuted Stinging Nettle with 60% alcohol or other suitable solvents. It contains NLT 5.0% of total amino acids, NLT 0.1% of β-sitosterol ($C_{29}H_{50}O$), and NLT 30 µg/g of scopoletin ($C_{10}H_8O_4$). The ratio of the starting crude plant material to Powdered Extract is 10:1.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution: 0.05 mg/mL of USP Scopoletin RS and 0.5 mg/mL of USP β-Sitosterol RS in methanol
Sample solution: Dissolve 0.6 g of Powdered Extract, in a mixture of toluene, ethyl acetate, and methanol (7:2:1). Filter, and dry under reduced pressure at a temperature below 40°. Dissolve the residue in 2.0 mL of the mixture containing toluene, ethyl acetate, and methanol.
Chromatographic system
(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
Adsorbent: 0.50-mm layer of chromatographic silica gel mixture
Application volume: 20 µL for the *Sample solution*; 10 µL for the *Standard solution*
Developing solvent system: Diethyl ether and methanol (9:1)
Spray reagent: 85% phosphoric acid, 10% vanillin in 96% ethanol, and water (4.5: 1: 4.5)
Analysis
Samples: *Standard solution* and *Sample solution*
Proceed as directed in the chapter. Examine the plates under UV light at 365 nm. Spray the plate with 10 mL of *Spray reagent*, heat between 100° and 105° for 10 min, and examine under daylight.
Acceptance criteria: The chromatogram of the *Sample solution* exhibits a violet-red zone corresponding to β-sitosterol at the same R_F value as β-sitosterol from the *Standard solution*, weakly visible zones above and below β-sitosterol, and a violet-red zone corresponding to β-sitosterolglucoside.

- **B. GAS CHROMATOGRAPHY IDENTIFICATION TEST**

Analysis: Proceed as directed in the test for *Content of β-Sitosterol*.
Acceptance criteria: The retention time of β-sitosterol of the *Sample solution* corresponds to that of the *Standard solution*.

- **C. HPLC IDENTIFICATION TEST**

Analysis: Proceed as directed in the test for *Content of Scopoletin*.
Acceptance criteria: The retention time of scopoletin of the *Sample solution* corresponds to that of the *Standard solution*.

COMPOSITION

- **CONTENT OF β-SITOSTEROL**

Derivatizing reagent: A solution containing equal volumes (1:1:1) of BSTFA [*N,O*-bis(trimethylsilyl)trifluoroacetamide], anhydrous pyridine, and a mixture of BSA [*N,O*-(trimethylsilyl)acetamide], TMSI (*N*-trimethylsilylimidazole), and TMCS (trimethylchlorosilane) (3:3:2)

Supplements

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Internal standard solution: 10 mg/mL of cholesterol in chloroform
Standard solution: Dissolve 50.0 mg of USP β-Sitosterol RS in 2 mL of chloroform, add 1.0 mL of *Internal standard solution*, and dilute to 5 mL with chloroform. Dry 0.5 mL under reduced pressure, and add 1 mL of *Derivatizing reagent*.
Sample solution: Transfer 20.0 g of Powdered Extract to a Soxhlet apparatus, treat with chloroform, and extract for 6 h. The volume of chloroform used is at least twice the volume of the thimble with an appropriately sized flask. Dry the solvent under reduced pressure, add 1.0 mL of *Internal standard solution*, and dilute with chloroform to 10 mL. Transfer 0.5 mL of this solution to a 10-mL round-bottom flask, dry the solvent under reduced pressure, and add 0.5 mL of *Derivatizing reagent*.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.20-mm × 25-m fused-silica capillary; 0.35-µm film of phase G2 coating
Temperature
Injector: 325°
Detector: 325°
Column: 300°, for 60 min
Carrier gas: Helium
Flow rate: 0.5 mL/min
Injection size: 1 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Tailing factor: NMT 2.0 for each sterol peak
Relative standard deviation: NMT 5.0% determined from each sterol peak
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of β-sitosterol in the portion of Powdered Extract taken:

$$\text{Result} = (R_U/R_S) \times (W_S/W_U) \times 100$$

R_U = peak response ratio of β-sitosterol to the internal standard from the *Sample solution*
R_S = peak response ratio of β-sitosterol to the internal standard from the *Standard solution*
W_S = weight of USP β-Sitosterol RS used to prepare the *Standard solution* (mg)
W_U = weight of Powdered Extract in the *Sample solution* (mg)

Acceptance criteria: NLT 0.1%

• **CONTENT OF SCOPOLETIN**
Solution A: Water
Solution B: Methanol
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	75	25
2	60	40
8	60	40
10	0	100
15	0	100
20	75	25

Table 1 *(continued)*

Time (min)	Solution A (%)	Solution B (%)
30	75	25

Standard solution: 0.02 µg/mL of USP Scopoletin RS in methanol
Sample stock solution: 8 mg/mL of Powdered Extract in methanol. Place in an ultrasonic bath for 25 min, and centrifuge.
Sample solution: *Sample stock solution* diluted with methanol 1 in 20
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Fluorescence; set at an excitation wavelength of 366 nm and an emission wavelength of 420 nm
Column: 4.6-mm × 25-cm; packing L1
Flow rate: 1 mL/min
Injection size: 10 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Capacity factor (k′): NLT 5 determined from the scopoletin peak
Tailing factor: NMT 2.0 for the scopoletin peak
Relative standard deviation: NMT 5.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak responses for scopoletin.
Calculate the content of scopoletin ($C_{10}H_8O_4$), in µg/g, in the portion of Powdered Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U)$$

r_U = peak area of scopoletin from the *Sample solution*
r_S = peak area of scopoletin from the *Standard solution*
C_S = concentration of USP Scopoletin RS in the *Standard solution* (µg/mL)
C_U = concentration of Powdered Extract in the *Sample solution* (g/mL)

Acceptance criteria: NLT 30 µg/g

• **CONTENT OF TOTAL AMINO ACIDS**
Buffer: Mix 5.40 g of anhydrous sodium acetate, 0.3 mL of glacial acetic acid, and water to a final volume of 100 mL. Adjust to pH 5.5.
Reagent solution: Solution containing 1.00 g of ninhydrin, 150 mg of hydrindantin, and 37.5 mL of propylene glycol. Adjust with *Buffer* to 50.0 mL. [NOTE—Prepare the *Reagent solution* daily.]
Standard solution: 20 µg/mL each of USP Glutamic Acid RS and USP Aspartic Acid RS in water
Sample solution: Dissolve 50 mg of Powdered Extract in 80 mL of water, shake for 10 min, dilute with water to 100 mL, and filter.
Blank: Water
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: Visible
Analytical wavelength: 570 nm
Cell: 1 cm
Analysis
Samples: *Standard solution*, *Sample solution*, and *Blank*
Transfer 5.0 mL of the *Standard solution*, *Sample solution*, and *Blank* to separate 50-mL volumetric flasks. Add 5.0 mL of *Reagent solution* to each flask. Heat in a boiling water

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bath for 30 min, cool, and adjust with a mixture of ethanol and water (1:1) to volume.

Calculate the percentage of total amino acids in the portion of Powdered Extract taken:

$$\text{Result} = (A_U/A_S) \times (C_S/C_U) \times 100$$

A_U = absorbance of the *Sample solution*
A_S = absorbance of the *Standard solution*
C_S = sum of the concentration of USP Glutamic Acid RS and USP Aspartic Acid RS in the *Standard solution* (mg/mL)
C_U = concentration of Powdered Extract in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 5.0%

CONTAMINANTS

Change to read:

- **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020)**:** Meets the requirements
- **ALCOHOL DETERMINATION,** *Method II* ⟨611⟩**:** NMT 1.0%, if present
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli.*

SPECIFIC TESTS

- **LOSS ON DRYING** ⟨731⟩**:** Dry 1.0 g of Powdered Extract at 105° for 2 h: it loses NMT 8.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 20.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light. Store at controlled room temperature.
- **LABELING:** The label states the official name of the article, the Latin binomial, and, following the official name, the part of the plant from which the article was prepared. Label it to indicate the content of total amino acids, β-sitosterol, scopoletin, the extracting solvent used for preparation, and the ratio of the starting crude plant material to Powdered Extract.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Aspartic Acid RS
  USP Glutamic Acid RS
  USP Scopoletin RS
  USP β-Sitosterol RS

Tangerine Peel

DEFINITION

Tangerine Peel consists of the dried exocarp and mesocarp of the ripe fruit of *Citrus reticulata* Blanco (Fam. Rutaceae), partly freed from the white spongy tissue of the mesocarp. It contains NLT 5.0% of total dihydroflavone glycosides, calculated as the sum of narirutin ($C_{27}H_{32}O_{14}$), hesperidin ($C_{28}H_{34}O_{15}$), and didymin ($C_{28}H_{34}O_{14}$) on the anhydrous basis, and NLT 0.1% of total polymethoxylated flavones, calculated as the sum of nobiletin ($C_{21}H_{22}O_8$), 3,5,6,7,8,3′,4′-heptamethoxyflavone ($C_{22}H_{24}O_9$), and tangeretin ($C_{20}H_{20}O_7$) on the anhydrous basis.

IDENTIFICATION

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 1.0 mg/mL of USP Hesperidin RS in methanol. Sonicate to dissolve.

Standard solution B: 50 mg/mL of USP *Citrus reticulata* Pericarp Dry Extract RS in methanol. Sonicate for 20 min, centrifuge, and use the supernatant.

Sample solution: 500 mg of Tangerine Peel, finely powdered, in 5 mL of methanol. Sonicate for 20 min, centrifuge, and use the supernatant.

Chromatographic system

Adsorbent: Chromatographic silica gel F_{254} mixture

Application volume: *Standard solution A,* 10 µL; *Standard solution B* and *Sample solution,* 5 µL for each, as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

Temperature: About 25°

Developing solvent system: Ethyl acetate, formic acid, and water (100:15:13)

Derivatization reagent A: 10 mg/mL of 2-aminoethyl diphenylborinate in methanol

Derivatization reagent B: 50 mg/mL of polyethylene glycol 4000 in alcohol

Analysis

Samples: *Standard solution A, Standard solution B,* and *Sample solution*

Apply the *Samples* as bands and dry in air. Develop in a saturated chamber, remove the plate from the chamber, and dry the plate at 100° for 3 min. Treat the plate with *Derivatization reagent A* and dry for 5 min with a stream of cool air. Immediately, treat the plate with *Derivatization reagent B,* dry for 5 min with a stream of cool air, and examine under UV light at 366 nm.

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements: *Standard solution A* exhibits a yellowish-brown band due to hesperidin in the lower half section. *Standard solution B* exhibits a band corresponding in R_F and color to the band due to hesperidin in *Standard solution A,* a yellow band below hesperidin, a blue band below the yellow band, another blue band above hesperidin, and a light yellowish-brown band between hesperidin and the blue band above. In the upper-third section, *Standard solution B* exhibits a bright blue band due to the coelution of nobiletin with some other components, and a faint green band above the bright blue band.

Acceptance criteria: The *Sample solution* exhibits a yellowish-brown band corresponding in R_F and color to the band due to hesperidin in *Standard solution A* and *Standard solution B,* a yellow band below hesperidin, a blue band below the yellow band, another blue band above hesperidin, and a light yellowish-brown band between hesperidin and the blue band above corresponding in R_F and color to the same bands in *Standard solution B.* In the upper-third section, the *Sample solution* exhibits a bright blue band and a faint green band above the bright blue band corresponding in R_F and color to the same bands in *Standard solution B.* In the lower-third section, the *Sample solution* exhibits a couple of yellow bands close to the starting position and a couple of faint bands above the yellow bands corresponding in R_F and color to the same bands in *Standard solution B.*

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• **B. HPLC**

Analysis: Proceed as directed in the test for *Content of Dihydroflavone Glycosides and Polymethoxylated Flavones*.

Acceptance criteria: The *Sample solution* exhibits the most intense peak at the retention time corresponding to hesperidin in *Standard solution A* and peaks due to narirutin, didymin, nobiletin, 3,5,6,7,8,3′,4′-heptamethoxyflavone, and tangeretin at retention times corresponding to the same constituents in *Standard solution B*. No other peak between narirutin and tangeretin is more intense than the peak corresponding to didymin (a distinction from other *Citrus* species; *Citrus maxima* peel and *Citrus wilsonii* fruit show a principal peak for naringin). The content ratios of narirutin and didymin relative to hesperidin are within the ranges listed in *Table 2*.

COMPOSITION

• **CONTENT OF DIHYDROFLAVONE GLYCOSIDES AND POLYMETHOXYLATED FLAVONES**

Solution A: 0.1% formic acid in water

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	85	15
8	81	19
10	81	19
17	60	40
28	56	44

[NOTE—The *Standard solutions* and the *Sample solution* are stable for 24 h at room temperature.]

Standard solution A: 0.40 mg/mL of USP Hesperidin RS in methanol

Standard solution B: 0.05 mg/mL of USP Nobiletin RS in methanol

Standard solution C: 5 mg/mL of USP *Citrus reticulata* Pericarp Dry Extract RS in methanol. Sonicate for 15 min, centrifuge, and pass through a suitable membrane filter of 0.22-µm pore size.

Sample solution: Accurately transfer about 100 mg of finely powdered Tangerine Peel to a suitable flask, accurately add 10.0 mL of methanol, and close tightly. Weigh the filled flask accurately and sonicate for 30 min. Cool to room temperature and adjust to the initial weight by adding methanol if needed. Before injection, pass through a suitable membrane filter of 0.22-µm pore size and discard the first portion of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 283 nm (0–17 min) and 330 nm (17–28 min)

Column: 4.6-mm × 5-cm; 1.8-µm packing L1

Column temperature: 25°

Flow rate: 0.7 mL/min

Injection volume: 2 µL

System suitability

Samples: *Standard solution A*, *Standard solution B*, and *Standard solution C*

Suitability requirements

Resolution: NLT 1.5 between the peak of hesperidin and the small peak before it, *Standard solution C*

Tailing factor: NMT 1.5 for the hesperidin and nobiletin peaks, *Standard solution A* and *Standard solution B*

Relative standard deviation: NMT 2.0% for the hesperidin and nobiletin peaks in repeated injections, *Standard solution A* and *Standard solution B*

Chromatographic similarity: The chromatogram of *Standard solution C* is similar to the reference chromatogram provided with the lot of USP *Citrus reticulata* Pericarp Dry Extract RS being used.

Analysis

For dihydroflavone glycosides

Samples: *Standard solution A*, *Standard solution C*, and *Sample solution*

Using the chromatograms of *Standard solution A*, *Standard solution C*, and the reference chromatogram provided with the lot of USP *Citrus reticulata* Pericarp Dry Extract RS being used, identify the peaks corresponding to narirutin, hesperidin, and didymin in the *Sample solution*. [NOTE—See *Table 2* for the relative retention times.]

Table 2

Analyte	Approximate Relative Retention Time	Conversion Factor	Content Ratio Relative to Hesperidin
Narirutin	0.79	1.17	0.1–0.3
Naringin	0.90	—	<0.02
Hesperidin	1.00	1.00	1.0
Didymin	1.70	1.00	0.02–0.06

Separately calculate the percentage of narirutin, hesperidin, and didymin in the portion of Tangerine Peel taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*

r_S = peak area of hesperidin from *Standard solution A*

C_S = concentration of USP Hesperidin RS in *Standard solution A* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Tangerine Peel taken to prepare the *Sample solution* (mg)

F = conversion factor for the analyte (see *Table 2*)

Calculate the content of the total dihydroflavone glycosides as the sum of the percentages of narirutin, hesperidin, and didymin.

For polymethoxylated flavones

Samples: *Standard solution B*, *Standard solution C*, and *Sample solution*

Using the chromatograms of *Standard solution B*, *Standard solution C*, and the reference chromatogram provided with the lot of USP *Citrus reticulata* Pericarp Dry Extract RS being used, identify the peaks corresponding to nobiletin, 3,5,6,7,8,3′,4′-heptamethoxyflavone, and tangeretin in the *Sample solution*. [NOTE—See *Table 3* for the relative retention times.]

Table 3

Analyte	Approximate Relative Retention Time	Conversion Factor
Nobiletin	1.00	1.00
3,5,6,7,8,3′,4′-Heptamethoxyflavone	1.06	1.32
Tangeretin	1.12	0.88

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Separately calculate the percentage of nobiletin, 3,5,6,7,8,3′,4′-heptamethoxyflavone, and tangeretin in the portion of Tangerine Peel taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of nobiletin from *Standard solution B*
C_S = concentration of USP Nobiletin RS in *Standard solution B* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Tangerine Peel taken to prepare the *Sample solution* (mg)
F = conversion factor for the analyte (see *Table 3*)

Calculate the content of the total polymethoxylated flavones as the sum of the percentages of nobiletin, 3,5,6,7,8,3′,4′-heptamethoxyflavone, and tangeretin.

Acceptance criteria

Total dihydroflavone glycosides: NLT 5.0% on the anhydrous basis

Total polymethoxylated flavones: NLT 0.1% on the anhydrous basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Limits of Elemental Impurities*: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Pesticide Residue Analysis*: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures*, *Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Test for Aflatoxins*: Meets the requirements

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: Usually in several lobes connected at the end, or in irregular slices with a thickness of 1–4 mm. External surface orange-red or reddish brown with fine wrinkles and sunken dotted oil cavity; interior surface pale yellowish white, rough, with yellowish-white or yellowish-brown vein-like vascular bundles. Texture is hard and fragile.

Microscopic

Transverse section: Epidermal cells subsquare, covered with cuticle, sometimes stomata visible. Parenchymatous cells with thickened walls containing calcium oxalate prisms and hesperidin crystals in spheroid or amorphous mass, sometimes vessels visible. Inner cells subround, loosely arranged with uneven thickened walls. Oil cavities consisting of 1–2 layers of cells, ovate or elliptical, arranged irregularly and mainly scattered at the outer side of mesocarp.

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Foreign Organic Matter*: NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Alcohol-Soluble Extractives, Method 1*: NLT 15.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Water-Soluble Extractives, Method 2*: NLT 30.0%
- **WATER DETERMINATION** ⟨921⟩, *Method II*: NMT 12.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Total Ash*: NMT 5.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Acid-Insoluble Ash*: NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial following the official name of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP *Citrus reticulata* Pericarp Dry Extract RS
  USP Hesperidin RS
  USP Nobiletin RS

Tangerine Peel Dry Extract

DEFINITION

Tangerine Peel Dry Extract is prepared from the dried exocarp and mesocarp of the ripe fruit of *Citrus reticulata* Blanco (Fam. Rutaceae), partially freed from the white spongy tissue of the mesocarp, by extraction with hydroalcoholic mixtures. It contains NLT 90.0% and NMT 110.0% of the labeled amount of total dihydroflavone glycosides, calculated as the sum of narirutin ($C_{27}H_{32}O_{14}$), hesperidin ($C_{28}H_{34}O_{15}$), and didymin ($C_{28}H_{34}O_{14}$) on the anhydrous basis, and NLT 90.0% and NMT 110.0% of the labeled amount of total polymethoxylated flavones, calculated as the sum of nobiletin ($C_{21}H_{22}O_8$), 3,5,6,7,8,3′,4′-heptamethoxyflavone ($C_{22}H_{24}O_9$), and tangeretin ($C_{20}H_{20}O_7$) on the anhydrous basis. It may contain suitable added substances as carriers.

IDENTIFICATION

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 1.0 mg/mL of USP Hesperidin RS in methanol. Sonicate to dissolve.

Standard solution B: 50 mg/mL of USP *Citrus reticulata* Pericarp Dry Extract RS in methanol. Sonicate for 20 min, centrifuge, and use the supernatant.

Sample solution: 50 mg/mL of Tangerine Peel Dry Extract in methanol. Sonicate for 20 min, centrifuge, and use the supernatant.

Chromatographic system

Adsorbent: Chromatographic silica gel F_{254} mixture

Application volume: *Standard solution A*, 10 µL; *Standard solution B* and *Sample solution*, 5 µL for each, as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

Temperature: About 25°

Developing solvent system: Ethyl acetate, formic acid, and water (100:15:13)

Derivatization reagent A: 10 mg/mL of 2-aminoethyl diphenylborinate in methanol

Derivatization reagent B: 50 mg/mL of polyethylene glycol 4000 in alcohol

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands and dry in air. Develop in a saturated chamber, remove the plate from the chamber, and dry the plate at 100° for 3 min. Treat the plate with *Derivatization reagent A* and dry for 5 min with a stream of cool air. Immediately, treat the plate with *Derivatization reagent B*, dry for 5 min with a stream of cool air, and examine under UV light at 366 nm.

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements: *Standard solution A* exhibits a yellowish-brown band due to hesperidin in the lower half

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section. *Standard solution B* exhibits a band corresponding in R_F and color to the band due to hesperidin in *Standard solution A*, a yellow band below hesperidin, a blue band below the yellow band, another blue band above hesperidin, and a light yellowish-brown band between hesperidin and the blue band above. In the upper-third section, *Standard solution B* exhibits a bright blue band due to the coelution of nobiletin with some other components, and a faint green band above the bright blue band.

Acceptance criteria: The *Sample solution* exhibits a yellowish-brown band corresponding in R_F and color to the band due to hesperidin in *Standard solution A* and *Standard solution B*, a yellow band below hesperidin, a blue band below the yellow band, another blue band above hesperidin, and a light yellowish-brown band between hesperidin and the blue band above corresponding in R_F and color to the same bands in *Standard solution B*. In the upper-third section, the *Sample solution* exhibits a bright blue band and a faint green band above the bright blue band corresponding in R_F and color to the same bands in *Standard solution B*. In the lower-third section, the *Sample solution* exhibits a couple of yellow bands close to the starting position and a couple of faint bands above the yellow bands corresponding in R_F and color to the same bands in *Standard solution B*.

• **B. HPLC**

Analysis: Proceed as directed in the test for *Content of Dihydroflavone Glycosides and Polymethoxylated Flavones*.

Acceptance criteria: The *Sample solution* exhibits the most intense peak at the retention time corresponding to hesperidin in *Standard solution A* and peaks due to narirutin, didymin, nobiletin, 3,5,6,7,8,3′,4′-heptamethoxyflavone, and tangeretin at retention times corresponding to the same constituents in *Standard solution B*. No other peak between narirutin and tangeretin is more intense than the peak corresponding to didymin (a distinction from other *Citrus* species; *Citrus maxima* peel and *Citrus wilsonii* fruit show a principal peak for naringin). Sometimes, the peaks of nobiletin, 3,5,6,7,8,3′,4′-heptamethoxyflavone, and tangeretin are much smaller, such that these peaks could not be detected.

COMPOSITION

• **CONTENT OF DIHYDROFLAVONE GLYCOSIDES AND POLYMETHOXYLATED FLAVONES**

Solution A: 0.1% formic acid in water

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	85	15
8	81	19
10	81	19
17	60	40
28	56	44

[NOTE—The *Standard solutions* and the *Sample solution* are stable for 24 h at room temperature.]

Standard solution A: 0.40 mg/mL of USP Hesperidin RS in methanol

Standard solution B: 0.05 mg/mL of USP Nobiletin RS in methanol

Standard solution C: 5 mg/mL of USP *Citrus reticulata* Pericarp Dry Extract RS in methanol. Sonicate for 15 min, centrifuge, and pass through a suitable membrane filter of 0.22-µm pore size.

Sample solution: 5 mg/mL of Tangerine Peel Dry Extract in methanol with a suitable flask, closed tightly. Sonicate for 15 min, centrifuge, and pass through a suitable membrane filter of 0.22-µm pore size and discard the first portion of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 283 nm (0–17 min) and 330 nm (17–28 min)

Column: 4.6-mm × 5-cm; 1.8-µm packing L1

Column temperature: 25°

Flow rate: 0.7 mL/min

Injection volume: 2 µL

System suitability

Samples: *Standard solution A*, *Standard solution B*, and *Standard solution C*

Suitability requirements

Resolution: NLT 1.5 between the peak of hesperidin and the small peak before it, *Standard solution C*

Tailing factor: NMT 1.5 for the hesperidin and nobiletin peaks, *Standard solution A* and *Standard solution B*

Relative standard deviation: NMT 2.0% for the hesperidin and nobiletin peaks in repeated injections, *Standard solution A* and *Standard solution B*

Chromatographic similarity: The chromatogram of *Standard solution C* is similar to the reference chromatogram provided with the lot of USP *Citrus reticulata* Pericarp Dry Extract RS being used.

Analysis

For dihydroflavone glycosides

Samples: *Standard solution A*, *Standard solution C*, and *Sample solution*

Using the chromatograms of *Standard solution A*, *Standard solution C*, and the reference chromatogram provided with the lot of USP *Citrus reticulata* Pericarp Dry Extract RS being used, identify the peaks corresponding to narirutin, hesperidin, and didymin in the *Sample solution*. [NOTE—See *Table 2* for the relative retention times.]

Table 2

Analyte	Approximate Relative Retention Time	Conversion Factor
Narirutin	0.79	1.17
Naringin	0.90	—
Hesperidin	1.00	1.00
Didymin	1.70	1.00

Separately calculate the percentage of narirutin, hesperidin, and didymin in the portion of Tangerine Peel Dry Extract taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*

r_S = peak area of hesperidin from *Standard solution A*

C_S = concentration of USP Hesperidin RS in *Standard solution A* (mg/mL)

V = volume of the *Sample solution* (mL)

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W = weight of Tangerine Peel Dry Extract taken to prepare the *Sample solution* (mg)
F = conversion factor for the analyte (see *Table 2*)

Calculate the content of total dihydroflavone glycosides as the sum of the percentages of narirutin, hesperidin, and didymin.
Calculate the percentage of the labeled amount of total dihydroflavone glycosides in the portion of Tangerine Peel Dry Extract taken:

$$\text{Result} = (P/L) \times 100$$

P = content of total dihydroflavone glycosides as determined above (%)
L = labeled amount of total dihydroflavone glycosides (%)

For polymethoxylated flavones
Samples: *Standard solution B*, *Standard solution C*, and *Sample solution*
Using the chromatograms of *Standard solution B*, *Standard solution C*, and the reference chromatogram provided with the lot of USP *Citrus reticulata* Pericarp Dry Extract RS being used, identify the peaks corresponding to nobiletin, 3,5,6,7,8,3′,4′-heptamethoxyflavone, and tangeretin in the *Sample solution*. [NOTE—See *Table 3* for the relative retention times.]

Table 3

Analyte	Approximate Relative Retention Time	Conversion Factor
Nobiletin	1.00	1.00
3,5,6,7,8,3′,4′-Heptamethoxyflavone	1.06	1.32
Tangeretin	1.12	0.88

Separately calculate the percentage of nobiletin, 3,5,6,7,8,3′,4′-heptamethoxyflavone, and tangeretin in the portion of Tangerine Peel Dry Extract taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of nobiletin from *Standard solution B*
C_S = concentration of USP Nobiletin RS in *Standard solution B* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Tangerine Peel Dry Extract taken to prepare the *Sample solution* (mg)
F = conversion factor for the analyte (see *Table 3*)

Calculate the content of total polymethoxylated flavones as the sum of the percentages of nobiletin, 3,5,6,7,8,3′,4′-heptamethoxyflavone, and tangeretin.
Calculate the percentage of the labeled amount of total polymethoxylated flavones in the portion of Tangerine Peel Dry Extract taken:

$$\text{Result} = (P/L) \times 100$$

P = content of total polymethoxylated flavones as determined above (%)
L = labeled amount of total polymethoxylated flavones (%)

Acceptance criteria
Total dihydroflavone glycosides: 90.0%–110.0% of the labeled amount on the anhydrous basis
Total polymethoxylated flavones: 90.0%–110.0% of the labeled amount on the anhydrous basis

CONTAMINANTS
- **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩
Acceptance criteria
Arsenic: NMT 2.0 µg/g
Cadmium: NMT 0.5 µg/g
Lead: NMT 5.0 µg/g
Mercury: NMT 0.2 µg/g
- **BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^4 cfu/g and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS
- **WATER DETERMINATION** ⟨921⟩, *Method I*: NMT 8.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Total Ash*: NMT 4.0%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial following the official name of the plant from which the article was prepared. It meets other labeling requirements in *Botanical Extracts* ⟨565⟩.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP *Citrus reticulata* Pericarp Dry Extract RS
USP Hesperidin RS
USP Nobiletin RS

Tangerine Peel Powder

DEFINITION
Tangerine Peel Powder consists of the dried exocarp and mesocarp of the ripe fruit of *Citrus reticulata* Blanco (Fam. Rutaceae), partly freed from the white spongy tissue of the mesocarp, reduced to a fine or very fine powder. It contains NLT 5.0% of total dihydroflavone glycosides, calculated as the sum of narirutin ($C_{27}H_{32}O_{14}$), hesperidin ($C_{28}H_{34}O_{15}$), and didymin ($C_{28}H_{34}O_{14}$) on the anhydrous basis, and NLT 0.1% of total polymethoxylated flavones, calculated as the sum of nobiletin ($C_{21}H_{22}O_8$), 3,5,6,7,8,3′,4′-heptamethoxyflavone ($C_{22}H_{24}O_9$), and tangeretin ($C_{20}H_{20}O_7$) on the anhydrous basis.

IDENTIFICATION
- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩
Standard solution A: 1.0 mg/mL of USP Hesperidin RS in methanol. Sonicate to dissolve.
Standard solution B: 50 mg/mL of USP *Citrus reticulata* Pericarp Dry Extract RS in methanol. Sonicate for 20 min, centrifuge, and use the supernatant.
Sample solution: 500 mg of Tangerine Peel Powder in 5 mL of methanol. Sonicate for 20 min, centrifuge, and use the supernatant.

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Chromatographic system
Adsorbent: Chromatographic silica gel F_{254} mixture
Application volume: *Standard solution A*, 10 µL; *Standard solution B* and *Sample solution*, 5 µL for each, as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
Temperature: About 25°
Developing solvent system: Ethyl acetate, formic acid, and water (100:15:13)
Derivatization reagent A: 10 mg/mL of 2-aminoethyl diphenylborinate in methanol
Derivatization reagent B: 50 mg/mL of polyethylene glycol 4000 in alcohol
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Apply the *Samples* as bands and dry in air. Develop in a saturated chamber, remove the plate from the chamber, and dry the plate at 100° for 3 min. Treat the plate with *Derivatization reagent A* and dry for 5 min with a stream of cool air. Immediately, treat the plate with *Derivatization reagent B*, dry for 5 min with a stream of cool air, and examine under UV light at 366 nm.
System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements: *Standard solution A* exhibits a yellowish-brown band due to hesperidin in the lower half section. *Standard solution B* exhibits a band corresponding in R_F and color to the band due to hesperidin in *Standard solution A*, a yellow band below hesperidin, a blue band below the yellow band, another blue band above hesperidin, and a light yellowish-brown band between hesperidin and the blue band above. In the upper-third section, *Standard solution B* exhibits a bright blue band due to the coelution of nobiletin with some other components, and a faint green band above the bright blue band.
Acceptance criteria: The *Sample solution* exhibits a yellowish-brown band corresponding in R_F and color to the band due to hesperidin in *Standard solution A* and *Standard solution B*, a yellow band below hesperidin, a blue band below the yellow band, another blue band above hesperidin, and a light yellowish-brown band between hesperidin and the blue band above corresponding in R_F and color to the same bands in *Standard solution B*. In the upper-third section, the *Sample solution* exhibits a bright blue band and a faint green band above the bright blue band corresponding in R_F and color to the same bands in *Standard solution B*. In the lower-third section, the *Sample solution* exhibits a couple of yellow bands close to the starting position and a couple of faint bands above the yellow bands corresponding in R_F and color to the same bands in *Standard solution B*.

• **B. HPLC**
Analysis: Proceed as directed in the test for *Content of Dihydroflavone Glycosides and Polymethoxylated Flavones*.
Acceptance criteria: The *Sample solution* exhibits the most intense peak at the retention time corresponding to hesperidin in *Standard solution A* and peaks due to narirutin, didymin, nobiletin, 3,5,6,7,8,3′,4′-heptamethoxyflavone, and tangeretin at retention times corresponding to the same constituents in *Standard solution B*. No other peak between narirutin and tangeretin is more intense than the peak corresponding to didymin (a distinction from other *Citrus* species; *Citrus maxima* peel and *Citrus wilsonii* fruit show a principal peak for naringin). The content ratios of narirutin and didymin relative to hesperidin are within the ranges listed in *Table 2*.

COMPOSITION

• **CONTENT OF DIHYDROFLAVONE GLYCOSIDES AND POLYMETHOXYLATED FLAVONES**
Solution A: 0.1% formic acid in water
Solution B: Acetonitrile
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	85	15
8	81	19
10	81	19
17	60	40
28	56	44

[NOTE—The *Standard solutions* and the *Sample solution* are stable for 24 h at room temperature.]
Standard solution A: 0.40 mg/mL of USP Hesperidin RS in methanol
Standard solution B: 0.05 mg/mL of USP Nobiletin RS in methanol
Standard solution C: 5 mg/mL of USP *Citrus reticulata* Pericarp Dry Extract RS in methanol. Sonicate for 15 min, centrifuge, and pass through a suitable membrane filter of 0.22-µm pore size.
Sample solution: Accurately transfer about 100 mg of Tangerine Peel Powder to a suitable flask, accurately add 10.0 mL of methanol, and close tightly. Weigh the filled flask accurately and sonicate for 30 min. Cool to room temperature and adjust to the initial weight by adding methanol if needed. Before injection, pass through a suitable membrane filter of 0.22-µm pore size and discard the first portion of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 283 nm (0–17 min) and 330 nm (17–28 min)
Column: 4.6-mm × 5-cm; 1.8-µm packing L1
Column temperature: 25°
Flow rate: 0.7 mL/min
Injection volume: 2 µL
System suitability
Samples: *Standard solution A*, *Standard solution B*, and *Standard solution C*
Suitability requirements
Resolution: NLT 1.5 between the peak of hesperidin and the small peak before it, *Standard solution C*
Tailing factor: NMT 1.5 for the hesperidin and nobiletin peaks, *Standard solution A* and *Standard solution B*
Relative standard deviation: NMT 2.0% for the hesperidin and nobiletin peaks in repeated injections, *Standard solution A* and *Standard solution B*
Chromatographic similarity: The chromatogram of *Standard solution C* is similar to the reference chromatogram provided with the lot of USP *Citrus reticulata* Pericarp Dry Extract RS being used.
Analysis
For dihydroflavone glycosides
Samples: *Standard solution A*, *Standard solution C*, and *Sample solution*
Using the chromatograms of *Standard solution A*, *Standard solution C*, and the reference chromatogram provided with the lot of USP *Citrus reticulata* Pericarp Dry Extract RS being used, identify the peaks corresponding

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to narirutin, hesperidin, and didymin in the *Sample solution*. [NOTE—See *Table 2* for the relative retention times.]

Table 2

Analyte	Approximate Relative Retention Time	Conversion Factor	Content Ratio Relative to Hesperidin
Narirutin	0.79	1.17	0.1–0.3
Naringin	0.90	—	<0.02
Hesperidin	1.00	1.00	1.0
Didymin	1.70	1.00	0.02–0.06

Separately calculate the percentage of narirutin, hesperidin, and didymin in the portion of Tangerine Peel Powder taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of hesperidin from *Standard solution A*
C_S = concentration of USP Hesperidin RS in *Standard solution A* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Tangerine Peel Powder taken to prepare the *Sample solution* (mg)
F = conversion factor for the analyte (see *Table 2*)

Calculate the content of total dihydroflavone glycosides as the sum of the percentages of narirutin, hesperidin, and didymin.

For polymethoxylated flavones

Samples: *Standard solution B*, *Standard solution C*, and *Sample solution*

Using the chromatograms of *Standard solution B*, *Standard solution C*, and the reference chromatogram provided with the lot of USP *Citrus reticulata* Pericarp Dry Extract RS being used, identify the peaks corresponding to nobiletin, 3,5,6,7,8,3′,4′-heptamethoxyflavone, and tangeretin in the *Sample solution*. [NOTE—See *Table 3* for the relative retention times.]

Table 3

Analyte	Approximate Relative Retention Time	Conversion Factor
Nobiletin	1.00	1.00
3,5,6,7,8,3′,4′-Heptamethoxyflavone	1.06	1.32
Tangeretin	1.12	0.88

Separately calculate the percentage of nobiletin, 3,5,6,7,8,3′,4′-heptamethoxyflavone, and tangeretin in the portion of Tangerine Peel Powder taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of nobiletin from *Standard solution B*
C_S = concentration of USP Nobiletin RS in *Standard solution B* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Tangerine Peel Powder taken to prepare the *Sample solution* (mg)
F = conversion factor for the analyte (see *Table 3*)

Calculate the content of total polymethoxylated flavones as the sum of the percentages of nobiletin, 3,5,6,7,8,3′,4′-heptamethoxyflavone, and tangeretin.

Acceptance criteria

Total dihydroflavone glycosides: NLT 5.0% on the anhydrous basis

Total polymethoxylated flavones: NLT 0.1% on the anhydrous basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Limits of Elemental Impurities*: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Pesticide Residue Analysis*: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures*, *Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Test for Aflatoxins*: Meets the requirements

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

Macroscopic: Yellowish-white powder

Microscopic: Mesocarp parenchymatous cells, numerous, irregular with unevenly thickened walls. Epidermal cells polygonal, sub-square or rectangular in surface view, 18–26 µm in diameter, anticlinal walls thickened, stomata sub-rounded, subsidiary cells indistinct; in lateral view, covered with cuticle, the outer radial wall thickened. Mesocarp parenchymatous cells containing calcium oxalate prisms in polyhedral, rhombic or biconical, 3–34 µm in diameter, 5–53 µm long; some cells containing two parallel polyhedral crystals or 3–5 prisms. Yellow or colorless hesperidin crystals in spheroid or amorphous masses and mainly present in parenchymatous cells, some of them with radial striations. Spiral, pitted and reticulated vessels and tracheids are small.

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Alcohol-Soluble Extractives, Method 1*: NLT 15.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Water-Soluble Extractives, Method 2*: NLT 30.0%
- **WATER DETERMINATION** ⟨921⟩, *Method II*: NMT 12.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Total Ash*: NMT 5.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Acid-Insoluble Ash*: NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial following the official name of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP *Citrus reticulata* Pericarp Dry Extract RS
USP Hesperidin RS
USP Nobiletin RS

Thiamine Hydrochloride—*see Thiamine Hydrochloride General Monographs*

DS Monographs

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Thiamine Hydrochloride Oral Solution—*see Thiamine Hydrochloride Oral Solution General Monographs*

Thiamine Hydrochloride Tablets—*see Thiamine Hydrochloride Tablets General Monographs*

Thiamine Mononitrate—*see Thiamine Mononitrate General Monographs*

Thiamine Mononitrate Oral Solution—*see Thiamine Mononitrate Oral Solution General Monographs*

Threonine—*see Threonine General Monographs*

Tienchi Ginseng Root and Rhizome

DEFINITION

Tienchi Ginseng Root and Rhizome consists of the dried roots and rhizomes of *Panax notoginseng* (Burkill) F.H. Chen ex C.Y. Wu & K.M. Feng (Fam. Araliaceae) collected before flowering in autumn. It contains NLT 5.0% of total ginsenosides calculated on the dried basis as the sum of notoginsenoside R1 ($C_{47}H_{80}O_{18}$), ginsenoside Rg1 ($C_{42}H_{72}O_{14}$), ginsenoside Re ($C_{48}H_{82}O_{18}$), ginsenoside Rb1 ($C_{54}H_{92}O_{23}$), and ginsenoside Rd ($C_{48}H_{82}O_{18}$).

IDENTIFICATION

Delete the following:

- **▲A.** It meets the requirements in *Specific Tests* for *Botanic Characteristics.*▲ (USP 1-May-2019)

Change to read:

- **▲A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩▲ (USP 1-May-2019)

Standard solution A: 0.5 mg/mL of USP Ginsenoside Rg1 RS in methanol

Standard solution B: 10 mg/mL of USP *Panax notoginseng* Root and Rhizome Dry Extract RS in methanol. Sonicate for about 10 min, centrifuge, and use the supernatant.

Sample solution: Sonicate about 0.2 g of Tienchi Ginseng Root and Rhizome, finely powdered, in 5 mL of methanol for 20 min. Centrifuge, and use the supernatant. [NOTE—The *Sample solution* is stable for 6 h at room temperature.]

Chromatographic system

▲▲ (USP 1-May-2019)

Adsorbent: Chromatographic silica gel F_{254} mixture

Application volume: 4 µL, as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

Developing solvent system: Methylene chloride, dehydrated alcohol, and water ▲(60: 45: 6.5)▲ (USP 1-May-2019)

Developing distance: 6–8 cm

Derivatization reagent: A solution of 10% sulfuric acid in alcohol. Prepare fresh. Keep alcohol cold over ice. Carefully and gradually add sulfuric acid.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands to a suitable HPTLC plate, and dry in air. Develop the chromatograms in a saturated chamber, remove the plate from the chamber, and dry. Treat with *Derivatization reagent*, heat at 105° for 5–10 min, and examine immediately under white light and under UV light at 366 nm.

System suitability

Under white light: The chromatogram of *Standard solution B* exhibits five main reddish-violet bands in the following order with increasing R_F: a band in the lower-third section due to ginsenoside Rb1; three less intense bands clearly separated in the middle-third section —the lower due to ginsenoside Rd, the middle due to ginsenoside Re, and the upper due to notoginsenoside R1; and a band at an R_F corresponding to ginsenoside Rg1 in *Standard solution A*. The two most intense bands are due to ginsenoside Rb1 and ginsenoside Rg1.

Under UV light at 366 nm: The chromatogram of *Standard solution B* exhibits bands in the following order with increasing R_F: a blue fluorescent band in the lower-third section due to ginsenoside Rb1; three less intense bands clearly separated in the middle-third section —the lower due to ginsenoside Rd, the middle due to ginsenoside Re, and the upper due to notoginsenoside R1 (the band due to ginsenoside Rd is blue fluorescent, while the other two bands are pinkish violet); and a pinkish-violet band at an R_F corresponding to ginsenoside Rg1 in *Standard solution A*. The two most intense bands are due to ginsenoside Rb1 and ginsenoside Rg1.

Acceptance criteria

Under white light: The chromatogram of the *Sample solution* exhibits five main reddish-violet bands corresponding in R_F to similar bands in *Standard solution B*. These bands appear in the following order of increasing R_F: a band in the lower-third section due to ginsenoside Rb1; three less intense bands clearly separated in the middle-third section due to ginsenoside Rd, ginsenoside Re, and notoginsenoside R1, respectively; and a band at an R_F corresponding to ginsenoside Rg1 in *Standard solution A*. The two most intense bands are due to ginsenoside Rb1 and ginsenoside Rg1.

Under UV light at 366 nm: The chromatogram of the *Sample solution* exhibits the following bands, with increasing R_F, corresponding to similar bands in *Standard solution B*: a blue fluorescent band in the lower-third section due to ginsenoside Rb1; three less intense bands clearly separated in the middle-third section due to ginsenoside Rd, ginsenoside Re, and notoginsenoside R1, respectively (the band due to ginsenoside Rd is blue fluorescent, while the other two bands are pinkish violet); and a pinkish-violet band at an R_F corresponding to ginsenoside Rg1 in *Standard solution A*. The two most intense bands are due to ginsenoside Rb1 and ginsenoside Rg1.

Change to read:

- **▲B.**▲ (USP 1-May-2019) **UHPLC**

Analysis: Proceed as directed in *Content of Ginsenosides*.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks at the retention times corresponding

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to notoginsenoside R1 ▲(a distinction from *P. ginseng* and *P. quinquefolius*),▲ (USP 1-May-2019) ginsenoside Rg1, ginsenoside Re, ginsenoside Rb1, and ginsenoside Rd in *Standard solution B*. The two most intense peaks are due to ginsenoside Rg1 and ginsenoside Rb1. The content of notoginsenoside R1 is NLT 0.6% as determined in *Composition*.

COMPOSITION

Change to read:

- **CONTENT OF GINSENOSIDES**

Solution A: 0.03% phosphoric acid in water (v/v)
Solution B: Acetonitrile
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	83	17
2.4	80	20
3.5	70	30
4.2	69	31
5.0	58	42
5.1	0	100
6.0	0	100
6.1	83	17
7.5	83	17

Solvent: Methanol and water (7:3)
Standard solution A: 0.04 mg/mL of USP Ginsenoside Rg1 RS in ▲*Solvent*▲ (USP 1-May-2019)
Standard solution B: 3.0 mg/mL of USP *Panax notoginseng* Root and Rhizome Dry Extract RS in *Solvent*. Sonicate for about 10 min, centrifuge, and use the supernatant. Before injection, pass through a polytetrafluoroethylene (PTFE) filter of 0.2-µm pore size. ▲▲ (USP 1-May-2019)
Sample solution: Transfer 0.3 g of ▲finely powdered▲ (USP 1-May-2019) Tienchi Ginseng Root and Rhizome (capable of passing through a 250-µm sieve), accurately weighed, to a 50-mL centrifuge tube. Add 10 mL of *Solvent*, and sonicate for 10 min. Centrifuge, and transfer the supernatant to a 25-mL volumetric flask. Repeat this extraction two more times, each with 5 mL of *Solvent*. Combine the extracts in the volumetric flask, dilute with *Solvent* to volume, and mix. Before injection, pass through a PTFE filter of 0.2-µm pore size, and discard the first portion of the filtrate. [NOTE—The *Sample solution* is stable for 24 h at room temperature.]
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: UHPLC
Detector: UV 203 nm
Column: 2.1-mm × 5-cm; 1.7-µm packing L1▲▲ (USP 1-May-2019)
Column temperature: 30 ± 1°
Flow rate: 0.8 mL/min
Injection volume: 5 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP *Panax notoginseng* Root and Rhizome Dry Extract RS being used.
Resolution: NLT 1.5 between the ginsenoside Rg1 and ginsenoside Re peaks, *Standard solution B*
Tailing factor: NMT 2.0 for the ginsenoside Rg1 peak, *Standard solution A*
Relative standard deviation: NMT 2.0%, determined from the ginsenoside Rg1 peak in repeated injections, *Standard solution A*
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP *Panax notoginseng* Root and Rhizome Dry Extract RS being used, identify the retention times of the peaks corresponding to relevant ginsenosides in the *Sample solution*.
Separately calculate the percentage of each of the ginsenosides in the portion of Tienchi Ginseng Root and Rhizome taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of ginsenoside Rg1 from *Standard solution A*
C_S = concentration of USP Ginsenoside Rg1 RS in *Standard solution A* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Tienchi Ginseng Root and Rhizome taken to prepare the *Sample solution* (mg)
F = conversion factor for the relevant analyte (see *Table 2*)

Table 2

Analyte	Conversion Factor
Notoginsenoside R1	1.09
Ginsenoside Rg1	1.00
Ginsenoside Re	1.02
Ginsenoside Rb1	1.26
Ginsenoside Rd	1.03

Calculate the content of total ginsenosides as the sum of the percentages of notoginsenoside R1, ginsenoside Rg1, ginsenoside Re, ginsenoside Rb1, and ginsenoside Rd.
Acceptance criteria: NLT 5.0% on the dried basis

CONTAMINANTS

Change to read:

- ▲**ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Limits of Elemental Impurities*: Meets the requirements, except for *Arsenic* and *Cadmium*▲ (USP 1-May-2019)
Arsenic: NMT 3.0 µg/g
Cadmium: NMT 1.0 µg/g
▲▲ (USP 1-May-2019)

Change to read:

- ▲**ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩▲ (CN 1-May-2019): Meets the requirements

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- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacterial count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Test for Aflatoxins*: Meets the requirements

SPECIFIC TESTS

Change to read:

- **BOTANICAL CHARACTERISTICS**

Macroscopic

Root: Subconical or cylindrical; 1–6 cm long, 1–4 cm in diameter; externally grayish brown or grayish yellow; showing interrupted longitudinal wrinkles, scars of rootlets, and scar of the stem at the apex surrounded by irregular-shaped protrusions; texture heavy and compact, fracture grayish green, yellowish green, or grayish white

Rootlets: Cylindrical or conical; 2–6 cm long, the upper end 0.8 cm in diameter, the lower end 0.3 cm in diameter

Rhizomes: Irregular lumps; showing several stem scars and annulations; fracture grayish green or grayish white in the center and deep green or gray at the margin

Microscopic

Transverse section: Cork consisting of several rows of radially arranged, thin-walled cells; phelloderm narrow; layers of parenchyma cells, cluster crystals of calcium oxalate rare and mostly distributed in parenchyma close to cork; phloem parenchyma showing resin canals containing yellow brown masses; cambium in a ring sometimes undulant; and xylem broad, vessels 1–2 rows in groups, arranged radially

▲▲ (USP 1-May-2019)

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Foreign Organic Matter*: NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Alcohol-Soluble Extractives, Method 2*: NLT 16.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Water-Soluble Extractives, Method 2*: NLT 26.0%

Change to read:

- **LOSS ON DRYING** ⟨731⟩

▲▲ (USP 1-May-2019)

Analysis: Dry at 105° for 2 h.

Acceptance criteria: NMT 14%

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Total Ash*

Sample: 4.0 g of Tienchi Ginseng Root and Rhizome, finely powdered

Acceptance criteria: NMT 6%

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Acid-Insoluble Ash*

Sample: 4.0 g of Tienchi Ginseng Root and Rhizome, finely powdered

Acceptance criteria: NMT 3.0%

ADDITIONAL REQUIREMENTS

Change to read:

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at ▲controlled▲ (USP 1-May-2019) room temperature.

Change to read:

- **LABELING:** The label states the Latin binomial ▲▲ (USP 1-May-2019) following the official name ▲▲ (USP 1-May-2019) of the plant from which the article was derived.

Change to read:

- **USP REFERENCE STANDARDS** ⟨11⟩

▲▲ (USP 1-May-2019)

USP Ginsenoside Rg1 RS

USP *Panax notoginseng* Root and Rhizome Dry Extract RS

Tienchi Ginseng Root and Rhizome Powder

DEFINITION

Change to read:

Tienchi Ginseng Root and Rhizome Powder consists of the dried roots and rhizomes of *Panax notoginseng* (Burkill) F.H. Chen ex C.Y. Wu & K.M. Feng (Fam. Araliaceae), collected before flowering in autumn and reduced to a ▲fine or very fine▲ (USP 1-May-2019) powder. It contains NLT 5.0% of ▲total▲ (USP 1-May-2019) ginsenosides calculated on the dried basis as the sum of notoginsenoside R1 ($C_{47}H_{80}O_{18}$), ginsenoside Rg1 ($C_{42}H_{72}O_{14}$), ginsenoside Re ($C_{48}H_{82}O_{18}$), ginsenoside Rb1 ($C_{54}H_{92}O_{23}$), and ginsenoside Rd ($C_{48}H_{82}O_{18}$).

IDENTIFICATION

Delete the following:

- ▲**A.** It meets the requirements in *Specific Tests* for *Botanic Characteristics*.▲ (USP 1-May-2019)

Change to read:

- ▲**A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩▲ (USP 1-May-2019)

Standard solution A: 0.5 mg/mL of USP Ginsenoside Rg1 RS in methanol

Standard solution B: 10 mg/mL of USP *Panax notoginseng* Root and Rhizome Dry Extract RS in methanol. Sonicate for about 10 min, centrifuge, and use the supernatant.

Sample solution: Sonicate about 0.2 g of ▲Tienchi Ginseng Root and Rhizome▲ (USP 1-May-2019) Powder ▲▲ (USP 1-May-2019) in 5 mL of methanol for 20 min. Centrifuge, and use the supernatant. [NOTE—The *Sample solution* is stable for 6 h at room temperature.]

Chromatographic system

▲▲ (USP 1-May-2019)

Adsorbent: Chromatographic silica gel F_{254} mixture

Application volume: 4 µL, as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

Developing solvent system: Methylene chloride, dehydrated alcohol, and water ▲(60: 45: 6.5)▲ (USP 1-May-2019)

Developing distance: 6–8 cm

Derivatization reagent: A solution of 10% sulfuric acid in alcohol. Prepare fresh. Keep alcohol cold over ice. Carefully and gradually add sulfuric acid.

DS Monographs

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Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Apply the *Samples* as bands to a suitable HPTLC plate, and dry in air. Develop the chromatograms in a saturated chamber, remove the plate from the chamber, and dry. Treat with *Derivatization reagent*, heat at 105° for 5–10 min, and examine immediately under white light and under UV light at 366 nm.
System suitability
Under white light: The chromatogram of *Standard solution B* exhibits five main reddish-violet bands in the following order with increasing R_F: a band in the lower-third section due to ginsenoside Rb1; three less intense bands clearly separated in the middle-third section —the lower due to ginsenoside Rd, the middle due to ginsenoside Re, and the upper due to notoginsenoside R1; and a band at an R_F corresponding to ginsenoside Rg1 in *Standard solution A*. The two most intense bands are due to ginsenoside Rb1 and ginsenoside Rg1.
Under UV light at 366 nm: The chromatogram of *Standard solution B* exhibits bands in the following order with increasing R_F: a blue fluorescent band in the lower-third section due to ginsenoside Rb1; three less intense bands clearly separated in the middle-third section —the lower due to ginsenoside Rd, the middle due to ginsenoside Re, and the upper due to notoginsenoside R1 (the band due to ginsenoside Rd is blue fluorescent, while the other two bands are pinkish violet); and a pinkish-violet band at an R_F corresponding to ginsenoside Rg1 in *Standard solution A*. The two most intense bands are due to ginsenoside Rb1 and ginsenoside Rg1.
Acceptance criteria
Under white light: The chromatogram of the *Sample solution* exhibits five main reddish-violet bands corresponding in R_F to similar bands in *Standard solution B*. These bands appear in the following order of increasing R_F: a band in the lower-third section due to ginsenoside Rb1; three less intense bands clearly separated in the middle-third section due to ginsenoside Rd, ginsenoside Re, and notoginsenoside R1, respectively; and a band at an R_F corresponding to ginsenoside Rg1 in *Standard solution A*. The two most intense bands are due to ginsenoside Rb1 and ginsenoside Rg1.
Under UV light at 366 nm: The chromatogram of the *Sample solution* exhibits the following bands, with increasing R_F, corresponding to similar bands in *Standard solution B*: a blue fluorescent band in the lower-third section due to ginsenoside Rb1; three less intense bands clearly separated in the middle-third section due to ginsenoside Rd, ginsenoside Re, and notoginsenoside R1, respectively (the band due to ginsenoside Rd is blue fluorescent, while the other two bands are pinkish violet); and a pinkish-violet band at an R_F corresponding to ginsenoside Rg1 in *Standard solution A*. The two most intense bands are due to ginsenoside Rb1 and ginsenoside Rg1.

Change to read:

• **▲B.▲ (USP 1-May-2019) UHPLC**
Analysis: Proceed as directed in *Content of Ginsenosides*.
Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks at the retention times corresponding to notoginsenoside R1 ▲(a distinction from *P. ginseng* and *P. quinquefolius*),▲ (USP 1-May-2019) ginsenoside Rg1, ginsenoside Re, ginsenoside Rb1, and ginsenoside Rd in *Standard solution B*. The two most intense peaks are due to ginsenoside Rg1 and ginsenoside Rb1. The content of notoginsenoside R1 is NLT 0.6% as determined in the test for *Composition*.

COMPOSITION

Change to read:

• **CONTENT OF GINSENOSIDES**
Solution A: 0.03% phosphoric acid in water (v/v)
Solution B: Acetonitrile
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	83	17
2.4	80	20
3.5	70	30
4.2	69	31
5.0	58	42
5.1	0	100
6.0	0	100
6.1	83	17
7.5	83	17

Solvent: Methanol and water (7:3)
Standard solution A: 0.04 mg/mL of USP Ginsenoside Rg1 RS in ▲*Solvent*▲ (USP 1-May-2019)
Standard solution B: 3.0 mg/mL of USP *Panax notoginseng* Root and Rhizome Dry Extract RS in *Solvent*. Sonicate for about 10 min, centrifuge, and use the supernatant. Before injection, pass through a polytetrafluoroethylene (PTFE) filter of 0.2-µm pore size. ▲▲ (USP 1-May-2019)
Sample solution: Transfer 0.3 g of ▲Tienchi Ginseng Root and Rhizome▲ (USP 1-May-2019) Powder (capable of passing through a 250-µm sieve), accurately weighed, to a 50-mL centrifuge tube. Add 10 mL of *Solvent*, and sonicate for 10 min. Centrifuge, and transfer the supernatant to a 25-mL volumetric flask. Repeat this extraction two more times, each with 5 mL of *Solvent*. Combine the extracts in the volumetric flask, dilute with *Solvent* to volume, and mix. Before injection, pass through a PTFE filter of 0.2-µm pore size, and discard the first portion of the filtrate. [NOTE—The *Sample solution* is stable for 24 h at room temperature.]
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: UHPLC
Detector: UV 203 nm
Column: 2.1-mm × 5-cm; 1.7-µm packing L1▲▲ (USP 1-May-2019)
Column temperature: 30 ± 1°
Flow rate: 0.8 mL/min
Injection volume: 5 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP *Panax notoginseng* Root and Rhizome Dry Extract RS being used.
Resolution: NLT 1.5 between the ginsenoside Rg1 and ginsenoside Re peaks, *Standard solution B*

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Tailing factor: NMT 2.0 for the ginsenoside Rg1 peak, *Standard solution A*
Relative standard deviation: NMT 2.0%, determined from the ginsenoside Rg1 peak in repeated injections, *Standard solution A*

Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP *Panax notoginseng* Root and Rhizome Dry Extract RS being used, identify the retention times of the peaks corresponding to relevant ginsenosides in the *Sample solution*.
Separately calculate the percentage of each of the ginsenosides in the portion of ▲Tienchi Ginseng Root and Rhizome▲ (USP 1-May-2019) Powder taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of ginsenoside Rg1 from *Standard solution A*
C_S = concentration of USP Ginsenoside Rg1 RS in *Standard solution A* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of ▲Tienchi Ginseng Root and Rhizome▲ (USP 1-May-2019) Powder taken to prepare the *Sample solution* (mg)
F = conversion factor for the relevant analyte (see *Table 2*)

Table 2

Analyte	Conversion Factor
Notoginsenoside R1	1.09
Ginsenoside Rg1	1.00
Ginsenoside Re	1.02
Ginsenoside Rb1	1.26
Ginsenoside Rd	1.03

Calculate the content of total ginsenosides as the sum of the percentages of notoginsenoside R1, ginsenoside Rg1, ginsenoside Re, ginsenoside Rb1, and ginsenoside Rd.
Acceptance criteria: NLT 5.0% on the dried basis

CONTAMINANTS

Change to read:

- **▲ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Limits of Elemental Impurities*: Meets the requirements, except for *Arsenic* and *Cadmium*▲ (USP 1-May-2019)
Arsenic: NMT 3.0 µg/g
Cadmium: NMT 1.0 µg/g
▲▲ (USP 1-May-2019)

Change to read:

- **▲ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Pesticide Residue Analysis*▲ (CN 1-May-2019): Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacterial count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures*, *Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Test for Aflatoxins*: Meets the requirements

SPECIFIC TESTS

Change to read:

- **BOTANICAL CHARACTERISTICS**
Macroscopic: Grayish yellow in color
Microscopic: Fragments of cork cells; fragments of parenchyma cells, crystals of calcium oxalate clusters rare; fragments of secretory ducts containing yellow secretions; vessels, scalariform, reticulated and spiral, 15–55 µm in diameter; starch granules fairly abundant, simple granules spherical, semispherical, or round-polygonal, 5–40 µm in diameter; compound granules of 2–10 or more components; showing a black, cross shape when examined under a polarizing microscope
▲▲ (USP 1-May-2019)
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis*, *Foreign Organic Matter*: NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis*, *Alcohol-Soluble Extractives, Method 2*: NLT 16.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis*, *Water-Soluble Extractives, Method 2*: NLT 26.0%

Change to read:

- **LOSS ON DRYING** ⟨731⟩
▲▲ (USP 1-May-2019)
Analysis: Dry at 105° for 2 h.
Acceptance criteria: NMT 14%

Change to read:

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis*, *Total Ash*
Sample: 4.0 g of ▲Tienchi Ginseng Root and Rhizome▲ (USP 1-May-2019) Powder ▲▲ (USP 1-May-2019)
Acceptance criteria: NMT 6%

Change to read:

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis*, *Acid-Insoluble Ash*
Sample: 4.0 g of ▲Tienchi Ginseng Root and Rhizome▲ (USP 1-May-2019) Powder ▲▲ (USP 1-May-2019)
Acceptance criteria: NMT 3.0%

ADDITIONAL REQUIREMENTS

Change to read:

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at ▲controlled▲ (USP 1-May-2019) room temperature.

Change to read:

- **LABELING:** The label states the Latin binomial ▲▲ (USP 1-May-2019) following the official name ▲▲ (USP 1-May-2019) of the plant contained in the article.

Change to read:

- **USP REFERENCE STANDARDS** ⟨11⟩
▲▲ (USP 1-May-2019)

DS Monographs

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USP Ginsenoside Rg1 RS
USP *Panax notoginseng* Root and Rhizome Dry Extract RS

Tienchi Ginseng Root and Rhizome Powder Capsules

DEFINITION

Tienchi Ginseng Root and Rhizome Powder Capsules contain Tienchi Ginseng Root and Rhizome Powder. They contain NLT 5.0% of ginsenosides calculated as the sum of notoginsenoside R1 ($C_{47}H_{80}O_{18}$), ginsenoside Rg1 ($C_{42}H_{72}O_{14}$), ginsenoside Re ($C_{48}H_{82}O_{18}$), ginsenoside Rb1 ($C_{54}H_{92}O_{23}$), and ginsenoside Rd ($C_{48}H_{82}O_{18}$) from the labeled amount of Tienchi Ginseng Root and Rhizome Powder.

IDENTIFICATION

Change to read:

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 0.5 mg/mL of USP Ginsenoside Rg1 RS in methanol

Standard solution B: 5 mg/mL of USP *Panax notoginseng* Root and Rhizome Dry Extract RS in methanol. Sonicate for about 10 min, centrifuge, and use the supernatant.

Sample solution: Transfer a finely powdered portion of the contents of the Capsules, equivalent to 0.4 g of Tienchi Ginseng Root and Rhizome Powder, to a suitable container, add 10.0 mL of methanol, and sonicate for 20 min. Centrifuge, and use the supernatant.

Chromatographic system

Adsorbent: Chromatographic silica gel F_{254} mixture

Application volume: 4 µL, as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

Developing solvent system: Methylene chloride, dehydrated alcohol, and water ▲(60: 45: 6.5)▲ (USP 1-May-2019)

Developing distance: 6 cm

Derivatization reagent: 10% sulfuric acid in alcohol. Prepare fresh. Keep alcohol cold over ice. Carefully and gradually add sulfuric acid.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands to a suitable HPTLC plate, and dry in air. Develop in a saturated chamber, remove the plate from the chamber, and dry in air. Treat the plate with *Derivatization reagent*, heat at 105° for 5 min, and examine immediately under white light and under UV light at 366 nm.

System suitability

Under white light: The chromatogram of *Standard solution B* exhibits five main reddish-violet bands. A band in the upper-half section corresponding in R_F to the band of ginsenoside Rg1 in *Standard solution A*; a band in the lower-third section due to ginsenoside Rb1; three less intense bands clearly separated between the bands of ginsenoside Rg1 and ginsenoside Rb1—the lower band due to ginsenoside Rd, the middle due to ginsenoside Re, and the upper due to notoginsenoside R1. The two most intense bands are due to ginsenoside Rb1 and ginsenoside Rg1.

Under UV light at 366 nm: The chromatogram of *Standard solution B* exhibits a pinkish-violet band at an R_F corresponding to the ginsenoside Rg1 band in *Standard solution A*; a blue fluorescent band in the lower-third section due to ginsenoside Rb1; three less intense bands clearly separated between the bands of ginsenoside Rg1 and ginsenoside Rb1—the lower due to ginsenoside Rd, the middle due to ginsenoside Re, and the upper due to notoginsenoside R1 (the band due to ginsenoside Rd is blue fluorescent, while the other two bands are pinkish violet). The two most intense bands in the chromatogram are due to ginsenoside Rb1 and ginsenoside Rg1.

Acceptance criteria

Under white light: The chromatogram of the *Sample solution* exhibits five main reddish-violet bands corresponding in R_F to similar bands in *Standard solution B*: a band in the upper-half section corresponding in R_F to ginsenoside Rg1 in *Standard solution A*; a band in the lower-third section due to ginsenoside Rb1; three less intense bands clearly separated between the bands of ginsenoside Rg1 and ginsenoside Rb1—the lower band due to ginsenoside Rd, the middle due to ginsenoside Re, and the upper due to notoginsenoside R1. The two most intense bands are due to ginsenoside Rb1 and ginsenoside Rg1.

Under UV light at 366 nm: The chromatogram of the *Sample solution* exhibits bands corresponding in R_F to similar bands in *Standard solution B*: a pinkish-violet band at an R_F corresponding to the ginsenoside Rg1 band in *Standard solution A*; a blue fluorescent band in the lower-third section due to ginsenoside Rb1; three less intense bands clearly separated between the bands of ginsenoside Rg1 and ginsenoside Rb1—the lower due to ginsenoside Rd, the middle due to ginsenoside Re, and the upper due to notoginsenoside R1 (the band due to ginsenoside Rd is blue fluorescent, while the other two bands are pinkish violet). The two most intense bands are due to ginsenoside Rb1 and ginsenoside Rg1.

- **B. LC**

Analysis: Proceed as directed in the test for *Content of Ginsenosides*.

Acceptance criteria: The retention times of the notoginsenoside R1, ginsenoside Rg1, ginsenoside Re, ginsenoside Rb1, and ginsenoside Rd peaks of the *Sample solution* correspond to those of *Standard solution B*. The two most intense peaks are due to ginsenoside Rg1 and ginsenoside Rb1.

STRENGTH

Change to read:

- **CONTENT OF GINSENOSIDES**

Extraction solvent: Methanol and water (70:30)

Solution A: 0.03% phosphoric acid in water (v/v)

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	83	17
2.4	80	20
3.5	70	30
4.2	69	31
5.0	58	42
5.1	0	100
6.0	0	100

DS Monographs

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Table 1 *(continued)*

Time (min)	Solution A (%)	Solution B (%)
6.1	83	17
7.5	83	17

Standard solution A: 0.04 mg/mL of USP Ginsenoside Rg1 RS in ▲*Extraction solvent*.▲ (USP 1-May-2019) Sonicate to dissolve, if necessary.

Standard solution B: 3.0 mg/mL of USP *Panax notoginseng* Root and Rhizome Dry Extract RS in *Extraction solvent*. Sonicate for about 10 min, centrifuge, and use the supernatant. Before injection, pass through a polytetrafluoroethylene (PTFE) membrane filter of 0.2-µm pore size, and discard the first portion of the filtrate.

Sample solution: Determine the total weight of 20 Capsules. Open the Capsules and combine their contents in an appropriate container. Weigh the empty Capsule shells and calculate the average fill weight per Capsule. Thoroughly mix and finely powder the contents of the Capsules. Transfer a portion of the Capsule contents, equivalent to 0.3 g of Tienchi Ginseng Root and Rhizome Powder to a 50-mL centrifuge tube. Add 10 mL of *Extraction solvent*, and sonicate for 20 min. Centrifuge, and transfer the supernatant to a 25-mL volumetric flask. Repeat this extraction with 10 mL of *Extraction solvent*, and sonicate for 10 min. Combine the extracts in a volumetric flask, dilute with *Extraction solvent* to volume, and mix. Before injection, pass through a PTFE membrane filter of 0.2-µm pore size, and discard the first portion of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 203 nm

Column: 2.1-mm × 5-cm; 1.7-µm packing L1

Column temperature: 30°

Flow rate: 0.8 mL/min

Injection volume: 5 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP *Panax notoginseng* Root and Rhizome Dry Extract RS being used.

Resolution: NLT 1.5 between the ginsenoside Rg1 and ginsenoside Re peaks, *Standard solution B*

Relative standard deviation: NMT 2.0%, determined from the ginsenoside Rg1 peak in repeated injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP *Panax notoginseng* Root and Rhizome Dry Extract RS being used, identify the peaks corresponding to notoginsenoside R1, ginsenoside Rg1, ginsenoside Re, ginsenoside Rb1, and ginsenoside Rd in the *Sample solution*. Measure the areas of the analyte peaks.

Separately calculate the quantity, in mg, of notoginsenoside R1, ginsenoside Rg1, ginsenoside Re, ginsenoside Rb1, and ginsenoside Rd, in each Capsule:

$$\text{Result} = (r_U/r_S) \times C_S \times V \times F \times (W_{av}/W)$$

r_U = peak area of the relevant analyte from the *Sample solution*

r_S = peak area of ginsenoside Rg1 from *Standard solution A*

C_S = concentration of USP Ginsenoside Rg1 RS in *Standard solution A* (mg/mL)

V = volume of the *Extraction solvent* taken to prepare the *Sample solution* (mL)

F = response factor for the relevant analyte (see *Table 2*)

W_{av} = average fill weight per Capsule (mg)

W = weight of the sample taken to prepare the *Sample solution* (mg)

Table 2

Analyte	Response Factor
Notoginsenoside R1	1.09
Ginsenoside Rg1	1.00
Ginsenoside Re	1.02
Ginsenoside Rb1	1.26
Ginsenoside Rd	1.03

Calculate the percentage of ginsenosides, as the sum of notoginsenoside R1, ginsenoside Rg1, ginsenoside Re, ginsenoside Rb1, and ginsenoside Rd, of the labeled amount of Tienchi Ginseng Root and Rhizome Powder in each Capsule:

$$\text{Result} = (\Sigma Q_i/L) \times 100$$

Q_i = sum of the calculated quantities of ginsenosides (mg)

L = labeled amount of Tienchi Ginseng Root and Rhizome Powder (mg)

Acceptance criteria: NLT 5.0%

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩, *Disintegration*: Meet the requirements
- **WEIGHT VARIATION** ⟨2091⟩: Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures*, *Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meet the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and the official name. The label states the amount of Tienchi Ginseng Root and Rhizome Powder in mg/Capsule.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Ginsenoside Rg1 RS

USP *Panax notoginseng* Root and Rhizome Dry Extract RS

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Tienchi Ginseng Root and Rhizome Powder Tablets

DEFINITION

Tienchi Ginseng Root and Rhizome Powder Tablets contain Tienchi Ginseng Root and Rhizome Powder. They contain NLT 5.0% of ginsenosides calculated as the sum of notoginsenoside R1 ($C_{47}H_{80}O_{18}$), ginsenoside Rg1 ($C_{42}H_{72}O_{14}$), ginsenoside Re ($C_{48}H_{82}O_{18}$), ginsenoside Rb1 ($C_{54}H_{92}O_{23}$), and ginsenoside Rd ($C_{48}H_{82}O_{18}$) from the labeled amount of Tienchi Ginseng Root and Rhizome Powder.

IDENTIFICATION

Change to read:

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 0.5 mg/mL of USP Ginsenoside Rg1 RS in methanol

Standard solution B: 5 mg/mL of USP *Panax notoginseng* Root and Rhizome Dry Extract RS in methanol. Sonicate for about 10 min, centrifuge, and use the supernatant.

Sample solution: Transfer a portion of the finely powdered Tablets, equivalent to 0.4 g of Tienchi Ginseng Root and Rhizome Powder, to a suitable container, add 10.0 mL of methanol, and sonicate for 20 min. Centrifuge and use the supernatant.

Chromatographic system

Adsorbent: Chromatographic silica gel F_{254} mixture

Application volume: 4 µL, as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

Developing solvent system: Methylene chloride, dehydrated alcohol, and water ▲(60: 45: 6.5)▲ (USP 1-May-2019)

Developing distance: 6 cm

Derivatization reagent: 10% sulfuric acid in alcohol. Prepare fresh. Keep alcohol cold over ice. Carefully and gradually add sulfuric acid.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands to a suitable HPTLC plate, and dry in air. Develop in a saturated chamber, remove the plate from the chamber, and dry in air. Treat the plate with *Derivatization reagent*, heat at 105° for 5 min, and examine immediately under white light and under UV light at 366 nm.

System suitability

Under white light: The chromatogram of *Standard solution B* exhibits five main reddish-violet bands. A band in the upper-half section corresponding in R_F to the band of ginsenoside Rg1 in *Standard solution A*; a band in the lower-third section due to ginsenoside Rb1; three less intense bands clearly separated between the bands of ginsenoside Rg1 and ginsenoside Rb1—the lower band due to ginsenoside Rd, the middle due to ginsenoside Re, and the upper due to notoginsenoside R1. The two most intense bands are due to ginsenoside Rb1 and ginsenoside Rg1.

Under UV light at 366 nm: The chromatogram of *Standard solution B* exhibits a pinkish-violet band at an R_F corresponding to the ginsenoside Rg1 band in *Standard solution A*; a blue fluorescent band in the lower-third section due to ginsenoside Rb1; three less intense bands clearly separated between the bands of ginsenoside Rg1 and ginsenoside Rb1—the lower due to ginsenoside Rd, the middle due to ginsenoside Re, and the upper due to notoginsenoside R1 (the band due to ginsenoside Rd is blue fluorescent, while the other two bands are pinkish violet). The two most intense bands are due to ginsenoside Rb1 and ginsenoside Rg1.

Acceptance criteria

Under white light: The chromatogram of the *Sample solution* exhibits five main reddish-violet bands corresponding in R_F to similar bands in *Standard solution B*: a band in the upper-half section corresponding in R_F to the band of ginsenoside Rg1 in *Standard solution A*; a band in the lower-third section due to ginsenoside Rb1; three less intense bands clearly separated between the bands of ginsenoside Rg1 and ginsenoside Rb1—the lower band due to ginsenoside Rd, the middle due to ginsenoside Re, and the upper due to notoginsenoside R1. The two most intense bands are due to ginsenoside Rb1 and ginsenoside Rg1.

Under UV light at 366 nm: The chromatogram of the *Sample solution* exhibits bands corresponding in R_F to similar bands in *Standard solution B*: a pinkish-violet band at an R_F corresponding to the ginsenoside Rg1 band in *Standard solution A*; a blue fluorescent band in the lower-third section due to ginsenoside Rb1; three less intense bands clearly separated between the bands of ginsenoside Rg1 and ginsenoside Rb1—the lower due to ginsenoside Rd, the middle due to ginsenoside Re, and the upper due to notoginsenoside R1 (the band due to ginsenoside Rd is blue fluorescent, while the other two bands are pinkish violet). The two most intense bands are due to ginsenoside Rb1 and ginsenoside Rg1.

- **B. LC**

Analysis: Proceed as directed in the test for *Content of Ginsenosides*.

Acceptance criteria: The retention times of the notoginsenoside R1, ginsenoside Rg1, ginsenoside Re, ginsenoside Rb1, and ginsenoside Rd peaks of the *Sample solution* correspond to those of *Standard solution B*. The two most intense peaks are due to ginsenoside Rg1 and ginsenoside Rb1.

STRENGTH

Change to read:

- **CONTENT OF GINSENOSIDES**

Extraction solvent: Methanol and water (70:30)

Solution A: 0.03% phosphoric acid in water (v/v)

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	83	17
2.4	80	20
3.5	70	30
4.2	69	31
5.0	58	42
5.1	0	100
6.0	0	100
6.1	83	17
7.5	83	17

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Standard solution A: 0.04 mg/mL of USP Ginsenoside Rg1 RS in ▲*Extraction solvent*.▲ (USP 1-May-2019) Sonicate to dissolve, if necessary.

Standard solution B: 2.0 mg/mL of USP *Panax notoginseng* Root and Rhizome Dry Extract RS in *Extraction solvent*. Sonicate for about 10 min, centrifuge, and use the supernatant. Before injection, pass through a polytetrafluoroethylene (PTFE) membrane filter of 0.2-µm pore size and discard the first portion of the filtrate.

Sample solution: Weigh NLT 20 Tablets, determine the average Tablet weight, and finely powder. Transfer a portion of finely powdered Tablets, equivalent to 0.3 g of Tienchi Ginseng Root and Rhizome Powder to a 50-mL centrifuge tube. Add 10 mL of *Extraction solvent*, and sonicate for 20 min. Centrifuge, and transfer the supernatant to a 25-mL volumetric flask. Repeat this extraction with 10 mL of *Extraction solvent*, and sonicate for 10 min. Combine the extracts in a volumetric flask, dilute with *Extraction solvent* to volume, and mix. Before injection, pass through a PTFE membrane filter of 0.2-µm pore size, and discard the first portion of the filtrate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 203 nm
Column: 2.1-mm × 5-cm; 1.7-µm packing L1
Column temperature: 30°
Flow rate: 0.8 mL/min
Injection volume: 5 µL

System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP *Panax notoginseng* Root and Rhizome Dry Extract RS being used.
Resolution: NLT 1.5 between the ginsenoside Rg1 and ginsenoside Re peaks, *Standard solution B*
Relative standard deviation: NMT 2.0%, determined from the ginsenoside Rg1 peak in repeated injections, *Standard solution A*

Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP *Panax notoginseng* Root and Rhizome Dry Extract RS being used, identify the peaks corresponding to notoginsenoside R1, ginsenoside Rg1, ginsenoside Re, ginsenoside Rb1, and ginsenoside Rd in the *Sample solution*.
Separately calculate the quantity, in mg, of notoginsenoside R1, ginsenoside Rg1, ginsenoside Re, ginsenoside Rb1, and ginsenoside Rd in each Tablet:

$$\text{Result} = (r_U/r_S) \times C_S \times V \times F \times (W_{av}/W)$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of ginsenoside Rg1 from *Standard solution A*
C_S = concentration of USP Ginsenoside Rg1 RS in *Standard solution A* (mg/mL)
V = volume of the *Extraction solvent* taken to prepare the *Sample solution* (mL)
F = response factor for the relevant analyte (see *Table 2*)
W_{av} = average Tablet weight (mg)
W = weight of the sample taken to prepare the *Sample solution* (mg)

Table 2

Analyte	Response Factor
Notoginsenoside R1	1.09
Ginsenoside Rg1	1.00
Ginsenoside Re	1.02
Ginsenoside Rb1	1.26
Ginsenoside Rd	1.03

Calculate the percentage of ginsenosides, as the sum of notoginsenoside R1, ginsenoside Rg1, ginsenoside Re, ginsenoside Rb1, and ginsenoside Rd, of the labeled amount of Tienchi Ginseng Root and Rhizome Powder in each Tablet:

$$\text{Result} = (\Sigma Q_i/L) \times 100$$

Q_i = sum of the calculated quantities of ginsenosides (mg)
L = labeled amount of Tienchi Ginseng Root and Rhizome Powder (mg)

Acceptance criteria: NLT 5.0%

PERFORMANCE TESTS
- **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩, *Disintegration*: Meet the requirements
- **WEIGHT VARIATION** ⟨2091⟩: Meet the requirements

CONTAMINANTS
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures*, *Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and the official name. The label states the amount of Tienchi Ginseng Root and Rhizome Powder in mg/Tablet.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Ginsenoside Rg1 RS
USP *Panax notoginseng* Root and Rhizome Dry Extract RS

Tienchi Ginseng Root and Rhizome Dry Extract

DEFINITION
Tienchi Ginseng Root and Rhizome Dry Extract is prepared from the dried roots and rhizomes of *Panax notoginseng* (Burkill) F.H. Chen ex C.Y. Wu & K.M. Feng (Fam. Araliaceae), collected before flowering in autumn, by extraction with hydroalcoholic mixtures. It contains NLT 90.0%–110.0% of the labeled amount of ginsenosides calculated on the dried basis as the sum of notoginsenoside R1 ($C_{47}H_{80}O_{18}$), ginsenoside Rg1 ($C_{42}H_{72}O_{14}$), ginsenoside

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Re ($C_{48}H_{82}O_{18}$), ginsenoside Rb1 ($C_{54}H_{92}O_{23}$), and ginsenoside Rd ($C_{48}H_{82}O_{18}$).

IDENTIFICATION

• **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 0.5 mg/mL of USP Ginsenoside Rg1 RS in methanol

Standard solution B: 10 mg/mL of USP *Panax notoginseng* Root and Rhizome Dry Extract RS in methanol. Sonicate for about 10 min, centrifuge, and use the supernatant.

Sample solution: Sonicate about 50 mg of Dry Extract, finely powdered, in 5 mL of methanol for 10 min. Centrifuge, and use the supernatant. [NOTE—The *Sample solution* is stable for 6 h at room temperature.]

Chromatographic system

Adsorbent: Chromatographic silica gel F_{254} mixture

Application volume: 4 µL, as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

Developing solvent system: Methylene chloride, dehydrated alcohol, and water (60: 45: 6.5)

Developing distance: 6–8 cm

Derivatization reagent: A solution of 10% sulfuric acid in alcohol. Prepare fresh. Keep alcohol cold over ice. Carefully and gradually add sulfuric acid.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands to a suitable HPTLC plate, and dry in air. Develop the chromatograms in a saturated chamber, remove the plate from the chamber, and dry. Treat with *Derivatization reagent*, heat at 105° for 5–10 min, and examine immediately under white light and under UV light at 366 nm.

System suitability

Under white light: The chromatogram of *Standard solution B* exhibits five main reddish-violet bands in the following order with increasing R_F: a band in the lower-third section due to ginsenoside Rb1; three less intense bands clearly separated in the middle-third section —the lower due to ginsenoside Rd, the middle due to ginsenoside Re, and the upper due to notoginsenoside R1; and a band at an R_F corresponding to ginsenoside Rg1 in *Standard solution A*. The two most intense bands are due to ginsenoside Rb1 and ginsenoside Rg1.

Under UV light at 366 nm: The chromatogram of *Standard solution B* exhibits bands in the following order with increasing R_F: a blue fluorescent band in the lower-third section due to ginsenoside Rb1; three less intense bands clearly separated in the middle-third section —the lower due to ginsenoside Rd, the middle due to ginsenoside Re, and the upper due to notoginsenoside R1 (the band due to ginsenoside Rd is blue fluorescent, while the other two bands are pinkish violet); and a pinkish-violet band at an R_F corresponding to ginsenoside Rg1 band in *Standard solution A*. The two most intense bands are due to ginsenoside Rb1 and ginsenoside Rg1.

Acceptance criteria

Under white light: The chromatogram of the *Sample solution* exhibits five main reddish-violet bands corresponding in R_F to similar bands in *Standard solution B*. These bands appear in the following order of increasing R_F: a band in the lower-third section due to ginsenoside Rb1; three less intense bands clearly separated in the middle-third section due to ginsenoside Rd, ginsenoside Re, and notoginsenoside R1, respectively; and a band at an R_F corresponding to ginsenoside Rg1 in *Standard solution A*. The two most intense bands are due to ginsenoside Rb1 and ginsenoside Rg1.

Under UV light at 366 nm: The chromatogram of the *Sample solution* exhibits the following bands, with increasing R_F, corresponding to similar bands in *Standard solution B*: a blue fluorescent band in the lower-third section due to ginsenoside Rb1; three less intense bands clearly separated in the middle-third section due to ginsenoside Rd, ginsenoside Re, and notoginsenoside R1, respectively (the band due to ginsenoside Rd is blue fluorescent, while the other two bands are pinkish violet); and a pinkish-violet band at an R_F corresponding to ginsenoside Rg1 in *Standard solution A*. The two most intense bands are due to ginsenoside Rb1 and ginsenoside Rg1.

• **B. UHPLC**

Analysis: Proceed as directed in *Content of Ginsenosides*.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks at the retention times corresponding to notoginsenoside R1 (a distinction from *P. ginseng* and *P. quinquefolius*), ginsenoside Rg1, ginsenoside Re, ginsenoside Rb1, and ginsenoside Rd in *Standard solution B*. The two most intense peaks are due to ginsenoside Rg1 and ginsenoside Rb1.

COMPOSITION

• **CONTENT OF GINSENOSIDES**

Solution A: 0.03% phosphoric acid in water (v/v)

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	83	17
2.4	80	20
3.5	70	30
4.2	69	31
5.0	58	42
5.1	0	100
6.0	0	100
6.1	83	17
7.5	83	17

Solvent: Methanol and water (7:3)

Standard solution A: 0.04 mg/mL of USP Ginsenoside Rg1 RS in *Solvent*

Standard solution B: 3.0 mg/mL of USP *Panax notoginseng* Root and Rhizome Dry Extract RS in *Solvent*. Sonicate for about 10 min, centrifuge, and use the supernatant. Before injection, pass through a polytetrafluoroethylene (PTFE) filter of 0.2-µm pore size.

Sample solution: Transfer 75 mg of Dry Extract (capable of passing through a 250-µm sieve), accurately weighed, to a 50-mL centrifuge tube. Add 10 mL of *Solvent*, and sonicate for 10 min. Centrifuge, and transfer the supernatant to a 25-mL volumetric flask. Repeat this extraction two more times, each with 5 mL of *Solvent*. Combine the extracts in the volumetric flask, dilute with *Solvent* to volume, and mix. Before injection, pass through a PTFE filter of 0.2-µm pore size, and discard the first portion of the filtrate. [NOTE—The *Sample solution* is stable for 24 h at room temperature.]

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: UHPLC

Detector: UV 203 nm

Column: 2.1-mm × 5-cm; 1.7-µm packing L1

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Column temperature: 30 ± 1°
Flow rate: 0.8 mL/min
Injection volume: 5 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP *Panax notoginseng* Root and Rhizome Dry Extract RS being used.
Resolution: NLT 1.5 between the ginsenoside Rg1 and ginsenoside Re peaks, *Standard solution B*
Tailing factor: NMT 2.0 for the ginsenoside Rg1 peak, *Standard solution A*
Relative standard deviation: NMT 2.0%, determined from the ginsenoside Rg1 peak in repeated injections, *Standard solution A*
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP *Panax notoginseng* Root and Rhizome Dry Extract RS being used, identify the retention times of the peaks corresponding to relevant ginsenosides in the *Sample solution*.
Separately calculate the percentage of each of the ginsenosides in the portion of Dry Extract taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of ginsenoside Rg1 from *Standard solution A*
C_S = concentration of USP Ginsenoside Rg1 RS in *Standard solution A* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Dry Extract taken to prepare the *Sample solution* (mg)
F = conversion factor for the relevant analyte (see *Table 2*)

Table 2

Analyte	Conversion Factor
Notoginsenoside R1	1.09
Ginsenoside Rg1	1.00
Ginsenoside Re	1.02
Ginsenoside Rb1	1.26
Ginsenoside Rd	1.03

Calculate the content of ginsenosides as the sum of the percentages of notoginsenoside R1, ginsenoside Rg1, ginsenoside Re, ginsenoside Rb1, and ginsenoside Rd.
Calculate the percentage of the labeled amount of ginsenosides in the portion of Dry Extract taken:

$$\text{Result} = (P/L) \times 100$$

P = content of ginsenosides as determined above (%)
L = labeled amount of ginsenosides (%)

Acceptance criteria: 90.0%–110.0% on the dried basis

CONTAMINANTS

Change to read:

- **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020) **:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic bacterial count does not exceed 10^4 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacterial count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli***:** Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Test for Aflatoxins***:** Meets the requirements

SPECIFIC TESTS
- **LOSS ON DRYING** ⟨731⟩
Analysis: Dry at 105° for 2 h.
Acceptance criteria: NMT 5%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial following the official name of the plant from which the article was prepared. It meets the other labeling requirements in *Botanical Extracts* ⟨565⟩.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Ginsenoside Rg1 RS
USP *Panax notoginseng* Root and Rhizome Dry Extract RS

Tienchi Ginseng Root and Rhizome Dry Extract Capsules

DEFINITION
Tienchi Ginseng Root and Rhizome Dry Extract Capsules contain Tienchi Ginseng Root and Rhizome Dry Extract. They contain NLT 90.0% and NMT 110.0% of the labeled amount of ginsenosides calculated as the sum of notoginsenoside R1 ($C_{47}H_{80}O_{18}$), ginsenoside Rg1 ($C_{42}H_{72}O_{14}$), ginsenoside Re ($C_{48}H_{82}O_{18}$), ginsenoside Rb1 ($C_{54}H_{92}O_{23}$), and ginsenoside Rd ($C_{48}H_{82}O_{18}$).

IDENTIFICATION

Change to read:

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩
Standard solution A: 0.5 mg/mL of USP Ginsenoside Rg1 RS in methanol
Standard solution B: 5 mg/mL of USP *Panax notoginseng* Root and Rhizome Dry Extract RS in methanol. Sonicate for about 10 min, centrifuge, and use the supernatant.
Sample solution: Transfer a portion of the contents of the Capsules, equivalent to 50 mg of Tienchi Ginseng Root and Rhizome Dry Extract, to a conical flask, add 10 mL of methanol, mix and sonicate for 20 min, centrifuge, and use the supernatant.
Chromatographic system
Adsorbent: Chromatographic silica gel F_{254} mixture
Application volume: 4 µL, as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

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Developing solvent system: Methylene chloride, dehydrated alcohol, and water ▲(60: 45: 6.5)▲ (USP 1-May-2019)
Developing distance: 6 cm
Derivatization reagent: 10% sulfuric acid in alcohol. Prepare fresh. Keep alcohol cold over ice. Carefully and gradually add sulfuric acid.

Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Apply the *Samples* as bands to a suitable HPTLC plate, and dry in air. Develop the chromatograms in a saturated chamber, remove the plate from the chamber, and dry. Treat with *Derivatization reagent*, heat at 105° for 5–10 min, and examine immediately under white light and under UV light at 366 nm.

System suitability
Under white light: The chromatogram of *Standard solution B* exhibits five main reddish-violet bands. A band in the upper-half section corresponding in R_F to the band of ginsenoside Rg1 in *Standard solution A*; a band in the lower-third section due to ginsenoside Rb1; three less intense bands clearly separated between the bands of ginsenoside Rg1 and ginsenoside Rb1—the lower band due to ginsenoside Rd, the middle due to ginsenoside Re, and the upper due to notoginsenoside R1. The two most intense bands are due to ginsenoside Rb1 and ginsenoside Rg1.
Under UV light at 366 nm: The chromatogram of *Standard solution B* exhibits a pinkish-violet band at an R_F corresponding to the ginsenoside Rg1 band in *Standard solution A*; a blue fluorescent band in the lower-third section due to ginsenoside Rb1; three less intense bands clearly separated between the bands of ginsenoside Rg1 and ginsenoside Rb1—the lower due to ginsenoside Rd, the middle due to ginsenoside Re, and the upper due to notoginsenoside R1 (the band due to ginsenoside Rd is blue fluorescent, while the other two bands are pinkish violet). The two most intense bands are due to ginsenoside Rb1 and ginsenoside Rg1.

Acceptance criteria
Under white light: The chromatogram of the *Sample solution* exhibits five main reddish-violet bands corresponding in R_F to similar bands in *Standard solution B*: a band in the upper-half section corresponding in R_F to the band of ginsenoside Rg1 in *Standard solution A*; a band in the lower-third section due to ginsenoside Rb1; three less intense bands clearly separated between the bands of ginsenoside Rg1 and ginsenoside Rb1—the lower band due to ginsenoside Rd, the middle one due to ginsenoside Re, and the upper due to notoginsenoside R1. The two most intense bands are due to ginsenoside Rb1 and ginsenoside Rg1.
Under UV light at 366 nm: The chromatogram of the *Sample solution* exhibits bands corresponding in R_F to similar bands in *Standard solution B*: a pinkish-violet band at an R_F corresponding to the ginsenoside Rg1 band in *Standard solution A*; a blue fluorescent band in the lower-third section due to ginsenoside Rb1; three less intense bands clearly separated between the bands of ginsenoside Rg1 and ginsenoside Rb1—the lower due to ginsenoside Rd, the middle due to ginsenoside Re, and the upper due to notoginsenoside R1 (the band due to ginsenoside Rd is blue fluorescent, while the other two bands are pinkish violet). The two most intense bands are due to ginsenoside Rb1 and ginsenoside Rg1.

• **B. LC**
Analysis: Proceed as directed in the test for *Content of Ginsenosides*.
Acceptance criteria: The retention times of the notoginsenoside R1, ginsenoside Rg1, ginsenoside Re, ginsenoside Rb1, and ginsenoside Rd peaks of the *Sample solution* correspond to those of *Standard solution B*. The two most intense peaks are due to ginsenoside Rg1 and ginsenoside Rb1.

STRENGTH

Change to read:

• **CONTENT OF GINSENOSIDES**
Extraction solvent: Methanol and water (70:30)
Solution A: 0.03% phosphoric acid in water (v/v)
Solution B: Acetonitrile
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	83	17
2.4	80	20
3.5	70	30
4.2	69	31
5.0	58	42
5.1	0	100
6.0	0	100
6.1	83	17
7.5	83	17

Standard solution A: 0.04 mg/mL of USP Ginsenoside Rg1 RS in ▲*Extraction solvent*.▲ (USP 1-May-2019) Sonicate to dissolve, if necessary.
Standard solution B: 3.0 mg/mL of USP *Panax notoginseng* Root and Rhizome Dry Extract RS in *Extraction solvent*. Sonicate for about 10 min, centrifuge, and use the supernatant. Before injection, pass through a polytetrafluoroethylene (PTFE) membrane filter of 0.2-µm pore size and discard the first portion of the filtrate.
Sample solution: Determine the total weight of 20 Capsules. Open the Capsules and combine their contents in an appropriate container. Weigh the empty Capsule shells and calculate the average fill weight per Capsule. Transfer a portion of the Capsule contents, equivalent to 35 mg of ginsenosides (sum of notoginsenoside R1, ginsenoside Rg1, ginsenoside Re, ginsenoside Rb1, and ginsenoside Rd) into a 25-mL volumetric flask. Add 20 mL of *Extraction solvent*, and sonicate for 30 min with occasional shaking. Cool to room temperature, dilute with *Extraction solvent* to volume, mix well, and centrifuge. Before injection, pass through a PTFE membrane filter of 0.2-µm pore size and discard the first portion of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 203 nm
Column: 2.1-mm × 5-cm; 1.7-µm packing L1
Column temperature: 30°
Flow rate: 0.8 mL/min
Injection volume: 5 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference

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chromatogram provided with the lot of USP *Panax notoginseng* Root and Rhizome Dry Extract RS being used.

Resolution: NLT 1.5 between the ginsenoside Rg1 and ginsenoside Re peaks, *Standard solution B*

Relative standard deviation: NMT 2.0%, determined from the ginsenoside Rg1 peak in repeated injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP *Panax notoginseng* Root and Rhizome Dry Extract RS being used, identify the peaks corresponding to notoginsenoside R1, ginsenoside Rg1, ginsenoside Re, ginsenoside Rb1, and ginsenoside Rd in the *Sample solution*. Measure the areas of the analyte peaks.

Separately calculate the quantity, in mg, of notoginsenoside R1, ginsenoside Rg1, ginsenoside Re, ginsenoside Rb1, and ginsenoside Rd in each Capsule:

$$\text{Result} = (r_U/r_S) \times C_S \times V \times F \times (W_{av}/W)$$

r_U = peak area of the relevant analyte from the *Sample solution*

r_S = peak area of ginsenoside Rg1 from *Standard solution A*

C_S = concentration of USP Ginsenoside Rg1 RS in *Standard solution A* (mg/mL)

V = volume of the *Extraction solvent* taken to prepare the *Sample solution* (mL)

F = response factor for the relevant analyte (see *Table 2*)

W_{av} = average fill weight per Capsule (mg)

W = weight of the sample taken to prepare the *Sample solution* (mg)

Table 2

Analyte	Response Factor
Notoginsenoside R1	1.09
Ginsenoside Rg1	1.00
Ginsenoside Re	1.02
Ginsenoside Rb1	1.26
Ginsenoside Rd	1.03

Calculate the percentage of the labeled amount of ginsenosides, as the sum of notoginsenoside R1, ginsenoside Rg1, ginsenoside Re, ginsenoside Rb1, and ginsenoside Rd, in each Capsule:

$$\text{Result} = (\Sigma Q_i/L) \times 100$$

Q_i = sum of the calculated quantities of ginsenosides (mg)

L = labeled amount of ginsenosides (mg)

Acceptance criteria: 90.0%–110.0%

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩, *Disintegration*: Meet the requirements
- **WEIGHT VARIATION** ⟨2091⟩: Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures*, *Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meet the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and the official name. The label states the amount of ginsenosides as the sum of notoginsenoside R1, ginsenoside Rg1, ginsenoside Re, ginsenoside Rb1, and ginsenoside Rd; and the amount of Tienchi Ginseng Root and Rhizome Dry Extract in mg/Capsule.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Ginsenoside Rg1 RS
  USP *Panax notoginseng* Root and Rhizome Dry Extract RS

Tienchi Ginseng Root and Rhizome Dry Extract Tablets

DEFINITION

Tienchi Ginseng Root and Rhizome Dry Extract Tablets contain Tienchi Ginseng Root and Rhizome Dry Extract. They contain NLT 90.0% and NMT 110.0% of the labeled amount of ginsenosides calculated as the sum of notoginsenoside R1 ($C_{47}H_{80}O_{18}$), ginsenoside Rg1 ($C_{42}H_{72}O_{14}$), ginsenoside Re ($C_{48}H_{82}O_{18}$), ginsenoside Rb1 ($C_{54}H_{92}O_{23}$), and ginsenoside Rd ($C_{48}H_{82}O_{18}$).

IDENTIFICATION

Change to read:

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩

Standard solution A: 0.5 mg/mL of USP Ginsenoside Rg1 RS in methanol

Standard solution B: 5 mg/mL of USP *Panax notoginseng* Root and Rhizome Dry Extract RS in methanol. Sonicate for about 10 min, centrifuge, and use the supernatant.

Sample solution: Transfer a portion of the powdered Tablets, equivalent to 50 mg of Tienchi Ginseng Root and Rhizome Dry Extract, to a conical flask, add 10 mL of methanol, mix and sonicate for 20 min, centrifuge, and filter.

Chromatographic system

Adsorbent: Chromatographic silica gel F_{254} mixture

Application volume: 4 µL, as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

Developing solvent system: Methylene chloride, dehydrated alcohol, and water ▲(60: 45: 6.5)▲ (USP 1-May-2019)

Developing distance: 6 cm

Derivatization reagent: 10% sulfuric acid in alcohol. Prepare fresh. Keep alcohol cold over ice. Carefully and gradually add sulfuric acid.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

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Apply the *Samples* as bands to a suitable HPTLC plate, and dry in air. Develop the chromatograms in a saturated chamber, remove the plate from the chamber, and dry. Treat with *Derivatization reagent*, heat at 105° for 5–10 min, and examine immediately under visible light and under UV light at 366 nm.

System suitability

Under visible light: The chromatogram of *Standard solution B* exhibits five main reddish-violet bands. A band in the upper-half section corresponding in R_F to the band of ginsenoside Rg1 in *Standard solution A*; a band in the lower-third section due to ginsenoside Rb1; three less intense bands clearly separated between the bands of ginsenoside Rg1 and ginsenoside Rb1—the lower band due to ginsenoside Rd, the middle due to ginsenoside Re, and the upper due to notoginsenoside R1. The two most intense bands are due to ginsenoside Rb1 and ginsenoside Rg1.

Under UV light at 366 nm: The chromatogram of *Standard solution B* exhibits a pinkish-violet band at an R_F corresponding to the ginsenoside Rg1 band in *Standard solution A*; a blue fluorescent band in the lower-third section due to ginsenoside Rb1; three less intense bands clearly separated between the bands of ginsenoside Rg1 and ginsenoside Rb1—the lower due to ginsenoside Rd, the middle due to ginsenoside Re, and the upper due to notoginsenoside R1 (the band due to ginsenoside Rd is blue fluorescent, while the other two bands are pinkish violet). The two most intense bands are due to ginsenoside Rb1 and ginsenoside Rg1.

Acceptance criteria

Under visible light: The chromatogram of the *Sample solution* exhibits five main reddish-violet bands corresponding in R_F to similar bands in *Standard solution B*: a band in the upper-half section corresponding in R_F to the band of ginsenoside Rg1 in *Standard solution A*; a band in the lower-third section due to ginsenoside Rb1; three less intense bands clearly separated between the bands of ginsenoside Rg1 and ginsenoside Rb1—the lower band due to ginsenoside Rd, the middle one due to ginsenoside Re, and the upper due to notoginsenoside R1. The two most intense bands are due to ginsenoside Rb1 and ginsenoside Rg1.

Under UV light at 366 nm: The chromatogram of the *Sample solution* exhibits the bands corresponding in R_F to similar bands in *Standard solution B*: a pinkish-violet band at an R_F corresponding to the ginsenoside Rg1 band in *Standard solution A*; a blue fluorescent band in the lower-third section due to ginsenoside Rb1; three less intense bands clearly separated between the bands of ginsenoside Rg1 and ginsenoside Rb1—the lower due to ginsenoside Rd, the middle due to ginsenoside Re, and the upper due to notoginsenoside R1 (the band due to ginsenoside Rd is blue fluorescent, while the other two bands are pinkish-violet). The two most intense bands are due to ginsenoside Rb1 and ginsenoside Rg1.

• **B. LC**

Analysis: Proceed as directed in the test for *Content of Ginsenosides*.

Acceptance criteria: The retention times of the notoginsenoside R1, ginsenoside Rg1, ginsenoside Re, ginsenoside Rb1, and ginsenoside Rd peaks of the *Sample solution* correspond to those of *Standard solution B*. The two most intense peaks are due to ginsenoside Rg1 and ginsenoside Rb1.

STRENGTH

Change to read:

• **CONTENT OF GINSENOSIDES**

Extraction solvent: Methanol and water (70:30)

Solution A: 0.03% phosphoric acid in water (v/v)

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	83	17
2.4	80	20
3.5	70	30
4.2	69	31
5.0	58	42
5.1	0	100
6.0	0	100
6.1	83	17
7.5	83	17

Standard solution A: 0.04 mg/mL of USP Ginsenoside Rg1 RS in ▲*Extraction solvent.*▲ (USP 1-May-2019) Sonicate to dissolve, if necessary.

Standard solution B: 3.0 mg/mL of USP *Panax notoginseng* Root and Rhizome Dry Extract RS in *Extraction solvent*. Sonicate for about 10 min, centrifuge, and use the supernatant. Before injection, pass through a polytetrafluoroethylene (PTFE) membrane filter of 0.2-µm pore size and discard the first portion of the filtrate.

Sample solution: Weigh NLT 20 Tablets, determine the average Tablet weight, and finely powder. Transfer a portion of finely powdered Tablets, nominally equivalent to 35 mg of ginsenosides (sum of notoginsenoside R1, ginsenoside Rg1, ginsenoside Re, ginsenoside Rb1, and ginsenoside Rd) into a 25-mL volumetric flask. Add 20 mL of *Extraction solvent*, and sonicate for 30 min with occasional shaking. Cool to room temperature, dilute with *Extraction solvent* to volume, mix well, centrifuge, pass the supernatant through a PTFE membrane filter of 0.2-µm pore size, and discard the first portion of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 203 nm

Column: 2.1-mm × 5-cm; 1.7-µm packing L1

Column temperature: 30°

Flow rate: 0.8 mL/min

Injection volume: 5 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP *Panax notoginseng* Root and Rhizome Dry Extract RS being used.

Resolution: NLT 1.5 between the ginsenoside Rg1 and ginsenoside Re peaks, *Standard solution B*

Relative standard deviation: NMT 2.0%, determined from the ginsenoside Rg1 peak in repeated injections, *Standard solution A*

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Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP *Panax notoginseng* Root and Rhizome Dry Extract RS being used, identify the peaks corresponding to notoginsenoside R1, ginsenoside Rg1, ginsenoside Re, ginsenoside Rb1, and ginsenoside Rd in the *Sample solution*. Measure the areas of the analyte peaks.

Separately calculate the quantity, in mg, of notoginsenoside R1, ginsenoside Rg1, ginsenoside Re, ginsenoside Rb1, and ginsenoside Rd in each Tablet:

$$\text{Result} = (r_U/r_S) \times C_S \times V \times F \times (W_{av}/W)$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of ginsenoside Rg1 from *Standard solution A*
C_S = concentration of USP Ginsenoside Rg1 RS in *Standard solution A* (mg/mL)
V = volume of the *Extraction solvent* taken to prepare the *Sample solution* (mL)
F = response factor for the relevant analyte (see *Table 2*)
W_{av} = average Tablet weight (mg)
W = weight of the sample taken to prepare the *Sample solution* (mg)

Table 2

Analyte	Response Factor
Notoginsenoside R1	1.09
Ginsenoside Rg1	1.00
Ginsenoside Re	1.02
Ginsenoside Rb1	1.26
Ginsenoside Rd	1.03

Calculate the percentage of the labeled amount of ginsenosides, as the sum of notoginsenoside R1, ginsenoside Rg1, ginsenoside Re, ginsenoside Rb1, and ginsenoside Rd, in each Tablet:

$$\text{Result} = (\Sigma Q_i/L) \times 100$$

Q_i = sum of the calculated quantities of ginsenosides (mg)
L = labeled amount of ginsenosides (mg)

Acceptance criteria: 90.0%–110.0%

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩, *Disintegration*: Meet the requirements
- **WEIGHT VARIATION** ⟨2091⟩: Meet the requirements

CONTAMINANTS

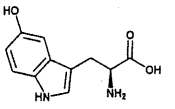
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures*, *Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meet the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and the official name. The label states the amount of ginsenosides, as the sum of notoginsenoside R1, ginsenoside Rg1, ginsenoside Re, ginsenoside Rb1, and ginsenoside Rd; and the amount of Tienchi Ginseng Root and Rhizome Dry Extract in mg/Tablet.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Ginsenoside Rg1 RS
  USP *Panax notoginseng* Root and Rhizome Dry Extract RS

Tryptophan—*see Tryptophan General Monographs*

5-Hydroxy- L-tryptophan



$C_{11}H_{12}N_2O_3$ 220.23

(*S*)-2-Amino-3-(5-hydroxy-1*H*-indol-3-yl)propanoic acid [4350-09-8].

DEFINITION

5-Hydroxy-L-tryptophan contains NLT 98.5% and NMT 101.5% of 5-hydroxy-L-tryptophan ($C_{11}H_{12}N_2O_3$), calculated on the dried basis.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

ASSAY

- **PROCEDURE**

Sample: 200 mg of 5-Hydroxy-L-tryptophan

Blank: Mix 3 mL of formic acid and 50 mL of glacial acetic acid.

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Direct titration

Titrant: 0.1 N perchloric acid VS

Endpoint detection: Potentiometric

Analysis

Samples: *Sample* and *Blank*

Dissolve the *Sample* in a mixture of 3 mL of formic acid and 50 mL of glacial acetic acid, and titrate with the *Titrant*. Perform a *Blank* titration, and make any necessary correction.

Calculate the percentage of 5-hydroxy-L-tryptophan ($C_{11}H_{12}N_2O_3$) in the *Sample* taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

V_S = *Titrant* volume consumed by the *Sample* (mL)
V_B = *Titrant* volume consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 220.2 mg/mEq
W = *Sample* weight (mg)

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Acceptance criteria: 98.5%–101.5% on the dried basis

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.2%
- **CHLORIDE AND SULFATE,** *Chloride* ⟨221⟩
  Standard solution: 0.50 mL of 0.020 N hydrochloric acid
  Sample: 0.73 g of 5-Hydroxy-L-tryptophan
  Acceptance criteria: NMT 0.05%
- **CHLORIDE AND SULFATE,** *Sulfate* ⟨221⟩
  Standard solution: 0.10 mL of 0.020 N sulfuric acid
  Sample: 0.33 g of 5-Hydroxy-L-tryptophan
  Acceptance criteria: NMT 0.03%
- **ORGANIC IMPURITIES**
  Solution A: 1 mL/L of trifluoroacetic acid in water
  Solution B: 1 mL/L of trifluoroacetic acid in a mixture of acetonitrile and water (80:20)
  Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	95	5
2	95	5
37	35	65
42	0	100
47	0	100
50	95	5
60	95	5

Standard solution: 1.0 µg/mL of USP 5-Hydroxy-L-tryptophan RS and 50 µg/mL of USP L-Tryptophan RS in water
Sample solution: 10.0 mg/mL of 5-Hydroxy-L-tryptophan in water
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 220 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 30°
Flow rate: 1 mL/min
Injection volume: 20 µL
System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for 5-hydroxy-L-tryptophan and L-tryptophan are 1.0 and 1.6, respectively.]
Suitability requirements
Relative standard deviation: NMT 5.0% for the 5-hydroxy-L-tryptophan and L-tryptophan peaks
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of each unspecified impurity in the portion of 5-Hydroxy-L-tryptophan taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of each unspecified impurity from the *Sample solution*
r_S = peak response of 5-hydroxy-L-tryptophan from the *Standard solution*
C_S = concentration of USP 5-Hydroxy-L-tryptophan RS in the *Standard solution* (µg/mL)
C_U = concentration of 5-Hydroxy-L-tryptophan in the *Sample solution* (µg/mL)

Calculate the percentage of tryptophan in the portion of 5-Hydroxy-L-tryptophan taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of tryptophan from the *Sample solution*
r_S = peak response of tryptophan from the *Standard solution*
C_S = concentration of USP L-Tryptophan RS in the *Standard solution* (µg/mL)
C_U = concentration of 5-Hydroxy-L-tryptophan in the *Sample solution* (µg/mL)

Acceptance criteria
Total impurities 1: NMT 0.01% of the total impurities eluting prior to the 5-hydroxy-L-tryptophan peak
Total impurities 2: NMT 0.03% of the total impurities eluting after the 5-hydroxy-L-tryptophan peak.
[NOTE—Exclude the peak for tryptophan.]
Tryptophan: NMT 0.5%

SPECIFIC TESTS

- **OPTICAL ROTATION,** *Specific Rotation* ⟨781⟩
  Sample solution: 10 mg/mL in water
  Acceptance criteria: −30.0° to −38.0°
- **PH** ⟨791⟩
  Sample solution: 10 mg/mL in water
  Acceptance criteria: 4.0–6.0
- **LOSS ON DRYING** ⟨731⟩
  Analysis: Dry at 105° for 3 h.
  Acceptance criteria: NMT 2.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP 5-Hydroxy-L-tryptophan RS
  USP L-Tryptophan RS

Turmeric

DEFINITION

Turmeric is the dried rhizome of *Curcuma longa* L., also known as *C. domestica* Val. (Fam. Zingiberaceae). It is commonly known as Curcuma, Curcum, Haridra, and Indian Saffron. It contains NLT 3.0% of curcuminoids, calculated on the anhydrous basis.

IDENTIFICATION

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩
  Standard solution: 1 mg/mL of USP Curcuminoids RS in methanol
  Sample solution: Suspend about 200 mg of Turmeric, finely powdered, in 3 mL of methanol, and sonicate for 10 min. Centrifuge or filter, and use the supernatant or the filtrate.
  Chromatographic system
  Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plate)[1]
  Application volume: 2 µL each of the *Standard solution* and the *Sample solution* as 8-mm bands
  Relative humidity: Condition the plate to a relative humidity of 33%.
  Temperature: Ambient, not to exceed 30°

[1] Suitable commercially available plates are HPTLC Silica Gel 60 F_{254} from EMD Millipore (e.g., Part No. 1.05642.0001).

Supplement 3

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Developing solvent system: Toluene and glacial acetic acid (4:1)
Developing distance: 6 cm
Derivatization reagent: 85 mL of ice-cold methanol combined with 10 mL of glacial acetic acid, 5 mL of sulfuric acid, and 0.5 mL of *p*-anisaldehyde
Analysis
Samples: *Standard solution* and *Sample solution*
Apply the *Samples* as bands and dry in air. Develop in a saturated chamber and dry in air. Treat with *Derivatization reagent*, heat at 100° for 3 min, and examine under long-wave UV light (365 nm) and under white light.
System suitability: Under long-wave UV light (365 nm), the derivatized chromatogram of the *Standard solution* exhibits, in its lower half, three bands in the order of increasing R_F: an orange band due to bisdesmethoxycurcumin, an orange band due to desmethoxycurcumin, and the red band due to curcumin. Under white light, the two lower bands appear orange, while the topmost band is reddish-pink.
Acceptance criteria: Under long-wave UV light (365 nm), the derivatized chromatogram of the *Sample solution* displays two orange bands and one red band, similar in position and color to those observed in the *Standard solution*. At the bottom part of the upper half of the plate, two purple bands are seen. Under white light, two orange bands and a darker red band are seen coincident with the bands due to bisdesmethoxycurcumin, desmethoxycurcumin, and curcumin in the *Standard solution*, in the order of increasing R_F. In the upper half of the plate, the lower of the two bands appears purple, while the upper band is brown. No bands appear in the topmost quarter of the plate, which is characteristic of *Curcuma zanthorrhiza* Roxb. and *Curcuma aromatica* Salisb. These confounders, and occasional adulterants, of Turmeric also lack the lower orange band corresponding to bisdesmethoxycurcumin. Additional weak bands may be observed in the *Sample solution* under either illumination condition.

• **B. HPLC**
Analysis: Proceed as directed in the test for *Content of Curcuminoids*.
Acceptance criteria: The retention times of the peaks for curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin of the *Sample solution* correspond to those of *Standard solution A* and *Standard solution B*.

COMPOSITION

• **CONTENT OF CURCUMINOIDS**
Mobile phase: Tetrahydrofuran and 1 mg/mL of citric acid in water (4:6)
Standard solution A: 40 µg/mL of USP Curcuminoids RS in *Mobile phase*
Standard solution B: A composite solution containing 40 µg/mL of USP Curcumin RS, 10 µg/mL of USP Desmethoxycurcumin RS, and 2.0 µg/mL of USP Bisdesmethoxycurcumin RS in *Mobile phase*. Use sonication if necessary. Before injection, pass through a filter of 0.45-µm pore size, and discard the initial 10 mL of the filtrate.
Sample stock solution: Pulverize about 5.0 g of Turmeric. Transfer about 0.5 g of the pulverized sample to a 50-mL volumetric flask, add 30 mL of acetone, and sonicate for 30 min. Dilute with acetone to volume, mix, and centrifuge.
Sample solution: Transfer 5 mL of the *Sample stock solution* to a 50-mL volumetric flask, dilute with *Mobile phase* to volume, and mix. Before injection, pass through a filter of 0.45-µm pore size, and discard the initial 10 mL of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Vis 420 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Flow rate: 1 mL/min
Injection volume: 20 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
[NOTE—The relative retention times for the curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin peaks are 1.0, 1.2, and 1.4, respectively.]
Suitability requirements
Chromatogram similarity: The chromatogram of *Standard solution A* is similar to the reference chromatogram provided with USP Curcuminoids RS.
Resolution: NLT 2.0 between the curcumin and desmethoxycurcumin peaks and the desmethoxycurcumin and bisdesmethoxycurcumin peaks, *Standard solution B*
Tailing factor: NMT 1.5 for bisdesmethoxycurcumin, desmethoxycurcumin, and curcumin peaks, *Standard solution B*
Relative standard deviation: NMT 2.0% for the desmethoxycurcumin peak, in repeated injections, *Standard solution B*
Analysis
Samples: *Standard solution B* and *Sample solution*
Calculate the percentages of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin in the portion of Turmeric taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of the relevant analyte from *Standard solution B*
C_S = concentration of the relevant analyte in *Standard solution B* (mg/mL)
V = volume of the *Sample stock solution* (mL)
W = weight of Turmeric used to prepare the *Sample stock solution* (mg)
D = dilution factor to obtain the *Sample solution* from the *Sample stock solution*, 10

Acceptance criteria: NLT 3.0% as the sum of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin on the anhydrous basis

CONTAMINANTS

• **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Limits of Elemental Impurities*: Meets the requirements
• **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Pesticide Residue Analysis*: Meets the requirements
• **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Test for Aflatoxins*: Meets the requirements
• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacterial count does not exceed 10^3 cfu/g.
• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures*, *Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS

• **BOTANICAL CHARACTERISTICS**
Macroscopic: Turmeric occurs as ovate, oblong, or pear-shaped primary rhizomes, also known as bulb or

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round turmeric, about 3 cm in diameter and 4–5 cm long and showing transverse annular leaf scars, and as cylindrical, sometimes short-branched secondary rhizomes, also known as finger or long turmeric, about 1 cm in diameter and 2–7 cm long and showing scars of lateral branches. The cured and dried turmeric of commerce is bright yellow to dull yellow in appearance, with a rough or polished surface and a characteristic aromatic odor. The texture is hard and uneasily broken, and the fracture is smooth and finely granular. Internally it is orange-yellow to orange, showing a cortex separated from a central cylinder by a distinct endodermis.

Microscopic: Transverse section of rhizome shows a row of thin-walled, flattened epidermal cells; a few layers of thin-walled, brick-shaped parenchyma cells of the cork; a broad cortex consisting of multiple layers of thin-walled parenchyma cells showing scattered vascular bundles; a thin layer of oblong cells of the endodermis; pericycle consisting of one to two rows of parenchyma cells; and a pith consisting of parenchyma cells showing scattered vascular bundles, most of them forming discontinuous rings near the endodermis and fewer inward. The vascular bundles are of the collateral type; the vessels have mainly spiral thickening, and a few have reticulate and annular thickening. Scattered throughout the parenchyma of the pith and cortex are oleoresin cells containing oil and scattered particles of an orange-yellow pigment, and prisms of calcium oxalate, which are usually obscured due to the bright yellow color of the pigment content. The parenchyma cells are full of starch granules, 15–30 µm in size, and flat or disk shaped. Bast fibers are absent.

- **Articles of Botanical Origin** ⟨561⟩, *Methods of Analysis, Volatile Oil Determination*: NLT 3.0 mL/100 g
- **Articles of Botanical Origin** ⟨561⟩, *Methods of Analysis, Foreign Organic Matter*: NMT 2.0%
- **Articles of Botanical Origin** ⟨561⟩, *Methods of Analysis, Alcohol-Soluble Extractives, Method 2*: NLT 100 mg/g
- **Articles of Botanical Origin** ⟨561⟩, *Methods of Analysis, Water-Soluble Extractives, Method 2*: NLT 9.0%
- **Water Determination** ⟨921⟩, *Method I, Method Ia*: NMT 10%
- **Articles of Botanical Origin** ⟨561⟩, *Methods of Analysis, Total Ash*: NMT 7.0%
- **Articles of Botanical Origin** ⟨561⟩, *Methods of Analysis, Acid-Insoluble Ash*: NMT 1.0%

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in well-closed containers. Protect from light and moisture, and store at room temperature.
- **Labeling:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP Reference Standards** ⟨11⟩
  USP Bisdesmethoxycurcumin RS
  USP Curcumin RS
  USP Curcuminoids RS
  USP Desmethoxycurcumin RS

Powdered Turmeric

DEFINITION

Powdered Turmeric is Turmeric reduced to a fine or very fine powder. It contains NLT 3.0% of curcuminoids, calculated on the anhydrous basis.

IDENTIFICATION

- **A. HPTLC for Articles of Botanical Origin** ⟨203⟩
  Standard solution: 1 mg/mL of USP Curcuminoids RS in methanol
  Sample solution: Suspend about 200 mg of Powdered Turmeric in 3 mL of methanol, and sonicate for 10 min. Centrifuge or filter, and use the supernatant or the filtrate.
  Chromatographic system
  Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plate)[1]
  Application volume: 2 µL each of the *Standard solution* and the *Sample solution* as 8-mm bands
  Relative humidity: Condition the plate to a relative humidity of 33%.
  Temperature: Ambient, not to exceed 30°
  Developing solvent system: Toluene and glacial acetic acid (4:1)
  Developing distance: 6 cm
  Derivatization reagent: 85 mL of ice-cold methanol combined with 10 mL of glacial acetic acid, 5 mL of sulfuric acid, and 0.5 mL of *p*-anisaldehyde
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Apply the *Samples* as bands and dry in air. Develop in a saturated chamber and dry in air. Treat with *Derivatization reagent*, heat at 100° for 3 min, and examine under long-wave UV light (365 nm) and under white light.
  System suitability: Under long-wave UV light (365 nm), the derivatized chromatogram of the *Standard solution* exhibits, in its lower half, three bands in the order of increasing R_F: an orange band due to bisdesmethoxycurcumin, an orange band due to desmethoxycurcumin, and the red band due to curcumin. Under white light, the two lower bands appear orange, while the topmost band is reddish-pink.
  Acceptance criteria: Under long-wave UV light (365 nm), the derivatized chromatogram of the *Sample solution* displays two orange bands and one red band, similar in position and color to those observed in the *Standard solution*. At the bottom part of the upper half of the plate, two purple bands are seen. Under white light, two orange bands and a darker red band are seen coincident with the bands due to bisdesmethoxycurcumin, desmethoxycurcumin, and curcumin in the *Standard solution*, in the order of increasing R_F. In the upper half of the plate, the lower of the two bands appears purple, while the upper band is brown. No bands appear in the topmost quarter of the plate, which is characteristic of *Curcuma zanthorrhiza* Roxb. and *Curcuma aromatica* Salisb. These confounders, and occasional adulterants, of Powdered Turmeric also lack the lower orange band corresponding to bisdesmethoxycurcumin. Additional weak bands may be observed in the *Sample solution* under either illumination condition.
- **B. HPLC**
  Analysis: Proceed as directed in the test for *Content of Curcuminoids*.
  Acceptance criteria: The retention times of the peaks for curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin of the *Sample solution* correspond to those of *Standard solution A* and *Standard solution B*.

COMPOSITION

- **Content of Curcuminoids**
  Mobile phase: Tetrahydrofuran and 1 mg/mL of citric acid in water (4:6)

[1] Suitable commercially available plates are HPTLC Silica Gel 60 F_{254} from EMD Millipore (e.g., Part No. 1.05642.0001).

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Standard solution A: 40 µg/mL of USP Curcuminoids RS in *Mobile phase*

Standard solution B: A composite solution containing 40 µg/mL of USP Curcumin RS, 10 µg/mL of USP Desmethoxycurcumin RS, and 2.0 µg/mL of USP Bisdesmethoxycurcumin RS in *Mobile phase*. Use sonication if necessary. Before injection, pass through a filter of 0.45-µm pore size, and discard the initial 10 mL of the filtrate.

Sample stock solution: Transfer about 0.5 g of Powdered Turmeric, accurately weighed, to a 50-mL volumetric flask, add 30 mL of acetone, and sonicate for 30 min. Dilute with acetone to volume, mix, and centrifuge.

Sample solution: Transfer 5.0 mL of the *Sample stock solution* to a 50-mL volumetric flask. Dilute with *Mobile phase* to volume, and mix. Before injection, pass through a filter of 0.45-µm pore size, and discard the initial 10 mL of the filtrate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Vis 420 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Flow rate: 1.0 mL/min
Injection volume: 20 µL

System suitability
Samples: *Standard solution A* and *Standard solution B*
[NOTE—The relative retention times for the curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin peaks are 1.0, 1.2, and 1.4, respectively.]
Suitability requirements
Chromatogram similarity: The chromatogram of *Standard solution A* is similar to the reference chromatogram provided with USP Curcuminoids RS.
Resolution: NLT 2.0 between curcumin and desmethoxycurcumin peaks and desmethoxycurcumin and bisdesmethoxycurcumin peaks, *Standard solution B*
Tailing factor: NMT 1.5 for bisdesmethoxycurcumin, desmethoxycurcumin, and curcumin peaks, *Standard solution B*
Relative standard deviation: NMT 2.0% for the desmethoxycurcumin peak, in replicate injections, *Standard solution B*

Analysis
Samples: *Standard solution B* and *Sample solution*
Calculate the percentages of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin in the portion of Powdered Turmeric taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of the relevant analyte from *Standard solution B*
C_S = concentration of the relevant analyte in *Standard solution B* (mg/mL)
V = volume of the *Sample stock solution* (mL)
W = weight of Powdered Turmeric used to prepare the *Sample stock solution* (mg)
D = dilution factor to obtain the *Sample solution* from the *Sample stock solution*, 10

Acceptance criteria: NLT 3.0% as the sum of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin on the anhydrous basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Limits of Elemental Impurities*: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Pesticide Residue Analysis*: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Test for Aflatoxins*: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacterial count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS:** Powdered Turmeric is deep yellow in color with a characteristic aromatic odor. Under a microscope, Powdered Turmeric reveals thin-walled parenchyma cells containing starch granules, 15–30 µm in size, flat or disk-shaped, gelatinized or ungelatinized; oil cells full of oil and scattered particles of orange-yellow pigments; prisms of calcium oxalate, usually obscured due to the bright yellow color of the pigment content, detected as bright orange prisms under a polarizing microscope; fragments of spiral vessels and a few reticulate and annular vessels; fragments of epidermal and cork cells; starch granules; scattered unicellular nonglandular trichomes; and the absence of bast fibers.
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Volatile Oil Determination*: NLT 3.0 mL/100 g
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Alcohol-Soluble Extractives, Method 2*: NLT 100 mg/g
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Water-Soluble Extractives, Method 2*: NLT 9.0%
- **WATER DETERMINATION** ⟨921⟩, *Method I, Method Ia*: NMT 10%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Total Ash*: NMT 7.0%
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Acid-Insoluble Ash*: NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. Protect from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Bisdesmethoxycurcumin RS
  USP Curcumin RS
  USP Curcuminoids RS
  USP Desmethoxycurcumin RS

Powdered Turmeric Extract

DEFINITION

Powdered Turmeric Extract is prepared from the pulverized rhizomes of *Curcuma longa* L. (Fam. Zingiberaceae), using acetone, methanol, or other suitable solvents. It contains NLT 20% of curcuminoids, calculated on the dried basis. It may contain other added substances.

IDENTIFICATION

- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩
  Standard solution: 1 mg/mL of USP Curcuminoids RS in methanol

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Sample solution: 50 mg/mL of Powdered Turmeric Extract in methanol. Sonicate to disperse, centrifuge or filter, and use the supernatant or the filtrate.

Chromatographic system

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plate)[1]

Application volume: 2 µL each of the *Standard solution* and the *Sample solution* as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of 33%.

Temperature: Ambient, not to exceed 30°

Developing solvent system: Toluene and glacial acetic acid (4:1)

Developing distance: 6 cm

Derivatization reagent: 85 mL of ice-cold methanol combined with 10 mL of glacial acetic acid, 5 mL of sulfuric acid, and 0.5 mL of *p*-anisaldehyde

Analysis

Samples: *Standard solution* and *Sample solution*

Apply the *Samples* as bands and dry in air. Develop in a saturated chamber and dry in air. Treat with *Derivatization reagent*, heat at 100° for 3 min, and examine under long-wave UV light (365 nm) and under white light.

System suitability: Under long-wave UV light (365 nm), the derivatized chromatogram of the *Standard solution* exhibits, in its lower half, three bands in the order of increasing R_F: an orange band due to bisdesmethoxycurcumin, an orange band due to desmethoxycurcumin, and the red band due to curcumin. Under white light, the two lower bands appear orange, while the topmost band is reddish-pink.

Acceptance criteria: Under long-wave UV light (365 nm), the derivatized chromatogram of the *Sample solution* displays two orange bands and one red band, similar in position and color to those observed in the *Standard solution*. At the bottom part of the upper half of the plate, two purple bands are seen. Under white light, two orange bands and a darker red band are seen coincident with the bands due to bisdesmethoxycurcumin, desmethoxycurcumin, and curcumin in the *Standard solution*, in the order of increasing R_F. In the upper half of the plate, the lower of the two bands appears purple, while the upper band is brown. No bands appear in the topmost quarter of the plate, which is characteristic of *Curcuma zanthorrhiza* Roxb. and *Curcuma aromatica* Salisb. These confounders, and occasional adulterants, of Powdered Turmeric Extract also lack the lower orange band corresponding to bisdesmethoxycurcumin. Additional weak bands may be observed in the *Sample solution* under either illumination condition.

• **B. HPLC**

Analysis: Proceed as directed in the test for *Content of Curcuminoids*.

Acceptance criteria: The retention times of the peaks for curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin of the *Sample solution* correspond to those of *Standard solution A* and *Standard solution B*.

COMPOSITION

• **CONTENT OF CURCUMINOIDS**

Mobile phase: Tetrahydrofuran and 1 mg/mL of citric acid in water (4:6)

Standard solution A: 40 µg/mL of USP Curcuminoids RS in *Mobile phase*

Standard solution B: A composite solution containing 40 µg/mL of USP Curcumin RS, 10 µg/mL of USP Desmethoxycurcumin RS, and 2.0 µg/mL of USP Bisdesmethoxycurcumin RS in *Mobile phase*. Use sonication if necessary. Before injection, pass through a filter of 0.45-µm pore size, and discard the initial 10 mL of the filtrate.

Sample stock solution: Transfer about 100 mg of Powdered Turmeric Extract, accurately weighed, to a 50-mL volumetric flask, add 30 mL of acetone, and sonicate for 30 min. Dilute with acetone to volume, mix, and centrifuge.

Sample solution: Transfer 5.0 mL of the *Sample stock solution* to a 50-mL volumetric flask. Dilute with *Mobile phase* to volume, and mix. Before injection, pass through a filter of 0.45-µm pore size, and discard the initial 10 mL of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Vis 420 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Flow rate: 1.0 mL/min

Injection volume: 20 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

[NOTE—The relative retention times for the curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin peaks are 1.0, 1.2, and 1.4, respectively.]

Suitability requirements

Chromatogram similarity: The chromatogram of *Standard solution A* is similar to the reference chromatogram provided with USP Curcuminoids RS.

Resolution: NLT 2.0 between curcumin and desmethoxycurcumin peaks and desmethoxycurcumin and bisdesmethoxycurcumin peaks, *Standard solution B*

Tailing factor: NMT 1.5 for bisdesmethoxycurcumin, desmethoxycurcumin, and curcumin peaks, *Standard solution B*

Relative standard deviation: NMT 2.0% for the desmethoxycurcumin peak, in replicate injections, *Standard solution B*

Analysis

Samples: *Standard solution B* and *Sample solution*

Calculate the percentages of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin in the portion of Powdered Turmeric Extract taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*

r_S = peak area of the relevant analyte from *Standard solution B*

C_S = concentration of the relevant analyte in *Standard solution B* (mg/mL)

V = volume of the *Sample stock solution* (mL)

W = weight of Powdered Turmeric Extract used to prepare the *Sample stock solution* (mg)

D = dilution factor to obtain the *Sample solution* from the *Sample stock solution*, 10

Add the percentages due to curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin.

Acceptance criteria: NLT 20% on the dried basis

CONTAMINANTS

Change to read:

• **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020)

: Meets the requirements

[1] Suitable commercially available plates are HPTLC Silica Gel 60 F_{254} from EMD Millipore (e.g., Part No. 1.05642.0001).

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- **BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Residual Solvents*: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Test for Aflatoxins*: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS

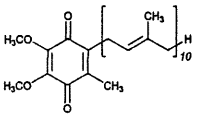
- **LOSS ON DRYING** ⟨731⟩
  Sample: 1.0 g of Powdered Turmeric Extract
  Analysis: Dry the *Sample* at 105° for 2 h.
  Acceptance criteria: NMT 7.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. Protect from light and moisture, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. It meets other labeling requirements in *Botanical Extracts* ⟨565⟩.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Bisdesmethoxycurcumin RS
  USP Curcumin RS
  USP Curcuminoids RS
  USP Desmethoxycurcumin RS

Tyrosine—*see Tyrosine General Monographs*

Ubidecarenone



$C_{59}H_{90}O_4$ 863.34

2,5-Cyclohexadiene-1,4-dione, 2-[(2*E*,6*E*,10*E*,14*E*,18*E*,22*E*,26*E*,30*E*,34*E*)-3,7,11,15,19,23,27,31,35,39-decamethyl-2,6,10,14,18,22,26,30,34,38-tetracontadecaenyl]-5,6-dimethoxy-3-methyl;
2-[(*all-E*)-3,7,11,15,19,23,27,31,35,39-Decamethyl-2,6,10,14,18,22,26,30,34,38-tetracontadecaenyl)-5,6-dimethoxy-3-methyl-*p*-benzoquinone [303-98-0].

DEFINITION

Ubidecarenone (Coenzyme Q_{10}) contains NLT 98.0% and NMT 101.0% of ubidecarenone ($C_{59}H_{90}O_4$), calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
- **B.**

Analysis: Dissolve 50 mg of Ubidecarenone in 1 mL of ethyl ether, and add 10 mL of dehydrated alcohol. To 2 mL of this solution add 3 mL of dehydrated alcohol and 2 mL of dimethyl malonate. Add 1 mL of potassium hydroxide solution (1 in 5) dropwise.

Acceptance criteria: A blue color appears.

ASSAY

- **PROCEDURE**

Mobile phase: Methanol and dehydrated alcohol (65:35)

System suitability solution: 0.5 mg/mL each of USP Ubidecarenone RS and USP Ubidecarenone Related Compound A RS in dehydrated alcohol. Heat at 50° for 2 min, if necessary, for complete dissolution.

Standard solution: 1.0 mg/mL of USP Ubidecarenone RS in dehydrated alcohol. Heat at 50° for 2 min, if necessary, for complete dissolution.

Sample solution: 1.0 mg/mL of Ubidecarenone in dehydrated alcohol. Heat at 50° for 2 min, if necessary, for complete dissolution.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 275 nm
Column: 4.6-mm × 15-cm; packing L1
Column temperature: 35°
Flow rate: Adjust to obtain a retention time of about 11 min for ubidecarenone.
Injection size: 5 µL

System suitability
Sample: *System suitability solution*
[NOTE—The relative retention times for ubidecarenone related compound A and ubidecarenone are about 0.75 and 1.0, respectively.]
Suitability requirements
Resolution: NLT 4 between ubidecarenone related compound A and ubidecarenone
Relative standard deviation: NMT 0.8% for ubidecarenone

Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of ubidecarenone ($C_{59}H_{90}O_4$) in the portion of Ubidecarenone taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP Ubidecarenone RS in the *Standard solution* (mg/mL)
C_U = concentration of Ubidecarenone in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–101.0% on the anhydrous basis

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%
- **CHROMATOGRAPHIC PURITY**

Procedure 1: Coenzymes Q_7, Q_8, Q_9, Q_{11}, and Related Impurities
Mobile phase, System suitability solution, Sample solution, Chromatographic system, and **System suitability:** Proceed as directed in the *Assay*.
Analysis
Sample: *Sample solution*
Calculate the percentage of impurities in the portion of Ubidecarenone taken:

$$\text{Result} = (r_{T1}/r_{T2}) \times 100$$

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r_{T1} = sum of all peak responses, other than that for ubidecarenone
r_{T2} = sum of all peak responses

Acceptance criteria: NMT 1.0%

Procedure 2: Ubidecarenone (*2Z*)-Isomer and Related Impurities
Mobile phase: *n*-Hexane and ethyl acetate (97:3)
System suitability solution: 1 mg/mL of USP Ubidecarenone for System Suitability RS in *n*-hexane
Sample solution: 1 mg/mL of Ubidecarenone in *n*-hexane
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 275 nm
Column: 4.6-mm × 25-cm; packing L3
Flow rate: 2 mL/min
Injection size: 20 µL
System suitability
Sample: *System suitability solution*
[NOTE—The relative retention times for ubidecarenone (*2Z*)-isomer and ubidecarenone are about 0.85 and 1.0, respectively.]
Suitability requirements
Resolution: NLT 1.5 between the ubidecarenone (*2Z*)-isomer and ubidecarenone
Analysis
Sample: *Sample solution*
Calculate the percentage of impurities in the portion of Ubidecarenone taken:

$$\text{Result} = (r_{T1}/r_{T2}) \times 100$$

r_{T1} = sum of all peak responses, other than that for ubidecarenone
r_{T2} = sum of all peak responses

Acceptance criteria: NMT 1.0%
Total impurities: NMT 1.5%, obtained from *Chromatographic Purity Procedures 1* and *2*

SPECIFIC TESTS
- **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 0.2%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed, light-resistant containers.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Ubidecarenone RS
USP Ubidecarenone Related Compound A RS [coenzyme Q_9]
USP Ubidecarenone for System Suitability RS

Ubidecarenone Capsules

DEFINITION
Ubidecarenone Capsules contain NLT 90.0% and NMT 115.0% of the labeled amount of ubidecarenone ($C_{59}H_{90}O_4$).

IDENTIFICATION
- **A.** The retention time of the major peak of either *Sample solution 1* or *Sample solution 2* corresponds to that of the *Standard solution*, as obtained in the *Procedure* for *Strength*.

STRENGTH
- **PROCEDURE**
[NOTE—Conduct this test promptly with minimum exposure to actinic light.]

Solvent: *n*-Hexane and dehydrated alcohol (5:2)
Mobile phase: Acetonitrile, tetrahydrofuran, and water (55:40:5)
Standard stock solution: 1.0 mg/mL of USP Ubidecarenone RS in *Solvent*
Standard solution: 40 µg/mL in dehydrated alcohol, from the *Standard stock solution*
System suitability stock solution: 1.0 mg/mL of USP Ubidecarenone Related Compound A RS in *Solvent*. Dilute a portion of this solution with dehydrated alcohol to obtain a concentration of 40 µg/mL.
System suitability solution: *Standard solution* and *System suitability stock solution* (1:1)
Sample solution 1 (for soft gelatin Capsules)**:** Open a number of Capsules equivalent to 200 mg of ubidecarenone, quantitatively transfer the shells and contents to a container, add 100 mL of *Solvent*, and shake by mechanical means for 30 min. Using small portions of *Solvent*, quantitatively transfer this mixture to a 200-mL volumetric flask, and dilute with *Solvent* to volume. Centrifuge a portion of this solution, transfer 1.0 mL of the supernatant to a 25-mL volumetric flask, add 2.5 mL of a 0.1% solution of anhydrous ferric chloride in alcohol, and dilute with alcohol to volume.
Sample solution 2 (for hard gelatin Capsules)**:** Empty and thoroughly mix the contents of NLT 20 Capsules. Transfer a portion of the powder, equivalent to 100 mg of ubidecarenone, to a 100-mL volumetric flask, add 60 mL of *Solvent*, and shake by mechanical means for 30 min. Dilute with *Solvent* to volume. Centrifuge a portion of this solution, transfer 1.0 mL of the supernatant to a 25-mL volumetric flask, add 2.5 mL of a 0.1% solution of anhydrous ferric chloride in alcohol, and dilute with alcohol to volume.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 280 nm
Column: 8-mm × 10-cm; packing L1
Flow rate: 2.5 mL/min
Injection size: 15 µL
System suitability
Samples: *Standard solution* and *System suitability solution*
Suitability requirements
Resolution: NLT 2.5 between ubidecarenone and ubidecarenone related compound A, *System suitability solution*
Tailing factor: NMT 1.5, *Standard solution*
Relative standard deviation: NMT 2.0% for ubidecarenone, *Standard solution*
Analysis
Samples: *Sample solution 1* or *Sample solution 2*, and *Standard solution*
Calculate the percentage of the labeled amount of ubidecarenone ($C_{59}H_{90}O_4$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of ubidecarenone from *Sample solution 1* or *Sample solution 2*
r_S = peak area of ubidecarenone from the *Standard solution*
C_S = concentration of USP Ubidecarenone RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of ubidecarenone in *Sample solution 1* or *Sample solution 2* (mg/mL)

Acceptance criteria: 90.0%–115.0%

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PERFORMANCE TESTS

• **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩: Meet the requirements of the test for *Disintegration*, except where the product is labeled to contain a water-soluble form of ubidecarenone. Capsules labeled to contain a water-soluble form of ubidecarenone meet the requirements for the test for *Dissolution*, as follows.

Medium: Water; 500 mL
Apparatus 2: 75 rpm
Time: 60 min
Standard solution: Dissolve 25 mg of USP Ubidecarenone RS in 1 mL of ethyl ether, and dilute with alcohol to obtain a concentration of 2.5 µg/mL. [NOTE—Use a freshly prepared solution only.]
Sample solution: Dilute with alcohol a volume of the solution under test, previously passed through a suitable filter of 0.45-µm pore size, to obtain a concentration of 2.5 µg/mL of ubidecarenone.
Mobile phase and **Chromatographic system:** Proceed as directed in the *Procedure* for *Strength*, except for *Injection size*.
Injection size: 100 µL
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of ubidecarenone ($C_{59}H_{90}O_4$) dissolved:

$$\text{Result} = (r_U/r_S) \times (C_S \times V \times D/L) \times 100$$

r_U = peak area of ubidecarenone from the *Sample solution*
r_S = peak area of ubidecarenone from the *Standard solution*
C_S = concentration of USP Ubidecarenone RS in the *Standard solution* (mg/mL)
V = volume of *Medium*, 500 mL
D = dilution factor for the *Sample solution*
L = label claim (mg/Capsule)

Tolerances: NLT 75% of the labeled amount of ubidecarenone ($C_{59}H_{90}O_4$) is dissolved.

SPECIFIC TESTS

• **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩ : Meet the requirements

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
• **LABELING:** Where the product contains a water-soluble form of ubidecarenone, this is so stated on the label.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Ubidecarenone RS
USP Ubidecarenone Related Compound A RS
Coenzyme Q_9.

Ubidecarenone Tablets

DEFINITION

Ubidecarenone Tablets contain NLT 90.0% and NMT 115.0% of the labeled amount of ubidecarenone ($C_{59}H_{90}O_4$).

IDENTIFICATION

• **A.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Procedure* for *Strength*.

STRENGTH

• **PROCEDURE**
[NOTE—Conduct this test promptly with minimum exposure to actinic light.]
Solvent: *n*-Hexane and dehydrated alcohol (5:2)
Mobile phase: Acetonitrile, tetrahydrofuran, and water (11:8:1)
Standard stock solution: 1.0 mg/mL of USP Ubidecarenone RS in *Solvent*
Standard solution: 40 µg/mL from *Standard stock solution* in dehydrated alcohol
System suitability stock solution: 1.0 mg/mL of USP Ubidecarenone Related Compound A RS in *Solvent*. Dilute a portion of this solution with dehydrated alcohol to obtain a concentration of 40 µg/mL.
System suitability solution: *Standard solution* and *System suitability stock solution* (1:1)
Sample stock solution: Weigh and finely powder NLT 20 Tablets. Transfer a quantity of powder, equivalent to about 100 mg of ubidecarenone, to a 100-mL volumetric flask, add 60 mL of *Solvent*, and shake by mechanical means for 30 min. Dilute with *Solvent* to volume, and mix. Centrifuge a portion of this solution, transfer 1.0 mL of the supernatant to a 25-mL volumetric flask, and add 2.5 mL of a 0.1% solution of anhydrous ferric chloride in alcohol. Dilute with alcohol to volume, and mix.
Sample solution: Centrifuge a portion of *Sample stock solution*, transfer 1.0 mL of the supernatant to a 25-mL volumetric flask, add 2.5 mL of a 0.1% solution of anhydrous ferric chloride in alcohol, and dilute with alcohol to volume.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 280 nm
Column: 8-mm × 10-cm; packing L1
Flow rate: 2.5 mL/min
Injection size: 15 µL
System suitability
Samples: *Standard solution* and *System suitability solution*
Suitability requirements
Resolution: NLT 2.5 between ubidecarenone and ubidecarenone related compound A, *System suitability solution*
Tailing factor: NMT 1.5, *Standard solution*
Relative standard deviation: NMT 2.0% for ubidecarenone, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of ubidecarenone ($C_{59}H_{90}O_4$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of ubidecarenone from the *Sample solution*
r_S = peak area of ubidecarenone from the *Standard solution*
C_S = concentration of USP Ubidecarenone RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of ubidecarenone in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–115.0%

PERFORMANCE TESTS

• **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩: Meet the requirements of the test for *Disintegration*, except where the product is labeled to contain a water-soluble form of

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ubidecarenone. Tablets labeled to contain a water-soluble form of ubidecarenone meet the requirements for the test for *Dissolution*, as follows.

Medium: Water; 500 mL
Apparatus 2: 75 rpm
Time: 60 min
Standard solution: Dissolve 25 mg of USP Ubidecarenone RS in 1 mL of ethyl ether, and dilute with alcohol to obtain a concentration of 2.5 µg/mL. [NOTE—Use a freshly prepared solution only.]
Sample solution: Dilute with alcohol a volume of the solution under test, previously passed through a suitable filter of 0.45-µm pore size, to obtain a concentration of 2.5 µg/mL of ubidecarenone.
Mobile phase and **Chromatographic system:** Proceed as directed in the *Procedure* for *Strength*, except for *Injection size*.
Injection size: 100 µL
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of ubidecarenone ($C_{59}H_{90}O_4$) dissolved:

$$\text{Result} = (r_U/r_S) \times (C_S \times V \times D/L) \times 100$$

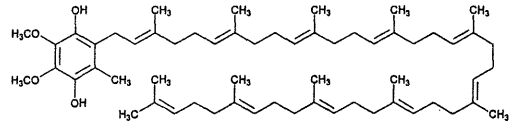
r_U = peak area of ubidecarenone from the *Sample solution*
r_S = peak area of ubidecarenone from the *Standard solution*
C_S = concentration of USP Ubidecarenone RS in the *Standard solution* (mg/mL)
V = volume of *Medium*, 500 mL
D = dilution factor for the *Sample solution*
L = label claim (mg/Tablet)

Tolerances: NLT 75% of the labeled amount of ubidecarenone ($C_{59}H_{90}O_4$) is dissolved.

SPECIFIC TESTS
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩ : Meet the requirements

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** Where the product contains a water-soluble form of ubidecarenone, this is so stated on the label.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Ubidecarenone RS
USP Ubidecarenone Related Compound A RS
Coenzyme Q_9.

Ubiquinol



$C_{59}H_{92}O_4$ 865.37
2-[(2*E*,6*E*,10*E*,14*E*,18*E*,22*E*,26*E*,30*E*,34*E*)-3,7,11,15,19,23,27,31,35,39-Decamethyltetraconta-2,6,10,14,18,22,26,30,34,38-decaenyl]-5,6-dimethoxy-3-methylbenzene-1,4-diol [992-78-9].

DEFINITION
Ubiquinol contains NLT 96.0% and NMT 102.0% of ubiquinol ($C_{59}H_{92}O_4$), calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

- **A.** ▲**SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

ASSAY
- **PROCEDURE**
Mobile phase: Acetonitrile and alcohol (50:50)
[NOTE—Protect the following solutions from exposure to air after preparation, because ubiquinol is readily oxidized to ubidecarenone when exposed to air.]
Diluent: Methanol and *n*-hexane (85:15)
System suitability solution: 0.5 mg/mL each of USP Ubiquinol RS and USP Ubidecarenone RS in *Diluent*. Sonicate, if necessary, for complete dissolution.
Standard solution: 0.5 mg/mL of USP Ubiquinol RS in *Diluent*. Sonicate, if necessary, for complete dissolution.
Sample solution: 0.5 mg/mL of Ubiquinol in *Diluent*. Sonicate, if necessary, for complete dissolution.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 290 nm
Column: 4.6-mm × 10-cm; 3-µm packing L1
Column temperature: 35°
Flow rate: 1.5 mL/min
Injection volume: 20 µL
System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The relative retention times for ubiquinol and ubidecarenone are about 1.0 and 1.2, respectively.]
Suitability requirements
Resolution: NLT 2.0 between ubiquinol and ubidecarenone, *System suitability solution*
Relative standard deviation: NMT 2.0%, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of ubiquinol ($C_{59}H_{92}O_4$) in the portion of Ubiquinol taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP Ubiquinol RS in the *Standard solution* (mg/mL)
C_U = concentration of Ubiquinol in the *Sample solution* (mg/mL)

Acceptance criteria: 96.0%–102.0% on the anhydrous basis

IMPURITIES
- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.2%
- **UBIDECARENONE AND OTHER RELATED COMPOUNDS**
Mobile phase, System suitability solution, Chromatographic system, and **System suitability:** Proceed as directed in the *Assay*.
Standard solution: 0.02 mg/mL of USP Ubidecarenone RS in *Diluent*

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Sample solution: 1.0 mg/mL of Ubiquinol in *Diluent*
Analysis
Samples: *Standard solution* and *Sample solution*
[NOTE—Protect the *Sample solution* from exposure to air after preparation, because ubiquinol is readily oxidized to ubidecarenone when exposed to air.]
Calculate the percentage of ubidecarenone in the portion of Ubiquinol taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of ubidecarenone from the *Sample solution*
r_S = peak response of ubidecarenone from the *Standard solution*
C_S = concentration of USP Ubidecarenone RS in the *Standard solution* (mg/mL)
C_U = concentration of Ubiquinol in the *Sample solution* (mg/mL)

Calculate the percentage of other related compounds in the portion of Ubiquinol taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of individual related compounds from the *Sample solution*
r_T = sum of all the peak responses from the *Sample solution*

Acceptance criteria
Ubidecarenone: NMT 2.0%
Other individual related compounds: NMT 0.5%
Sum of other related compounds: NMT 1.0%

SPECIFIC TESTS
• **WATER DETERMINATION,** *Method Ic* ⟨921⟩**:** NMT 0.3%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed, light-resistant containers.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Ubidecarenone RS
USP Ubiquinol RS

Ubiquinol Capsules

DEFINITION
Ubiquinol Capsules contain NLT 90.0% and NMT 115.0% of the labeled amount of ubiquinol ($C_{59}H_{92}O_4$).

IDENTIFICATION
• **A.** The retention time of the major peak of either *Sample solution 1* or *Sample solution 2* corresponds to that of the *Standard solution*, as obtained in the *Procedure* for *Strength*.

STRENGTH
• **PROCEDURE**
[NOTE—Conduct this test promptly with minimal exposure to air. Ubiquinol is readily oxidized to ubidecarenone when exposed to air.]
Mobile phase: Acetonitrile and alcohol (50:50)
Diluent: Methanol and *n*-hexane (85:15)
System suitability solution: 0.5 mg/mL each of USP Ubiquinol RS and USP Ubidecarenone RS in *Diluent*. Sonicate, if necessary, for complete dissolution.
Standard solution: 0.5 mg/mL of USP Ubiquinol RS in *Diluent*. Sonicate, if necessary, for complete dissolution.
Sample solution 1 (for soft gelatin Capsules): Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without the loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing, if necessary, with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the average net weight per Capsule. Transfer a portion of the Capsule contents, equivalent to 250 mg of ubiquinol into a 50-mL volumetric flask. Add 40 mL of *Diluent* and shake by mechanical means for 10 min. Sonicate additionally for about 15 min, cool to room temperature, dilute with *Diluent* to volume, and mix well. Dilute a portion of the resultant solution with *Diluent* to obtain a concentration of 0.5 mg/mL of ubiquinol. Mix well and pass through a membrane filter of 0.45-µm pore size.
Sample solution 2 (for hard gelatin Capsules): Empty and thoroughly mix the contents of NLT 20 Capsules. Transfer a portion of the contents, equivalent to 250 mg of ubiquinol into a 50-mL volumetric flask. Add 40 mL of *Diluent* and shake by mechanical means for 20 min. Sonicate additionally for about 15 min, cool to room temperature, dilute with *Diluent* to volume, and mix well. Dilute a portion of the resultant solution with *Diluent* to obtain a concentration of 0.5 mg/mL of ubiquinol. Mix well and pass through a membrane filter of 0.45-µm pore size.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 290 nm
Column: 4.6-mm × 10-cm; 3.5-µm packing L1
Flow rate: 1.5 mL/min
Injection volume: 20 µL
System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The relative retention times for ubiquinol and ubidecarenone are about 1.0 and 1.2, respectively.]
Suitability requirements
Resolution: NLT 2.0 between ubiquinol and ubidecarenone, *System suitability solution*
Relative standard deviation: NMT 2.0%, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution 1* or *Sample solution 2*
Calculate the percentage of ubiquinol ($C_{59}H_{92}O_4$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of ubiquinol from the *Sample solution*
r_S = peak area of ubiquinol from the *Standard solution*
C_S = concentration of USP Ubiquinol RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of ubiquinol in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–115.0%

PERFORMANCE TESTS
• **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩**:** Meet the requirements for *Disintegration*
• **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩**:** Meet the requirements

CONTAMINANTS
• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 3×10^3 cfu/g, and the

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total combined molds and yeasts count does not exceed 3 × 10^2 cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩: Meet the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Ubidecarenone RS

USP Ubiquinol RS

Valerian

DEFINITION

Valerian consists of the subterranean parts of *Valeriana officinalis* L. (Fam. Valerianaceae) including the rhizome, roots, and stolons. It contains NLT 0.5% of volatile oil, NLT 0.05% of valerenic acid ($C_{15}H_{22}O_2$), and NLT 0.17% of total valerenic acids, calculated as the sum of hydroxyvalerenic acid, acetoxyvalerenic acid, and valerenic acid, on the dried basis.

IDENTIFICATION

• **A.** Meets the requirements for *Specific Tests, Botanic Characteristics*

• **B.**

Sample solution: 0.2 g of freshly powdered Valerian in 5 mL of methylene chloride. Shake several times, and allow to stand for 5 min. Filter, wash the filter with 2 mL of methylene chloride, combine the filtrate and washings, and evaporate to dryness. Dissolve the residue in 0.2 mL of methylene chloride.

Analysis: To 0.1 mL of the *Sample solution* add 3 mL of a mixture of equal volumes of glacial acetic acid and 25% hydrochloric acid, and shake several times.

Acceptance criteria: A blue color develops within 15 min.

• **C. THIN-LAYER CHROMATOGRAPHY**

Standard solution A: 0.25 mg/mL of USP Valerenic Acid RS in methanol

Standard solution B: 40 mg/mL of USP Powdered Valerian Extract RS in methanol. Sonicate for 10 min, centrifuge, and use the supernatant.

Sample solution: About 0.5 g of Valerian, finely powdered, in 5 mL of methanol. Sonicate for 10 min, centrifuge, and use the supernatant.

Chromatographic system

Adsorbent: Chromatographic silica gel mixture with an average particle size of 2–10 µm (HPTLC plates)

Application volume: 5 µL, as 8-mm bands

Developing solvent system: A mixture of cyclohexane, ethyl acetate, and glacial acetic acid (60:38:2)

Derivatization reagent A: A mixture of glacial acetic acid and hydrochloric acid (1:4)

Derivatization reagent B: 0.5 mL of *p*-anisaldehyde, 10 mL of glacial acetic acid, and 5 mL of sulfuric acid. Add to 85 mL of ice-cold methanol, and mix.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands to a suitable high-performance thin-layer chromatographic plate. Use a saturated chamber, and condition the plate to a relative humidity of about 33% using a suitable device. Develop the chromatograms over a distance of 6 cm. Remove the plate from the chamber, dry, derivatize with *Derivatization reagent A*, heat at 120° for 5 min, and examine under white light. Derivatize with *Derivatization reagent B*, heat at 100° for 3 min, and examine under white light.

Acceptance criteria: After treatment with *Derivatization reagent A* and heating, the *Sample solution* does not exhibit an intense blue band at about the middle of the chromatogram nor any other significant bands [distinction from Mexican valerian (*Valeriana edulis*)], though minor bands may be observed.

After treatment with *Derivatization reagent B* and heating, the *Sample solution* exhibits three violet bands in positions and colors similar to the bands of *Standard solution B*. These bands include a minor band in the lower third of the chromatogram due to hydroxyvalerenic acid, a major band at about the middle the chromatogram due to acetoxyvalerenic acid [distinction from Scouler's valerian (*Valeriana wallichii*)], and a major band at an R_F corresponding to the valerenic acid band of *Standard solution A* and *Standard solution B*. Other minor bands may be observed in the *Sample solution* and in *Standard solution B*.

• **D. HPLC**

Analysis: Proceed as directed in the test for *Content of Valerenic Acids*.

Acceptance criteria: The *Sample solution* exhibits a peak at a retention time corresponding to the valerenic acid peak of *Standard solution A*. The *Sample solution* shows additional peaks corresponding to hydroxyvalerenic acid and acetoxyvalerenic acid.

COMPOSITION

• **CONTENT OF VALERENIC ACIDS**

Solution A: Mix 6 mL of 85% phosphoric acid with 900 mL of water, dilute with water to 1000 mL, mix, filter, and degas.

Solution B: Mix 6 mL of 85% phosphoric acid with 900 mL of methanol, dilute with methanol to 1000 mL, mix, filter, and degas.

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	40	60
15	5	95
25	5	95
30	40	60

Solvent: A mixture of methanol and a solution of 0.1% phosphoric acid in water (3:1)

Standard solution A: 0.02 mg/mL of USP Valerenic Acid RS in methanol. Sonicate if necessary.

Standard solution B: Sonicate a portion of USP Powdered Valerian Extract RS in *Solvent* to obtain a solution having a concentration of about 20 mg/mL. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.

Sample solution: To a 50-mL volumetric flask, transfer about 1.0 g of Valerian, finely powdered and accurately weighed, add 10.0 mL of water, and shake for 2 min while heating in a water bath maintained at about 50°. Sonicate for 15 min, add 35 mL of methanol, and sonicate for 15 min. Cool, dilute with methanol to volume, and mix. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.

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Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 225 nm
Column: 4.6-mm × 25-cm; end-capped, 5-µm 100 Å packing L1
Column temperature: 40°
Flow rate: 1.0 mL/min
Injection volume: 25 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Valerian Extract RS being used.
Tailing factor: NMT 2.0 for the valerenic acid peak, *Standard solution A*
Relative standard deviation: NMT 2.0% for the valerenic acid peak in repeated injections, *Standard solution A*
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Identify the valerenic acids in the *Sample solution* chromatogram by comparison with the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Valerian Extract RS being used.
Calculate the percentages of hydroxyvalerenic acid, acetoxyvalerenic acid, and valerenic acid in the portion of Valerian taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of valerenic acid from *Standard solution A*
C_S = concentration of valerenic acid in *Standard solution A* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Valerian taken to prepare the *Sample solution* (mg)
F = conversion factor for each analyte (1.10 for hydroxyvalerenic acid, 1.25 for acetoxyvalerenic acid, and 1.00 for valerenic acid)

Acceptance criteria: NLT 0.05% of valerenic acid ($C_{15}H_{22}O_2$), and NLT 0.17% of total valerenic acids, calculated as the sum of hydroxyvalerenic acid, acetoxyvalerenic acid, and valerenic acid on the dried basis

- **Articles of Botanical Origin,** *Volatile Oil Determination* ⟨561⟩
Sample: 100 g, freshly and coarsely comminuted
Acceptance criteria: NLT 0.5% on the dried basis

CONTAMINANTS

- **Elemental Impurities—Procedures** ⟨233⟩
Acceptance criteria
Arsenic: NMT 0.5 µg/g
Cadmium: NMT 1.0 µg/g
Lead: NMT 5.0 µg/g
Mercury: NMT 0.1 µg/g

Change to read:

- **▲Articles of Botanical Origin,** *Pesticide Residue Analysis* ⟨561⟩▲ (CN 1-May-2019)**:** Meets the requirements

- **Microbial Enumeration Tests** ⟨2021⟩**:** The total bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and bile-tolerant Gram-negative bacteria does not exceed 10^3 cfu/g.
- **Absence of Specified Microorganisms** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

- **Botanic Characteristics**
Macroscopic: Rhizomes: yellowish-gray to pale grayish-brown; entire or cut longitudinally; up to 5 cm in length and up to 3 cm in diameter; base elongated or compressed, covered by and merging with numerous roots; apices usually bearing a cup-shaped scar from aerial parts, stem base rarely present, fracture reveals pith with a central cavity, transverse septum observed in longitudinal cut. Roots: yellowish-gray to pale grayish-brown with longitudinal stripes, numerous, cylindrical, slender, about 10 cm in length and up to 3 mm in diameter, a few filiform, fragile secondary roots, fracture short. Stolons: pale yellowish-gray, showing prominent nodes separated by longitudinally striated internodes each 2–5 cm in length, fracture fibrous.
Microscopic: Roots: epidermis, pilliferous layer with papillose cells, some developed into root hairs and exodermis consisting of a single layer of quadrangular to polygonal cells with suberized walls; oil globules scattered through the epidermis and cortex; cortex, occupying most of the root, outer part consisting of 2–4 layers of resin-containing cells, inner part consisting of numerous layers of polygonal to subrounded cells filled with starch granules, clefts present; endodermis consisting of a single layer of slightly lignified cells, Casparian dots distinct; pericycle consisting of 1–2 layers of tangentially elongated cells, sometimes indistinct; phloem, vascular bundles forming an interrupted ring; cambium frequently indistinct; xylem, continuously distributed, with polygonal vessels, surrounding a central pith; pith showing cells with slightly thickened walls containing starch granules; starch granules, simple, hilum dotted, stellate or cleft-shaped, 5–15 µm in diameter; compound starch granules of 2–6 components. Rhizomes: similar to roots except epidermis and exodermis partially replaced by poorly developed periderm; presence of numerous vascular bundles; central pith wider, including clefts of various sizes, the larger being separated by groups of stone cells.
- **Articles of Botanical Origin,** *Foreign Organic Matter* ⟨561⟩**:** NMT 2.0%
- **Extractable Matter**
Sample: 2 g, carefully dried at 40° and coarsely powdered
Analysis: Mix the *Sample* with 20 mL of 70% alcohol, and allow to stand for 2 h, shaking frequently. Filter, evaporate 5 mL of the filtrate on a water bath to dryness, and dry the residue at 105°.
Acceptance criteria: NLT 20%
- **Loss on Drying** ⟨731⟩
Sample: 1.0 g of finely powdered Valerian
Analysis: Dry at 105° for 2 h.
Acceptance criteria: NMT 12%
- **Articles of Botanical Origin,** *Total Ash* ⟨561⟩**:** NMT 12.0%
- **Articles of Botanical Origin,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 5.0%

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in tight containers, store at room temperature, and protect from light and moisture.

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• **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.

• **USP REFERENCE STANDARDS** ⟨11⟩
USP Powdered Valerian Extract RS
USP Valerenic Acid RS

Powdered Valerian

DEFINITION

Powdered Valerian is Valerian reduced to a fine or a very fine powder. It contains no calcium oxalate crystals and no foreign starch granules. It contains NLT 0.3% of volatile oil, NLT 0.04% of valerenic acid ($C_{15}H_{22}O_2$), and NLT 0.1% of total valerenic acids, calculated as the sum of hydroxyvalerenic acid, acetoxyvalerenic acid, and valerenic acid, on the dried basis.

IDENTIFICATION

• **A.** Meets the requirements for *Specific Tests, Botanic Characteristics*

• **B.**

Sample solution: 0.2 g of Powdered Valerian in 5 mL of methylene chloride. Shake several times, and allow to stand for 5 min. Filter, wash the filter with 2 mL of methylene chloride, combine the filtrate and washings, and evaporate to dryness. Dissolve the residue in 0.2 mL of methylene chloride.

Analysis: To 0.1 mL of the *Sample solution* add 3 mL of a mixture of equal volumes of glacial acetic acid and 25% hydrochloric acid, and shake several times.

Acceptance criteria: A blue color develops within 15 min.

• **C. THIN-LAYER CHROMATOGRAPHY**

Standard solution A: 0.25 mg/mL of USP Valerenic Acid RS in methanol

Standard solution B: 40 mg/mL of USP Powdered Valerian Extract RS in methanol. Sonicate for 10 min, centrifuge, and use the supernatant.

Sample solution: About 0.5 g of Powdered Valerian in 5 mL of methanol. Sonicate for 10 min, centrifuge, and use the supernatant.

Chromatographic system

Adsorbent: Chromatographic silica gel mixture with an average particle size of 2–10 µm (HPTLC plates)

Application volume: 5 µL, as 8-mm bands

Developing solvent system: A mixture of cyclohexane, ethyl acetate, and glacial acetic acid (60:38:2)

Derivatization reagent A: A mixture of glacial acetic acid and hydrochloric acid (1:4)

Derivatization reagent B: 0.5 mL of *p*-anisaldehyde, 10 mL of glacial acetic acid, and 5 mL of sulfuric acid. Add to 85 mL of ice-cold methanol, and mix.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands to a suitable high-performance thin-layer chromatographic plate. Use a saturated chamber, and condition the plate to a relative humidity of about 33% using a suitable device. Develop the chromatograms over a distance of 6 cm. Remove the plate from the chamber, dry, derivatize with *Derivatization reagent A*, heat at 120° for 5 min, and examine under white light. Derivatize with *Derivatization reagent B*, heat at 100° for 3 min, and examine under white light.

Acceptance criteria: After treatment with *Derivatization reagent A* and heating, the *Sample solution* does not exhibit an intense blue band at about the middle of the chromatogram nor any other significant bands [distinction from Mexican valerian (*Valeriana edulis*)], though minor bands may be observed.

After treatment with *Derivatization reagent B* and heating, the *Sample solution* exhibits three violet bands in positions and colors similar to the bands of *Standard solution B*. These bands include a minor band in the lower third of the chromatogram due to hydroxyvalerenic acid, a major band at about the middle the chromatogram due to acetoxyvalerenic acid [distinction from Scouler's valerian (*Valeriana wallichii*)], and a major band at an R_F corresponding to the valerenic acid band of *Standard solution A* and *Standard solution B*. Other minor bands may be observed in the *Sample solution* and in *Standard solution B*.

• **D. HPLC**

Analysis: Proceed as directed in the test for *Content of Valerenic Acids*.

Acceptance criteria: The *Sample solution* exhibits a peak at a retention time corresponding to the valerenic acid peak of *Standard solution A*. The *Sample solution* shows additional peaks corresponding to hydroxyvalerenic acid and acetoxyvalerenic acid.

COMPOSITION

• **CONTENT OF VALERENIC ACIDS**

Solution A: Mix 6 mL of 85% phosphoric acid with 900 mL of water, dilute with water to 1000 mL, mix, filter, and degas.

Solution B: Mix 6 mL of 85% phosphoric acid with 900 mL of methanol, dilute with methanol to 1000 mL, mix, filter, and degas.

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	40	60
15	5	95
25	5	95
30	40	60

Solvent: A mixture of methanol and a solution of 0.1% phosphoric acid in water (3:1)

Standard solution A: 0.02 mg/mL of USP Valerenic Acid RS in methanol. Sonicate if necessary.

Standard solution B: Sonicate a portion of USP Powdered Valerian Extract RS in *Solvent* to obtain a solution having a concentration of about 20 mg/mL. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.

Sample solution: To a 50-mL volumetric flask, transfer about 1.0 g of Powdered Valerian, accurately weighed, add 10.0 mL of water, and shake for 2 min while heating in a water bath maintained at about 50°. Sonicate for 15 min, add 35 mL of methanol, and sonicate for 15 min. Cool, dilute with methanol to volume, and mix. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 225 nm

Column: 4.6-mm × 25-cm; end-capped, 5-µm 100 Å packing L1

Column temperature: 40°

Flow rate: 1.0 mL/min

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Injection volume: 25 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Valerian Extract RS being used.
Tailing factor: NMT 2.0 for the valerenic acid peak, *Standard solution A*
Relative standard deviation: NMT 2.0% for the valerenic acid peak in repeated injections, *Standard solution A*
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Identify the valerenic acids in the *Sample solution* chromatogram by comparison with the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Valerian Extract RS being used.
Calculate the percentages of hydroxyvalerenic acid, acetoxyvalerenic acid, and valerenic acid in the portion of Powdered Valerian taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of valerenic acid from *Standard solution A*
C_S = concentration of valerenic acid in *Standard solution A* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Powdered Valerian taken to prepare the *Sample solution* (mg)
F = conversion factor for each analyte (1.10 for hydroxyvalerenic acid, 1.25 for acetoxyvalerenic acid, and 1.00 for valerenic acid)

Acceptance criteria: NLT 0.04% of valerenic acid ($C_{15}H_{22}O_2$), and NLT 0.1% of total valerenic acids, calculated as the sum of hydroxyvalerenic acid, acetoxyvalerenic acid, and valerenic acid on the dried basis

- **ARTICLES OF BOTANICAL ORIGIN,** *Volatile Oil Determination* ⟨561⟩
Sample: 100 g of Powdered Valerian
Acceptance criteria: NLT 0.3%

CONTAMINANTS

- **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩
Acceptance criteria
Arsenic: NMT 0.5 µg/g
Cadmium: NMT 1.0 µg/g
Lead: NMT 5.0 µg/g
Mercury: NMT 0.1 µg/g

Change to read:

- **▲ARTICLES OF BOTANICAL ORIGIN,** *Pesticide Residue Analysis* ⟨561⟩▲ (CN 1-May-2019)**:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and bile-tolerant Gram-negative bacteria does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

- **BOTANIC CHARACTERISTICS**
Microscopic: Numerous starch granules, simple, hilum dotted, stellate or cleft-shaped, 5–15 µm in diameter; compound starch granules of 2–6 components; black, cruciate shape under a polarizing microscope. Scattered stone cells, single or aggregated, with cell lumina of various sizes, bright yellowish-white or bright white under a polarizing microscope; numerous fragments of parenchyma cells containing globules of volatile oil and starch granules; fragments of pale yellow lignified fibers, scattered, single or aggregated; fragments of vessels, reticulate, bordered pitted and spiral.
- **EXTRACTABLE MATTER**
Sample: 2 g of Powdered Valerian, carefully dried at 40°
Analysis: Mix the *Sample* with 20 mL of 70% alcohol, and allow to stand for 2 h, shaking frequently. Filter, evaporate 5 mL of the filtrate on a water bath to dryness, and dry the residue at 105°.
Acceptance criteria: NLT 20%
- **LOSS ON DRYING** ⟨731⟩
Sample: 1.0 g of Powdered Valerian
Analysis: Dry at 105° for 2 h.
Acceptance criteria: NMT 12%
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 12.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 5.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, store at room temperature, and protect from light and moisture.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant from which the article was derived.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Powdered Valerian Extract RS
USP Valerenic Acid RS

Powdered Valerian Extract

DEFINITION

Powdered Valerian Extract is prepared from comminuted Valerian using hydroalcoholic mixtures. It contains NLT 0.3% of valerenic acid ($C_{15}H_{22}O_2$), and NLT 0.6% of total valerenic acids, calculated as the sum of hydroxyvalerenic acid, acetoxyvalerenic acid, and valerenic acid, on the dried basis. The ratio of the starting crude plant material to the Extract is between 4:1 and 7:1.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHY**
Standard solution A: 0.25 mg/mL of USP Valerenic Acid RS in methanol
Standard solution B: 40 mg/mL of USP Powdered Valerian Extract RS in methanol. Sonicate for 10 min, centrifuge, and use the supernatant.
Sample solution: 40 mg/mL of Extract in methanol. Sonicate for 10 min, centrifuge, and use the supernatant.
Chromatographic system
Adsorbent: Chromatographic silica gel mixture with an average particle size of 2–10 µm (HPTLC plates)
Application volume: 5 µL, as 8-mm bands
Developing solvent system: A mixture of cyclohexane, ethyl acetate, and glacial acetic acid (60:38:2)

USP Monographs

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Derivatization reagent A: A mixture of glacial acetic acid and hydrochloric acid (1:4)
Derivatization reagent B: 0.5 mL of *p*-anisaldehyde, 10 mL of glacial acetic acid, and 5 mL of sulfuric acid. Add to 85 mL of ice-cold methanol, and mix.
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Apply the *Samples* as bands to a suitable high-performance thin-layer chromatographic plate. Use a saturated chamber, and condition the plate to a relative humidity of about 33% using a suitable device. Develop the chromatograms over a distance of 6 cm. Remove the plate from the chamber, dry, derivatize with *Derivatization reagent A*, heat at 120° for 5 min, and examine under white light. Derivatize with *Derivatization reagent B*, heat at 100° for 3 min, and examine under white light.
Acceptance criteria: After treatment with *Derivatization reagent A* and heating, the *Sample solution* does not exhibit an intense blue band at about the middle of the chromatogram nor any other significant bands [distinction from Mexican valerian (*Valeriana edulis*)], though minor bands may be observed.
After treatment with *Derivatization reagent B* and heating, the *Sample solution* exhibits three violet bands in positions and colors similar to the bands of *Standard solution B*. These bands include a minor band in the lower third of the chromatogram due to hydroxyvalerenic acid, a major band at about the middle the chromatogram due to acetoxyvalerenic acid [distinction from Scouler's valerian (*Valeriana wallichii*)], and a major band at an R_F corresponding to the valerenic acid band of *Standard solution A* and *Standard solution B*. Other minor bands may be observed in the *Sample solution* and in *Standard solution B*.

• **B. HPLC**
Analysis: Proceed as directed in the test for *Content of Valerenic Acids*.
Acceptance criteria: The *Sample solution* exhibits a peak at a retention time corresponding to the valerenic acid peak of *Standard solution A*. The *Sample solution* shows additional peaks corresponding to hydroxyvalerenic acid and acetoxyvalerenic acid.

COMPOSITION

• **CONTENT OF VALERENIC ACIDS**
Solution A: Mix 6 mL of 85% phosphoric acid with 900 mL of water, dilute with water to 1000 mL, mix, filter, and degas.
Solution B: Mix 6 mL of 85% phosphoric acid with 900 mL of methanol, dilute with methanol to 1000 mL, mix, filter, and degas.
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	40	60
15	5	95
25	5	95
30	40	60

Solvent: A mixture of methanol and a solution of 0.1% phosphoric acid in water (3:1)
Standard solution A: 0.05 mg/mL of USP Valerenic Acid RS in methanol. Sonicate if necessary.
Standard solution B: Sonicate a portion of USP Powdered Valerian Extract RS in *Solvent* to obtain a solution having a concentration of about 20 mg/mL. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.
Sample solution: Sonicate a portion of Extract in *Solvent* to obtain a solution having a concentration of about 20 mg/mL. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 225 nm
Column: 4.6-mm × 25-cm; end-capped, 5-µm 100 Å packing L1
Column temperature: 40°
Flow rate: 1.0 mL/min
Injection volume: 25 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Valerian Extract RS being used.
Tailing factor: NMT 2.0 for the valerenic acid peak, *Standard solution A*
Relative standard deviation: NMT 2.0% for the valerenic acid peak in repeated injections, *Standard solution A*
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Identify the valerenic acids in the *Sample solution* chromatogram by comparison with the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Valerian Extract RS being used.
Calculate the percentages of hydroxyvalerenic acid, acetoxyvalerenic acid, and valerenic acid in the portion of Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of valerenic acid from *Standard solution A*
C_S = concentration of valerenic acid in *Standard solution A* (mg/mL)
C_U = concentration of the Extract in the *Sample solution* (mg/mL)
F = conversion factor for each analyte (1.10 for hydroxyvalerenic acid, 1.25 for acetoxyvalerenic acid, and 1.00 for valerenic acid)

Acceptance criteria: NLT 0.3% of valerenic acid ($C_{15}H_{22}O_2$), and NLT 0.6% of total valerenic acids, calculated as the sum of hydroxyvalerenic acid, acetoxyvalerenic acid, and valerenic acid on the dried basis

CONTAMINANTS

• **ELEMENTAL IMPURITIES—PROCEDURES** ⟨233⟩
Acceptance criteria
Arsenic: NMT 0.5 µg/g
Cadmium: NMT 1.0 µg/g
Lead: NMT 5.0 µg/g
Mercury: NMT 0.1 µg/g

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Change to read:

- **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020) : Meets the requirements
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total bacterial count does not exceed 10^4 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, the coliform count does not exceed 10^3 cfu/g, and the *Enterobacteriaceae* count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩: It meets the requirements of the tests for absence of *Salmonella* species, *Escherichia coli*, and *Staphylococcus aureus*.

SPECIFIC TESTS

- **WATER DETERMINATION,** *Method Ia* ⟨921⟩: NMT 5%
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩: NMT 7.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, store at controlled room temperature, and protect from moisture and light.
- **LABELING:** The label states the official name of the article, the Latin binomial, and the part of the plant from which the article was prepared. Label it to indicate the content of valerenic acid and total valerenic acids, and the ratio of the starting crude plant material to the Extract. It meets other labeling requirements in *Botanical Extracts* ⟨565⟩.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Powdered Valerian Extract RS
  USP Valerenic Acid RS

Valerian Tablets

DEFINITION

Valerian Tablets contain Powdered Valerian Extract. Tablets contain NLT 90.0% and NMT 120.0% of the labeled amount of Powdered Valerian Extract, calculated as valerenic acids equivalent to the sum of hydroxyvalerenic acid, acetoxyvalerenic acid, and valerenic acid.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHY**

Standard solution A: 0.25 mg/mL of USP Valerenic Acid RS in methanol

Standard solution B: 40 mg/mL of USP Powdered Valerian Extract RS in methanol. Sonicate for 10 min, centrifuge, and use the supernatant.

Sample solution: Finely pulverize NLT 10 Tablets. Transfer an amount of the powder equivalent to 100 mg of Powdered Valerian Extract to a suitable flask. Add 5 mL of water and 3 mL of a 10% aqueous solution of potassium hydroxide, extract with two 5-mL portions of methylene chloride, and discard the organic phase. Heat the aqueous phase in a water bath at 40° for 10 min, cool, acidify with 7% hydrochloric acid, and extract with two 5-mL portions of methylene chloride. Dry the organic phase over anhydrous sodium sulfate, filter, evaporate the filtrate to dryness, and dissolve the residue in 2.0 mL of methylene chloride.

Chromatographic system

Adsorbent: Chromatographic silica gel mixture with an average particle size of 2–10 µm (HPTLC plates)

Application volume: 5 µL, as 8-mm bands

Developing solvent system: A mixture of cyclohexane, ethyl acetate, and glacial acetic acid (60:38:2)

Derivatization reagent A: A mixture of glacial acetic acid and hydrochloric acid (1:4)

Derivatization reagent B: 0.5 mL of *p*-anisaldehyde, 10 mL of glacial acetic acid, and 5 mL of sulfuric acid. Add to 85 mL of ice-cold methanol, and mix.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands to a suitable high-performance thin-layer chromatographic plate. Use a saturated chamber, and condition the plate to a relative humidity of about 33% using a suitable device. Develop the chromatograms over a distance of 6 cm. Remove the plate from the chamber, dry, derivatize with *Derivatization reagent A*, heat at 120° for 5 min, and examine under white light. Derivatize with *Derivatization reagent B*, heat at 100° for 3 min, and examine under white light.

Acceptance criteria: After treatment with *Derivatization reagent A* and heating, the *Sample solution* does not exhibit an intense blue band at about the middle of the chromatogram nor any other significant bands [distinction from Mexican valerian (*Valeriana edulis*)], though minor bands may be observed.

After treatment with *Derivatization reagent B* and heating, the *Sample solution* exhibits three violet bands in positions and colors similar to the bands of *Standard solution B*. These bands include a minor band in the lower third of the chromatogram due to hydroxyvalerenic acid, a major band at about the middle the chromatogram due to acetoxyvalerenic acid [distinction from Scouler's valerian (*Valeriana wallichii*)], and a major band at an R_F corresponding to the valerenic acid band of *Standard solution A* and *Standard solution B*. Other minor bands may be observed in the *Sample solution* and in *Standard solution B*.

- **B. HPLC**

Analysis: Proceed as directed in the test for *Content of Valerenic Acids*.

Acceptance criteria: The *Sample solution* exhibits a peak at a retention time corresponding to the valerenic acid peak of *Standard solution A*. The *Sample solution* shows additional peaks corresponding to hydroxyvalerenic acid and acetoxyvalerenic acid.

STRENGTH

- **CONTENT OF VALERIAN EXTRACT**

Solution A: Mix 6 mL of 85% phosphoric acid with 900 mL of water, dilute with water to 1000 mL, mix, filter, and degas.

Solution B: Mix 6 mL of 85% phosphoric acid with 900 mL of methanol, dilute with methanol to 1000 mL, mix, filter, and degas.

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	40	60
15	5	95
25	5	95
30	40	60

Solvent: A mixture of methanol and a solution of 0.1% phosphoric acid in water (3:1)

Standard solution A: 0.02 mg/mL of USP Valerenic Acid RS in methanol. Sonicate if necessary.

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Standard solution B: Sonicate a portion of USP Powdered Valerian Extract RS in *Solvent* to obtain a solution having a concentration of about 20 mg/mL. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.
Sample solution: Weigh NLT 20 Tablets and pulverize. Transfer a portion of the powder, nominally equivalent to about 3.0 mg of valerenic acids, to a suitable flask. Add 25.0 mL of *Solvent*, shake to disperse the powder, sonicate for 10 min, and centrifuge. Use the clear supernatant.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 225 nm
Column: 4.6-mm × 25-cm; end-capped, 5-µm 100 Å packing L1
Column temperature: 40°
Flow rate: 1.0 mL/min
Injection volume: 25 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Valerian Extract RS being used.
Tailing factor: NMT 2.0 for the valerenic acid peak, *Standard solution A*
Relative standard deviation: NMT 2.0% for the valerenic acid peak in repeated injections, *Standard solution A*
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Identify the valerenic acids in the *Sample solution* chromatogram by comparison with the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Valerian Extract RS being used.
Calculate the percentage of the labeled amount of Powdered Valerian Extract as valerenic acids (sum of hydroxyvalerenic acid, acetoxyvalerenic acid, and valerenic acid) in the portion of Tablets taken:

$$\text{Result} = \{[\Sigma(r_U \times F)]/r_S\} \times (C_S \times V/W) \times (100/L_E) \times (A_W \times 100/L)$$

r_U = peak areas of the relevant analytes from the *Sample solution*
F = conversion factor for each analyte (1.10 for hydroxyvalerenic acid, 1.25 for acetoxyvalerenic acid, and 1.00 for valerenic acid)
r_S = peak area of valerenic acid from *Standard solution A*
C_S = concentration of valerenic acid in *Standard solution A* (mg/mL)
V = volume of the *Sample solution* (mL)
W = weight of the sample of powdered Tablets used to prepare the *Sample solution* (mg)
A_W = average weight of the Tablets (mg/Tablet)
L_E = labeled amount of valerenic acids in 100 mg of the Powdered Valerian Extract used to prepare the Tablets (mg)
L = labeled amount of Powdered Valerian Extract per Tablet (mg/Tablet)

Acceptance criteria: 90.0%–120.0%

PERFORMANCE TESTS
- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** ⟨2040⟩**:** Meet the requirements for *Disintegration*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩**:** Meet the requirements

CONTAMINANTS
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **MICROBIAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meet the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the article from which the Tablets were prepared. The label also indicates the quantity, in mg, of Powdered Valerian Extract per Tablet and the content, as a percentage, of valerenic acids in the Extract.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Powdered Valerian Extract RS
USP Valerenic Acid RS

Valerian Tincture

DEFINITION
Valerian Tincture is prepared as follows.

Valerian	200 g
A mixture of Alcohol and Water (6:4) to (8:2), a sufficient quantity to make	1000 mL

Prepare the Tincture as directed for *Botanical Extracts* ⟨565⟩, *Tinctures, Maceration Process*. Valerian Tincture contains NLT 0.015% of valerenic acids, calculated as the sum of hydroxyvalerenic acid, acetoxyvalerenic acid, and valerenic acid.

IDENTIFICATION
- **A. THIN-LAYER CHROMATOGRAPHY**
Standard solution A: 0.25 mg/mL of USP Valerenic Acid RS in methanol
Standard solution B: 40 mg/mL of USP Powdered Valerian Extract RS in methanol. Sonicate for 10 min, centrifuge, and use the supernatant.
Sample solution: Centrifuge the Tincture, and use the supernatant.
Chromatographic system
Adsorbent: Chromatographic silica gel mixture with an average particle size of 2–10 µm (HPTLC plates)
Application volume: 5 µL of *Standard solution A* and *Standard solution B* and 10 µL of the *Sample solution*, as 8-mm bands
Developing solvent system: A mixture of cyclohexane, ethyl acetate, and glacial acetic acid (60:38:2)
Derivatization reagent A: A mixture of glacial acetic acid and hydrochloric acid (1:4)
Derivatization reagent B: 0.5 mL of *p*-anisaldehyde, 10 mL of glacial acetic acid, and 5 mL of sulfuric acid. Add to 85 mL of ice-cold methanol, and mix.

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Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Apply the *Samples* as bands to a suitable high-performance thin-layer chromatographic plate. Use a saturated chamber, and condition the plate to a relative humidity of about 33% using a suitable device. Develop the chromatograms over a distance of 6 cm. Remove the plate from the chamber, dry, derivatize with *Derivatization reagent A*, heat at 120° for 5 min, and examine under white light. Derivatize with *Derivatization reagent B*, heat at 100° for 3 min, and examine under white light.
Acceptance criteria: After treatment with *Derivatization reagent A* and heating, the *Sample solution* does not exhibit an intense blue band at about the middle of the chromatogram nor any other significant bands [distinction from Mexican valerian (*Valeriana edulis*)], though minor bands may be observed.
After treatment with *Derivatization reagent B* and heating, the *Sample solution* exhibits three violet bands in positions and colors similar to the bands of *Standard solution B*. These bands include a minor band in the lower third of the chromatogram due to hydroxyvalerenic acid, a major band at about the middle of the chromatogram due to acetoxyvalerenic acid [distinction from Scouler's valerian (*Valeriana wallichii*)], and a major band at an R_F corresponding to the valerenic acid band of *Standard solution A* and *Standard solution B*. Other minor bands may be observed in the *Sample solution* and in *Standard solution B*.

• **B. HPLC**
Analysis: Proceed as directed in the test for *Content of Valerenic Acids*.
Acceptance criteria: The *Sample solution* exhibits a peak at a retention time corresponding to the valerenic acid peak of *Standard solution A*. The *Sample solution* shows additional peaks corresponding to hydroxyvalerenic acid and acetoxyvalerenic acid.

STRENGTH

• **CONTENT OF VALERENIC ACIDS**
Solution A: Mix 6 mL of 85% phosphoric acid with 900 mL of water, dilute with water to 1000 mL, mix, filter, and degas.
Solution B: Mix 6 mL of 85% phosphoric acid with 900 mL of methanol, dilute with methanol to 1000 mL, mix, filter, and degas.
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	40	60
15	5	95
25	5	95
30	40	60

Solvent: A mixture of methanol and a solution of 0.1% phosphoric acid in water (3:1)
Standard solution A: 0.02 mg/mL of USP Valerenic Acid RS in methanol. Sonicate if necessary.
Standard solution B: Sonicate a portion of USP Powdered Valerian Extract RS in *Solvent* to obtain a solution having a concentration of about 20 mg/mL. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.
Sample solution: Use Tincture. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 225 nm
Column: 4.6-mm × 25-cm; end-capped, 5-µm 100 Å packing L1
Column temperature: 40°
Flow rate: 1.0 mL/min
Injection volume: 25 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Valerian Extract RS being used.
Tailing factor: NMT 2.0 for the valerenic acid peak, *Standard solution A*
Relative standard deviation: NMT 2.0% for the valerenic acid peak in repeated injections, *Standard solution A*
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Identify the valerenic acids in the *Sample solution* chromatogram by comparison with the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Valerian Extract RS being used.
Calculate the percentage of valerenic acids (sum of hydroxyvalerenic acid, acetoxyvalerenic acid, and valerenic acid) in the portion of Tincture taken:

$$\text{Result} = \{[\Sigma(r_U \times F)]/r_S\} \times C_S \times 0.1$$

r_U = peak areas of the relevant analytes from the *Sample solution*
F = conversion factor for each analyte (1.10 for hydroxyvalerenic acid, 1.25 for acetoxyvalerenic acid, and 1.00 for valerenic acid)
r_S = peak area of valerenic acid from *Standard solution A*
C_S = concentration of valerenic acid in *Standard solution A* (mg/mL)

Acceptance criteria: NLT 0.015% of valerenic acids, calculated as the sum of hydroxyvalerenic acid, acetoxyvalerenic acid, and valerenic acid

OTHER COMPONENTS

• **ALCOHOL DETERMINATION,** *Method I* ⟨611⟩**:** NLT 90.0% and NMT 110.0% of the labeled amount of alcohol (C_2H_5OH)

CONTAMINANTS

Change to read:

• **▲BOTANICAL EXTRACTS** ⟨565⟩, *Preparations, General Pharmacopeial Requirements, Pesticide Residues*▲ (CN 1-May-2020)
: Meets the requirements

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at room temperature.
• **LABELING:** The label states the official name of the article, the Latin binomial, and the part of the plant from which the article was prepared. Label it to indicate the content of

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valerenic acids, the solvent mixture used for extraction, and the ratio of the starting crude plant material to Tincture.

• **USP Reference Standards** ⟨11⟩
USP Valerenic Acid RS
USP Powdered Valerian Extract RS

Valerian Root Dry Extract Capsules

DEFINITION

Valerian Root Dry Extract Capsules contain dry extract derived from the subterranean parts of *Valeriana officinalis* L. (Fam. Caprifoliaceae, formerly Valerianaceae) including rhizome, root, and stolon. They contain NLT 90% and NMT 120% of the labeled amount of valerenic acids calculated as the sum of hydroxyvalerenic acid ($C_{15}H_{22}O_3$), acetoxyvalerenic acid ($C_{17}H_{24}O_4$), and valerenic acid ($C_{15}H_{22}O_2$).

IDENTIFICATION

• **A. HPTLC for Articles of Botanical Origin** ⟨203⟩

Standard solution A: 0.25 mg/mL of USP Valerenic Acid RS in methanol

Standard solution B: 40 mg/mL of USP Powdered Valerian Extract RS in methanol. Sonicate for 10 min, centrifuge, and use the supernatant.

Sample solution: Transfer a portion of the Capsule contents, equivalent to 400 mg of valerian root dry extract, to a conical flask, add 10 mL of methanol, mix and sonicate for 20 min, centrifuge, and use the supernatant.

Chromatographic system

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)

Application volume: 5 µL, as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of 33%.

Temperature: Ambient, not to exceed 30°

Developing solvent system: Cyclohexane, ethyl acetate, and glacial acetic acid (60:38:2)

Developing distance: 6 cm

Derivatization reagent A: Glacial acetic acid and hydrochloric acid (1:4)

Derivatization reagent B: 0.5 mL of *p*-anisaldehyde, 10 mL of glacial acetic acid, and 5 mL of sulfuric acid. Add to 85 mL of ice-cold methanol, and mix.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands, and dry in air. Develop in a saturated chamber, remove the plate from the chamber, and dry in air. Treat the plate with *Derivatization reagent A*, heat at 120° for 5 min, and examine under white light. Then treat with *Derivatization reagent B*, heat at 100° for 3 min, and examine under white light.

System suitability: After treatment with *Derivatization reagent A* and heating, *Standard solution A* and *Standard solution B* do not exhibit an intense blue band at about the middle of the chromatogram nor any other significant bands. After treatment with *Derivatization reagent B* and heating, *Standard solution A* exhibits a violet band due to valerenic acid, while *Standard solution B* exhibits a band corresponding in R_F and color to that of valerenic acid in *Standard solution A* as well as a violet band below valerenic acid at about middle of the chromatogram due to acetoxyvalerenic acid and a minor violet band in the lower third of the chromatogram due to hydroxyvalerenic acid.

Acceptance criteria: After treatment with *Derivatization reagent A* and heating, the *Sample solution* does not exhibit an intense blue band at about the middle of the chromatogram nor any other significant bands [distinction from Mexican valerian (*Valeriana edulis*)], though minor bands may be observed. After treatment with *Derivatization reagent B* and heating, the *Sample solution* exhibits three violet bands in positions and colors similar to those in *Standard solution B*. These bands include a band corresponding in R_F to valerenic acid in *Standard solution A* and *Standard solution B*, a band below valerenic acid at about the middle of the chromatogram due to acetoxyvalerenic acid [distinction from Scouler's valerian (*Valeriana wallichii*)], and a minor violet band in the lower third of the chromatogram due to hydroxyvalerenic acid. Other faint bands may be observed.

• **B. HPLC**

Analysis: Proceed as directed in the test for *Content of Valerenic Acids*.

Acceptance criteria: The *Sample solution* exhibits a peak at a retention time corresponding to valerenic acid in *Standard solution A*. The *Sample solution* shows additional peaks corresponding to hydroxyvalerenic acid and acetoxyvalerenic acid.

STRENGTH

• **Content of Valerenic Acids**

Extraction solvent: Methanol and a solution of 0.1% phosphoric acid in water (3:1)

Solution A: Mix 6 mL of 85% phosphoric acid with 900 mL of water, dilute with water to 1000 mL, mix, filter, and degas.

Solution B: Mix 6 mL of 85% phosphoric acid with 900 mL of methanol, dilute with methanol to 1000 mL, mix, filter, and degas.

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	40	60
15	5	95
25	5	95
30	40	60

Standard solution A: 0.05 mg/mL of USP Valerenic Acid RS in methanol. Sonicate if necessary.

Standard solution B: Sonicate a portion of USP Powdered Valerian Extract RS in *Extraction solvent* to obtain a solution with a concentration of about 20 mg/mL. Before injection, pass through a membrane filter of 0.45-µm or finer pore size.

Sample solution: Determine the total weight of 20 Capsules. Empty the Capsules and combine their contents in an appropriate container. Weigh the empty Capsule shells and calculate the average fill weight per Capsule. Transfer a portion of the Capsule contents, equivalent to about 3.0 mg of valerenic acids, to a 25-mL volumetric flask. Add 20 mL of *Extraction solvent* and sonicate for 30 min with occasional shaking. Cool to room temperature, dilute with *Extraction solvent* to volume, mix well, and centrifuge. Pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few milliliters of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 225 nm

Column: 4.6-mm × 25-cm; end-capped, 5-µm, 100-Å packing L1

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Column temperature: 40°
Flow rate: 1.0 mL/min
Injection volume: 25 µL
System suitability
Samples: *Standard solution A* and *Standard solution B*
Suitability requirements
Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Valerian Extract RS being used.
Tailing factor: NMT 2.0 for the valerenic acid peak, *Standard solution A*
Relative standard deviation: NMT 2.0%, *Standard solution A*
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Using the chromatograms of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Valerian Extract RS being used, identify and measure the areas of the peaks corresponding to hydroxyvalerenic acid, acetoxyvalerenic acid, and valerenic acid in the *Sample solution*.
Calculate the quantity, in mg, of hydroxyvalerenic acid, acetoxyvalerenic acid, and valerenic acid in each Capsule:

$$\text{Result} = (r_U/r_S) \times C_S \times (V \times W_{AV}/W) \times F$$

r_U = peak area of the relevant analyte from the *Sample solution*
r_S = peak area of valerenic acid from *Standard solution A*
C_S = concentration of USP Valerenic Acid RS in *Standard solution A* (mg/mL)
V = volume of the solvent taken to prepare the *Sample solution* (mL)
W_{AV} = average Capsule fill weight (mg)
W = weight of the sample taken to prepare the *Sample solution* (mg)
F = conversion factor for analytes (1.10 for hydroxyvalerenic acid, 1.25 for acetoxyvalerenic acid, and 1.00 for valerenic acid)

Calculate the percentage of the labeled amount of valerenic acids, as the sum of hydroxyvalerenic acid, acetoxyvalerenic acid, and valerenic acid in each Capsule:

$$\text{Result} = (\Sigma Q_i/L) \times 100$$

Q_i = sum of the quantities of valerenic acids previously determined (mg)
L = labeled amount of valerenic acids (mg)

Acceptance criteria: 90.0%–120.0%

PERFORMANCE TESTS
- **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩, *Disintegration*: Meet the requirements
- **WEIGHT VARIATION** ⟨2091⟩: Meet the requirements

CONTAMINANTS
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and **TEST FOR ABSENCE OF EDCHERICHIA:** Meet the requirements

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial, the official name, and the article from which the Capsules were prepared. The label states the amount of valerenic acids (as the sum of hydroxyvalerenic acid, acetoxyvalerenic acid, and valerenic acid) in mg/Capsule.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Valerenic Acid RS
USP Powdered Valerian Extract RS

Valerian Root Powder Capsules

DEFINITION
Valerian Root Powder Capsules contain finely powdered dried subterranean parts of *Valeriana officinalis* L. (Fam. Caprifoliaceae, formerly Valerianaceae) including rhizome, root, and stolon. They contain NLT 0.04% of valerenic acid ($C_{15}H_{22}O_2$) and NLT 0.1% of total valerenic acids, calculated as the sum of hydroxyvalerenic acid ($C_{15}H_{22}O_3$), acetoxyvalerenic acid ($C_{17}H_{24}O_4$) and valerenic acid ($C_{15}H_{22}O_2$), from the labeled amount of valerian root powder.

IDENTIFICATION
- **A. HPTLC FOR ARTICLES OF BOTANICAL ORIGIN** ⟨203⟩
Standard solution A: 0.25 mg/mL of USP Valerenic Acid RS in methanol
Standard solution B: 40 mg/mL of USP Powdered Valerian Extract RS in methanol. Sonicate for 10 min, centrifuge, and use the supernatant.
Sample solution: Transfer a portion of the Capsule contents, equivalent to 0.5 g of valerian root powder, to a centrifuge tube, and add 5 mL of methanol. Sonicate for 10 min, centrifuge, and use the supernatant.
Chromatographic system
Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)
Application volume: 5 µL, as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
Temperature: Ambient, not to exceed 30°
Developing solvent system: Cyclohexane, ethyl acetate, and glacial acetic acid (60:38:2)
Developing distance: 6 cm
Derivatization reagent A: Glacial acetic acid and hydrochloric acid (1:4)
Derivatization reagent B: 0.5 mL of *p*-anisaldehyde, 10 mL of glacial acetic acid, and 5 mL of sulfuric acid. Add to 85 mL of ice-cold methanol, and mix.
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Apply the *Samples* as bands and dry in air. Develop in a saturated chamber, remove the plate from the chamber, and dry in air. Treat the plate with *Derivatization reagent A*, heat at 120° for 5 min, and examine under white light. Then treat the plate with *Derivatization reagent B*, heat at 100° for 3 min, and examine under white light.
System suitability: After treatment with *Derivatization reagent A* and heating, *Standard solution A* and *Standard solution B* do not exhibit an intense blue band at about the middle of the chromatogram nor any other significant bands. After treatment with *Derivatization reagent B* and heating, *Standard solution A* exhibits a violet band due to

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valerenic acid, while *Standard solution B* exhibits a band corresponding in R_F and color to that of valerenic acid in *Standard solution A* as well as a violet band below valerenic acid at about the middle of the chromatogram due to acetoxyvalerenic acid and a minor violet band in the lower third of the chromatogram due to hydroxyvalerenic acid.

Acceptance criteria: After treatment with *Derivatization reagent A* and heating, the *Sample solution* does not exhibit an intense blue band at about the middle of the chromatogram nor any other significant bands [distinction from Mexican valerian (*Valeriana edulis*)], though minor bands may be observed. After treatment with *Derivatization reagent B* and heating, the *Sample solution* exhibits three violet bands in positions and colors similar to the bands in *Standard solution B.* These bands include a band corresponding in R_F to valerenic acid in *Standard solution A* and *Standard solution B*, a band below valerenic acid at about middle of the chromatogram due to acetoxyvalerenic acid [distinction from Scouler's valerian (*Valeriana wallichii*)], and a minor violet band in the lower third of the chromatogram due to hydroxyvalerenic acid. Other minor bands may be observed.

• **B. HPLC**

Analysis: Proceed as directed in the test for *Content of Valerenic Acids.*

Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks at the retention times of those due to valerenic acid, hydroxyvalerenic acid, and acetoxyvalerenic acid in the chromatograms of *Standard solution A* and *Standard solution B.*

STRENGTH

• **CONTENT OF VALERENIC ACIDS**

Solvent: Methanol and a solution of 0.1% phosphoric acid in water (3:1)

Solution A: Mix 6 mL of 85% phosphoric acid with 900 mL of water, dilute with water to 1000 mL, mix, filter, and degas.

Solution B: Mix 6 mL of 85% phosphoric acid with 900 mL of methanol, dilute with methanol to 1000 mL, mix, filter, and degas.

Mobile phase: See *Table 1.*

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	40	60
15	5	95
25	5	95
30	40	60

Standard solution A: 0.02 mg/mL of USP Valerenic Acid RS in methanol. Sonicate if necessary.

Standard solution B: Sonicate a portion of USP Powdered Valerian Extract RS in *Solvent* to obtain a solution with a concentration of about 20 mg/mL. Before injection, pass through a membrane filter of 0.45-µm or finer pore size.

Sample solution: Determine the total weight of 20 Capsules. Empty the Capsules, combine and mix their contents to obtain a homogenous composite. Weigh the empty Capsule shells and calculate the average fill weight per Capsule. Transfer a portion of the Capsule contents, equivalent to 1.0 g of valerian root powder, to a 50-mL volumetric flask. Add 10.0 mL of water and shake for 2 min while heating in a water bath maintained at about 50°. Sonicate for 15 min, add 35 mL of methanol, and sonicate for 15 min. Cool, dilute with methanol to volume, and mix. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few milliliters of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability.*)

Mode: LC

Detector: UV 225 nm

Column: 4.6-mm × 25-cm; end-capped, 5-µm, 100-Å packing L1

Column temperature: 40°

Flow rate: 1.0 mL/min

Injection volume: 25 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Valerian Extract RS being used.

Tailing factor: NMT 2.0 for the valerenic acid peak, *Standard solution A*

Relative standard deviation: NMT 2.0%, *Standard solution A*

Analysis

Samples: *Standard solution A, Standard solution B,* and *Sample solution*

Using the chromatograms of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Valerian Extract RS being used, identify and measure the areas of the peaks corresponding to hydroxyvalerenic acid, acetoxyvalerenic acid, and valerenic acid in the *Sample solution.*

Calculate the quantity, in mg, of hydroxyvalerenic acid, acetoxyvalerenic acid, and valerenic acid in each Capsule:

$$\text{Result} = (r_U/r_S) \times C_S \times (V \times W_{AV}/W) \times F$$

r_U = peak area of the relevant analyte from the *Sample solution*

r_S = peak area of valerenic acid from *Standard solution A*

C_S = concentration of USP Valerenic Acid RS in *Standard solution A* (mg/mL)

V = volume of the solvent taken to prepare the *Sample solution* (mL)

W_{AV} = average Capsule fill weight (mg)

W = weight of the sample taken to prepare the *Sample solution* (mg)

F = conversion factor for analytes (1.10 for hydroxyvalerenic acid, 1.25 for acetoxyvalerenic acid, and 1.00 for valerenic acid)

Calculate the percentage of valerenic acid within the labeled amount of valerian rhizome, root, and stolon powder in each Capsule:

$$\text{Result} = (Q/L) \times 100$$

Q = amount of valerenic acid previously determined (mg)

L = labeled amount of valerian rhizome, root, and stolon powder (mg)

Calculate the percentage of valerenic acids, calculated as the sum of hydroxyvalerenic acid, acetoxyvalerenic acid and valerenic acid, within the labeled amount of valerian rhizome, root, and stolon powder in each Capsule:

$$\text{Result} = (\Sigma Q_i/L) \times 100$$

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Q_i = sum of the quantities of hydroxyvalerenic acid, acetoxyvalerenic acid, and valerenic acid previously determined (mg)
L = labeled amount of valerian rhizome, root, and stolon powder (mg)

Acceptance criteria: NLT 0.04% of valerenic acid and NLT 0.1% of total valerenic acids

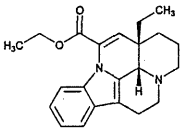
PERFORMANCE TESTS
- **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩, *Disintegration*: Meet the requirements
- **WEIGHT VARIATION** ⟨2091⟩: Meet the requirements

CONTAMINANTS
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, Test Procedures, Test for Absence of Salmonella Species and Test for Absence of Edcherichia: Meet the requirements

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and the official name, and the article from which the Capsules were prepared. The label states the amount of valerian root powder in mg/Capsule.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Valerenic Acid RS
  USP Powdered Valerian Extract RS

Valine—*see Valine General Monographs*

Vinpocetine



$C_{22}H_{26}N_2O_2$ 350.45
Eburnamenine-14-carboxylic acid, ethyl ester, (3α,16α)-; Ethyl apovincamin-22-oate [42971-09-5].

DEFINITION
Vinpocetine contains NLT 98.0% and NMT 102.0% of vinpocetine ($C_{22}H_{26}N_2O_2$), calculated on the dried basis.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
- **B.** The retention time of the main peak of the *Sample solution* corresponds to that of the main peak of *Standard solution 4*, as obtained in the *Assay*.

ASSAY
- **PROCEDURE**

Solution A: 15.4 g/L of ammonium acetate in water
Mobile phase: Acetonitrile and *Solution A* (55:45)
Standard solution 1: 0.02 mg/mL of USP Vinpocetine RS in *Mobile phase*
Standard solution 2: 0.12 mg/mL of USP Vinpocetine Related Compound A RS and 0.10 mg/mL each of USP Vinpocetine Related Compound B RS, USP Vinpocetine Related Compound C RS, and USP Vinpocetine Related Compound D RS in *Mobile phase*
Standard solution 3: Dilute 1.0 mL of *Standard solution 1* and 1.0 mL of *Standard solution 2* with *Mobile phase* to 20.0 mL.
Standard solution 4: 0.2 mg/mL of USP Vinpocetine RS in *Mobile phase*
Sample solution: 0.2 mg/mL of Vinpocetine in *Mobile phase*
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 280 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Flow rate: 1.0 mL/min
Injection volume: 15 µL
System suitability
Sample: *Standard solution 3*
[NOTE—The relative retention times for vinpocetine and its related compounds are shown in *Table 1*.]
Suitability requirements
Resolution: NLT 2.0 between vinpocetine related compound B and vinpocetine related compound D
Relative standard deviation: NMT 2.0%, *Standard solution 4*
Analysis
Samples: *Standard solution 4* and *Sample solution*
Calculate the percentage of vinpocetine ($C_{22}H_{26}N_2O_2$) in the portion of Vinpocetine taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from *Standard solution 4*
C_S = concentration of USP Vinpocetine RS in *Standard solution 4* (mg/mL)
C_U = concentration of Vinpocetine in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–102.0% on the dried basis

IMPURITIES
- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%
- **ORGANIC IMPURITIES**

Solution A, Mobile phase, Standard solution 1, Standard solution 2, Standard solution 3, Chromatographic system, and **System suitability:** Proceed as directed in the *Assay*.
Sample solution: 1 mg/mL of Vinpocetine in *Mobile phase*
Analysis
Samples: *Standard solution 3* and *Sample solution*
Record the chromatograms for up to a minimum of three times the retention time of vinpocetine. Disregard any peak with an area less than 0.5 times the area of the peak due to vinpocetine in *Standard solution 3*.
Calculate the percentage of vinpocetine related compounds A, B, C, and D in the portion of Vinpocetine taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of each of the known impurities from the *Sample solution*

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r_S = peak response of the corresponding Standard for each of the known impurities from *Standard solution 3*
C_S = concentration of the corresponding USP Reference Standard for each of the known impurities in *Standard solution 3* (mg/mL)
C_U = concentration of Vinpocetine in the *Sample solution* (mg/mL)

Calculate the percentage of any unspecified individual impurity, as vinpocetine, in the portion of Vinpocetine taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of each of the unspecified impurities from the *Sample solution*
r_S = peak response of vinpocetine from *Standard solution 3*
C_S = concentration of USP Vinpocetine RS in *Standard solution 3* (mg/mL)
C_U = concentration of Vinpocetine in the *Sample solution* (mg/mL)

Acceptance criteria: See *Table 1*.

Table 1

Name	Relative Retention Time	Acceptance Criteria, NMT (%)
Vinpocetine	1.00	—
Vinpocetine related compound A[a]	0.40	0.6
Vinpocetine related compound B[b]	0.75	0.5
Vinpocetine related compound C[c]	0.83	0.3
Vinpocetine related compound D[d]	0.68	0.5
Unspecified individual impurity	—	0.1
Total impurities	—	1.0

[a] Ethyl (12*S*,13a*S*,13b*S*)-13a-ethyl-12-hydroxy-2,3,5,6,12,13,13a,13b-octahydro-1*H*-indolo[3,2,1-*de*]pyrido[3,2,1-*ij*][1,5]naphthyridine-12-carboxylate (ethyl vincaminate).
[b] Methyl (13a*S*,13b*S*)-13a-ethyl-2,3,5,6,13a,13b-hexahydro-1*H*-indolo[3,2,1-*de*]pyrido[3,2,1-*ij*][1,5]naphthyridine-12-carboxylate (apovincamine).
[c] Ethyl (13a*S*,13b*S*)-13a-ethyl-10-methoxy-2,3,5,6,13a,13b-hexahydro-1*H*-indolo[3,2,1-*de*]pyrido[3,2,1-*ij*][1,5]naphthyridine-12-carboxylate (methoxyvinpocetine).
[d] Ethyl (12*R*,13a*S*,13b*S*)-13a-ethyl-2,3,5,6,12,13,13a,13b-octahydro-1*H*-indolo[3,2,1-*de*]pyrido[3,2,1-*ij*][1,5]naphthyridine-12-carboxylate (dihydrovinpocetine).

SPECIFIC TESTS

- **Loss on Drying** ⟨731⟩
  Analysis: Dry a sample in a vacuum at 100° for 3 h.
  Acceptance criteria: NMT 0.5%
- **Optical Rotation,** *Specific Rotation* ⟨781S⟩
  Sample solution: 10 mg/mL in dimethylformamide
  Acceptance criteria: From +127.0° to +134.0°, determined at 20°

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in well-closed containers, and store at controlled room temperature.
- **USP Reference Standards** ⟨11⟩
  USP Vinpocetine RS
  USP Vinpocetine Related Compound A RS
  USP Vinpocetine Related Compound B RS
  USP Vinpocetine Related Compound C RS
  USP Vinpocetine Related Compound D RS

Vinpocetine Capsules

DEFINITION

Vinpocetine Capsules contain NLT 90.0% and NMT 110.0% of the labeled amount of vinpocetine ($C_{22}H_{26}N_2O_2$).

IDENTIFICATION

Change to read:

- **A. ▲Spectroscopic Identification Tests** ⟨197⟩, *Ultraviolet-Visible Spectroscopy:* **197U**▲ (CN 1-May-2020)
  Medium: Alcohol
  Standard solution: 15 µg/mL of USP Vinpocetine RS in *Medium*
  Sample solution: Equivalent to 15 µg/mL of vinpocetine from Capsule contents in *Medium*
  Acceptance criteria: The spectra of the *Sample solution* and *Standard solution* exhibit maxima and minima at the same wavelengths.
- **B. HPLC:** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in *Strength*.

STRENGTH

- **Procedure**
  Solution A: 15.4 g/L of ammonium acetate in water
  Mobile phase: Acetonitrile and *Solution A* (55:45)
  Standard solution: 0.2 mg/mL of USP Vinpocetine RS in *Mobile phase*
  Sample solution: Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without the loss of shell material, and transfer the contents to a suitable beaker. Remove any contents adhering to the empty shells by washing, if necessary, with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the average net weight per Capsule. Dissolve a portion of the Capsule contents in acetonitrile with sonication for 5 min. Allow the solution to cool to room temperature, dilute with acetonitrile to get a solution with a nominal concentration of 0.2 mg/mL of vinpocetine, and filter.
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: LC
  Detector: UV 280 nm
  Column: 4.6-mm × 25-cm; 5-µm packing L1
  Flow rate: 1.0 mL/min
  Injection volume: 15 µL
  System suitability
  Sample: *Standard solution*
  Suitability requirements
  Relative standard deviation: NMT 2.0%
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Calculate the percentage of the labeled amount of vinpocetine ($C_{22}H_{26}N_2O_2$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP Vinpocetine RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of vinpocetine in the *Sample solution* (mg/mL)

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Acceptance criteria: 90.0%–110.0%

PERFORMANCE TESTS
- **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩**:** Meet the requirements for *Disintegration*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩ **:** Meet the requirements

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Vinpocetine RS

Vinpocetine Tablets

DEFINITION
Vinpocetine Tablets contain NLT 90.0% and NMT 110.0% of the labeled amount of vinpocetine ($C_{22}H_{26}N_2O_2$).

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩**,** *Ultraviolet-Visible Spectroscopy*: **197U▲** (CN 1-May-2020)
  Medium: Alcohol
  Standard solution: 15 µg/mL of USP Vinpocetine RS in *Medium*
  Sample solution: Equivalent to 15 µg/mL of vinpocetine from finely powdered Tablets in *Medium*
  Acceptance criteria: The spectra of the *Sample solution* and *Standard solution* exhibit maxima and minima at the same wavelengths.
- **B. HPLC:** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in *Strength*.

STRENGTH
- **PROCEDURE**
  Solution A: 15.4 g/L of ammonium acetate in water
  Mobile phase: Acetonitrile and *Solution A* (55:45)
  Standard solution: 0.2 mg/mL of USP Vinpocetine RS in *Mobile phase*
  Sample solution: Dissolve a portion from NLT 20 finely powdered Tablets in acetonitrile by shaking thoroughly and sonicating for 5 min. Allow the solution to cool to room temperature, dilute with acetonitrile to get a solution nominally equivalent to 0.2 mg/mL of vinpocetine, and filter.
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: LC
  Detector: UV 280 nm
  Column: 4.6-mm × 25-cm; 5-µm packing L1
  Flow rate: 1.0 mL/min
  Injection volume: 15 µL
  System suitability
  Sample: *Standard solution*
  Suitability requirements
  Relative standard deviation: NMT 2.0%
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Calculate the percentage of the labeled amount of vinpocetine ($C_{22}H_{26}N_2O_2$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP Vinpocetine RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of vinpocetine in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–110.0%

PERFORMANCE TESTS
- **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩**:** Meet the requirements for *Dissolution*
  Medium: 0.1 N hydrochloric acid; 900 mL
  Apparatus 1: 100 rpm
  Time: 30 min
  Sample solution: Withdraw a portion of the solution under test, pass through a suitable filter, and use the pooled sample as the test specimen.
  Standard solution: Dissolve a suitable amount of USP Vinpocetine RS in *Medium* to obtain a concentration similar to that of the *Sample solution*.
  Instrumental conditions
  (See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
  Mode: UV absorption spectroscopy
  Analytical wavelength: UV 268 nm
  Blank: *Medium*
  Analysis
  Samples: *Sample solution* and *Standard solution*
  Calculate the percentage of the labeled amount of vinpocetine ($C_{22}H_{26}N_2O_2$) dissolved:

$$\text{Result} = (A_U/A_S) \times [C_S \times (V/L)] \times 100$$

A_U = absorbance of vinpocetine from the *Sample solution*
A_S = absorbance of vinpocetine from the *Standard solution*
C_S = concentration of USP Vinpocetine RS in the *Standard solution* (mg/mL)
V = volume of *Medium*, 900 mL
L = label claim of vinpocetine (mg/Tablet)

Tolerances: NLT 75% of the labeled amount of vinpocetine ($C_{22}H_{26}N_2O_2$) is dissolved.
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩ **:** Meet the requirements

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Vinpocetine RS

Vitamin A—*see Vitamin A General Monographs*

Vitamin A Capsules—*see Vitamin A Capsules General Monographs*

Vitamin A Oral Liquid Preparation—*see Vitamin A Oral Liquid Preparation General Monographs*

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Vitamin A Tablets—*see Vitamin A Tablets General Monographs*

Vitamin B_6 Tablets—*see Pyridoxine Hydrochloride Tablets General Monographs*

Vitamin C Tablets—*see Ascorbic Acid Tablets General Monographs*

Vitamin E—*see Vitamin E General Monographs*

Vitamin E Preparation—*see Vitamin E Preparation General Monographs*

Vitamin E Capsules—*see Vitamin E Capsules General Monographs*

Vitamin E Polyethylene Glycol Succinate
—*see Vitamin E Polyethylene Glycol Succinate NF*

Add the following:

▲Vitamins with Minerals Oral Powder

DEFINITION

Vitamins with Minerals Oral Powder contains one or more of the following oil-soluble vitamins: Vitamin A as retinyl acetate or retinyl palmitate, Vitamin D as cholecalciferol, Vitamin E as *RRR*-alpha-tocopheryl acetate or *all-rac*-alpha-tocopheryl acetate; one or more of the following water-soluble vitamins: Vitamin C as ascorbic acid, sodium ascorbate or calcium ascorbate, Vitamin B1 as thiamine mononitrate, Vitamin B2 as riboflavin, Vitamin B3 as niacin or niacinamide, Vitamin B6 as pyridoxine hydrochloride, Vitamin B12 as cyanocobalamin, and Folic Acid; and one or more minerals: copper, iodine, iron, selenium, and zinc, derived from substances generally recognized as safe and furnishing the elements in ionizable form. [NOTE—NMT 25% of elemental iron from the labeled amount may derive from edetate iron (III) sodium when used as a source of iron.] Vitamins with Minerals Oral Powder contains NLT 90.0% and NMT 165.0% of the labeled amount of Vitamin A as retinol ($C_{20}H_{30}O$); NLT 90.0% and NMT 150.0% of the labeled amount of Vitamin D as cholecalciferol ($C_{27}H_{44}O$), and Vitamin E as 2*R*-alpha-tocopherol ($C_{29}H_{50}O_2$); NLT 90.0% and NMT 150.0% of the labeled amount of Vitamin C as ascorbic acid ($C_6H_8O_6$), Vitamin B1 as thiamine ion ($C_{12}H_{17}N_4OS^+$), Vitamin B2 as riboflavin ($C_{17}H_{20}N_4O_6$), Vitamin B3 as niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), Vitamin B6 as pyridoxine ($C_8H_{11}NO_3$), Vitamin B12 as cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$), and folic acid ($C_{19}H_{19}N_7O_6$); NLT 90.0% and NMT 125.0% of the labeled amounts of copper (Cu), iron (Fe), and zinc (Zn); and NLT 90.0% and NMT 160.0% of the labeled amounts of iodine (I) and selenium (Se).

IDENTIFICATION

- **A.** The retention times of the main peaks of the *Sample solutions* as obtained in *Strength*, correspond to those of the vitamins claimed in their respective *Standard solutions*.
- **B.** The *Sample solution* presents significant emission at the characteristic wavelengths or characteristic absorption as obtained in *Strength* for each mineral claimed.
- **C.** When iodine is claimed: the *Sample solution* turns blue upon the addition of starch TS in the test for *Content of Iodine*.

STRENGTH

[NOTE—In the following assays, where more than one assay method is given for an individual ingredient, the requirements may be met by following any one of the specified methods, the method used being stated in the labeling only if *Method 1* is not used.]

- **CONTENT OF VITAMIN A AND VITAMIN E**

[NOTE—Use low-actinic glassware.]

For Oral Powders containing retinyl acetate

Mobile phase: Methanol

Standard stock solution: A solution containing the equivalent of 0.15 mg/mL of retinol from USP Retinyl Acetate RS in isopropyl alcohol

Standard solution: A solution in methanol of USP Retinyl Acetate RS from *Standard stock solution* and USP Alpha Tocopheryl Acetate RS at concentrations similar to those obtained for the corresponding vitamins in the *Sample solution*

Sample solution: Empty 20 containers and combine and mix their contents to obtain a homogenous composite. Weigh the composite and calculate the average fill weight per container. Transfer a calculated amount of accurately weighed composite into a 200- or 250-mL volumetric flask to obtain a solution with a known nominal concentration of vitamins in the range of 2.5–56 µg/mL of retinol from retinyl acetate and 0.2–2.0 mg/mL of alpha tocopheryl acetate. Add 20 mL of water and immediately swirl the sample to slurry it and prevent clumping. Add about 500 units of leucine amino peptidase from a suitable enzyme preparation from *Aspergillus oryzae* containing NLT 500 units of LAP/g[1] to the bottom of the flask using a plastic pipette and gently swirl the contents to mix. Place the sample in a water bath set to 60°–65° for 5 min. Swirl the flask 3–4 times during the 5 min to ensure the sample is completely dispersed in the water and that it has not formed clumps or stuck to the walls of the flask. Remove the flask from the water bath, add 80 mL of isopropyl alcohol, and swirl for 1 min to dissolve the vitamins. Place the sample in a sonicator for 5 min, then cool the flask to room temperature. Add 50 mL of methanol and swirl to mix. Dilute with methanol to volume and mix well. Pass a portion of the solution through a 0.45-µm PTFE membrane filter, and discard the first 1 mL of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 325 nm for 11 min, then switch to 280 nm

Column: 4.6-mm × 25-cm; 4-µm packing L1

[1] A suitable grade is available from Sigma: Protease from *Aspergillus oryzae*, catalog #P6110.

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Temperatures
Sample: 8°. [NOTE—Use a thermostatic autosampler or a cooled bath.]
Column: 30°
Flow rate: 1.0 mL/min
Injection volume: 10 µL
System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for retinyl acetate and alpha tocopheryl acetate are 1.0 and 2.2, respectively.]
Suitability requirements
Relative standard deviation: NMT 2.0% for each peak

For Oral Powders containing retinyl palmitate
Mobile phase: Methanol
Standard stock solution: A solution containing the equivalent of 0.15 mg/mL of retinol from USP Retinyl Palmitate RS in isopropyl alcohol
Standard solution: A solution in methanol of USP Retinyl Palmitate RS from *Standard stock solution* and USP Alpha Tocopheryl Acetate RS at concentrations similar to those obtained for the corresponding vitamins in the *Sample solution*
Sample solution: Prepare as directed in *For Oral Powders containing retinyl acetate*, except replace retinyl acetate with retinyl palmitate throughout the text.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 280 nm for 10 min, then switch to 325 nm
Column: 4.6-mm × 15-cm; 4-µm packing L1
Temperatures
Sample: 8°. [NOTE—Use a thermostatic autosampler or a cooled bath.]
Column: 30°
Flow rate: 2.0 mL/min
Injection volume: 10 µL
System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for alpha tocopheryl acetate and retinyl palmitate are 1.0 and 2.6, respectively.]
Suitability requirements
Relative standard deviation: NMT 2.0% for each peak

Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$), in the portion of Oral Powder taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the all-*trans*-retinyl ester from the *Sample solution*
r_S = peak area of the all-*trans*-retinyl ester from the *Standard solution*
C_S = concentration of retinol in the *Standard solution* (µg/mL)
C_U = nominal concentration of vitamin A, as retinol, in the *Sample solution* (µg/mL)

Calculate the percentage of the labeled amount of vitamin E as 2*R*-alpha-tocopherol ($C_{29}H_{50}O_2$), in the portion of the Oral Powder taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of the alpha-tocopheryl acetate from the *Sample solution*
r_S = peak area of the alpha-tocopheryl acetate from the *Standard solution*
C_S = concentration of *all-rac*-alpha-tocopherol from USP Alpha Tocopheryl Acetate RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of vitamin E, as 2*R*-alpha-tocopherol, in the *Sample solution* (mg/mL)
F = conversion factor for the content of alpha tocopherol to 2*R*-alpha-tocopherol equivalent: 1/2 for products labeled to contain *all-rac*-alpha-tocopheryl acetate and 1 for products labeled to contain *RRR*-alpha-tocopheryl acetate

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A as retinol and 90.0%–150.0% of the labeled amount of vitamin E as 2*R*-alpha-tocopherol ($C_{29}H_{50}O_2$)

• **CONTENT OF CHOLECALCIFEROL (VITAMIN D)**
[NOTE—Use amber, low-actinic glassware.]

For Oral Powders containing less than 10 µg/g of vitamin D
Solution A: Dimethyl sulfoxide in water (10:2)
Extraction solvent: 0.02% butylated hydroxytoluene (BHT) in *n*-hexane
Mobile phase: Methanol and water (84:16)
[NOTE—Wash the column periodically with 100% methanol to eliminate retained substances and avoid carryover interferences.]
Standard stock solution: 0.04 mg/mL of cholecalciferol from USP Cholecalciferol RS in methanol
Standard solution: Dilute the *Standard stock solution* with methanol to obtain a solution with an accurate vitamin concentration as close as possible to that in the *Sample solution*.
System suitability solution: Heat a volume of the *Standard stock solution* at 60° for 1 h to partially isomerize vitamin D to pre-vitamin D.
Sample solution: Empty 20 containers and combine and mix their contents to obtain a homogenous composite. Weigh the composite and calculate the average fill weight per container. Transfer a calculated amount of accurately weighed composite (NMT 3 g) into a 50-mL centrifuge tube. Add 15 mL of *Solution A*, insert the stopper, and shake the sample on a horizontal shaker for 60 min. Add 15 mL of *Extraction solvent* into the tube and shake on a horizontal shaker for 30 min. Centrifuge the sample for 10 min at about 1500 rpm. Accurately transfer 10 mL of the upper hexane layer into centrifuge tube. Gently evaporate the hexane to dryness using a stream of nitrogen. [NOTE—Some samples may result in an oil residue after the hexane evaporates.] Redissolve the residue in an accurately pipetted volume of absolute alcohol to obtain a solution with a known nominal concentration in the range of 1.0–8.0 µg/mL of cholecalciferol and mix well. Pass a portion of the solution through a 0.2-µm PTFE membrane filter, and discard the first 1 mL of the filtrate.

For Oral Powders containing 10 µg/g or more of vitamin D
Solution A: 50 mg/mL of sodium dodecyl sulfate in water
Mobile phase, Standard stock solution, Standard solution, and **System suitability solution:** Prepare as directed in *For Oral Powders containing less than 10 µg/g of vitamin D*.

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Sample solution: Empty 20 containers and combine and mix their contents to obtain a homogenous composite. Weigh the composite and calculate the average fill weight per container. Transfer a calculated amount of accurately weighed composite (NMT 5 g) into a 50-mL volumetric flask. [NOTE—A larger flask may be used in order to eliminate additional dilutions. The final content of water in the *Sample solution* is NMT 20%.] Add 10 mL of *Solution A* and immediately swirl the flask to mix the contents, and sonicate for 15 min swirling occasionally. Add 5 mL of methyl tert-butyl ether, swirl to mix, add 10 mL of absolute alcohol, and swirl to mix. Continue to add absolute alcohol in 10-mL aliquots, with swirling, almost to volume. Cool to room temperature, and dilute with absolute alcohol to volume to obtain a solution with a known nominal concentration in the range of 1.0–8.0 µg/mL of cholecalciferol. Mix well. [NOTE—Excessive amounts of dissolved solids may be precipitated by placing the flask in a freezer for 15 min, if necessary.] Pass a portion of the solution through a 0.2-µm PTFE membrane filter, and discard the first 1 mL of the filtrate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: 265 nm
Column: 2.1-mm × 10-cm; 1.7-µm packing L1
Sample temperature: 8°
Flow rate: 0.4 mL/min
Injection volume: 1 µL

System suitability
Samples: *Standard solution* and *System suitability solution*
[NOTE—The relative retention times for pre-cholecalciferol and cholecalciferol are 1.0 and 1.1, respectively.]
Suitability requirements
Column efficiency: NLT 14,000 theoretical plates, *Standard solution*
Resolution: NLT 3 between pre-cholecalciferol and cholecalciferol, *System suitability solution*
Relative standard deviation: NMT 2.0%, *Standard solution*

Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of vitamin D, as cholecalciferol ($C_{27}H_{44}O$), in the portion of Oral Powder taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of cholecalciferol from the *Sample solution*
r_S = peak area of cholecalciferol from the *Standard solution*
C_S = concentration of cholecalciferol from USP Cholecalciferol RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of cholecalciferol in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0%

• **CONTENT OF VITAMINS B1, B2, B3, B6, AND FOLIC ACID**
[NOTE—Use low-actinic glassware.]
Solution A: 10 mg/mL of ascorbic acid in water. Prepare fresh at the time of use.
Solution B: Phosphoric acid and water (50:50)
Solution C: 1% sodium bicarbonate in water
Solution D: 0.1 M edetate disodium in water
Mobile phase A: 0.1% trifluoroacetic acid in water
Mobile phase B: 0.1% trifluoroacetic acid in mixture of water and acetonitrile (88:12)
Mobile phase: See *Table 1*.

Table 1

Time (min)	Flow Rate (mL/min)	Mobile Phase A (%)	Mobile Phase B (%)
0	1	100	0
8	1	100	0
20	1	0	100
26	1.5	0	100
40	1.5	0	100
41	1.5	100	0
50	1.5	100	0

[NOTE—For preparation of the following *Standard stock solutions*, dissolve the related Reference Standard in water first. The solution can be swirled or placed on a steam bath for about 5–10 min with frequent swirling to assist in dissolution. When the vitamin has completely dissolved, cool the solution to room temperature, add *Solution B*, and dilute stepwise with water to final volume. *Solution B* should be added in an amount resulting in a concentration of 0.5% *Solution B* in the final volume of stock solution.]
Thiamine standard stock solution: 150 µg/mL of thiamine from USP Thiamine Hydrochloride RS in water containing 0.5% *Solution B*
Riboflavin standard stock solution: 120 µg/mL of USP Riboflavin RS in water containing 0.5% *Solution B*
Niacin (or Niacinamide) standard stock solution: 1.5 mg/mL of USP Niacin RS or USP Niacinamide RS in water containing 0.5% *Solution B*
Pyridoxine standard stock solution: 150 µg/mL of pyridoxine from USP Pyridoxine Hydrochloride RS in water containing 0.5% *Solution B*
Folic acid standard stock solution: 55 µg/mL of USP Folic Acid RS in *Solution C*
Standard solution: Accurately transfer calculated volumes of *Thiamine standard stock solution*, *Riboflavin standard stock solution*, *Niacin (or Niacinamide) standard stock solution*, *Pyridoxine standard stock solution*, and *Folic acid standard stock solution* into a 200-mL volumetric flask to obtain a solution with final vitamin concentrations similar to the nominal concentrations in the *Sample solution* for the corresponding vitamins. Add 1.0 mL of *Solution B* and 1.0 mL of *Solution A*, and dilute with water almost to volume. Add 2–3 drops of polysorbate 80 and add water to final volume. Mix well by completely inverting the flask at least 20 times. [NOTE—After inverting the flask 10–12 times, carefully remove the stopper to release the built-up pressure.]
Sample solution: Empty 20 containers and combine and mix their contents to obtain a homogenous composite. Weigh the composite and calculate the average fill weight per container. Transfer a calculated amount of accurately weighed composite into a suitable volumetric flask to obtain a solution containing known nominal concentrations of vitamins in the range of 2–130 µg/mL for thiamine, 1.5–110 µg/mL for riboflavin, 10–800 µg/mL for niacin, 10–500 µg/mL for niacinamide, 2–120 µg/mL for pyridoxine, and 0.5–2.0 µg/mL for folic acid. Add about half volume of water and an aliquot of *Solution D* equivalent

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to 2% the volume of the flask, and swirl to mix thoroughly. Place the flask on a steam bath for 2–3 min, swirl frequently, and avoid overheating. Cool the flask to room temperature and add an aliquot of *Solution C* equivalent to 10% the volume of the flask, mix well, and sonicate for 1 min. Cool to room temperature, add an aliquot of *Solution A* equivalent to 0.5% the volume of the flask, and an aliquot of *Solution B* equivalent to 0.3% the volume of the flask, swirl to mix and dilute almost to volume with water. Add 2–3 drops of polysorbate 80 and bring to final volume with water. [NOTE—If more than one dilution is necessary to obtain the required concentrations of vitamins make the dilutions with water first and add *Solution A*, *Solution B*, and polysorbate 80 to the last dilution.] Mix well by completely inverting the flask at least 20 times with caution to release the built up pressure. Pass a portion of the solution through a suitable filter of 0.45-µm pore size and discard the first milliliter of the filtrate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm for 13 min, switch to 280 nm for 33 min, then return to 254 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Flow rate: See *Table 1*.
Injection volume: 20 µL

System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for ascorbic acid, niacinamide, thiamine, pyridoxine, folic acid, and riboflavin are 0.8, 1.0, 1.8, 3.3, 5.0, and 6.2, respectively.]
Suitability requirements
Resolution: NLT 1.5 between the ascorbic acid and niacinamide peaks
Relative standard deviation: NMT 2.0% for each individual peak: niacinamide (or niacin), thiamine, pyridoxine, riboflavin, and folic acid

Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of vitamin B1 as thiamine ion ($C_{12}H_{17}N_4OS^+$), vitamin B2 as riboflavin ($C_{17}H_{20}N_4O_6$), vitamin B3 as niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), vitamin B6 as pyridoxine ($C_8H_{11}NO_3$), and folic acid ($C_{19}H_{19}N_7O_6$), in the portion of Oral Powder taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin from the *Sample solution*
r_S = peak area of the relevant vitamin from the *Standard solution*
C_S = concentration of the relevant vitamin Reference Standard in the *Standard solution* (µg/mL)
C_U = nominal concentration of the relevant vitamin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of each individual vitamin

• **CONTENT OF VITAMIN B12, *Method 1***
[NOTE—Use low-actinic glassware throughout this procedure. Inject samples within 30 min.]
Chelating reagent: A powdered mixture of ammonium pyrrolidinedithiocarbamate, edetate disodium, and diethylenetriaminepentaacetic acid (1:1:1)
Buffer: Dissolve 470.5 mg of hexanesulfonic acid sodium salt in water, add 1 mL of phosphoric acid, dilute with water to 1000 mL, and mix. Adjust with 50% potassium hydroxide to a pH of 3.5.
Mobile phase: Acetonitrile and *Buffer*. See *Table 2* for gradient.

Table 2

Time (min)	Acetonitrile (%)	Buffer (%)
0	1.0	99.0
0.5	1.0	99.0
1.2	2.3	97.7
1.4	5.0	95.0
2.5	7.0	93.0
5.0	18.0	82.0
5.5	25.0	75.0
6.5	25.0	75.0
7.0	1.0	99.0
15.0	1.0	99.0

Cyanocobalamin standard stock solution: 10 µg/mL of USP Cyanocobalamin (Crystalline) RS in water.
[NOTE—Store this stock solution in a dark place and discard after 1 week.]
Standard solution: 0.3 µg/mL of cyanocobalamin from *Cyanocobalamin standard stock solution* in water
Riboflavin solution: 80 µg/mL of USP Riboflavin RS in water.
[NOTE—The solution can be swirled or placed on a steam bath for about 5–10 min with frequent swirling to assist in dissolution.]
System suitability solution: Transfer 1.5 mL of *Cyanocobalamin standard stock solution* into 50-mL volumetric flask and dilute with *Riboflavin solution* to volume.
Sample solution: Empty 20 containers and combine and mix their contents to obtain a homogenous composite. Weigh the composite and calculate the average fill weight per container. Transfer a portion of the composite, nominally equivalent to 10 µg of cyanocobalamin, to a flask. Add 25 mg of *Chelating reagent* and 40 mL of water accurately measured. Record the weight of the flask, sonicate for 5 min, and shake vigorously for 2 min. Add water to the previously recorded weight to compensate for any solvent loss. Immediately pass a portion of the solution through a polysulfone membrane filter of 0.45-µm pore size, and discard the first portion of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 361 nm
Column: 2.1-mm × 10-cm; 1.7-µm packing L1
Column temperature: 35°
Flow rate: 0.5 mL/min
Injection volume: 15 µL

System suitability
Sample: *Standard solution* and *System suitability solution*
[NOTE—The relative retention times for cyanocobalamin and riboflavine are 1.0 and 1.04, respectively.]

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Suitability requirements
Resolution: NLT 1.5 between cyanocobalamin and riboflavine, *System suitability solution*
Relative standard deviation: NMT 2.0%, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of vitamin B12, as cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$), in the portion of Oral Powder taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of cyanocobalamin from the *Sample solution*
r_S = peak area of cyanocobalamin from the *Standard solution*
C_S = concentration of cyanocobalamin from USP Cyanocobalamin (Crystalline) RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of cyanocobalamin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0%

• **CONTENT OF VITAMIN B12, *Method 2***
[NOTE—Use low-actinic glassware throughout this procedure.]
Mobile phase: Methanol and water (7:13)
Standard stock solution: 10 µg/mL of USP Cyanocobalamin (Crystalline) RS in water. [NOTE—Store this stock solution in a dark place, and discard after 1 week.]
Standard solution: 1 µg/mL of USP Cyanocobalamin (Crystalline) RS from the *Standard stock solution* diluted with water
Sample solution: Empty 20 containers and combine and mix their contents to obtain a homogenous composite. Weigh the composite and calculate the average fill weight per container. Transfer a portion of the composite, nominally equivalent to 10 µg of cyanocobalamin, to a flask. Add 25 mg of *Chelating reagent* and 25 mL of water accurately measured. Record the weight of the flask, sonicate for 5 min, and shake vigorously for 2 min. Add water to the previously recorded weight to compensate for any solvent loss. Immediately pass a portion of the solution through a polysulfone membrane filter of 0.45-µm pore size, and discard the first portion of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 550 nm
Column: 4.6-mm × 15-cm; 5-µm packing L1
Flow rate: 0.5 mL/min
Injection volume: 200 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 3.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas of cyanocobalamin.
Calculate the percentage of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$) in the portion of Oral Powder taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*
r_S = peak area from the *Standard solution*
C_S = concentration of USP Cyanocobalamin (Crystalline) RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of cyanocobalamin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$)

• **CONTENT OF VITAMIN C**
[NOTE—Protect samples from air, light, and heat.]
Buffer: 2.04 g/L of monobasic potassium phosphate in water. Adjust with phosphoric acid to a pH of 3.0.
Mobile phase: *Buffer*
Diluent: 0.56 g of edetate disodium and 2.04 g of monobasic potassium phosphate per 1000 mL of water. Adjust with phosphoric acid to a pH of 3.0.
Standard solution: 0.25 mg/mL of USP Ascorbic Acid RS in *Diluent*
Sample solution: Empty 20 containers and combine and mix their contents to obtain a homogenous composite. Weigh the composite and calculate the average fill weight per container. Transfer a portion of the composite, nominally equivalent to about 25 mg of ascorbic acid, into a 100-mL volumetric flask. Add 60 mL of *Diluent*, shake mechanically for 15 min, dilute with *Diluent* to volume, mix well, and pass through a membrane filter of 0.45-µm pore size, discarding the first 4 mL of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 245 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Flow rate: 1.0 mL/min
Injection volume: 5 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 2.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of vitamin C, as ascorbic acid ($C_6H_8O_6$), in the portion of Oral Powder taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the ascorbic acid from the *Sample solution*
r_S = peak area of the ascorbic acid from the *Standard solution*
C_S = concentration of ascorbic acid from USP Ascorbic Acid RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of ascorbic acid in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0%

• **CONTENT OF COPPER, IRON, SELENIUM, AND ZINC, *Method 1***
Stock aqua regia solution: Prepare a mixture of hydrochloric acid and nitric acid (3:1) by adding the nitric acid to the hydrochloric acid. [NOTE—Periodically vent the solution in an appropriate fume hood.]
Diluent: Prepare a mixture of the *Stock aqua regia solution* and water (1:9) by adding 1 volume of *Stock aqua regia solution* to 2 volumes of water. Dilute with additional water to volume, and mix well.
Solution A: 1 mg/mL of yttrium in 5% nitric acid solution

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Solution B: 1 mg/mL of scandium in 5% nitric acid solution
System suitability solution: A mixture of *Solution A*, *Solution B*, and *Diluent* (1:1:198)
Standard stock solution 1 [copper (Cu), iron (Fe), and zinc (Zn)]: Use commercially available element standard solutions in 5% nitric acid solution as follows. Pipet the appropriate amount of element standard solution into a volumetric flask, and dilute with 5% nitric acid solution to obtain a solution with final concentrations of about 0.1 mg/mL of copper, 0.25 mg/mL of iron, and 0.25 mg/mL of zinc.
Standard stock solution 2 [selenium (Se)]: Use commercially available element standard solutions in 20% hydrochloric acid solution as follows. Pipet the appropriate amount of element standard solution into a volumetric flask, and dilute with 20% hydrochloric acid solution to obtain a solution with a final concentration of 0.1 mg/mL of selenium.
Standard curve solutions: Prepare a mixture of *Standard stock solution 1* and *Standard stock solution 2*, as required, in *Diluent* to generate a six-point calibration curve to bracket the concentration range of each mineral of interest.
Sample solution: Empty 20 containers and combine and mix their contents to obtain a homogenous composite. Weigh the composite and calculate the average fill weight per container. Transfer a calculated amount of accurately weighed composite into a 250-mL volumetric flask. Slowly add 25 mL of the *Stock aqua regia solution* in 5-mL increments, followed by mixing. Bring the solution to a boil on a hot plate. Continue to heat gently for NMT 15 min. Remove from heat, cool, and dilute with water to volume. Pass about 30 mL through a 5-µm pore size nylon syringe filter into a centrifuge tube. If necessary, make any further dilutions using the *Diluent* to concentrations fitting within the range of the *Standard curve solutions* used.
Instrumental conditions
(See *Plasma Spectrochemistry* ⟨730⟩.)
Mode: Inductively coupled plasma spectrometry, using a spectrophotometer set to measure the emission of each mineral of interest at about the corresponding wavelength. [NOTE—The operating conditions may be developed and optimized based on the manufacturer's recommendation. The wavelengths selected should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision.]
System suitability
Sample: *System suitability solution*
[NOTE—Analyze the *System suitability solution*, and obtain the response as directed in the *Analysis*.]
Suitability requirements
Relative standard deviation: NMT 2.0%
Analysis
Samples: *Standard curve solutions* and *Sample solution*
Determine the emission of each mineral of interest in the *Standard curve solutions* and *Sample solution* with an inductively coupled plasma system using the *Diluent* as the blank. Plot the emission of the *Standard curve solutions* versus the concentration, in µg/mL, of the minerals of interest, and draw the straight line best fitting the plotted points. From the graph, determine the concentration (C), in µg/mL, for each mineral of interest in the *Sample solution*.
Calculate the percentage of the labeled amount of each mineral in the portion of Oral Powder taken:

$$\text{Result} = C \times (V/W) \times F \times (W_{AV}/L) \times 100$$

C = concentration of relevant mineral in the *Sample solution* determined from the standard curve (µg/mL)
V = volume of the *Sample solution* (mL)
W = weight of the portion of Oral Powder taken (mg)
F = dilution factor for the *Sample solution*
W_{AV} = average fill weight per container (mg)
L = label claim for each mineral (µg/container)

Acceptance criteria: 90.0%–125.0% of the labeled amounts of copper (Cu), iron (Fe), and zinc (Zn); 90.0%–160.0% of the labeled amount of selenium (Se)

• **COPPER, *Method 2***
Copper standard solution: Dissolve 1.00 g of copper foil in a minimum volume of a 50% solution of nitric acid, and dilute with a 1% solution of nitric acid to 1000 mL. The solution contains 1000 µg/mL of copper.
Standard stock solution: 100 µg/mL of copper from the *Copper standard solution* diluted with 0.125 N hydrochloric acid
Standard solutions: To separate 200-mL volumetric flasks transfer 1.0, 2.0, 4.0, 6.0, and 8.0 mL of the *Standard stock solution*. Dilute with water to volume to obtain concentrations of 0.5, 1.0, 2.0, 3.0, and 4.0 µg/mL of copper.
Sample solution: Empty 20 containers and combine and mix their contents to obtain a homogenous composite. Weigh the composite and calculate the average fill weight per container. Transfer a portion of the powder, equivalent to 5 containers, to a porcelain crucible. Heat the crucible in a muffle furnace maintained at 550° for 6–12 h, and cool. Add 60 mL of hydrochloric acid, and boil gently on a hot plate or steam bath for 30 min, intermittently rinsing the inner surface of the crucible with 6 N hydrochloric acid. Cool, and quantitatively transfer the contents of the crucible to a 100-mL volumetric flask. Rinse the crucible with small portions of 6 N hydrochloric acid, and add the rinsings to the flask. Dilute with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute this solution quantitatively, with 0.125 N hydrochloric acid, to obtain a concentration of 2 µg/mL of copper.
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Analytical wavelength: Copper emission line at 324.7 nm
Lamp: Copper hollow-cathode
Flame: Air–acetylene
Blank: 0.125 N hydrochloric acid
Analysis
Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of copper, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C, in µg/mL, of copper in the *Sample solution*.
Calculate the percentage of the labeled amount of copper (Cu) in the portion of Oral Powder taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of copper in the *Sample solution* from the graph (µg/mL)
C_U = nominal concentration of copper in the *Sample solution* (µg/mL)

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Acceptance criteria: 90.0%–125.0% of the labeled amount of copper (Cu)

• **IRON, *Method 2***

Iron standard stock solution: Transfer 100 mg of iron powder to a 1000-mL volumetric flask. Dissolve in 25 mL of 6 N hydrochloric acid, and dilute with water to volume.

Standard solutions: To separate 100-mL volumetric flasks, transfer 2.0, 4.0, 5.0, 6.0, and 8.0 mL of the *Iron standard stock solution*. Dilute the contents of each flask with water to volume to obtain concentrations of 2.0, 4.0, 5.0, 6.0, and 8.0 µg/mL of iron.

Sample solution: Empty 20 containers and combine and mix their contents to obtain a homogenous composite. Weigh the composite and calculate the average fill weight per container. Transfer a portion of the powder, equivalent to 5 containers, to a porcelain crucible. Heat the crucible in a muffle furnace maintained at 550° for 6–12 h, and cool. Add 60 mL of hydrochloric acid, and boil gently on a hot plate or steam bath for 30 min, intermittently rinsing the inner surface of the crucible with 6 N hydrochloric acid. Cool, and quantitatively transfer the contents of the crucible to a 100-mL volumetric flask. Rinse the crucible with small portions of 6 N hydrochloric acid, and add the rinsings to the flask. Dilute with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute this solution quantitatively, with 0.125 N hydrochloric acid, to obtain a concentration of 5 µg/mL of iron.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Copper emission line at 248.3 nm

Lamp: Iron hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of iron, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C, in µg/mL, of iron in the *Sample solution*.

Calculate the percentage of the labeled amount of iron (Fe) in the portion of Oral Powder taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of iron in the *Sample solution* from the graph (µg/mL)

C_U = nominal concentration of iron in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of iron (Fe)

• **SELENIUM, *Method 2***

Diluent: 20 mg/mL of ammonium chloride in water

Selenium standard solution: [**CAUTION**—Selenium is toxic; handle it with care.] Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness, add 2 mL of water, and evaporate to dryness. Repeat the addition of water and the evaporation to dryness three times. Dissolve the residue in 3 N hydrochloric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume to obtain a concentration of 1000 µg/mL of selenium.

Standard stock solution: 50 µg/mL of selenium from the *Selenium standard solution* diluted with water

Standard solutions: To separate 100-mL volumetric flasks transfer 5.0, 10.0, and 25.0 mL of the *Standard stock solution*, and add 5.0 mL of perchloric acid to each flask. Gently boil the solutions for 15 min, cool to room temperature, and dilute each with *Diluent* to volume to obtain solutions with concentrations of 2.5, 5.0, and 12.5 µg/mL of selenium.

Sample solution: Transfer a portion of the powder, equivalent to 250 µg of selenium, to a suitable flask, and add 12 mL of nitric acid. [NOTE—The volume of nitric acid may be varied to ensure that the powder is uniformly dispersed.] Carefully swirl the flask to disperse the test specimen. Sonicate for 10 min or until the test specimen is completely dissolved. Gently boil the solution for 15 min, and cool to room temperature. Carefully add 8 mL of perchloric acid to the flask, heat the flask until perchloric acid fumes appear, and swirl the flask to dissipate the fumes. Repeat the heating and swirling until the fumes appear again. Cool to room temperature. Transfer the contents of the flask to a 50-mL volumetric flask with the aid of the *Diluent*, and dilute with *Diluent* to volume.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Selenium emission line at 196.0 nm

Lamp: Selenium hollow-cathode

Flame: Air–acetylene

Blank: *Diluent* and perchloric acid (20:1)

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of selenium, and draw the straight line best fitting the three plotted points. From the graph so obtained, determine the concentration, C, in µg/mL, of selenium in the *Sample solution*.

Calculate the percentage of the labeled amount of selenium (Se) in the portion of Oral Powder taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of selenium in the *Sample solution* from the graph (µg/mL)

C_U = nominal concentration of selenium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of selenium (Se)

• **ZINC, *Method 2***

Zinc standard stock solution: 1000 µg/mL of zinc from zinc oxide in 5 M hydrochloric acid (3.89 mg/mL) and diluted with water to final volume. [NOTE—Dissolve in 5 M hydrochloric acid by warming, if necessary, cool, and then dilute to final volume.]

Standard stock solution: 50 µg/mL of zinc from the *Zinc standard stock solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Transfer 1.0, 2.0, 3.0, 4.0, and 5.0 mL of *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of zinc.

Sample solution: Empty 20 containers and combine and mix their contents to obtain a homogenous composite. Weigh the composite and calculate the average fill weight per container. Transfer a portion of the powder, equivalent

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to 5 containers, to a porcelain crucible. Heat the crucible in a muffle furnace maintained at 550° for 6–12 h, and cool. Add 60 mL of hydrochloric acid, and boil gently on a hot plate or steam bath for 30 min, intermittently rinsing the inner surface of the crucible with 6 N hydrochloric acid. Cool, and quantitatively transfer the contents of the crucible to a 100-mL volumetric flask. Rinse the crucible with small portions of 6 N hydrochloric acid, and add the rinsings to the flask. Dilute with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute this solution quantitatively, with 0.125 N hydrochloric acid, to obtain a concentration of 2 µg/mL of zinc.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Zinc emission line at 213.8 nm

Lamp: Zinc hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of zinc, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of zinc in the *Sample solution*.

Calculate the percentage of the labeled amount of zinc (Zn) in the portion of Oral Powder taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of zinc in the *Sample solution* (µg/mL)

C_U = nominal concentration of zinc in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of zinc (Zn)

• **CONTENT OF IODINE**

Bromine water: To 20 mL of bromine in a glass-stoppered bottle add 100 mL of water. Insert the stopper into the bottle, and shake. Allow to stand for 30 min, and use the supernatant.

Sample solution: Empty 20 containers and combine and mix their contents to obtain a homogenous composite. Weigh the composite and calculate the average fill weight per container. Transfer accurately weighed composite, nominally equivalent to 1 mg of iodide, to a nickel crucible. Add 5 g of sodium carbonate, 5 mL of 50% sodium hydroxide solution, and 10 mL of alcohol, taking care that the entire specimen is moistened. Heat the crucible on a steam bath to evaporate the alcohol, then dry the crucible at 100° for 30 min to prevent spattering upon subsequent heating. Transfer the crucible with its contents to a furnace heated to 500°, and heat the crucible for 15 min. [NOTE—Heating at 500° is necessary to carbonize any organic matter present; a higher temperature may be used, if necessary, to ensure complete carbonization of all organic matter.]

Cool the crucible, add 25 mL of water, cover the crucible with a watch glass, and boil gently for 10 min. Filter the solution, and wash the crucible with boiling water, collecting the filtrate and washings in a beaker. Add phosphoric acid until the solution is neutral to methyl orange TS, then add 1 mL excess of phosphoric acid. Add excess of *Bromine water*, and boil the solution gently until colorless and then for 5 min longer. Add a few crystals of salicylic acid, and cool the solution to 20°.

Titrimetric system

Mode: Direct titration

Titrant: 0.005 N sodium thiosulfate from 0.1 N sodium thiosulfate VS

Analysis

Sample: *Sample solution*

Add 1 mL of phosphoric acid and 0.5 g of potassium iodide, and titrate the liberated iodine with *Titrant*, adding starch TS when the liberated iodine color has nearly disappeared.

Calculate the percentage of the labeled amount of iodine (I) in the portion of Oral Powder taken:

$$\text{Result} = V \times N_A \times F \times I_{me} \times (W_{AV}/W) \times (100/L)$$

V = volume of sodium thiosulfate consumed (mL)

N_A = actual normality of the sodium thiosulfate solution used

F = correction factor to convert mg to µg, 1000 µg/mL

I_{me} = milliequivalent of I, 21.16 mg/mEq

W_{AV} = average fill weight per container (mg)

W = weight of the portion of Oral Powder taken (mg)

L = label claim for iodine (µg/container)

Acceptance criteria: 90.0%–160.0%

PERFORMANCE TESTS

• **WEIGHT VARIATION** ⟨2091⟩

Procedure: Empty and weigh individually the net contents from 20 containers. Determine the average net content from the sum of the individual net weights. Determine the difference between each individual net content and the average net content.

Acceptance criteria: The requirements are met if (a) NMT 2 of the differences are greater than 10% of the average net content and (b) in no case is the difference greater than 25%.

CONTAMINANTS

• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed $\times 10^2$ cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meets the requirements

SPECIFIC TESTS

• **LOSS ON DRYING** ⟨731⟩

Sample: 2.0 g

Analysis: Dry the *Sample* at 105° for 2 h.

Acceptance criteria: NMT 6%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light- and moisture-resistant packages.

• **LABELING:** The label states the quantity of each vitamin and mineral in terms of metric units per dosage unit and, where necessary, the chemical form in which a vitamin is present, and also states the salt form of the mineral used as the source of each element. Where the product contains vitamin E, the label indicates whether it contains *RRR*-alpha-tocopheryl acetate or *all-rac*-alpha-tocopheryl acetate. Where more than one assay method is given for a

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particular vitamin, the labeling states with which assay method the product complies only if *Method 1* is not used.

• **USP REFERENCE STANDARDS** ⟨11⟩
USP Alpha Tocopheryl Acetate RS
USP Ascorbic Acid RS
USP Cholecalciferol RS
USP Cyanocobalamin (Crystalline) RS
USP Folic Acid RS
USP Niacin RS
USP Niacinamide RS
USP Pyridoxine Hydrochloride RS
USP Riboflavin RS
USP Thiamine Hydrochloride RS
USP Retinyl Acetate RS
USP Retinyl Palmitate RS▲ (USP 1-May-2019)

Oil-Soluble Vitamins Capsules

DEFINITION

Oil-Soluble Vitamins Capsules contain two or more of the following oil-soluble vitamins: Vitamin A, Vitamin D as Ergocalciferol (Vitamin D_2) or Cholecalciferol (Vitamin D_3), Vitamin E, Phytonadione (Vitamin K_1), and Beta Carotene. Capsules contain NLT 90.0% and NMT 165.0% of the labeled amounts of vitamin A ($C_{20}H_{30}O$) as retinol or esters of retinol in the form of retinyl acetate ($C_{22}H_{32}O_2$) or retinyl palmitate ($C_{36}H_{60}O_2$); vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$); vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$); phytonadione ($C_{31}H_{46}O_2$); and beta carotene ($C_{40}H_{56}$).

Oil-Soluble Vitamins Capsules contain no other vitamins or any minerals. They may contain other labeled added substances that are generally recognized as safe, in amounts that are unobjectionable.

STRENGTH

[NOTE—In the following assays, where more than one assay method is given for an individual ingredient, the requirements may be met by following any one of the specified methods, the method used being stated in the labeling only if *Method 1* is not used.]

• **VITAMIN A, *Method 1***

[NOTE—Where the use of a vitamin A ester (retinyl acetate or retinyl palmitate) is specified in the following procedure, use the chemical form present in the formulation. USP Vitamin A RS is retinyl acetate. It is to be used where USP Vitamin A RS is specified. Use low-actinic glassware throughout this procedure.]

Mobile phase: *n*-Hexane

Standard solution: 15 µg/mL of retinyl acetate from USP Vitamin A RS in *n*-hexane

System suitability stock solution: 15 µg/mL of retinyl palmitate in *n*-hexane

System suitability solution: Mix equal volumes of the *System suitability stock solution* and the *Standard solution* to obtain concentrations of 7.5 µg/mL each of retinyl acetate and retinyl palmitate.

Sample stock solution: Transfer the contents of NLT 20 Capsules to a suitable container, mix, and weigh. Transfer a portion of the mixture, equivalent to 5 Capsules, to a container having a polytef-lined screw cap. [NOTE—For hard gelatin Capsules, remove, as completely as possible, the contents of NLT 20 Capsules by cutting open the Capsule shells, transferring the shells and their contents to a suitable container, and triturating to a homogeneous mass. Transfer a portion of the mass, equivalent to 5 Capsules, to a container having a polytef-lined screw cap.] Add 10 mL of dimethyl sulfoxide and 15 mL of *n*-hexane, and shake for 45 min on a wrist-action shaker in a water bath maintained at 60°. [NOTE—Set up the wrist-action shaker to ensure that the contents of the container are mixed vigorously and thoroughly.] Centrifuge at 3000 rpm for 10 min, and transfer the hexane layer by means of a pipet to a 100-mL volumetric flask. Add 15 mL of *n*-hexane to the dimethyl sulfoxide layer, shake thoroughly for 5 min, and transfer the hexane layer by means of a pipet to the 100-mL volumetric flask. Repeat this extraction with three additional 15-mL portions of *n*-hexane. Dilute the extracts in the volumetric flask with *n*-hexane to volume.

Sample solution: Dilute a volume of the *Sample stock solution* with *n*-hexane to obtain a solution with a concentration of 15 µg/mL of vitamin A as retinol ($C_{20}H_{30}O$).

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 325 nm
Column: 4.6-mm × 15-cm; 3-µm packing L8
Flow rate: 1 mL/min
Injection size: 40 µL

System suitability
Sample: *System suitability solution*
Suitability requirements
Resolution: NLT 10 between all-*trans*-retinyl acetate and all-*trans*-retinyl palmitate
Relative standard deviation: NMT 3.0%

Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak area of all-*trans*-retinyl acetate from the *Standard solution* and the peak area of all-*trans*-retinyl acetate or all-*trans*-retinyl palmitate from the *Sample solution*.

For products containing vitamin A acetate or vitamin A palmitate, calculate the percentage of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$), in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of the all-*trans*-retinyl ester from the *Sample solution*
r_S = peak area of the all-*trans*-retinyl ester from the *Standard solution*
C_S = concentration of retinyl acetate ($C_{22}H_{32}O_2$) from USP Vitamin A RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of vitamin A, as retinol ($C_{20}H_{30}O$) in the *Sample solution* (µg/mL)
F = factor used to convert retinyl acetate, the ester form present in USP Vitamin A RS, to retinol, 0.872

[NOTE—The molar responses of retinyl acetate and retinyl palmitate are equivalent.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$)

• **VITAMIN A, *Method 2***

[NOTE—Where a vitamin A ester (retinyl acetate or retinyl palmitate) is indicated in the following procedure, use the chemical form present in the formulation. USP Vitamin A RS is retinyl acetate. It is to be used where USP Vitamin A RS is specified. Use low-actinic glassware throughout this procedure.]

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3 N methanolic sulfuric acid solution: Cautiously add 9 mL of sulfuric acid to 80 mL of methanol in a 100-mL volumetric flask. Cool, and dilute with methanol to volume.
Sodium ascorbate–pyrogallol solution: Transfer 10 g of sodium ascorbate and 5 g of pyrogallol to a 100-mL volumetric flask, and add sufficient water to dissolve. Add 1.7 mL of sulfuric acid, and dilute with water to volume.
Lecithin solution: 5 mg/mL of lecithin in 2,2,4-trimethylpentane
Mobile phase: *n*-Hexane and ethyl acetate (99.7: 0.3)
Standard solution: 15 µg/mL of retinyl acetate from USP Vitamin A RS in 2,2,4-trimethylpentane
System suitability stock solution: 15 µg/mL of retinyl palmitate in 2,2,4-trimethylpentane
System suitability solution: Mix equal volumes of the *System suitability stock solution* and the *Standard solution* to obtain concentrations of 7.5 µg/mL each of retinyl acetate and retinyl palmitate.
Sample solution: [NOTE—This preparation is suitable for the determination of vitamin A, vitamin D, and vitamin E, when present in the formulation.] Weigh NLT 20 Capsules in a tared weighing bottle. Using a sharp blade if necessary, carefully open the Capsules, without loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to 30 µg of the labeled amount of cholecalciferol or ergocalciferol (vitamin D), to a container with a polytef-lined screw cap. If vitamin D is not present in the formulation, use a portion, equivalent to 90 mg of the labeled amount of vitamin E. If vitamin E is not present in the formulation, use a portion, equivalent to 2.5 mg of the labeled amount of vitamin A, as retinol. Add 0.5 g of sodium bicarbonate, 1.5 mL of *Lecithin solution*, and 12.5 mL of 2,2,4-trimethylpentane, and disperse on a vortex mixer. Add 6 mL of *Sodium ascorbate–pyrogallol solution*, shake slowly, and allow the solution to degas. Continue shaking until the evolution of gas has ceased, and then shake for an additional 12 min. Add 6 mL of dimethyl sulfoxide, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 6 mL of *3 N methanolic sulfuric acid solution*, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 12.5 mL of 2,2,4-trimethylpentane, mix on a vortex mixer to form a suspension, and shake for 10 min. Centrifuge for 10 min to break up the emulsion and to clarify the supernatant. [NOTE—The supernatant is used for the determination of vitamin A, and also vitamin D and vitamin E, if present in the formulation.] If necessary, quantitatively dilute a volume of the supernatant with 2,2,4-trimethylpentane to obtain a concentration close to that of the *Standard solution*.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 325 nm
Column: 4.6-mm × 25-cm; 5-µm packing L24
Flow rate: 1.5 mL/min
Injection size: 40 µL
System suitability
Sample: *System suitability solution*
Suitability requirements
Resolution: NLT 8.0 between all-*trans*-retinyl acetate and all-*trans*-retinyl palmitate
Relative standard deviation: NMT 3.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak area of all-*trans*-retinyl acetate from the *Standard solution* and the peak area of all-*trans*-retinyl acetate or all-*trans*-retinyl palmitate from the *Sample solution*.
Calculate the percentage of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$), in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of the all-*trans*-retinyl ester from the *Sample solution*
r_S = peak area of the all-*trans*-retinyl ester from the *Standard solution*
C_S = concentration of retinyl acetate ($C_{22}H_{32}O_2$) from USP Vitamin A RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of vitamin A, as retinol ($C_{20}H_{30}O$) in the *Sample solution* (µg/mL)
F = factor used to convert retinyl acetate, the ester form present in USP Vitamin A RS, to retinol, 0.872

[NOTE—Account for the initial extraction volume of 26.5 mL of 2,2,4-trimethylpentane to calculate the nominal concentration. The molar responses of retinyl acetate and retinyl palmitate are equivalent.]
Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$)

• **VITAMIN A, *Method 3***
[NOTE—Where a vitamin A ester (retinyl acetate or retinyl palmitate) is indicated in the following procedure, use the chemical form present in the formulation. USP Vitamin A RS is retinyl acetate. It is to be used where USP Vitamin A RS is specified. Use low-actinic glassware throughout this procedure.]
Extraction solvent: *n*-Hexane and methylene chloride (3:1)
Potassium hydroxide solution: 800 mg/mL of potassium hydroxide in water. [NOTE—Cautiously add the potassium hydroxide to the water. Mix, and cool.]
Diluent: 10 mg/mL of pyrogallol in alcohol
Mobile phase: *n*-Hexane and isopropyl alcohol (92:8)
Standard stock solution: 30 µg/mL of retinyl acetate from USP Vitamin A RS in *Diluent*. [NOTE—This solution may be stored in a refrigerator for 1 week.]
Standard solution: Dilute a volume of *Standard stock solution* with *Diluent* to obtain a concentration of 1 µg/mL of retinyl acetate from USP Vitamin A RS. Transfer 10.0 mL of this solution to a stoppered 125-mL flask, and add 5 mL of water, 5 mL of *Diluent*, and 3 mL of *Potassium hydroxide solution*. Insert the stopper tightly, shake for 15 min over a water bath maintained at 60 ± 5°, and cool to room temperature. Add 7 mL of water and 25.0 mL of *Extraction solvent*. Insert the stopper tightly, and shake vigorously for 60 s. Rinse the sides of the flask with 60 mL of water, and allow to stand for 10 min until the layers separate. Withdraw a portion of the organic layer for injection into the chromatograph. This *Standard solution* contains 0.34 µg/mL of retinol.
Sample solution: Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the

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Capsule contents. Transfer a portion of the Capsule contents, equivalent to 1.5 mg of retinyl acetate, to a stoppered 125-mL flask. Add 5 mL of water, 15 mL of *Diluent*, and 3 mL of *Potassium hydroxide solution*. Insert the stopper tightly, shake for 15 min over a water bath maintained at 60 ± 5°, and cool to room temperature. Add 7 mL of water and 25.0 mL of *Extraction solvent*. Insert the stopper tightly, and shake vigorously for 60 s or longer, if necessary, for complete extraction. Rinse the sides of the flask with 60 mL of water, and allow to stand for 10 min until the layers separate. [NOTE—Do not shake, because an emulsion may form.] Withdraw a portion of the organic layer, and dilute quantitatively, and stepwise if necessary, with *Extraction solvent*, to obtain a concentration of 0.34 µg/mL of retinol.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 335 nm
Column: 6.2-mm × 8-cm; packing L3
Column temperature: 40°
Flow rate: 4 mL/min
Injection size: 50 µL

System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for 13-*cis*-retinol and all-*trans*-retinol are about 0.92 and 1.0, respectively.]
Suitability requirements
Relative standard deviation: NMT 5.0%

Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas for all-*trans*-retinol and 13-*cis*-retinol.
Calculate the percentage of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$), in the portion of Capsules taken:

$$\text{Result} = (r_{T1}/r_{T2}) \times (C_S/C_U) \times F \times 100$$

r_{T1} = sum of the areas of the all-*trans*-retinol and 13-*cis*-retinol peaks from the *Sample solution*
r_{T2} = sum of the areas of all-*trans*-retinol and 13-*cis*-retinol peaks from the *Standard solution*
C_S = concentration of retinyl acetate ($C_{23}H_{32}O_2$) from USP Vitamin A RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of vitamin A, as retinol ($C_{20}H_{30}O$) in the *Sample solution* (µg/mL)
F = factor used to convert retinyl acetate, the ester form present in USP Vitamin A RS, to retinol, 0.872

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$)

• **CHOLECALCIFEROL OR ERGOCALCIFEROL (VITAMIN D), *Method 1***
[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Mobile phase: *n*-Hexane and isopropyl alcohol (99:1)
Standard solution: 2 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in *n*-hexane
System suitability solution: Heat a volume of the *Standard solution* at 60° for 1 h to partially isomerize vitamin D (cholecalciferol or ergocalciferol) to its corresponding precursor.
Sample solution: Proceed as directed for the *Sample stock solution* in *Vitamin A, Method 1*. Transfer NLT 20 mL of this solution retained as specified in the directions for the *Sample solution* in *Vitamin A, Method 1* to a suitable container, and evaporate, if necessary, in vacuum at room temperature to obtain a solution with a concentration of 2 µg/mL of cholecalciferol or ergocalciferol.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 265 nm
Column: 4.6-mm × 15-cm; 3-µm packing L8
Flow rate: 1 mL/min
Injection size: 100 µL

System suitability
Samples: *Standard solution* and *System suitability solution*
Suitability requirements
Resolution: NLT 10 between the vitamin D form present and its corresponding precursor, *System suitability solution*
Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas for vitamin D.
Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*
r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*
C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)
F = correction factor to account for the average amount of previtamin D present in the *Sample solution*, 1.09

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

• **CHOLECALCIFEROL OR ERGOCALCIFEROL (VITAMIN D), *Method 2***
[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

3 N methanolic sulfuric acid solution, Sodium ascorbate–pyrogallol solution, Lecithin solution, and **Sample solution:** Proceed as directed in *Vitamin A, Method 2*.
Mobile phase: *n*-Hexane and tertiary butyl alcohol (98.75:1.25)
Standard solution: 1 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in 2,2,4-trimethylpentane
System suitability solution: Heat a volume of the *Standard solution* at 60° for 1 h to partially isomerize vitamin D (cholecalciferol or ergocalciferol) to its corresponding precursor.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 265 nm

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Column: 4.6-mm × 25-cm; 5-µm packing L24
Flow rate: 1 mL/min
Injection size: 40 µL
System suitability
Samples: *Standard solution* and *System suitability solution*
Suitability requirements
Resolution: NLT 4.0 between the vitamin D form present and its corresponding precursor, *System suitability solution*
Relative standard deviation: NMT 3.0%, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas for vitamin D.
Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of Capsules taken:

$$\text{Result} = (r_S/r_U) \times (C_S/C_U) \times 100$$

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*
r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*
C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

• **CHOLECALCIFEROL OR ERGOCALCIFEROL (VITAMIN D), *Method 3***

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Diluted acetic acid: Glacial acetic acid solution (1 in 10) in water
Phenolphthalein solution: 10 mg/mL of phenolphthalein in alcohol
Potassium hydroxide solution: Slowly dissolve 14 g of potassium hydroxide in a mixture of 31 mL of dehydrated alcohol and 5 mL of water. Prepare fresh daily.
Extraction solvent: Methylene chloride and isopropyl alcohol (99.8: 0.2)
Mobile phase: Acetonitrile and methanol (91:9)
Standard stock solution: 0.2 mg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in dehydrated alcohol. [NOTE—Prepare fresh every 4 weeks. Store in a freezer.]
Standard solution: [NOTE—Condition the solid-phase extraction column specified for use in the *Standard solution* and the *Sample solution* by initially washing the column with 4.0 mL of a mixture of methylene chloride and isopropyl alcohol (4:1), followed by 5.0 mL of *Extraction solvent*. Do not allow the column to dry.]
Dilute a volume of the *Standard stock solution* with dehydrated alcohol to obtain a concentration of 5 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS. Prepare this solution fresh daily. Transfer 2.0 mL of this solution to a stoppered 125-mL flask. Add 15.0 mL of water and 15.0 mL of *Potassium hydroxide solution*, insert the stopper, and shake for 30 min in a water bath maintained at 60°. Allow to cool to room temperature, and transfer the contents of the flask to a 250-mL separatory funnel. Add 15.0 mL of water to the flask, insert the stopper, shake vigorously, and transfer this solution to the separatory funnel. Rinse the flask with 60 mL of *n*-hexane, and transfer the rinsing to the separatory funnel. Insert the stopper, shake vigorously for 90 s, and allow to stand for 15 min until the layers separate. Drain and discard the aqueous layer. Add 15.0 mL of water to the hexane layer in the separatory funnel, insert the stopper, and shake vigorously. Allow to stand for 10 min until the layers separate, and discard the aqueous layer. Add 1 drop of *Phenolphthalein solution* and 15.0 mL of water to the separatory funnel. Add *Diluted acetic acid* dropwise, with shaking, until the washing is neutral. Allow to stand for 10 min until the layers separate. Drain and discard the aqueous layer. Filter the hexane layer through anhydrous sodium sulfate supported by a small pledget of cotton into a 100-mL round-bottom flask. Rinse the funnel and sodium sulfate with a few mL of *n*-hexane, and collect the rinsings in the same flask. Evaporate the hexane in the flask on a rotary evaporator at 50° to dryness. Immediately add 2.0 mL of *Extraction solvent* to dissolve the residue. Transfer this solution to a freshly conditioned solid-phase extraction column containing silica packing with a sorbent mass-to-column volume ratio of 500 mg to 2.8 mL or equivalent, rinse the round-bottom flask with 1.0 mL of *Extraction solvent*, and transfer to the column. Elute the column with 2.0 mL of *Extraction solvent*, and discard this fraction. Elute the column with 7.0 mL of *Extraction solvent*, and collect the eluate in a suitable flask. Place the flask in a warm water bath maintained at 42°, and evaporate the solvent with the aid of a stream of nitrogen. Immediately add 2.0 mL of acetonitrile to the residue, and use the solution for injection into the chromatograph.
Sample solution: Proceed as directed for the *Sample solution* in *Vitamin A, Method 3*, through "calculate the net weight of the Capsule contents." Transfer a portion of the Capsule contents, equivalent to 10 µg of ergocalciferol or cholecalciferol, to a stoppered 125-mL flask, and proceed as directed for the *Standard solution*, beginning with "Add 15.0 mL of water and 15.0 mL of *Potassium hydroxide solution*".
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 265 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 27°
Flow rate: 0.7 mL/min
Injection size: 15 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 4.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas for vitamin D.
Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*
r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*

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C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

• **VITAMIN E, *Method 1***

[NOTE—Where vitamin E (alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Solution A: Phosphoric acid solution (1 in 100) in water

Mobile phase: Methanol and *Solution A* (19:1)

Standard solution: 2 mg/mL of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, or USP Alpha Tocopheryl Acid Succinate RS in methanol

System suitability solution: Prepare a 0.65-mg/mL solution of USP Ergocalciferol RS in methanol. Transfer 1.0 mL of this solution to a 100-mL volumetric flask containing 100 mg of USP Alpha Tocopheryl Acetate RS.Dissolve in 30 mL of methanol, with the aid of sonication if necessary, and dilute with methanol to volume. Store this solution in a refrigerator.

Sample solution: Proceed as directed for the *Sample stock solution* in *Vitamin A, Method 1*. Transfer NLT 20 mL of this solution retained as specified in the directions for the *Sample solution* in *Vitamin A, Method 1* to a suitable container, and evaporate if necessary, in vacuum at room temperature to dryness. Transfer the contents of the flask to a suitable volumetric flask with the aid of methanol, and dilute with methanol to volume, to obtain a concentration of 2 mg/mL of alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 8-mm × 10-cm; 5-µm packing L1

Flow rate: 2 mL/min

Injection size: 100 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

[NOTE—The relative retention times for ergocalciferol and alpha tocopheryl acetate are about 0.5 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 12 between ergocalciferol and alpha tocopheryl acetate, *System suitability solution*

Tailing factor: Between 0.8 and 1.2, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas.

Calculate the percentage of the labeled amount of alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*
r_S = peak area of the relevant vitamin E form from the *Standard solution*
C_S = concentration of the corresponding USP Reference Standard in the *Standard solution* (mg/mL)
C_U = nominal concentration of the corresponding form of vitamin E in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$)

• **VITAMIN E, *Method 2***

[NOTE—Where vitamin E (alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Mobile phase: Mix 240 mL of methanol with 10 mL of water followed by 0.5 mL of 50% phosphoric acid, and dilute with acetonitrile to 1000 mL.

System suitability solution: 2 mg/mL each of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, and USP Alpha Tocopheryl Acid Succinate RS in methanol

Standard solution: 2 mg/mL of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, or USP Alpha Tocopheryl Acid Succinate RS in methanol

3 N methanolic sulfuric acid solution: Cautiously mix sulfuric acid and methanol (9 in 100) in a 100-mL volumetric flask. [NOTE—Dissolve in a portion of methanol, cool, and then dilute to final volume.]

Sodium ascorbate–pyrogallol solution: Transfer 10 g of sodium ascorbate and 5 g of pyrogallol to a 100-mL volumetric flask. Add sufficient water to dissolve. Add 1.7 mL of sulfuric acid, and dilute with water to volume.

Lecithin solution: 5 mg/mL of lecithin in 2,2,4-trimethylpentane

Sample solution: Proceed as directed for the *Sample solution* in *Vitamin A, Method 2*, through "calculate the net weight of the Capsule contents." Transfer a portion of the Capsule contents, equivalent to 55 mg of vitamin E, to a container having a polytef-lined screw cap. Add 0.5 g of sodium bicarbonate, 1.5 mL of *Lecithin solution*, and 12.5 mL of 2,2,4-trimethylpentane, and disperse on a vortex mixer. Add 6 mL of *Sodium ascorbate–pyrogallol solution*, shake slowly, and allow the solution to degas. Continue shaking until the evolution of gas has ceased, and then shake for an additional 12 min. Add 6 mL of dimethyl sulfoxide, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 6 mL of *3 N methanolic sulfuric acid solution*, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 12.5 mL of 2,2,4-trimethylpentane, mix on a vortex mixer to form a suspension, and shake for 10 min. Centrifuge for 10 min to break up the emulsion and to clarify the supernatant layer. Transfer a volume of the supernatant 2,2,4-trimethylpentane layer to a suitable volumetric flask, the volume of the specimen withdrawn from the 2,2,4-trimethylpentane layer and the size of the volumetric flask being such that the final concentration of the *Sample solution* is equivalent to that of the *Standard solution*. Evaporate nearly to dryness, add several mL of methanol, and evaporate the remaining 2,2,4-trimethylpentane. Dilute with methanol to volume.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

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Flow rate: 1.5 mL/min
Injection size: 25 µL
System suitability
Samples: *Standard solution* and *System suitability solution*
[NOTE—The relative retention times for alpha tocopheryl acid succinate, alpha tocopherol, and alpha tocopheryl acetate are about 0.6, 0.8, and 1.0, respectively.]
Suitability requirements
Resolution: NLT 4.0 between alpha tocopheryl acid succinate and alpha tocopherol and NLT 3.0 between alpha tocopherol and alpha tocopheryl acetate, *System suitability solution*
Relative standard deviation: NMT 3.0%, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas.
Calculate the percentage of the labeled amount of alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_S) \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*
r_S = peak area of the relevant vitamin E form from the *Standard solution*
C_S = concentration of the corresponding USP Reference Standard in the *Standard solution* (mg/mL)
C_U = nominal concentration of the corresponding form of vitamin E in the *Sample solution* (mg/mL)

[NOTE—Account for the initial extraction volume of 26.5 mL of 2,2,4-trimethylpentane and the dilution factor to exchange the solvent from 2,2,4-trimethylpentane to methanol to calculate the nominal concentration.]
Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$)

• **VITAMIN E, *Method 3***
[NOTE—Where vitamin E (alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]
Diluent: Acetonitrile and ethyl acetate (1:1)
Mobile phase: Methanol, acetonitrile, and *n*-hexane (46.5: 46.5: 7.0)
Standard solution: 0.3 mg/mL of USP Alpha Tocopherol RS in methanol
Sample solution: Proceed as directed for the *Sample solution* in *Vitamin A, Method 3* through "calculate the net weight of the Capsule contents". Transfer a portion of the Capsule contents, equivalent to 8.0 mg of alpha tocopherol, to a glass-stoppered conical flask. Add 25.0 mL of water, 25.0 mL of dehydrated alcohol, and 3.5 g of potassium hydroxide pellets. Shake for 1 h in a water bath maintained at 55°, cool, and transfer with the aid of a minimum volume of water to a 125-mL separatory funnel. Rinse the flask with 50 mL of *n*-hexane, and add the rinsing to the separatory funnel. Insert the stopper, shake vigorously for 60 s, and allow the layers to separate. Drain the aqueous layer into a second 250-mL separatory funnel, and repeat the extraction with 50 mL of *n*-hexane. Discard the aqueous layer and combine the hexane extracts. Wash the combined extracts with 25 mL of water, allow the layers to separate, and discard the aqueous layer. Add 3 drops of glacial acetic acid, and repeat the washing procedure two more times. Filter the washed hexane layer through anhydrous sodium sulfate into a 250-mL round-bottom flask. Rinse the funnel and sodium sulfate with a few mL of *n*-hexane, and add the rinsing to the hexane solution in the flask. Place the flask in a water bath maintained at 50°, and evaporate the hexane solution with the aid of a rotary evaporator to dryness. Immediately add 25.0 mL of *Diluent*, and swirl to dissolve the residue.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 291 nm
Column: 4.6-mm × 25-cm; packing L1
Column temperature: 40°
Flow rate: 3 mL/min
Injection size: 20 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 5.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of alpha tocopherol from the *Sample solution*
r_S = peak area of alpha tocopherol from the *Standard solution*
C_S = concentration of alpha tocopherol in the *Standard solution* (mg/mL)
C_U = nominal concentration of alpha tocopherol the *Sample solution* (mg/mL)

[NOTE—Calculate the content of alpha tocopheryl acetate ($C_{31}H_{52}O_3$) or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$) by dividing the content, in mg/Capsule of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$), by the factor 0.91 or 0.81, respectively.]
Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$) or alpha tocopheryl succinate ($C_{33}H_{54}O_5$)

• **PHYTONADIONE**
[NOTE—Use low-actinic glassware throughout this procedure.]
Mobile phase: Methanol and water (19:1)
Standard stock solution: 200 µg/mL of USP Phytonadione RS in methanol. Dissolve with the aid of sonication if necessary.
Standard solution: 20 µg/mL of USP Phytonadione RS from the *Standard stock solution* diluted with methanol
System suitability solution: 0.65 mg/mL of USP Alpha Tocopheryl Acetate RS and 20 µg/mL of USP Phytonadione RS from the *Standard stock solution* diluted with methanol. [NOTE—Dissolve USP Alpha Tocopheryl Acetate RS in a portion of methanol, add the *Standard stock solution*, and then dilute with methanol to volume.]
Sample solution: Proceed as directed for the *Sample stock solution* in *Vitamin A, Method 1*. Transfer NLT 20 mL of this solution retained as specified in the directions for the

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Sample solution in *Vitamin A, Method 1* to a suitable container, and evaporate, if necessary, in vacuum at room temperature to dryness. Transfer the contents of the flask to a suitable volumetric flask with the aid of methanol, and dilute with methanol to volume to obtain a concentration of 20 µg/mL of phytonadione.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 8-mm × 10-cm; 5-µm packing L1
Flow rate: 2 mL/min
Injection size: 100 µL

System suitability
Samples: *Standard solution* and *System suitability solution*
[NOTE—The relative retention times for alpha tocopheryl acetate and phytonadione are about 0.68 and 1.0, respectively.]
Suitability requirements
Resolution: NLT 5 between alpha tocopheryl acetate and phytonadione, *System suitability solution*
Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas.
Calculate the percentage of the labeled amount of phytonadione ($C_{31}H_{46}O_2$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of phytonadione from the *Sample solution*
r_S = peak area of phytonadione from the *Standard solution*
C_S = concentration of USP Phytonadione RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of phytonadione in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of phytonadione ($C_{31}H_{46}O_2$)

• **BETA CAROTENE**
[NOTE—Use low-actinic glassware throughout this procedure.]
Potassium hydroxide solution: Dissolve 58.8 g of potassium hydroxide in 50 mL of water.
Iodine solution: Transfer 10 mg of iodine to a 100-mL volumetric flask. Dissolve in cyclohexane, and dilute with cyclohexane to volume. Dilute 10 mL of this solution with cyclohexane to 100 mL. [NOTE—Prepare this solution fresh daily.]
Sample solution A (for preparations containing beta carotene in oil solutions): Proceed as directed in *Vitamin A, Method 1*, except use cyclohexane instead of *n*-hexane as the extraction solvent, and dilute the filtered extracts with cyclohexane to obtain a concentration of 2 µg/mL of beta carotene.
Sample solution B (for preparations containing beta carotene in dry powder): Remove the contents of NLT 20 Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the Capsule contents, equivalent to 2 mg of beta carotene, to a 500-mL saponification flask. Add 100 mL of alcohol, 6 mL of *Potassium hydroxide solution*, and a magnetic stirring bar. Attach an air condenser to the flask, and heat under reflux for 45 min with constant stirring. Cool to room temperature. Add 170 mL of solvent hexane, and stir for 30 min. Quantitatively transfer the contents of the flask to a 500-mL separatory funnel with portions of solvent hexane. Allow the layers to separate for 5–10 min, and transfer the upper organic layer to a 500-mL volumetric flask. Transfer the lower aqueous layer into the saponification flask. Add 170 mL of solvent hexane, and stir for an additional 20 min. Quantitatively transfer the contents of the saponification flask to the separatory funnel with the aid of portions of solvent hexane. Allow the layers to separate for 10 min. Drain the lower aqueous layer, and discard. Transfer the organic layer to the volumetric flask containing the previously collected organic layer. Rinse the separatory funnel with small portions of solvent hexane, and transfer the washings to the volumetric flask. Dilute the hexane extracts with solvent hexane to volume. Add 3 g of anhydrous sodium sulfate, shake, and allow to settle. Quantitatively transfer a volume of this solution, equivalent to 100 µg of beta carotene, to a 50-mL volumetric flask. Evaporate under a stream of nitrogen to dryness, and immediately add cyclohexane. Add 2 mL of *Iodine solution*, and heat for 15 min in a water bath maintained at 65°. Cool rapidly, and dilute with cyclohexane to volume.

Spectrometric conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: Vis
Analytical wavelength: 452 nm
Blank: Cyclohexane

Analysis
Sample: *Sample solution A* or *Sample solution B*
Determine the absorbance against the *Blank*.
Calculate the percentage of the labeled amount of beta carotene ($C_{40}H_{56}$) in the portion of Capsules taken:

$$\text{Result} = (A_U/F) \times (100/C_U)$$

A_U = absorbance of *Sample solution A* or *Sample solution B*
F = absorptivity of beta carotene at 452 nm, 223
C_U = nominal concentration of beta carotene in *Sample solution A* or *Sample solution B* (mg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of beta carotene ($C_{40}H_{56}$)

PERFORMANCE TESTS
• **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** ⟨2040⟩: Meet the requirements
• **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩: Meet the requirements

CONTAMINANTS
• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic microbial count does not exceed 3000 cfu/g, and the total combined molds and yeasts count does not exceed 300 cfu/g.
• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩: Meet the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

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• **LABELING:**[1] Label the Capsules to state that the product is Oil-Soluble Vitamins Capsules. The label also states the quantity of each vitamin/dosage unit and, where necessary, the chemical form in which it is present. Where the product contains vitamin E, the label also indicates whether it is the *d*- or *dl*- form. Where more than one assay method is given for a particular vitamin, the labeling states the assay method used only if *Method 1* is not used.

• **USP REFERENCE STANDARDS** ⟨11⟩
USP Alpha Tocopherol RS
USP Alpha Tocopheryl Acetate RS
USP Alpha Tocopheryl Acid Succinate RS
USP Cholecalciferol RS
USP Ergocalciferol RS
USP Phytonadione RS
USP Vitamin A RS

Oil-Soluble Vitamins Oral Solution

DEFINITION

Oil-Soluble Vitamins Oral Solution contains two or more of the following oil-soluble vitamins: Vitamin A, as retinol or esters of retinol in the form of retinyl acetate or retinyl palmitate; Vitamin D, as ergocalciferol (Vitamin D_2) or cholecalciferol (Vitamin D_3); Vitamin E, as alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate; Phytonadione (Vitamin K_1); and beta carotene. It contains NLT 90.0% and NMT 150.0% of the labeled amounts of vitamin A, as retinol equivalent ($C_{20}H_{30}O$); vitamin D, as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$); vitamin E, as alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$); phytonadione ($C_{31}H_{46}O_2$); and beta carotene ($C_{40}H_{56}$).

STRENGTH

• **VITAMIN A**
[NOTE—Use low-actinic glassware throughout this procedure.]
Mobile phase: *n*-Hexane
Standard solution 1: 13 µg/mL of retinol from USP Retinyl Acetate RS in *n*-hexane
Standard solution 2: 13 µg/mL of retinol from USP Retinyl Palmitate RS in *n*-hexane
System suitability solution: Mix equal volumes of *Standard solution 1* and *Standard solution 2*.
Sample solution: Equivalent to 13 µg/mL of retinol from an accurately measured volume of Oral Solution in *n*-hexane
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 325 nm
Column: 4.6-mm × 15-cm; packing L8
Flow rate: 1 mL/min
Injection volume: 40 µL
System suitability
Sample: *System suitability solution*
Suitability requirements
Resolution: NLT 10 between all-*trans*-retinyl acetate and all-*trans*-retinyl palmitate
Relative standard deviation: NMT 3.0%
Analysis
Samples: *Standard solution 1* or *Standard solution 2* and *Sample solution*
Calculate the percentage of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$), in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the all-*trans*-retinyl ester from the *Sample solution*
r_S = peak area of the all-*trans*-retinyl ester from the appropriate *Standard solution*
C_S = concentration of retinol in the appropriate *Standard solution* (µg/mL)
C_U = nominal concentration of vitamin A, as retinol, in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of vitamin A as retinol equivalent ($C_{20}H_{30}O$)

• **VITAMIN D**
[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]
Mobile phase: *n*-Hexane and isopropyl alcohol (99:1)
Standard solution: 2 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in *n*-hexane
System suitability solution: Heat a volume of the *Standard solution* at 60° for 1 h to partially isomerize vitamin D (cholecalciferol or ergocalciferol) to its corresponding precursor.
Sample stock solution: Equivalent to 20 µg/mL of cholecalciferol or ergocalciferol from an accurately measured volume of Oral Solution in *n*-hexane
Sample solution: Transfer 5.0 mL of the *Sample stock solution* to a container having a polytef-lined screw cap and heat, with constant shaking, for 1 h in a water bath maintained at 60° to obtain a solution containing vitamin D (cholecalciferol or ergocalciferol) and its corresponding precursor. Cool, and dilute with *n*-hexane to obtain a solution containing 2 µg/mL of cholecalciferol or ergocalciferol.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 265 nm
Column: 4.6-mm × 15-cm; 3-µm packing L8
Flow rate: 1 mL/min
Injection volume: 100 µL
System suitability
Sample: *System suitability solution*
Suitability requirements
Resolution: NLT 10 between the vitamin D form present and its corresponding precursor

[1] USP Units of activity for vitamins, where such exist or formerly existed, are equivalent to the corresponding international units, where such formerly existed. The USP Unit for Vitamin E has been discontinued. International units (IU) for vitamins also have been discontinued; however, the use of IU on the labels of vitamin products continues. Where articles are labeled in terms of Units in addition to the required labeling, the relationship of the USP Units or IU to mass is as follows. One USP Vitamin A Unit = 0.3 µg of all-*trans*-retinol (vitamin A alcohol) or 0.344 µg of all-*trans*-retinyl acetate (vitamin A acetate) or 0.55 µg of all-*trans*-retinyl palmitate (vitamin A palmitate), and 1 µg of retinol (3.3 USP Vitamin A Units) = 1 retinol equivalent (RE); 1 IU of beta carotene = 0.6 µg of all-*trans*-beta carotene; 1 USP Vitamin D Unit = 0.025 µg of ergocalciferol or cholecalciferol; and 1 mg of *dl*-alpha tocopherol = 1.1 former USP Vitamin E Units, 1 mg of *dl*-alpha tocopheryl acetate = 1 former USP Vitamin E Unit, 1 mg of *dl*-alpha tocopheryl acid succinate = 0.89 former USP Vitamin E Unit, 1 mg of *d*-alpha tocopherol = 1.49 former USP Vitamin E Units, and 1 mg of *d*-alpha tocopheryl acetate = 1.36 former USP Vitamin E Units, 1 mg of *d*-alpha tocopheryl acid succinate = 1.21 former USP Vitamin E Units. In terms of *d*-alpha tocopherol equivalents, 1 mg of *d*-alpha tocopheryl acetate = 0.91, 1 mg of *d*-alpha tocopheryl acid succinate = 0.81, 1 mg of *dl*-alpha tocopherol = 0.74, 1 mg of *dl*-alpha tocopheryl acetate = 0.67, and 1 mg of *dl*-alpha tocopheryl acid succinate = 0.60.

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Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for vitamin D. Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*

r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*

C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

F = correction factor to account for the average amount of pre-vitamin D present in the *Sample solution*, 1.09

Acceptance criteria: 90.0%–150.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

• **VITAMIN E**

[NOTE—Where vitamin E (alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Solution A: Phosphoric acid solution in water (1 in 100)

Mobile phase: Methanol and *Solution A* (19:1)

Standard solution: 2 mg/mL of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, or USP Alpha Tocopheryl Acid Succinate RS in methanol

Sample solution: Equivalent to 2.0 mg/mL of alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate from an accurately measured volume of Oral Solution in methanol

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 291 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Flow rate: 1 mL/min

Injection volume: 50 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Tailing factor: NMT 1.5

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*

r_S = peak area of the relevant vitamin E form from the *Standard solution*

C_S = concentration of the corresponding USP Reference Standard in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding form of vitamin E in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of vitamin E

• **PHYTONADIONE (VITAMIN K_1)**

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Methanol and water (19:1)

Standard stock solution: 200 µg/mL of USP Phytonadione RS in methanol. Dissolve with the aid of sonication if necessary.

Standard solution: 20 µg/mL of USP Phytonadione RS from the *Standard stock solution* diluted with methanol

System suitability solution: 0.65 mg/mL of USP Alpha Tocopheryl Acetate RS and 20 µg/mL of USP Phytonadione RS from the *Standard stock solution* diluted with methanol. [NOTE—Dissolve USP Alpha Tocopheryl Acetate RS in a portion of methanol, add the *Standard stock solution*, and then dilute with methanol to volume.]

Sample solution: Equivalent to 20 µg/mL of phytonadione from an accurately measured volume of Oral Solution in methanol

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Flow rate: 1 mL/min

Injection volume: 50 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 5 between alpha tocopheryl acetate and phytonadione, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of phytonadione ($C_{31}H_{46}O_2$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Phytonadione RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of phytonadione in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of phytonadione ($C_{31}H_{46}O_2$)

• **BETA CAROTENE**

[NOTE—Use low-actinic glassware.]

Mobile phase: Transfer 50 mg of butylated hydroxytoluene into a 1-L volumetric flask, and dissolve with 20 mL of 2-propanol. Add 0.2 mL of *N*-ethyldiisopropylamine, 25 mL of 0.2% ammonium acetate solution, 455 mL of acetonitrile, and about 450 mL of methanol. Allow the solution to reach room temperature, and dilute with methanol to volume.

Diluent: 50 µg/mL of butylated hydroxytoluene in alcohol

System suitability solution: Transfer 20 mg of USP Beta Carotene System Suitability RS to a 50-mL volumetric flask.

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Add 1 mL of water, 4 mL of tetrahydrofuran, and sonicate for 5 min. Dilute with *Diluent* to volume and sonicate for 5 min. Cool to room temperature, pass the suspension through a membrane filter of 0.45-µm pore size, and use the clear filtrate.

Standard stock solution: 60 µg/mL of USP Beta Carotene RS in tetrahydrofuran

Standard solution A: Transfer 5.0 mL of the *Standard stock solution* into a 100-mL volumetric flask, add 5.0 mL of tetrahydrofuran, and dilute with *Diluent* to volume. Determine the concentration of *Standard solution A* from the concentration of *Standard solution B* as described below.

Standard solution B: Transfer 5.0 mL of the *Standard stock solution* into a 100-mL volumetric flask, and dilute with cyclohexane to volume. Prepare in triplicate.

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Analytical wavelength: 457 nm

Cell path: 1 cm

Blank: Cyclohexane

Analysis

Sample: *Standard solution B*

Calculate the concentration of total beta carotene (mg/mL) as all-*trans*-beta carotene ($C_{40}H_{56}$) in *Standard solution B*. [NOTE—The concentration of *Standard solution B* equals the concentration of *Standard solution A*.]

$$\text{Result} = A/F$$

A = average absorbance of the three preparations of *Standard solution B*

F = absorptivity of pure all-*trans*-beta carotene in cyclohexane, 250

Sample stock solution: Transfer an accurately measured volume of Oral Solution equivalent to 20 mg of beta carotene to a 250-mL volumetric flask. Add 250 mg of butylated hydroxytoluene, 120 mL of methylene chloride, and 100 mL of alcohol. Shake the flask until the sample is completely dissolved or suspended. Let the mixture stand in the dark until it reaches room temperature (about 2 h). Add methylene chloride to volume, and shake again vigorously.

Sample solution: Dilute a volume of the *Sample stock solution* with a mixture of methylene chloride and *Diluent* (1:1) to obtain the final concentration of beta carotene of 3 µg/mL. Pass through a membrane filter of 0.45-µm pore size.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV-Vis 448 nm

Column: 4.6-mm × 25-cm; 5-µm packing L68

Column temperature: 30°

Flow rate: 0.6 mL/min

Injection volume: 20 µL

System suitability

Samples: *System suitability solution* and *Standard solution A*

[NOTE—The approximate relative retention times of the components in the *System suitability solution* are listed in *Table 1*.]

Table 1

Name	Relative Retention Time	Relative Response Factor
All-*trans*-alpha carotene	0.93	1.1
All-*trans*-beta carotene	1.00	1
9-*cis*-Beta carotene	1.07	1
13-*cis*-Beta carotene	1.17	1.2
15-*cis*-Beta carotene	1.21	1.4

Suitability requirements

Chromatogram similarity: The chromatogram from the *System suitability solution* is similar to the reference chromatogram provided with the lot of USP Beta Carotene System Suitability RS being used.

Resolution: NLT 1.5 between beta carotene and alpha carotene and between beta carotene and 9-*cis*-beta carotene, *System suitability solution*

Tailing factor: NMT 2.0 for the beta carotene peak, *Standard solution A*

Relative standard deviation: NMT 2.0% for the beta carotene peak from replicate injections, *Standard solution A*

Analysis

Samples: *Standard solution A* and *Sample solution*

Calculate the percentage of all-*trans*-beta carotene in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of all-*trans*-beta carotene from the *Sample solution*

r_S = peak area of all-*trans*-beta carotene from *Standard solution A*

C_S = concentration of all-*trans*-beta carotene in *Standard solution A* as determined by the spectrometric procedure

C_U = nominal concentration of beta carotene in the *Sample solution*

Acceptance criteria: 90.0%–150.0% of the labeled amount of beta carotene ($C_{40}H_{56}$)

OTHER COMPONENTS

- **ALCOHOL DETERMINATION,** *Method I* ⟨611⟩ (if present)**:** 90.0%–120.0% of the labeled amount of C_2H_5OH

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 3×10^3 cfu/mL, and the combined molds and yeasts count does not exceed 3×10^2 cfu/mL.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species, and *Escherichia coli*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, under an inert gas or with a minimum of headspace.

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- **LABELING:**[1] The label states that the product is Oil-Soluble Vitamins Oral Solution. The label states the quantity of each vitamin present in a given volume of Oral Solution and, where necessary, the chemical form in which a vitamin is present. Where the product contains vitamin E, the label indicates whether it is the *d-* or *dl-* form.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Alpha Tocopherol RS
  USP Alpha Tocopheryl Acetate RS
  USP Alpha Tocopheryl Acid Succinate RS
  USP Beta Carotene RS
  USP Beta Carotene System Suitability RS
  USP Cholecalciferol RS
  USP Ergocalciferol RS
  USP Phytonadione RS
  USP Retinyl Acetate RS
  USP Retinyl Palmitate RS

Oil-Soluble Vitamins Tablets

DEFINITION

Change to read:

Oil-Soluble Vitamins Tablets contain 2 or more of the following oil-soluble vitamins: Vitamin A ▲as retinyl acetate or retinyl palmitate,▲ (USP 1-May-2020) Vitamin D as Ergocalciferol (vitamin D_2) or Cholecalciferol (vitamin D_3), Vitamin E ▲as *RRR-* or *all-rac*-alpha-tocopherol, *RRR-* or *all-rac*-alpha-tocopheryl acetate, or *RRR-* or *all-rac*-alpha-tocopheryl acid succinate,▲ (USP 1-May-2020) Phytonadione (vitamin K_1), and Beta Carotene. Tablets contain NLT 90.0% and NMT 165.0% of the labeled amount of vitamin A ▲as retinol▲ (USP 1-May-2020) ($C_{20}H_{30}O$), vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$), vitamin E ▲as 2*R*-alpha-tocopherol▲ (USP 1-May-2020) ($C_{29}H_{50}O_2$), phytonadione ($C_{31}H_{46}O_2$), and beta carotene ($C_{40}H_{56}$).

Oil-Soluble Vitamins Tablets contain no other vitamins or any minerals. They may contain other labeled added substances that are generally recognized as safe, in amounts that are unobjectionable.

[1] USP Units of activity for vitamins, where such exist or formerly existed, are equivalent to the corresponding international units, where such formerly existed. The USP Unit for Vitamin E has been discontinued. International units (IU) for vitamins also have been discontinued; however, the use of IU on the labels of vitamin products continues. Where articles are labeled in terms of Units in addition to the required labeling, the relationship of the USP Units or IU to mass is as follows. One USP Vitamin A Unit = 0.3 µg of all-*trans*-retinol (vitamin A alcohol) or 0.344 µg of all-*trans*-retinyl acetate (vitamin A acetate) or 0.55 µg of all-*trans*-retinyl palmitate (vitamin A palmitate), and 1 µg of retinol (3.3 USP Vitamin A Units) = 1 retinol equivalent (RE); 1 IU of beta carotene = 0.6 µg of all-*trans*-beta carotene; 1 USP Vitamin D Unit = 0.025 µg of ergocalciferol or cholecalciferol; and 1 mg of *dl*-alpha tocopherol = 1.1 former USP Vitamin E Units, 1 mg of *dl*-alpha tocopheryl acetate = 1 former USP Vitamin E Unit, 1 mg of *dl*-alpha tocopheryl acid succinate = 0.89 former USP Vitamin E Unit, 1 mg of *d*-alpha tocopherol = 1.49 former USP Vitamin E Units, 1 mg of *d*-alpha tocopheryl acetate = 1.36 former USP Vitamin E Units, and 1 mg of *d*-alpha tocopheryl acid succinate = 1.21 former USP Vitamin E Units. In terms of *d*-alpha tocopherol equivalents, 1 mg of *d*-alpha tocopheryl acetate = 0.91, 1 mg of *d*-alpha tocopheryl acid succinate = 0.81, 1 mg of *dl*-alpha tocopherol = 0.74, 1 mg of *dl*-alpha tocopheryl acetate = 0.67, and 1 mg of *dl*-alpha tocopheryl acid succinate = 0.60.

IDENTIFICATION

Add the following:

▲• **A.** The retention times of the vitamin peaks of the *Sample solutions* correspond to those of the corresponding vitamin peaks of the *Standard solutions* as obtained in the tests for *Strength*.▲ (USP 1-May-2020)

STRENGTH

[NOTE—In the following assays, where more than one assay method is given for an individual ingredient, the requirements may be met by following any one of the specified methods, with the method used being stated in the labeling only if *Method 1* is not used.]

Change to read:

- **VITAMIN A, *Method 1***
  ▲Proceed as directed in *Vitamin A Assay* ⟨571⟩, *Assay, Chromatographic Methods, Procedure 1*, except for the *Sample stock solution* and *Sample solution*.▲ (USP 1-May-2020)
  Sample stock solution: Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 5 Tablets, to a container having a polytef-lined screw-cap. Add 10 mL of dimethyl sulfoxide and 15 mL of *n*-hexane, and shake for 45 min on a wrist-action shaker in a water bath maintained at 60°. [NOTE—Set up the wrist-action shaker to ensure that the contents of the container are mixed vigorously and thoroughly.] Centrifuge at 3000 rpm for 10 min, and transfer the hexane layer by means of a pipet to a 100-mL volumetric flask. Add 15 mL of *n*-hexane to the dimethyl sulfoxide layer, shake thoroughly for 5 min, and transfer the hexane layer by means of a pipet to the 100-mL volumetric flask. Repeat this extraction with 3 additional 15-mL portions of *n*-hexane. Dilute the extracts in the volumetric flask with *n*-hexane to volume.
  Sample solution: Dilute a 10-mL volume of the *Sample stock solution* with *n*-hexane to obtain a solution with a concentration of 15 µg/mL of vitamin A as retinol ($C_{20}H_{30}O$).
  ▲▲ (USP 1-May-2020)
  Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$)

Change to read:

- **VITAMIN A, *Method 2***
  ▲Proceed as directed in *Vitamin A Assay* ⟨571⟩, *Assay, Chromatographic Methods, Procedure 2*.▲ (USP 1-May-2020)
  Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$)

Change to read:

- **VITAMIN A, *Method 3***
  ▲Proceed as directed in *Vitamin A Assay* ⟨571⟩, *Assay, Chromatographic Methods, Procedure 3*.▲ (USP 1-May-2020)
  Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$)

Change to read:

- **VITAMIN D (CHOLECALCIFEROL OR ERGOCALCIFEROL), *Method 1***
  ▲Proceed as directed in *Vitamin D Assay* ⟨581⟩, *Assay, Chromatographic Methods, Procedure 1*, except for the *Sample solution*.▲ (USP 1-May-2020)

DS Monographs

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Sample solution: Prepare as directed for the *Sample stock solution* in *Vitamin A, Method 1*. Transfer NLT 20 mL of this solution to a suitable container, and, if necessary, evaporate under vacuum at room temperature to obtain a concentration of 2 µg/mL of cholecalciferol or ergocalciferol.
▲ ▲ (USP 1-May-2020)
Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

Change to read:

- **VITAMIN D (CHOLECALCIFEROL OR ERGOCALCIFEROL), *Method 2***

▲Proceed as directed in *Vitamin D Assay* ⟨581⟩, *Assay, Chromatographic Methods, Procedure 2*.▲ (USP 1-May-2020)
Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

Change to read:

- **VITAMIN D (CHOLECALCIFEROL OR ERGOCALCIFEROL), *Method 3***

▲Proceed as directed in *Vitamin D Assay* ⟨581⟩, *Assay, Chromatographic Methods, Procedure 3*, except for the *Analysis*.▲ (USP 1-May-2020)
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas of vitamin D.
Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*
r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*
C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

Change to read:

- **VITAMIN E, *Method 1***

▲Proceed as directed in *Vitamin E Assay* ⟨551⟩, *Assay, Procedure 1*, except for the *Sample solution* and *Analysis*.▲ (USP 1-May-2020)
Sample solution: Prepare as directed for the *Sample stock solution* in *Vitamin A, Method 1*. Transfer NLT 20 mL of this solution to a suitable container, and, if necessary, evaporate under vacuum at room temperature to dryness. Transfer the residue to a suitable volumetric flask with the aid of methanol, and dilute with methanol to volume to obtain a concentration of 2 mg/mL of alpha-tocopherol, alpha-tocopheryl acetate, or alpha-tocopheryl acid succinate.
▲ ▲ (USP 1-May-2020)
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas.
Calculate the percentage of the labeled amount of alpha-tocopherol ($C_{29}H_{50}O_2$), alpha-tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha-tocopheryl acid succinate ($C_{33}H_{54}O_5$) ▲as *2R*-alpha-tocopherol ($C_{29}H_{50}O_2$)▲ (USP 1-May-2020) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times {}^{\blacktriangle}F_{\blacktriangle\ (\text{USP 1-May-2020})} \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*
r_S = peak area of the relevant vitamin E form from the *Standard solution*
C_S = concentration of ▲*all-rac*-alpha-tocopherol from either USP Alpha Tocopherol RS or USP Alpha Tocopheryl Acetate RS or *RRR*-alpha-tocopherol from USP Alpha Tocopheryl Acid Succinate RS▲ (USP 1-May-2020) in the *Standard solution* (mg/mL)
C_U = nominal concentration of the corresponding form of vitamin E ▲as *2R*-alpha-tocopherol▲ (USP 1-May-2020) in the *Sample solution* (mg/mL)
▲F = conversion factor for the content of *all-rac*-alpha-tocopherol to *2R*-alpha-tocopherol, 1/2 (for products labeled to contain *all-rac* vitamin E sources) and 1 (for products labeled to contain *RRR* vitamin E sources)▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–165.0% of the labeled amount of alpha-tocopherol ($C_{29}H_{50}O_2$), alpha-tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha-tocopheryl acid succinate ($C_{33}H_{54}O_5$) as ▲*2R*-alpha-tocopherol▲ (USP 1-May-2020)

Change to read:

- **VITAMIN E, *Method 2***

▲Proceed as directed in *Vitamin E Assay* ⟨551⟩, *Assay, Procedure 2*, except for the *Analysis*.
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of alpha-tocopherol ($C_{29}H_{50}O_2$), alpha-tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha-tocopheryl acid succinate ($C_{33}H_{54}O_5$) as *2R*-alpha-tocopherol ($C_{29}H_{50}O_2$) in the portion of Tablets taken as follows:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*
r_S = peak area of the relevant vitamin E form from the *Standard solution*
C_S = concentration of *all-rac*–alpha-tocopherol from either USP Alpha Tocopherol RS or USP Alpha Tocopheryl Acetate RS or *RRR*-alpha-tocopherol from USP Alpha Tocopheryl Acid Succinate RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of the corresponding form of vitamin E as *2R*-alpha-tocopherol in the *Sample solution* (mg/mL)
F = conversion factor for the content of *all-rac*-alpha-tocopherol to *2R*-alpha-tocopherol, 1/2 (for products labeled to contain *all-rac* vitamin E sources) and 1 (for products labeled to contain *RRR* vitamin E sources)▲ (USP 1-May-2020)

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Acceptance criteria: 90.0%–165.0% of the labeled amount of alpha-tocopherol ($C_{29}H_{50}O_2$), alpha-tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha-tocopheryl acid succinate ($C_{33}H_{54}O_5$) as ▲2*R*-alpha-tocopherol▲ (USP 1-May-2020)

Change to read:

- **VITAMIN E, *Method 3***

▲Proceed as directed in *Vitamin E Assay* ⟨551⟩, *Assay, Procedure 3*, except for the *Analysis*.

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of alpha-tocopherol ($C_{29}H_{50}O_2$), alpha -ocopheryl acetate ($C_{31}H_{52}O_3$), or alpha-tocopheryl acid succinate ($C_{33}H_{54}O_5$) as 2*R*-alpha-tocopherol ($C_{29}H_{50}O_2$) in the portion of Tablets taken as follows:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*

r_S = peak area of the relevant vitamin E form from the *Standard solution*

C_S = concentration of *all-rac*-alpha-tocopherol from either USP Alpha Tocopherol RS or USP Alpha Tocopheryl Acetate RS or *RRR*-alpha-tocopherol from USP Alpha Tocopheryl Acid Succinate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding form of vitamin E as 2*R*-alpha-tocopherol in the *Sample solution* (mg/mL)

F = conversion factor for the content of *all-rac*-alpha-tocopherol to 2*R*-alpha-tocopherol, 1/2 (for products labeled to contain *all-rac* vitamin E sources) and 1 (for products labeled to contain *RRR* vitamin E sources)▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–165.0% of the labeled amount of alpha-tocopherol ($C_{29}H_{50}O_2$), alpha-tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha-tocopheryl acid succinate ($C_{33}H_{54}O_5$) as ▲2*R*-alpha-tocopherol▲ (USP 1-May-2020)

- **PHYTONADIONE, *Method 1***

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Methanol and water (19:1)

Standard stock solution: 200 µg/mL of USP Phytonadione RS in methanol. Dissolve with the aid of sonication if necessary.

Standard solution: 20 µg/mL of USP Phytonadione RS from *Standard stock solution* diluted with methanol

System suitability solution: 0.65 mg/mL of USP Alpha Tocopheryl Acetate RS and 20 µg/mL of USP Phytonadione RS from *Standard stock solution* diluted with methanol. [NOTE—Dissolve USP Alpha Tocopheryl Acetate RS in a portion of methanol, add the *Standard stock solution*, and then dilute with methanol to volume.]

Sample solution: Prepare as directed for the *Sample stock solution* in *Vitamin A, Method 1*. Transfer NLT 20 mL of this solution to a suitable container, and evaporate under vacuum at room temperature to dryness. Transfer the residue with the aid of methanol to a suitable volumetric flask, and dilute with methanol to volume to obtain a concentration of 20 µg/mL of phytonadione.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 8-mm × 10-cm; 5-µm packing L1

Flow rate: 2 mL/min

Injection volume: 100 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

[NOTE—The relative retention times for alpha-tocopheryl acetate and phytonadione are 0.68 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 5 between alpha-tocopheryl acetate and phytonadione, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas.

Calculate the percentage of the labeled amount of phytonadione ($C_{31}H_{46}O_2$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of phytonadione from the *Sample solution*

r_S = peak area of phytonadione from the *Standard solution*

C_S = concentration of USP Phytonadione RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of phytonadione in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of phytonadione ($C_{31}H_{46}O_2$)

- **PHYTONADIONE, *Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Solvent: Methanol and isopropyl alcohol (19:1)

Mobile phase: Mix 800 mL of methanol, 200 mL of methylene chloride, 0.1 mL of glacial acetic acid, 1.36 g of zinc chloride, and 0.41 g of sodium acetate.

Internal standard solution: 5 µg/mL of menaquinone 4 (vitamin K_2) in *Solvent*. [NOTE—A concentrated stock solution of menaquinone 4 (100 µg/mL) can be stored for 2 months in a refrigerator.]

Standard stock solution: 5 µg/mL of USP Phytonadione RS, prepared by dissolving in methylene chloride with the aid of sonication, and diluting with *Solvent* to final volume

Standard solution: Transfer 1.0 mL of the *Standard stock solution* and 1.0 mL of the *Internal standard solution* to a suitable flask and dilute with *Solvent* to 5 mL. Pass through a membrane filter of 0.45-µm or finer pore size.

Sample solution: Finely powder NLT 20 Tablets. To a centrifuge tube, fitted with a cap, transfer an amount of powder, not exceeding 800 mg and equivalent to an amount of phytonadione not exceeding 50 µg. Add 4 mL of water. Insert the stopper, and mix using a vortex mixer until the sample is dispersed. Place the tube in a water bath at 60° for 5 min. Remove from the bath, and again shake or mix using a vortex mixer for 1 min while the preparation is still hot. Add 8 mL of alcohol, and swirl the contents to mix. Place the tube in a water bath at 60° for 5 min. Remove from the bath, and again shake or mix using a vortex mixer for 2 min while the preparation is still hot. Cool to room temperature. Add a volume of *Internal standard solution*, equivalent to 1.0 mL per each 5 µg of the expected amount of phytonadione in the aliquot taken. Add 20.0 mL of petroleum ether, and cap the tube tightly. Shake or mix using a vortex mixer for 15 min to thoroughly mix the contents. Centrifuge to separate the 2 layers. Transfer a

Supplement 2

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volume of the top layer of petroleum ether, equivalent to 5–50 µg of the nominal amount of phytonadione, to an appropriate flask. Place the flask in a water bath at 35°–45°, and evaporate the solvent under a stream of nitrogen until an oily residue is left. Dissolve the residue in a volume of *Solvent* to obtain a concentration of 1 µg/mL of phytonadione.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Fluorometric
Excitation wavelength: 320 nm
Emission wavelength: 420 nm
Column: 4.6-mm × 25-cm; 5-µm, end-capped packing L1, and a postcolumn reactor constituted with a 4.6-mm × 3-cm PEEK column tightly packed with zinc powder. [NOTE—Prepare the postcolumn reactor daily, or as necessary, to meet the *System suitability* requirements.]
Flow rate: 1 mL/min
Injection volume: 25 µL
System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for the internal standard and phytonadione are 1.0 and 1.4, respectively.]
Suitability requirements
Column efficiency: NLT 2500 theoretical plates for the phytonadione peak
Tailing factor: NMT 1.5 for the phytonadione peak
Relative standard deviation: NMT 3.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of phytonadione ($C_{31}H_{46}O_2$) in the portion of Tablets taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of phytonadione to that of the internal standard from the *Sample solution*
R_S = peak response ratio of phytonadione to that of the internal standard from the *Standard solution*
C_S = concentration of USP Phytonadione RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of phytonadione in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of labeled amount of phytonadione ($C_{31}H_{46}O_2$)

Add the following:

▲• **VITAMIN A, VITAMIN D,** and **VITAMIN E,** ***Method 4;*** **PHYTONADIONE,** ***Method 3***
[NOTE—Where vitamin A (retinyl acetate or retinyl palmitate), vitamin D (cholecalciferol or ergocalciferol), or vitamin E (alpha-tocopherol, alpha-tocopheryl acetate, or alpha-tocopheryl acid succinate) are specified in the following procedure, use the chemical form present in the formulation and the relevant Reference Standard. Use low-actinic glassware]
Diluent: Methanol and water (90:10 v/v)
Solution A: 0.1% (v/v) trifluoroacetic acid in *Diluent*
Solution B: 0.1% (v/v) trifluoroacetic acid in methanol
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
19	0	100
27	0	100
28	100	0
30	100	0

Vitamin A standard stock solution: A solution containing the equivalent of 0.2 mg/mL of retinol from USP Retinyl Acetate RS or USP Retinyl Palmitate RS in isopropyl alcohol. Dissolve with the aid of sonication if necessary.
Vitamin D standard stock solution: 0.05 mg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in isopropyl alcohol. Dissolve with the aid of sonication if necessary.
Vitamin E standard stock solution: 3.0 mg/mL of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, or USP Alpha Tocopheryl Acid Succinate RS in isopropyl alcohol. Dissolve with the aid of sonication if necessary.
Phytonadione standard stock solution: 0.04 mg/mL of USP Phytonadione RS in isopropyl alcohol. Dissolve with the aid of sonication if necessary.
Sample solution 1: Finely powder NLT 20 Tablets. Transfer an accurately weighed portion of the powder into a 50-mL volumetric flask to obtain a solution containing known nominal concentrations in the ranges of 0.05–0.1 mg/mL of vitamin A and 0.5–0.15 mg/mL of vitamin E. Add 10 mL of dimethyl sulfoxide and sonicate for 30 min with vigorous intermittent shaking. Cool to room temperature, dilute with isopropyl alcohol to volume and mix well. Pass a portion of the solution through a nylon filter of 0.45-µm pore size, and discard the first milliliter of the filtrate.
Sample solution 2: Transfer an accurately weighed portion of the powder from NLT 20 finely powdered Tablets into a 50-mL volumetric flask to obtain a solution containing known nominal concentrations in the ranges of 1.0–3.5 µg/mL of vitamin D and 1.0–3.5 µg/mL of phytonadione. Add 5 mL of water and sonicate for 10 min with intermittent shaking. Add 10 mL of dimethyl sulfoxide and sonicate for 30 min with vigorous intermittent shaking. Cool to room temperature, dilute to volume with isopropyl alcohol, and mix well. Pass a portion of the solution through a nylon filter of 0.45-µm pore size, and discard the first milliliter of the filtrate.
Standard solution: Transfer calculated volumes of *Vitamin A standard stock solution*, Vitamin D standard stock solution, Vitamin E standard stock solution, and Phytonadione standard stock solution into a suitable volumetric flask and dilute to volume with isopropyl alcohol to obtain a solution with final vitamin concentrations similar to those obtained for the corresponding vitamins in the *Sample solution 1* and *Sample solution 2*.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detectors
For *Sample solution 1* containing retinyl acetate: UV 325 nm for 7 min, then switch to 265 nm
For *Sample solution 1* containing retinyl palmitate: UV 265 nm for 18 min, then switch to 325 nm
For *Sample solution 2*: UV 265 nm
Column: 4.6-mm × 10-cm; 3-µm packing L1
Temperatures
Sample: 4°

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Column: 35°
Flow rate: 1.5 mL/min
Injection volume: 20 µL
System suitability
Samples: *Standard solution, Sample solution 1, Sample solution 2*
Suitability requirements
Relative standard deviation: NMT 2.0% for each individual peak, *Standard solution*
Peak purity of vitamin D and vitamin A (as retinyl palmitate): Monitor the chromatograms of the *Sample solution 1* and *Sample solution 2* at 450 nm. The area of any peak at the retention time of retinyl palmitate from *Sample solution 1* and any peak at the retention time of vitamin D (as cholecalciferol or ergocalciferol) from *Sample solution 2* should be NMT 5% of the corresponding peak areas detected at 325 nm and 265 nm, respectively. [NOTE—This test is required due to possible interference with carotenoids (cryptoxanthin and lycopene) that may be present in the formulation. Use another procedure in the monograph for analysis of vitamin D and vitamin A (as retinyl palmitate) if the system is not suitable.]
Analysis
Samples: *Sample solution 1, Sample solution 2* and *Standard solution*
Calculate the percentage of the labeled amount of vitamin A activity, as retinol ($C_{20}H_{30}O$), in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the all-*trans*-retinyl ester from the *Sample solution*
r_S = peak area of the all-*trans*-retinyl ester from the *Standard solution*
C_S = concentration of retinol in the *Standard solution* (mg/mL)
C_U = nominal concentration of vitamin A, as retinol, in the *Sample solution* (mg/mL)

Calculate the percentage of the labeled amount of vitamin E, as 2*R*-alpha-tocopherol ($C_{29}H_{50}O_2$), in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*
r_S = peak area of the relevant vitamin E form from the *Standard solution*
C_S = concentration of *all-rac*-alpha-tocopherol from either USP Alpha Tocopherol RS or USP Alpha Tocopheryl Acetate RS or *RRR*-alpha-tocopherol from USP Alpha Tocopheryl Acid Succinate RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of vitamin E, as 2*R*-alpha-tocopherol, in the *Sample solution* (mg/mL)
F = conversion factor for the content of *all-rac*-alpha-tocopherol to *RRR*-alpha-tocopherol equivalent, 1/2 (for products labeled to contain *all-rac* vitamin E sources) and 1 (for products labeled to contain *RRR* vitamin E sources)

Calculate the percentage of the labeled amount of vitamin D, as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$), and phytonadione ($C_{31}H_{46}O_2$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the related vitamin from the *Sample solution*
r_S = peak area of the related vitamin from the *Standard solution*
C_S = concentration of related vitamin in the *Standard solution* (µg/mL)
C_U = nominal concentration of related vitamin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of each individual vitamin ▲ (USP 1-May-2020)

• **BETA CAROTENE**
[NOTE—Use low-actinic glassware throughout this procedure.]
Potassium hydroxide solution: Dissolve 58.8 g of potassium hydroxide in 50 mL of water.
Iodine solution: 0.01 mg/mL of iodine in cyclohexane. [NOTE—Prepare this solution fresh daily]
Sample solution: Weigh NLT 20 Tablets. Grind the Tablets to a fine powder, and transfer a quantity of the powder, equivalent to 2 mg of beta carotene, to a 500-mL saponification flask. Add 100 mL of alcohol, 6 mL of *Potassium hydroxide solution*, and a magnetic stirring bar. Attach an air condenser to the flask, and heat under reflux for 45 min with constant stirring. Cool to room temperature, add 170 mL of solvent hexane, and stir for 30 min. Quantitatively transfer the contents of the flask to a 500-mL separatory funnel with portions of solvent hexane. Allow the layers to separate for 5–10 min, and transfer the upper organic layer to a 500-mL volumetric flask. Transfer the lower aqueous layer into the saponification flask, add 170 mL of solvent hexane, and stir for an additional 20 min. Quantitatively transfer the contents of the saponification flask to the separatory funnel with the aid of portions of solvent hexane. Allow the layers to separate for 10 min. Drain the lower aqueous layer, and discard. Transfer the organic layer to the volumetric flask containing the previously collected organic layer. Rinse the separatory funnel with small portions of solvent hexane, and transfer the washings to the volumetric flask. Dilute the hexane extracts with solvent hexane to volume, add 3 g of anhydrous sodium sulfate, shake, and allow to settle. Quantitatively transfer a volume of this solution, equivalent to 100 µg of beta carotene, to a 50-mL volumetric flask. Evaporate under a stream of nitrogen to dryness, and immediately add cyclohexane. Add 2 mL of *Iodine solution*, and heat for 15 min in a water bath maintained at 65°. Cool rapidly, and dilute with cyclohexane to volume.
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: Vis
Analytical wavelength: 452 nm
Blank: Cyclohexane
Analysis
Sample: *Sample solution*
Determine the absorbance against the *Blank*.
Calculate the percentage of the labeled amount of beta carotene ($C_{40}H_{56}$) in the portion of Tablets taken:

$$\text{Result} = (A_U/F) \times (100/C_U)$$

A_U = absorbance of the *Sample solution*

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F = absorptivity of beta carotene at 452 nm, 223
C_U = nominal concentration of beta carotene in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of beta carotene ($C_{40}H_{56}$)

PERFORMANCE TESTS
- **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩, *Disintegration*: Meet the requirements
- **WEIGHT VARIATION** ⟨2091⟩: Meet the requirements

CONTAMINANTS
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic microbial count does not exceed 3000 cfu/g, and the combined molds and yeasts count does not exceed 300 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures*, *Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meet the requirements

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

Change to read:

- **LABELING:**[1] Label the Tablets to state that the product is Oil-Soluble Vitamins Tablets. The label also states the quantity of each vitamin ▲in mg/Tablet or µg/Tablet▲ (USP 1-May-2020) and where necessary the chemical form in which it is present. Where the product contains vitamin E, the label indicates whether it is the ▲*RRR*▲ (USP 1-May-2020) - or ▲*all-rac*▲ (USP 1-May-2020)- form. Where more than one assay method is given for a particular vitamin, the labeling states the assay method used only if *Method 1* is not used.

Change to read:

- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Alpha Tocopherol RS
  USP Alpha Tocopheryl Acetate RS
  USP Alpha Tocopheryl Acid Succinate RS
  USP Cholecalciferol RS
  USP Ergocalciferol RS
  USP Phytonadione RS
  ▲USP Retinyl Acetate RS
  USP Retinyl Palmitate RS▲ (USP 1-May-2020)

[1] ▲▲ (USP 1-May-2020) The USP Unit for Vitamin E has been discontinued. International units (IU) for vitamins also have been discontinued; however, the use of IU on the labels of vitamin products continues. Where articles are labeled in terms of Units in addition to the required labeling, the relationship of the USP Units or IU to mass is as follows. One USP Vitamin A Unit = 0.3 µg of all-*trans*-retinol (vitamin A alcohol) or 0.344 µg of all-*trans*-retinyl acetate (vitamin A acetate) or 0.55 µg of all-*trans*-retinyl palmitate (vitamin A palmitate), and 1 µg of retinol (3.3 USP Vitamin A Units) = 1 retinol equivalent (RE); 1 IU of beta carotene = 0.6 µg of all-*trans*-beta carotene; 1 USP Vitamin D Unit = 0.025 µg of ergocalciferol or cholecalciferol; and 1 mg of ▲*all-rac*▲ (USP 1-May-2020)-alpha-tocopherol = 1.1 former USP Vitamin E Units; 1 mg of -alpha-tocopheryl acetate = 1 former USP Vitamin E unit; 1 mg of ▲*all-rac*▲ (USP 1-May-2020)-alpha-tocopheryl acid succinate = 0.89 former USP Vitamin E Units; 1 mg of ▲*RRR*▲ (USP 1-May-2020)-alpha-tocopherol = 1.49 former USP Vitamin E Units; 1 mg of ▲*RRR*▲ (USP 1-May-2020)-alpha-tocopheryl acetate = 1.36 former USP Vitamin E Units; and 1 mg of ▲*RRR*▲ (USP 1-May-2020)-alpha-tocopheryl acid succinate = 1.21 former USP Vitamin E Units. In terms of ▲*RRR*▲ (USP 1-May-2020)-alpha-tocopherol equivalents, 1 mg of ▲*RRR*▲ (USP 1-May-2020)-alpha-tocopheryl acetate = 0.91; 1 mg of ▲*RRR*▲ (USP 1-May-2020)-alpha-tocopheryl acid succinate = 0.81; 1 mg of ▲*all-rac*▲ (USP 1-May-2020)-alpha-tocopherol = 0.74; 1 mg of ▲*all-rac*▲ (USP 1-May-2020)-alpha-tocopheryl acetate = 0.67; and 1 mg of ▲*all-rac*▲ (USP 1-May-2020)-alpha-tocopheryl acid succinate = 0.60. ▲Note that 1 mg of Institute of Medicine (IOM) alpha-tocopherol equivalent = 1 mg of 2*R*-alphatocopherol = 1 mg of *RRR*-alpha-tocopherol = 2 mg of *all-rac*-alpha-tocopherol.▲ (USP 1-May-2020)

Oil-Soluble Vitamins with Minerals Capsules

DEFINITION
Oil-Soluble Vitamins with Minerals Capsules contain two or more of the following oil-soluble vitamins: Vitamin A, as retinol or esters of retinol in the form of retinyl acetate or retinyl palmitate; Vitamin D as ergocalciferol (Vitamin D_2) or cholecalciferol (Vitamin D_3); Vitamin E, as alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate; Phytonadione (Vitamin K_1); beta carotene; and one or more minerals derived from substances generally recognized as safe, furnishing one or more of the following elements in ionizable form: boron, calcium, chromium, copper, fluorine, iodine, iron, magnesium, manganese, molybdenum, nickel, phosphorus, potassium, selenium, tin, vanadium, and zinc. Capsules contain NLT 90.0% and NMT 165.0% of the labeled amounts of vitamin A, as retinol equivalent ($C_{20}H_{30}O$); vitamin D, as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$); vitamin E, as alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$); phytonadione ($C_{31}H_{46}O_2$); and beta carotene ($C_{40}H_{56}$); NLT 90.0% and NMT 125.0% of the labeled amount of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), potassium (K), and zinc (Zn); and NLT 90.0% and NMT 160.0% of the labeled amounts of boron (B), chromium (Cr), fluorine (F), iodine (I), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), and vanadium (V).
They may contain other labeled added substances that are generally recognized as safe, in amounts that are unobjectionable.

STRENGTH
- **VITAMIN A**
  [NOTE—Use low-actinic glassware throughout this procedure.]
  Mobile phase: *n*-Hexane
  Standard solution 1: 13 µg/mL of retinol from USP Retinyl Acetate RS in *n*-hexane
  Standard solution 2: 13 µg/mL of retinol from USP Retinyl Palmitate RS in *n*-hexane
  System suitability solution: Mix equal volumes of *Standard solution 1* and *Standard solution 2*.
  Sample stock solution: Transfer the contents of NLT 20 Capsules to a suitable container, mix, and weigh. Transfer a portion of the mixture, equivalent to 5 Capsules, to a container having a polytef-lined screw cap. [NOTE—For hard gelatin Capsules, remove, as completely as possible, the contents of NLT 20 Capsules by cutting open the Capsule shells, transferring the shells and their contents to a suitable container, and triturating to a homogeneous mass. Transfer a portion of the mass, equivalent to 5 Capsules, to a container having a polytef-lined screw cap.] Add 10 mL of dimethyl sulfoxide and 15 mL of *n*-hexane, and shake for 45 min in a water bath maintained at 60°. Centrifuge at 3000 rpm for 10 min, and transfer the hexane layer by means of a pipet to a 100-mL volumetric flask. Add 15 mL of *n*-hexane to the dimethyl sulfoxide layer, shake thoroughly for 5 min, and transfer the hexane layer by means of a pipet to the 100-mL volumetric flask. Repeat this

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extraction with three additional 15-mL portions of *n*-hexane. Dilute the extracts in the volumetric flask with *n*-hexane to volume.

Sample solution: Dilute the *Sample stock solution* with *n*-hexane to obtain a solution with a concentration of 13 µg/mL of vitamin A as retinol ($C_{20}H_{30}O$).

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 325 nm
Column: 4.6-mm × 15-cm; packing L8
Flow rate: 1 mL/min
Injection volume: 40 µL

System suitability
Sample: *System suitability solution*
Suitability requirements
Resolution: NLT 10 between all-*trans*-retinyl acetate and all-*trans*-retinyl palmitate
Relative standard deviation: NMT 3.0%

Analysis
Samples: *Standard solution 1* or *Standard solution 2* and *Sample solution*
Calculate the percentage of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$), in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the all-*trans*-retinyl ester from the *Sample solution*
r_S = peak area of the all-*trans*-retinyl ester from the appropriate *Standard solution*
C_S = concentration of retinol in the appropriate *Standard solution* (µg/mL)
C_U = nominal concentration of vitamin A, as retinol, in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A as retinol equivalent ($C_{20}H_{30}O$)

• **VITAMIN D**
[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Mobile phase: *n*-Hexane and isopropyl alcohol (99:1)
Standard solution: 2 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in *n*-hexane
System suitability solution: Heat a volume of the *Standard solution* at 60° for 1 h to partially isomerize vitamin D (cholecalciferol or ergocalciferol) to its corresponding precursor.
Sample solution: Transfer NLT 20 mL of a solution prepared as directed for the *Sample stock solution* in *Vitamin A* to a suitable container, and concentrate, if necessary, in vacuum at room temperature to obtain a solution with an expected concentration of 2 µg/mL of cholecalciferol or ergocalciferol.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 265 nm
Column: 4.6-mm × 15-cm; 3-µm packing L8
Flow rate: 1 mL/min
Injection volume: 100 µL

System suitability
Sample: *System suitability solution*
Suitability requirements
Resolution: NLT 10 between the vitamin D form present and its corresponding precursor
Relative standard deviation: NMT 3.0%

Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*
r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*
C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)
F = correction factor to account for the average amount of pre-vitamin D present in the *Sample solution*, 1.09

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

• **VITAMIN E**
[NOTE—Where vitamin E (alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Solution A: Phosphoric acid solution (1 in 100) in water
Mobile phase: Methanol and *Solution A* (19:1)
Standard solution: 2 mg/mL of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, or USP Alpha Tocopheryl Acid Succinate RS in methanol
Sample solution: Transfer NLT 20 mL of the solution prepared as directed for the *Sample stock solution* in *Vitamin A* to a suitable container, and evaporate in vacuum at room temperature to dryness. Transfer the residue with the aid of methanol to a suitable volumetric flask, and dilute with methanol to volume to obtain a concentration of 2 mg/mL of alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 291 nm
Column: 4.6-mm × 15-cm; 5-µm packing L1
Flow rate: 1 mL/min
Injection volume: 50 µL

System suitability
Sample: *Standard solution*
Suitability requirements
Tailing factor: NMT 1.5
Relative standard deviation: NMT 3.0%

Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*

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r_S = peak area of the relevant vitamin E form from the *Standard solution*
C_S = concentration of the corresponding USP Reference Standard in the *Standard solution* (mg/mL)
C_U = nominal concentration of the corresponding form of vitamin E in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin E

• **PHYTONADIONE (VITAMIN K_1)**

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Methanol and water (19:1)

Standard stock solution: 200 µg/mL of USP Phytonadione RS in methanol. Dissolve with the aid of sonication if necessary.

Standard solution: 20 µg/mL of USP Phytonadione RS from the *Standard stock solution* diluted with methanol

System suitability solution: 0.65 mg/mL of USP Alpha Tocopheryl Acetate RS and 20 µg/mL of USP Phytonadione RS from the *Standard stock solution* diluted with methanol. [NOTE—Dissolve USP Alpha Tocopheryl Acetate RS in a portion of methanol, add the *Standard stock solution*, and then dilute with methanol to volume.]

Sample solution: Transfer NLT 20 mL of the solution prepared as directed for the *Sample stock solution* in *Vitamin A* to a suitable container, and evaporate in vacuum at room temperature to dryness. Transfer the residue with the aid of methanol to a suitable volumetric flask, and dilute with methanol to volume to obtain a concentration of 20 µg/mL of phytonadione.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Flow rate: 1 mL/min

Injection volume: 50 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 5 between alpha tocopheryl acetate and phytonadione, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of phytonadione ($C_{31}H_{46}O_2$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*
r_S = peak area from the *Standard solution*
C_S = concentration of USP Phytonadione RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of phytonadione in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of phytonadione ($C_{31}H_{46}O_2$)

• **BETA CAROTENE**

[NOTE—Use low-actinic glassware.]

Mobile phase: Transfer 50 mg of butylated hydroxytoluene into a 1-L volumetric flask, and dissolve with 20 mL of 2-propanol. Add 0.2 mL of *N*-ethyldiisopropylamine, 25 mL of 0.2% ammonium acetate solution, 455 mL of acetonitrile, and about 450 mL of methanol. Allow the solution to reach to room temperature, and dilute with methanol to volume.

Diluent: 50 µg/mL of butylated hydroxytoluene in alcohol

System suitability solution: Transfer 20 mg of USP Beta Carotene System Suitability RS to a 50-mL volumetric flask. Add 1 mL of water, 4 mL of tetrahydrofuran, and sonicate for 5 min. Dilute with *Diluent* to volume, and sonicate for 5 min. Cool to room temperature, pass the suspension through a membrane filter of 0.45-µm pore size, and use the clear filtrate.

Standard stock solution: 60 µg/mL of USP Beta Carotene RS in tetrahydrofuran

Standard solution A: Transfer 5.0 mL of the *Standard stock solution* into a 100-mL volumetric flask, add 5.0 mL of tetrahydrofuran, and dilute with *Diluent* to volume. Determine the concentration of *Standard solution A* from the concentration of *Standard solution B* as described below.

Standard solution B: Transfer 5.0 mL of the *Standard stock solution* into a 100-mL volumetric flask, and dilute with cyclohexane to volume. Prepare in triplicate.

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Analytical wavelength: 457 nm

Cell path: 1 cm

Blank: Cyclohexane

Analysis

Sample: *Standard solution B*

Calculate the concentration of total beta carotene (mg/mL) as all-*trans*-beta carotene ($C_{40}H_{56}$) in *Standard solution B*. [NOTE—The concentration of *Standard solution B* equals the concentration of *Standard solution A*.]

$$\text{Result} = A/F$$

A = average absorbance of the three preparations of *Standard solution B*
F = absorptivity of pure all-*trans*-beta carotene in cyclohexane, 250

Sample solution: Transfer NLT 20 mL of the solution prepared as directed for the *Sample stock solution* in *Vitamin A* to a suitable container, and evaporate in vacuum at room temperature to dryness. Dissolve the residue in a mixture of methylene chloride and *Diluent* (1:1), and dilute with the same mixture to obtain a concentration of 3 µg/mL of beta carotene. Pass through a membrane filter of 0.45-µm pore size if necessary.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV-Vis 448 nm

Column: 4.6-mm × 25-cm; 5-µm packing L68

Column temperature: 30°

Flow rate: 0.6 mL/min

Injection volume: 20 µL

System suitability

Samples: *System suitability solution* and *Standard solution A*

[NOTE—The approximate relative retention times of the components in the *System suitability solution* are listed in *Table 1*.]

Table 1

Name	Relative Retention Time	Relative Response Factor
All-*trans*-Alpha carotene	0.93	1.1

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Table 1 *(continued)*

Name	Relative Retention Time	Relative Response Factor
All-*trans*-Beta carotene	1.00	1
9-*cis*-Beta carotene	1.07	1
13-*cis*-Beta carotene	1.17	1.2
15-*cis*-Beta carotene	1.21	1.4

Suitability requirements

Chromatogram similarity: The chromatogram from the *System suitability solution* is similar to the reference chromatogram provided with the lot of USP Beta Carotene System Suitability RS being used.

Resolution: NLT 1.5 between beta carotene and alpha carotene and between beta carotene and 9-*cis*-beta carotene, *System suitability solution*

Tailing factor: NMT 2.0 for the beta carotene peak, *Standard solution A*

Relative standard deviation: NMT 2.0% for the beta carotene peak from replicate injections, *Standard solution A*

Analysis

Samples: *Standard solution A* and *Sample solution*

Calculate the percentage of all-*trans*-beta carotene in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of all-*trans*-beta carotene from the *Sample solution*

r_S = peak area of all-*trans*-beta carotene from *Standard solution A*

C_S = concentration of all-*trans*-beta carotene in *Standard solution A* as determined by spectrometric procedure

C_U = nominal concentration of beta carotene in the *Sample solution*

Acceptance criteria: 90.0%–165.0% of the labeled amount of beta carotene ($C_{40}H_{56}$)

• **CALCIUM, *Method 1***

Lanthanum chloride solution: 267 mg/mL of lanthanum chloride heptahydrate in 0.125 N hydrochloric acid

Calcium standard solution: 400 µg/mL of calcium. Dissolve 1.001 g of calcium carbonate, previously dried at 300° for 3 h and cooled in a desiccator for 2 h, in 25 mL of 1 N hydrochloric acid. Boil to expel carbon dioxide, and dilute with water to 1000 mL.

Standard stock solution: 100 µg/mL of calcium from the *Calcium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Into separate 100-mL volumetric flasks pipet 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, and dilute with 0.125 N hydrochloric acid to volume to obtain concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 µg/mL of calcium.

Polysorbate 80 solution: Polysorbate 80 and alcohol (1:10)

Sample solution: Transfer 5 Capsules to a 100-mL volumetric flask. [NOTE—For hard gelatin Capsules, weigh NLT 20 Capsules. Open the Capsules, without loss of shell material, and transfer the contents to a suitable container. Remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and allow the Capsule shells to dry. Weigh the empty Capsule shells, calculate the net weight of the Capsule contents, and transfer a portion of the Capsule contents, equivalent to 5 Capsules, to a 100-mL volumetric flask.] Add 15 mL of water, 10 mL of 6 N hydrochloric acid, and 1 mL of *Polysorbate 80 solution* to the flask. Heat on a hot plate or steam bath, with intermittent swirling, until the Capsules are completely disintegrated or the contents are dissolved. Boil gently for an additional 15 min. Cool, dilute with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute this solution with 0.125 N hydrochloric acid, to obtain a concentration of 2 µg/mL of calcium, adding 1 mL of *Lanthanum chloride solution* per 100 mL of the final volume.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Calcium emission line at 422.7 nm

Lamp: Calcium hollow-cathode

Flame: Nitrous oxide–acetylene

Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution* per 100 mL

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of calcium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in mg/mL, of calcium in the *Sample solution*.

Calculate the percentage of the labeled amount of calcium (Ca) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of calcium in the *Sample solution* (µg/mL)

C_U = nominal concentration of calcium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of calcium (Ca)

• **CHROMIUM, *Method 1***

Chromium standard solution: 1000 µg/mL of chromium from potassium dichromate, previously dried at 120° for 4 h in water. Store in a polyethylene bottle.

Standard stock solution: 10 µg/mL of chromium from *Chromium standard solution* diluted with 6 N hydrochloric acid and water (1 in 20)

Standard solutions: Transfer 10.0 and 20.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks, and transfer 15.0 and 20.0 mL of the *Standard stock solution* to separate 50-mL volumetric flasks. Dilute the contents of each of the four flasks with 0.125 N hydrochloric acid to volume to obtain concentrations of 1.0, 2.0, 3.0, and 4.0 µg/mL of chromium.

Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain 2.5 µg/mL of chromium and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Chromium emission line at 357.9 nm

Lamp: Chromium hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

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Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of chromium, and draw the straight line best fitting the four plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of chromium in the *Sample solution*.

Calculate the percentage of the labeled amount of chromium (Cr) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of chromium in the *Sample solution* (µg/mL)

C_U = nominal concentration of chromium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of chromium (Cr)

• **COPPER, *Method 1***

Copper standard solution: Dissolve 1.00 g of copper foil in a minimum volume of a 50% solution of nitric acid, and dilute with a 1% solution of nitric acid to 1000 mL. This solution contains 1000 µg/mL of copper.

Standard stock solution: 100 µg/mL of copper from the *Copper standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 200-mL volumetric flasks transfer 1.0, 2.0, 4.0, 6.0, and 8.0 mL of the *Standard stock solution*. Dilute with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.5, 1.0, 2.0, 3.0, and 4.0 µg/mL of copper.

Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain 2 µg/mL of copper and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Copper emission line at 324.7 nm

Lamp: Copper hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of copper, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of copper in the *Sample solution*.

Calculate the percentage of the labeled amount of copper (Cu) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of copper in the *Sample solution* (µg/mL)

C_U = nominal concentration of copper in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of copper (Cu)

• **FLUORIDE, *Method 1***

[NOTE—Store all solutions in plastic containers.]

3 M sodium acetate solution: Dissolve 408 g of sodium acetate in 600 mL of water contained in a 1000-mL volumetric flask. Allow the solution to equilibrate to room temperature, and dilute with water to volume. Adjust with a few drops of acetic acid to a pH of 7.0.

Sodium citrate solution: Dissolve 222 g of sodium citrate in 250 mL of water in a 1000-mL volumetric flask. Add 28 mL of perchloric acid, and dilute with water to volume.

Fluoride standard stock solution: 500 µg/mL of fluoride from a quantity of sodium fluoride in water, previously dried at 100° for 4 h and cooled in a desiccator

Intermediate stock solution A: 100 µg/mL of fluoride from the *Fluoride standard stock solution* diluted with water

Intermediate stock solution B: 10 µg/mL of fluoride from the *Fluoride standard stock solution* diluted with water

Standard solutions: To five separate 100-mL volumetric flasks transfer 3.0, 5.0, and 10.0 mL of *Intermediate stock solution B* and 5.0 and 10.0 mL of *Intermediate stock solution A*. To each flask add 10.0 mL of 1 N hydrochloric acid, 25 mL of *3 M sodium acetate solution*, and 25.0 mL of *Sodium citrate solution*. Dilute the contents of each flask with water to volume to obtain concentrations of 0.3, 0.5, 1.0, 5.0, and 10.0 µg/mL of fluoride.

Sample solution: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the mixed Capsule contents, equivalent to 200 µg of fluoride, to a 100-mL volumetric flask. Add 10.0 mL of 1 N hydrochloric acid, 25.0 mL of *3 M sodium acetate solution*, and 25.0 mL of *Sodium citrate solution*. Dilute with water to volume.

Analysis

Samples: *Standard solutions* and *Sample solution*

To separate plastic beakers, each containing a plastic-coated stirring bar, transfer 50.0 mL each of the *Standard solutions* and the *Sample solution*. Measure the potentials (see *pH* ⟨791⟩), in mV, of the *Standard solutions* and the *Sample solution*, with a pH meter capable of a minimum reproducibility of ±0.2 mV and equipped with a fluoride-specific ion-indicating electrode and a calomel reference electrode. [NOTE—When taking measurements, immerse the electrodes in the solution, stir on a magnetic stirrer having an insulated top until equilibrium is attained (1–2 min), and record the potential. Rinse and dry the electrodes between measurements, taking care to avoid damaging the crystal of the specific-ion electrode.]

Plot the logarithms of fluoride concentrations, in µg/mL, of the *Standard solutions* versus potential, in mV. From the standard response curve so obtained and the measured potential of the *Sample solution*, determine the concentration, *C*, in mg/mL, of fluoride in the *Sample solution*.

Calculate the percentage of the labeled amount of fluorine (F) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of fluoride in the *Sample solution* (µg/mL)

C_U = nominal concentration of fluorine in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (F)

• **FLUORIDE, *Method 2***

[NOTE—Use plastic containers and deionized water throughout this procedure.]

pH 10.0 buffer: Add 214 mL of 0.1 N sodium hydroxide to 1000 mL of 0.05 M sodium bicarbonate.

Mobile phase: Alcohol, 0.1 N sulfuric acid, and water (20:5:175)

Standard stock solution: 220 µg/mL of USP Sodium Fluoride RS in water. This solution contains 100 µg/mL of fluoride.

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Standard solution: [NOTE—Condition the solid-phase extraction column specified for use in the *Standard solution* and the *Sample solution* in the following manner. Using a vacuum at a pressure not exceeding 5 mm of mercury, wash the column with one column volume of methanol followed by one column volume of *pH 10.0 buffer*. Do not allow the column top to dry. If the top of the column becomes dry, recondition the column.] Transfer 10.0 mL of the *Standard stock solution* to a 100-mL volumetric flask. Add 75 mL of water, and adjust with 0.1 N sodium hydroxide to a pH of 10.4 ± 0.1. Dilute with water to volume. Filter, discarding the first 15 mL of the filtrate. Transfer 25.0 mL of the filtrate to a 50-mL volumetric flask, add 15.0 mL of water, and adjust with 0.1 N sodium hydroxide to a pH of 10.0. Dilute with *pH 10.0 buffer* to volume. Elute a portion of this solution through a 3-mL solid-phase extraction column containing L1 packing that is connected through an adaptor to a second solid-phase extraction column containing sulfonylpropyl strong cation-exchange packing. Discard the first 3 mL of the eluate, and collect the rest of the eluate in a suitable flask for injection into the chromatograph.

Sample solution: Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents to a 100-mL container. If necessary, remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to 1 mg of fluorine, to a 100-mL volumetric flask. Add 15 mL of water, and shake vigorously. Rinse the sides of the flask with 15 mL of water, and allow to stand for 10 min. Dilute with water to 85 mL, adjust with 1 N sodium hydroxide to a pH of 10.4 ± 0.1, and dilute with water to 100 mL. Proceed as directed for the *Standard solution*, beginning with "Filter, discarding the first 15 mL of the filtrate."

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Conductivity
Columns
Guard: 4.6-mm × 3-cm; packing L17
Analytical: 7.8-mm × 30-cm; packing L17
Flow rate: 0.5 mL/min
Injection volume: 100 µL

System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 2.0%

Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas for fluoride.
Calculate the percentage of the labeled amount of fluorine (F) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*
r_S = peak area from the *Standard solution*
C_S = concentration of fluoride in the *Standard solution* (µg/mL)
C_U = nominal concentration of fluorine in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (F)

• **IODIDE, *Method 1***
Bromine water: To 20 mL of bromine in a glass-stoppered bottle add 100 mL of water. Insert the stopper into the bottle, and shake. Allow to stand for 30 min, and use the supernatant.

Analysis
Sample: Capsules
Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the contents, equivalent to 3 mg of iodine, to a nickel crucible. Add 5 g of sodium carbonate, 5 mL of 50% (w/v) sodium hydroxide solution, and 10 mL of alcohol, taking care that the entire specimen is moistened. Heat the crucible on a steam bath to evaporate the alcohol, then dry the crucible at 100° for 30 min to prevent spattering upon subsequent heating. Transfer the crucible with its contents to a furnace heated to 500°, and heat the crucible for 15 min. [NOTE—Heating at 500° is necessary to carbonize any organic matter present; a higher temperature may be used, if necessary, to ensure complete carbonization of all organic matter.]
Cool the crucible, add 25 mL of water, cover the crucible with a watchglass, and boil gently for 10 min. Filter the solution, and wash the crucible with boiling water, collecting the filtrate and washings in a beaker. Add phosphoric acid until the solution is neutral to methyl orange, then add 1 mL excess of phosphoric acid. Add excess of *Bromine water*, and boil the solution gently until colorless and then for 5 min longer. Add a few crystals of salicylic acid, and cool the solution to 20°. Add 1 mL of phosphoric acid and 0.5 g of potassium iodide, and titrate the liberated iodine with 0.005 N sodium thiosulfate VS, adding starch TS when the liberated iodine color has nearly disappeared.
Calculate the percentage of the labeled amount of iodine (I) in the portion of Capsules taken:

$$\text{Result} = V \times N_A \times F \times I_{me} \times (A_w/W) \times (100/L)$$

V = volume of sodium thiosulfate consumed (mL)
N_A = actual normality of the sodium thiosulfate solution used
F = correction factor to convert mg to µg, 1000 µg/mL
I_{me} = milliequivalent of I, 21.16 mg/meq
A_w = average weight of the Capsules content
W = weight of the sample of Capsules content taken
L = labeled amount of iodine (µg/Capsule)

Acceptance criteria: 90.0%–160.0% of the labeled amount of iodine (I)

• **IODIDE, *Method 2***
Analysis: Proceed as directed.
Acceptance criteria: 90.0%–160.0% of the labeled amount of iodine (I)

• **IRON, *Method 1***
Iron standard stock solution: Transfer 100 mg of iron powder to a 1000-mL volumetric flask. Dissolve in 25 mL of 6 N hydrochloric acid, dilute with water to volume, and mix.
Standard solutions: To separate 100-mL volumetric flasks transfer 2.0, 4.0, 5.0, 6.0, and 8.0 mL of *Iron standard stock solution*. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 2.0, 4.0, 5.0, 6.0, and 8.0 µg/mL of iron.
Polysorbate 80 solution: Prepare as directed in *Calcium, Method 1*.
Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain a

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concentration of 5 µg/mL of iron and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Iron emission line at 248.3 nm

Lamp: Iron hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of iron, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of iron in the *Sample solution*.

Calculate the percentage of the labeled amount of iron (Fe) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of iron in the *Sample solution* (µg/mL)

C_U = nominal concentration of iron in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of iron (Fe)

• **MAGNESIUM,** ***Method 1***

Lanthanum chloride solution: Prepare as directed in *Calcium, Method 1*.

Magnesium standard solution: Transfer 1.0 g of magnesium ribbon to a 1000-mL volumetric flask, dissolve in 50 mL of 6 N hydrochloric acid, dilute with water to volume, and mix to obtain a solution with a known concentration of 1000 µg/mL of magnesium.

Standard stock solution: 20 µg/mL of magnesium from *Magnesium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, and dilute with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.2, 0.3, 0.4, 0.5, and 0.6 µg/mL of magnesium.

Polysorbate 80 solution: Prepare as directed in *Calcium, Method 1*.

Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain 0.4 µg/mL of magnesium.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Magnesium emission line at 285.2 nm

Lamp: Magnesium hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution* per 100 mL

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of magnesium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of magnesium in the *Sample solution*.

Calculate the percentage of the labeled amount of magnesium (Mg) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of magnesium in the *Sample solution* (µg/mL)

C_U = nominal concentration of magnesium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of magnesium (Mg)

• **MANGANESE,** ***Method 1***

Manganese standard stock solution: Transfer 1.00 g of manganese to a 1000-mL volumetric flask. Dissolve in 20 mL of nitric acid, dilute with 6 N hydrochloric acid to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of manganese.

Standard stock solution: 50 µg/mL of manganese from the *Manganese standard stock solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Standard stock solution*. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions with known concentrations of 0.5, 0.75, 1.0, 1.5, and 2.0 µg/mL of manganese.

Polysorbate 80 solution: Prepare as directed in *Calcium, Method 1*.

Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain 1 µg/mL of manganese and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Manganese emission line at 279.5 nm

Lamp: Manganese hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of manganese, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of manganese in the *Sample solution*.

Calculate the percentage of the labeled amount of manganese (Mn) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of manganese in the *Sample solution* (µg/mL)

C_U = nominal concentration of manganese in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of manganese (Mn)

• **MOLYBDENUM,** ***Method 1***

Diluent: 20 mg/mL of ammonium chloride in water

Molybdenum standard solution: Transfer 1.0 g of molybdenum wire to a 1000-mL volumetric flask, and dissolve in 50 mL of nitric acid, warming if necessary. Dilute with water to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of molybdenum.

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Standard stock solution: 100 µg/mL of molybdenum from the *Molybdenum standard solution* diluted with water
Standard solutions: To separate 100-mL volumetric flasks transfer 2.0, 10.0, and 25.0 mL of the *Standard stock solution*, and add 5.0 mL of perchloric acid to each flask. Gently boil the solution in each flask for 15 min, cool to room temperature, and dilute each with *Diluent* to volume to obtain concentrations of 5.0, 10.0, and 25.0 µg/mL of molybdenum.
Polysorbate 80 solution: Prepare as directed in *Calcium, Method 1*.
Sample solution: Proceed as directed in *Calcium, Method 1*, except take a number of Capsules or a portion of Capsule contents equivalent to 1000 µg of molybdenum and make appropriate dilutions to obtain a final concentration of 10 µg/mL of molybdenum, omitting the addition of the *Lanthanum chloride solution*.
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Analytical wavelength: Molybdenum emission line at 313.3 nm
Lamp: Molybdenum hollow-cathode
Flame: Nitrous oxide–acetylene
Blank: *Diluent* and perchloric acid (20:1)
Analysis
Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of molybdenum, and draw the straight line best fitting the three plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of molybdenum in the *Sample solution*.
Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of molybdenum in the *Sample solution* (µg/mL)
C_U = nominal concentration of molybdenum in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

• **MOLYBDENUM, *Method 2***
Sodium fluoride solution: Add 200 mL of water to 10 g of sodium fluoride, stir until the solution is saturated, and filter. Store in a polyethylene bottle.
Ferrous sulfate solution: 4.98 mg/mL of ferrous sulfate in water
Potassium thiocyanate solution: 200 mg/mL of potassium thiocyanate in water
20% Stannous chloride solution: Transfer 40 mg of stannous chloride to a beaker, add 20 mL of 6.5 N hydrochloric acid solution, and heat the solution until the stannous chloride is dissolved. Cool, and dilute with water to 100 mL.
Diluted stannous chloride solution: *20% Stannous chloride solution* diluted with water (1 in 25). Prepare this solution fresh at the time of use.
Standard solution: 20 µg/mL of molybdenum in water
Sample: Remove the contents of a counted number of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Use a quantity of the Capsule contents, equivalent to 40 µg of molybdenum.
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: UV-Vis
Analytical wavelength: 465 nm
Cell: 1 cm
Blank: Amyl alcohol
Analysis
Samples: *Standard solution* and *Sample*
Transfer the *Sample* and 2.0 mL of the *Standard solution* to separate 200-mL beakers. Add 20 mL of nitric acid to each beaker. Cover each beaker with a watchglass, and boil slowly on a hot plate for 45 min. Cool to room temperature. Add 6 mL of perchloric acid, cover the beakers with a watchglass, and continue the heating until digestion is complete, as indicated when the liquid becomes colorless or pale yellow. Evaporate the solutions in the beakers to dryness. Rinse the sides of the beakers and the watchglasses with water, and add more water to complete 50 mL in each beaker. Gently boil the water solution for a few min. Cool to room temperature. Add 2 drops of methyl orange TS, and neutralize with ammonium hydroxide. Add 8.2 mL of hydrochloric acid. Quantitatively transfer the contents of the beakers to separate 100-mL volumetric flasks, rinse the beakers with water, transfer the rinsings to the corresponding volumetric flasks, and dilute with water to volume. Transfer 50.0 mL of each solution to separatory funnels. To each separatory funnel add 1.0 mL of *Sodium fluoride solution*, 0.5 mL of *Ferrous sulfate solution*, 4.0 mL of *Potassium thiocyanate solution*, 1.5 mL of *20% Stannous chloride solution*, and 15.0 mL of amyl alcohol, and shake the separatory funnel for 1 min. Allow the layers to separate, and discard the aqueous layers. Add 25 mL of *Diluted stannous chloride solution* to each separatory funnel, and shake gently for 15 s. Allow the layers to separate, and discard the aqueous layers. Transfer the organic layer from each separatory funnel to a centrifuge tube, and centrifuge at 2000 rpm for 10 min. Determine the absorbances of the organic phases obtained from the *Standard solution* and the *Sample*, and correct with the *Blank*.
Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Capsules taken:

$$\text{Result} = (A_U/A_S) \times [(V \times C_S)/M_U] \times 100$$

A_U = absorbance of the *Sample*
A_S = absorbance of the *Standard solution*
V = volume of the *Standard solution* analyzed, 2.0 mL
C_S = concentration of molybdenum in the *Standard solution* (µg/mL)
M_U = nominal amount of molybdenum in the *Sample* (µg)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

• **PHOSPHORUS, *Method 1***
Sulfuric acid solution: Cautiously add sulfuric acid to water (37.5: 100), and mix.
Ammonium molybdate solution: 50 mg/mL of ammonium molybdate in *Sulfuric acid solution* and water (2:3). [NOTE—Dissolve in water first, and then dilute with the *Sulfuric acid solution* to volume.]
Hydroquinone solution: 5 mg/mL of hydroquinone in water. Add one drop of sulfuric acid per 100 mL of solution.
Sodium bisulfite solution: 200 mg/mL of sodium bisulfite in water
Phosphorus standard stock solution: Weigh 4.395 g of monobasic potassium phosphate, previously dried at 105° for 2 h and stored in a desiccator, and transfer to a 1000-mL volumetric flask. Dissolve in water, add 6 mL of sulfuric acid

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as a preservative, dilute with water to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of phosphorus.

Standard solution: 20 µg/mL of phosphorus from the *Phosphorus standard stock solution* diluted with water

Sample solution: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the Capsule contents, equivalent to 100 mg of phosphorus, to 25 mL of nitric acid, and digest on a hot plate for 30 min. Add 15 mL of hydrochloric acid, and continue the digestion to the cessation of brown fumes. Cool, and transfer the contents of the flask to a 500-mL volumetric flask with the aid of small portions of water. Dilute with water to volume. Transfer 10.0 mL of this solution to a 100-mL volumetric flask, and dilute with water to volume.

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Mode: Vis

Analytical wavelength: 650 nm

Cell: 1 cm

Analysis

Samples: *Standard solution* and *Sample solution*

To three separate 25-mL volumetric flasks transfer 5.0 mL each of the *Standard solution*, the *Sample solution*, and water to provide the blank. To each of the three flasks add 1.0 mL each of *Ammonium molybdate solution*, *Hydroquinone solution*, and *Sodium bisulfite solution*, and swirl to mix. Dilute the contents of each flask with water to volume, and allow the flasks to stand for 30 min. Determine the absorbances of the solutions against the blank.

Calculate the percentage of the labeled amount of phosphorus (P) in the portion of Capsules taken:

$$\text{Result} = (A_U/A_S) \times (C_S/C_U) \times 100$$

A_U = absorbance of the *Sample solution*
A_S = absorbance of the *Standard solution*
C_S = concentration of phosphorus in the *Standard solution* (µg/mL)
C_U = nominal concentration of phosphorus in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of phosphorus (P)

• **POTASSIUM**

Potassium standard solution: 100 µg/mL of potassium from potassium chloride, previously dried at 105° for 2 h, in water

Standard stock solution: 10 µg/mL of potassium from the *Potassium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Transfer 5.0, 10.0, 15.0, 20.0, and 25.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions containing 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of potassium.

Polysorbate 80 solution: Prepare as directed in *Calcium, Method 1*.

Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain 1.5 µg/mL of potassium and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Potassium emission line at 766.5 nm

Lamp: Potassium hollow-cathode

Flame: Air–acetylene

Blank: Water

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of potassium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of potassium in the *Sample solution*.

Calculate the percentage of the labeled amount of potassium (K) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of potassium in the *Sample solution* (µg/mL)
C_U = nominal concentration of potassium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of potassium (K)

• **SELENIUM, *Method 1***

Diluent: Prepare as directed in *Molybdenum, Method 1*.

Selenium standard solution: [**CAUTION**—Selenium is toxic; handle it with care.] Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness, add 2 mL of water, and evaporate to dryness. Repeat the addition of water and the evaporation to dryness three times. Dissolve the residue in 3 N hydrochloric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume to obtain a concentration of 1000 µg/mL of selenium.

Standard stock solution: 100 µg/mL of selenium from the *Selenium standard solution* diluted with water

Standard solutions: To separate 100-mL volumetric flasks transfer 5.0, 10.0, and 25.0 mL of the *Standard stock solution*, and add 5.0 mL of perchloric acid to each flask. Gently boil the solutions for 15 min, cool to room temperature, and dilute each with *Diluent* to volume to obtain solutions with concentrations of 5.0, 10.0, and 25.0 µg/mL of selenium.

Sample solution: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the Capsule contents, equivalent to 1000 µg of selenium, to a suitable flask, and add 12 mL of nitric acid. [NOTE—The volume of nitric acid may be varied to ensure that the powder is uniformly dispersed.] Carefully swirl the flask to disperse the test specimen. Sonicate for 10 min or until the test specimen is completely dissolved. Gently boil the solution for 15 min, and cool to room temperature. Carefully add 8 mL of perchloric acid to the flask, heat the flask until perchloric acid fumes appear, and swirl the flask to dissipate the fumes. Repeat the heating and swirling until the fumes appear again. Cool to room temperature. Transfer the contents of the flask to a 50-mL volumetric flask with the aid of the *Diluent*, and dilute with *Diluent* to volume.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Selenium emission line at 196.0 nm

Lamp: Selenium hollow-cathode

Flame: Air–acetylene

Blank: *Diluent* and perchloric acid (20:1)

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Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of selenium, and draw the straight line best fitting the three plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of selenium in the *Sample solution*.

Calculate the percentage of the labeled amount of selenium (Se) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of selenium in the *Sample solution* (µg/mL)

C_U = nominal concentration of selenium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of selenium (Se)

• **SELENIUM, *Method 2***

Hydrochloric acid solution: Hydrochloric acid diluted with water (1 in 10)

50% Ammonium hydroxide solution: Ammonium hydroxide diluted with water (1 in 2)

Reagent A: 9 mg/mL of edetate disodium and 25 mg/mL of hydroxylamine hydrochloride in water. [NOTE—Dissolve edetate disodium in a portion of water first, then add hydroxylamine hydrochloride, and dilute with water to volume.]

Reagent B: Transfer 200 mg of 2,3-diaminonaphthalene to a 250-mL separatory funnel, and add 200 mL of 0.1 N hydrochloric acid. Wash the solution with three 40-mL portions of cyclohexane, and discard the cyclohexane layer. Filter the solution into a brown bottle, and cover the solution with a 1-cm layer of cyclohexane. This solution is stable for 1 week if stored in a refrigerator.

Standard stock solution: [**CAUTION**—Selenium is toxic; handle it with care.] Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness, add 2 mL of water, and evaporate to dryness. Repeat the addition of water and evaporation to dryness three times. Dissolve the residue in 3 N hydrochloric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume to obtain a solution with a concentration of 1000 µg/mL of selenium. Dilute a volume of the solution with 0.125 N hydrochloric acid to obtain a concentration of 2.0 µg/mL of selenium.

Standard solution: Transfer 10 mL of the *Standard stock solution* to a glass-stoppered flask. Add 1 mL of perchloric acid and 1 mL of *Hydrochloric acid solution*, and dilute with water to 20 mL.

Sample solution: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the Capsule contents, equivalent to 20 µg of selenium, to a suitable flask. Add 10 mL of nitric acid, and warm gently on a hot plate. Continue heating until the initial nitric acid reaction has subsided, then add 3 mL of perchloric acid. [**CAUTION**—Exercise care at this stage, because perchloric acid reaction becomes vigorous.]

Continue heating on the hot plate until the appearance of white fumes of perchloric acid or until the digest begins to darken. Add 0.5 mL of nitric acid, and resume heating, adding additional amounts of nitric acid if further darkening occurs. Digest for 10 min after the first appearance of perchloric acid fumes or until the digest becomes colorless. Cool the flask. Add 2.5 mL of *Hydrochloric acid solution*, and return the flask to the hot plate to expel residual nitric acid. Heat the mixture for 3 min after it begins to boil. Cool the flask to room temperature, and dilute with water to 20 mL.

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Mode: UV

Analytical wavelength: 380 nm

Cell: 1 cm

Blank: 1 mL of perchloric acid and 1 mL of *Hydrochloric acid solution* diluted with water to 20 mL

Analysis

Samples: *Standard solution* and *Sample solution*

Treat the *Sample solution*, the *Standard solution*, and the *Blank* as follows. Add 5 mL of *Reagent A* to each flask, and swirl gently to mix. Adjust the solution in each flask with *50% Ammonium hydroxide solution* to a pH of 1.1 ± 0.1. Add 5 mL of *Reagent B* to each flask, and swirl gently to mix. Place the flasks in a water bath maintained at 50°, and equilibrate for 30 min, taking care that the flasks are covered to protect them from light. Cool to room temperature, and transfer the contents of each flask to separate separatory funnels. Transfer 10.0 mL of cyclohexane to each separatory funnel, and extract vigorously for 1 min. Discard the aqueous layer. Transfer the cyclohexane layer to a centrifuge tube, and centrifuge at 1000 rpm for 1 min to remove any remaining water. Determine the absorbances of the solutions obtained from the *Samples* against the solution obtained from the *Blank*.

Calculate the percentage of the labeled amount of selenium (Se) in the portion of Capsules taken:

$$\text{Result} = (A_U/A_S) \times [(V \times C_S)/M_U] \times 100$$

A_U = absorbances of the cyclohexane layer from the *Sample solution*

A_S = absorbances of the cyclohexane layer from the *Standard solution*

V = volume of the *Standard stock solution* used to prepare the *Standard solution*, 10 mL

C_S = concentration of selenium in the *Standard stock solution* (µg/mL)

M_U = nominal amount of selenium in the *Sample solution* (µg)

Acceptance criteria: 90.0%–160.0% of the labeled amount of selenium (Se)

• **ZINC, *Method 1***

Zinc standard stock solution: 1000 µg/mL of zinc from zinc oxide in 5 M hydrochloric acid (3.89 mg/mL) and diluted with water to final volume. [NOTE—Dissolve in 5 M hydrochloric acid by warming, if necessary, cool, and then dilute to final volume.]

Standard stock solution: 50 µg/mL of zinc from *Zinc standard stock solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Transfer 1.0, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of zinc.

Polysorbate 80 solution: Prepare as directed in *Calcium, Method 1*.

Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain 2 µg/mL of zinc and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Zinc emission line at 213.8 nm

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Lamp: Zinc hollow-cathode
Flame: Air–acetylene
Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of zinc, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of zinc in the *Sample solution*.

Calculate the percentage of the labeled amount of zinc (Zn) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of zinc in the *Sample solution* (µg/mL)
C_U = nominal concentration of zinc in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of zinc (Zn)

- **BORON, NICKEL, TIN, and VANADIUM, *Method 1*; CALCIUM, CHROMIUM, COPPER, IRON, MAGNESIUM, MANGANESE, PHOSPHORUS, and ZINC, *Method 2*; MOLYBDENUM and SELENIUM, *Method 3***

Stock aqua regia solution: Prepare a mixture of hydrochloric acid and nitric acid (3:1) by adding the nitric acid to the hydrochloric acid. [NOTE—Periodically vent the solution in an appropriate fume hood.]

Diluent: Prepare a mixture of *Stock aqua regia solution* and water (1:9) by adding one volume of *Stock aqua regia solution* to two volumes of water. Dilute with additional water to volume, and mix well.

System suitability solution: Prepare a mixture of 1000 mg/L of yttrium in 5% nitric acid solution, 1000 mg/L of scandium in 5% nitric acid solution, and *Diluent* (1:1:198), and mix.

Standard stock solution 1 (Ca, Cu, Fe, Mg, Mn, P, and Zn): [NOTE—It is only necessary to include the minerals of interest in the solution.] Using commercially available element standard (single- or multi-element) solutions in 5% nitric acid solution, pipet the appropriate amount of element standard solution into a volumetric flask, and dilute with 5% nitric acid solution to obtain a solution having final concentrations of about 1000 mg/L of calcium, 100 mg/L of copper, 250 mg/L of iron, 500 mg/L of magnesium, 100 mg/L of manganese, 800 mg/L of phosphorus, and 250 mg/L of zinc.

Standard stock solution 2 (B, Cr, Mo, Ni, Se, Sn, and V): [NOTE—It is only necessary to include the minerals of interest in the solution.] Using commercially available element standard (single- or multi-element) solutions in 20% hydrochloric acid solution, pipet the appropriate amount of element standard solution into a volumetric flask, and dilute with 20% hydrochloric acid solution to obtain a solution having final concentrations of about 200 mg/L of boron, and 100 mg/L each of chromium, molybdenum, nickel, selenium, tin, and vanadium.

Standard solutions: Prepare a mixture of *Standard stock solution 1* and *Standard stock solution 2*, as required, in *Diluent*, to prepare a six-point calibration curve to bracket the concentration range of each mineral of interest.

Sample solution: Weigh, then transfer 5 Capsules to a 250-mL volumetric flask, and heat gently on a hot plate until the contents begin to release. Cautiously add 25 mL of *Stock aqua regia solution* in 5-mL increments, and swirl. Heat, continue to swirl until the Capsules dissolve into the acid, immediately remove from the heat source, and add 150 mL of water. Cool, and dilute with water to volume. Filter about 30 mL into a centrifuge tube, using a 5-µm pore size nylon syringe filter. If necessary, make any further adjustments using the *Diluent*.

Instrumental conditions

(See *Plasma Spectrochemistry* ⟨730⟩.)

Mode: Inductively coupled plasma spectrometry, using a spectrometer set to measure the emission of each mineral of interest at about the corresponding wavelength. [NOTE—The operating conditions may be developed and optimized based on the manufacturer's recommendation. The wavelengths selected should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision.]

System suitability

[NOTE—Analyze the *System suitability solution*, and obtain the response as directed in the *Analysis*.]

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the emission of each mineral of interest in the *Standard solutions* and *Sample solution* with an inductively coupled plasma system using the *Diluent* as the blank. Plot the emission of the *Standard solutions* versus the concentration, in mg/L, of the minerals of interest, and draw the straight line best fitting the plotted points. From the graph so obtained, determine the concentration, *C*, in mg/L, for each mineral of interest in the *Sample solution*.

Calculate the percentage of the labeled amount for each mineral taken:

$$\text{Result} = C \times (V/W) \times F \times (C_W/L) \times 100$$

C = measured concentration of the *Sample solution* (mg/L)
V = volume of the *Sample solution* (L)
W = sample weight (mg)
F = dilution factor of the *Sample solution*
C_W = average Capsule weight (mg)
L = labeled amount per Capsule (mg)

Acceptance criteria: 90.0%–125.0% of the labeled amount of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), and zinc (Zn); and 90.0%–160.0% of the labeled amounts of boron (B), chromium (Cr), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), and vanadium (V)

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩**:** Meet the requirements for *Dissolution*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩**:** Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 3×10^3 cfu/g, and the combined molds and yeasts count does not exceed 3×10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meet the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

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- **LABELING:**[1] The label states that the product is Oil-Soluble Vitamins with Minerals Capsules. The label also states the quantity of each vitamin and mineral per dosage unit and, where necessary, the chemical form in which a vitamin is present and also states the salt form of the mineral used as the source of each element. Where the product contains vitamin E, the label indicates whether it is the *d-* or *dl-* form. Where more than one assay method is given for a particular vitamin or mineral, the labeling states with which assay method the product complies only if *Method 1* is not used.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Alpha Tocopherol RS
  USP Alpha Tocopheryl Acetate RS
  USP Alpha Tocopheryl Acid Succinate RS
  USP Beta Carotene RS
  USP Beta Carotene System Suitability RS
  USP Cholecalciferol RS
  USP Ergocalciferol RS
  USP Phytonadione RS
  USP Retinyl Palmitate RS
  USP Sodium Fluoride RS
  USP Vitamin A RS

Oil-Soluble Vitamins with Minerals Oral Solution

DEFINITION

Oil-Soluble Vitamins with Minerals Oral Solution contains two or more of the following oil-soluble vitamins: Vitamin A, as retinol or esters of retinol in the form of retinyl acetate or retinyl palmitate; Vitamin D as ergocalciferol (Vitamin D_2) or cholecalciferol (Vitamin D_3); Vitamin E, as alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate; Phytonadione (Vitamin K_1); beta carotene; and one or more minerals derived from substances generally recognized as safe, furnishing one or more of the following elements in ionizable form: chromium, fluorine, iodine, iron, magnesium, manganese, molybdenum, and zinc. It contains NLT 90.0% and NMT 150.0% of the labeled amounts of vitamin A, as retinol equivalent ($C_{20}H_{30}O$); vitamin D, as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$); vitamin E, as alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$); phytonadione ($C_{31}H_{46}O_2$); and beta carotene ($C_{40}H_{56}$); NLT 90.0% and NMT 160.0% of the labeled amounts of chromium (Cr), fluorine (F), iodine (I), and molybdenum (Mo); and NLT 90.0% and NMT 125.0% of the labeled amounts of iron (Fe), magnesium (Mg), manganese (Mn), and zinc (Zn).

STRENGTH

- **VITAMIN A**

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: *n*-Hexane

Standard solution 1: 13 µg/mL of retinol from USP Retinyl Acetate RS in *n*-hexane

Standard solution 2: 13 µg/mL of retinol from USP Retinyl Palmitate RS in *n*-hexane

System suitability solution: Mix equal volumes of *Standard solution 1* and *Standard solution 2*.

Sample solution: Transfer an accurately measured volume of Oral Solution, equivalent to 3.25 mg of retinol, to a separatory funnel containing 10 mL of water and 20 mL of dehydrated alcohol. Add 100 mL of *n*-hexane, insert the stopper, and shake for 1 min. Allow the layers to separate, drain the aqueous layer into another separatory funnel, and repeat the extraction with 100 mL of *n*-hexane. Discard the aqueous layer, and combine the hexane extracts. Wash the combined extracts with 25 mL of water, allow the layers to separate, and discard the aqueous layer. Filter the washed hexane layer through anhydrous sodium sulfate into a 250-mL volumetric flask. Rinse the funnel and sodium sulfate with *n*-hexane, and add the rinsing to the hexane solution in the flask. Dilute the extracts in the volumetric flask with *n*-hexane to volume to obtain a solution with a concentration of 13 µg/mL of vitamin A as retinol ($C_{20}H_{30}O$).

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 325 nm
Column: 4.6-mm × 15-cm; packing L8
Flow rate: 1 mL/min
Injection volume: 40 µL

System suitability
Sample: *System suitability solution*
Suitability requirements
Resolution: NLT 10 between all-*trans*-retinyl acetate and all-*trans*-retinyl palmitate
Relative standard deviation: NMT 3.0%

Analysis
Samples: *Standard solution 1* or *Standard solution 2* and *Sample solution*

Calculate the percentage of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$), in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the all-*trans*-retinyl ester from the *Sample solution*
r_S = peak area of the all-*trans*-retinyl ester from the appropriate *Standard solution*
C_S = concentration of retinol in the appropriate *Standard solution* (µg/mL)
C_U = nominal concentration of vitamin A, as retinol, in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of vitamin A as retinol equivalent ($C_{20}H_{30}O$)

- **VITAMIN D**

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and

[1] USP Units of activity for vitamins, where such exist or formerly existed, are equivalent to the corresponding international units, where such formerly existed. The USP Unit for Vitamin E has been discontinued. International units (IU) for vitamins also have been discontinued; however, the use of IU on the labels of vitamin products continues. Where articles are labeled in terms of Units in addition to the required labeling, the relationship of the USP Units or IU to mass is as follows. One USP Vitamin A Unit = 0.3 µg of all-*trans*-retinol (vitamin A alcohol) or 0.344 µg of all-*trans*-retinyl acetate (vitamin A acetate) or 0.55 µg of all-*trans*-retinyl palmitate (vitamin A palmitate), and 1 µg of retinol (3.3 USP Vitamin A Units) = 1 retinol equivalent (RE); 1 IU of beta carotene = 0.6 µg of all-*trans*-beta carotene; 1 USP Vitamin D Unit = 0.025 µg of ergocalciferol or cholecalciferol; and 1 mg of *dl*-alpha tocopherol = 1.1 former USP Vitamin E Units, 1 mg of *dl*-alpha tocopheryl acetate = 1 former USP Vitamin E Unit, 1 mg of *dl*-alpha tocopheryl acid succinate = 0.89 former USP Vitamin E Unit, 1 mg of *d*-alpha tocopherol = 1.49 former USP Vitamin E Units, and 1 mg of *d*-alpha tocopheryl acetate = 1.36 former USP Vitamin E Units, 1 mg of *d*-alpha tocopheryl acid succinate = 1.21 former USP Vitamin E Units. In terms of *d*-alpha tocopherol equivalents, 1 mg of *d*-alpha tocopheryl acetate = 0.91, 1 mg of *d*-alpha tocopheryl acid succinate = 0.81, 1 mg of *dl*-alpha tocopherol = 0.74, 1 mg of *dl*-alpha tocopheryl acetate = 0.67, and 1 mg of *dl*-alpha tocopheryl acid succinate = 0.60.

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the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Mobile phase: *n*-Hexane and isopropyl alcohol (99:1)

Standard solution: 2 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in *n*-hexane

System suitability solution: Heat a volume of the *Standard solution* at 60° for 1 h to partially isomerize vitamin D (cholecalciferol or ergocalciferol) to its corresponding precursor.

Sample stock solution: Using an accurately measured volume of Oral Solution, equivalent to 5.0 mg of cholecalciferol or ergocalciferol, proceed as directed for the *Sample solution* in *Vitamin A* to obtain a solution with a concentration of 20 µg/mL of cholecalciferol or ergocalciferol in *n*-hexane.

Sample solution: Transfer 5.0 mL of the *Sample stock solution* to a container having a polytef-lined screw cap. Heat, with constant shaking, for 1 h in a water bath maintained at 60° to obtain a solution containing vitamin D (cholecalciferol or ergocalciferol) and its corresponding precursor. Cool, and dilute with *n*-hexane to obtain a solution containing 2 µg/mL of cholecalciferol or ergocalciferol.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 15-cm; 3-µm packing L8

Flow rate: 1 mL/min

Injection volume: 100 µL

System suitability

Sample: *System suitability solution*

Suitability requirements

Resolution: NLT 10 between the vitamin D form present and its corresponding precursor

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*

r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*

C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

F = correction factor to account for the average amount of pre-vitamin D present in the *Sample solution*, 1.09

Acceptance criteria: 90.0%–150.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

• **VITAMIN E**

[NOTE—Where vitamin E (alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Solution A: Phosphoric acid solution (1 in 100) in water

Mobile phase: Methanol and *Solution A* (19:1)

Standard solution: 2 mg/mL of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, or USP Alpha Tocopheryl Acid Succinate RS in methanol

Sample solution: Transfer NLT 20 mL of the solution prepared as directed for the *Sample solution* in *Vitamin A* to a suitable container, and evaporate in vacuum at room temperature to dryness. Transfer the residue with the aid of methanol to a suitable volumetric flask, and dilute with methanol to volume to obtain a concentration of 2 mg/mL of alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 291 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Flow rate: 1 mL/min

Injection volume: 50 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Tailing factor: NMT 1.5

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*

r_S = peak area of the relevant vitamin E form from the *Standard solution*

C_S = concentration of the corresponding USP Reference Standard in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding form of vitamin E in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of vitamin E

• **PHYTONADIONE (VITAMIN K_1)**

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Methanol and water (19:1)

Standard stock solution: 200 µg/mL of USP Phytonadione RS in methanol. Dissolve with the aid of sonication if necessary.

Standard solution: 20 µg/mL of USP Phytonadione RS from the *Standard stock solution* diluted with methanol

System suitability solution: 0.65 mg/mL of USP Alpha Tocopheryl Acetate RS and 20 µg/mL of USP Phytonadione RS from the *Standard stock solution* diluted with methanol. [NOTE—Dissolve USP Alpha Tocopheryl Acetate RS in a portion of methanol, add the *Standard stock solution*, and then dilute with methanol to volume.]

Sample solution: Transfer NLT 20 mL of the solution prepared as directed for the *Sample solution* in *Vitamin A* to a suitable container, and evaporate in vacuum at room temperature to dryness. Transfer the residue with the aid of methanol to a suitable volumetric flask, and dilute with methanol to volume to obtain a concentration of 20 µg/mL of phytonadione.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

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Detector: UV 254 nm
Column: 4.6-mm × 15-cm; 5-µm packing L1
Flow rate: 1 mL/min
Injection volume: 50 µL
System suitability
Samples: *Standard solution* and *System suitability solution*
Suitability requirements
Resolution: NLT 5 between alpha tocopheryl acetate and phytonadione, *System suitability solution*
Relative standard deviation: NMT 3.0%, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of phytonadione ($C_{31}H_{46}O_2$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*
r_S = peak area from the *Standard solution*
C_S = concentration of USP Phytonadione RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of phytonadione in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of phytonadione ($C_{31}H_{46}O_2$)

• **BETA CAROTENE**
[NOTE—Use low-actinic glassware.]
Mobile phase: Transfer 50 mg of butylated hydroxytoluene into a 1-L volumetric flask, and dissolve with 20 mL of 2-propanol. Add 0.2 mL of *N*-ethyldiisopropylamine, 25 mL of 0.2% ammonium acetate solution, 455 mL of acetonitrile, and about 450 mL of methanol. Allow the solution to reach room temperature, and dilute with methanol to volume.
Diluent: 50 µg/mL of butylated hydroxytoluene in alcohol
System suitability solution: Transfer 20 mg of USP Beta Carotene System Suitability RS to a 50-mL volumetric flask. Add 1 mL of water, 4 mL of tetrahydrofuran, and sonicate for 5 min. Dilute with *Diluent* to volume, and sonicate for 5 min. Cool to room temperature, pass the suspension through a membrane filter of 0.45-µm pore size, and use the clear filtrate.
Standard stock solution: 60 µg/mL of USP Beta Carotene RS in tetrahydrofuran
Standard solution A: Transfer 5.0 mL of the *Standard stock solution* into a 100-mL volumetric flask, add 5.0 mL of tetrahydrofuran, and dilute with *Diluent* to volume. Determine the concentration of *Standard solution A* from the concentration of *Standard solution B* as described below.
Standard solution B: Transfer 5.0 mL of the *Standard stock solution* into a 100-mL volumetric flask, and dilute with cyclohexane to volume. Prepare in triplicate.
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Analytical wavelength: 457 nm
Cell path: 1 cm
Blank: Cyclohexane
Analysis
Sample: *Standard solution B*
Calculate the concentration of total beta carotene (mg/mL) as all-*trans*-beta carotene ($C_{40}H_{56}$) in *Standard solution B*.
[NOTE—The concentration of *Standard solution B* equals the concentration of *Standard solution A*.]

$$\text{Result} = A/F$$

A = average absorbance of the three preparations of *Standard solution B*
F = absorptivity of pure all-*trans*-beta carotene in cyclohexane, 250

Sample solution: Transfer NLT 20 mL of the solution prepared as directed for the *Sample solution* in *Vitamin A* to a suitable container, and evaporate in vacuum at room temperature to dryness. Dissolve the residue in a mixture of methylene chloride and *Diluent* (1:1), and dilute with the same mixture to obtain a concentration of 3 µg/mL of beta carotene. Pass through a membrane filter of 0.45-µm pore size if necessary.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV-Vis 448 nm
Column: 4.6-mm × 25-cm; 5-µm packing L68
Column temperature: 30°
Flow rate: 0.6 mL/min
Injection volume: 20 µL
System suitability
Samples: *System suitability solution* and *Standard solution A*
[NOTE—The approximate relative retention times of the components in the *System suitability solution* are listed in *Table 1*.]

Table 1

Name	Relative Retention Time	Relative Response Factor
All-*trans*-alpha carotene	0.93	1.1
All-*trans*-beta carotene	1.00	1
9-*cis*-Beta carotene	1.07	1
13-*cis*-Beta carotene	1.17	1.2
15-*cis*-Beta carotene	1.21	1.4

Suitability requirements
Chromatogram similarity: The chromatogram from the *System suitability solution* is similar to the reference chromatogram provided with the lot of USP Beta Carotene System Suitability RS being used.
Resolution: NLT 1.5 between beta carotene and alpha carotene and between beta carotene and 9-*cis*-beta carotene, *System suitability solution*
Tailing factor: NMT 2.0 for the beta carotene peak, *Standard solution A*
Relative standard deviation: NMT 2.0% for the beta carotene peak from replicate injections, *Standard solution A*
Analysis
Samples: *Standard solution A* and *Sample solution*
Calculate the percentage of all-*trans*-beta carotene in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of all-*trans*-beta carotene from the *Sample solution*
r_S = peak area of all-*trans*-beta carotene from *Standard solution A*
C_S = concentration of all-*trans*-beta carotene in *Standard solution A* as determined by spectrometric procedure
C_U = nominal concentration of beta carotene in the *Sample solution*

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Acceptance criteria: 90.0%–150.0% of the labeled amount of beta carotene ($C_{40}H_{56}$)

• **CHROMIUM**

Chromium standard solution: 1000 µg/mL of chromium from potassium dichromate, previously dried at 120° for 4 h in water. Store in a polyethylene bottle.

Standard stock solution: 10 µg/mL of chromium from the *Chromium standard solution* diluted with 6 N hydrochloric acid and water (1 in 20)

Standard solutions: Transfer 10.0 and 20.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks, and transfer 15.0 and 20.0 mL of the *Standard stock solution* to separate 50-mL volumetric flasks. Dilute the contents of each of the four flasks with 0.125 N hydrochloric acid to volume to obtain concentrations of 1.0, 2.0, 3.0, and 4.0 µg/mL of chromium.

Sample solution: Dilute an accurately measured volume of Oral Solution to obtain a solution equivalent to 2.5 µg/mL of chromium in 0.125 N hydrochloric acid.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometer

Lamp: Chromium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: 357.9 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus concentration, in µg/mL, of chromium, and draw the straight line best fitting the four plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of chromium in the *Sample solution*.

Calculate the percentage of the labeled amount of chromium (Cr) in the portion of Oral Solution taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of chromium in the *Standard solution* (µg/mL)

C_U = nominal concentration of chromium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of chromium (Cr)

• **FLUORIDE**

[NOTE—Use plastic containers throughout this procedure.]

Ascorbic acid solution: 70 mg/mL of ascorbic acid in water

Mobile phase: Alcohol, water, and 1 N sulfuric acid (50:449:1)

Standard stock solution: 220 µg/mL of USP Sodium Fluoride RS in water. This solution contains 100 µg/mL of fluoride.

Standard solution: Transfer 5.0 mL of the *Standard stock solution* to a 100-mL volumetric flask. Add 2 mL of *Ascorbic acid solution*, 10 mL of alcohol, and about 70 mL of water, and mix. Adjust with 1 N sodium hydroxide to a pH of 4.25 ± 0.05. Dilute with water to volume, and mix to obtain 5 µg/mL of fluoride solution.

Sample solution: Transfer an accurately measured volume of the Oral Solution, equivalent to 0.5 mg of fluoride, to a 100-mL volumetric flask. Add 1 drop of hydrochloric acid, 10 mL of alcohol, and about 75 mL of water, and mix. Adjust with 1 N sodium hydroxide to a pH of 4.25 ± 0.05. Dilute with water to volume.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Conductivity

Columns

Guard: 4.6-mm × 3-cm; packing L17

Analytical: 7.8-mm × 30-cm; packing L17

Flow rate: 0.6 mL/min

Injection volume: 100 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of fluoride. Calculate the percentage of the labeled amount of fluorine (F) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of fluoride from the *Sample solution*

r_S = peak area of fluoride from the *Standard solution*

C_S = concentration of fluoride in the *Standard solution* (µg/mL)

C_U = nominal concentration of fluorine in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (F)

• **IODIDE, *Method 1***

Mobile phase: Dissolve 5.15 g of tetrabutylammonium bromide in 320 mL of acetonitrile. Dilute with water to 2000 mL.

System suitability solution: Transfer 0.13 g of potassium iodide and 0.5 g of potassium iodate to a 100-mL volumetric flask. Dissolve in *Mobile phase*, using sonication if necessary, dilute with *Mobile phase* to volume, and mix. Transfer 1.0 mL of this solution to a 100-mL volumetric flask, dilute with *Mobile phase* to volume, and mix. Transfer 25.0 mL of this solution to a 100-mL volumetric flask, and dilute with *Mobile phase* to volume.

Standard stock solution: 1.3 mg/mL of potassium iodide in *Mobile phase*. This solution has a concentration of 1 mg/mL of iodide.

Standard solution: 2.5 µg/mL of iodide from the *Standard stock solution* in *Mobile phase*

Sample solution: Dilute an accurately measured volume of the Oral Solution to obtain a solution with a concentration of 2.5 µg/mL of iodine in *Mobile phase*.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 225 nm

Column: 4.6-mm × 15-cm; packing L1

Flow rate: 1.5 mL/min

Injection volume: 30 µL

System suitability

Samples: *System suitability solution* and *Standard solution*

[NOTE—The relative retention times for iodate and iodide are about 0.32 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 2.5 between iodate and iodide, *System suitability solution*

Relative standard deviation: NMT 2.0% for the iodide peak, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of iodine (I) in the portion of Oral Solution taken:

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$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of iodide from the *Sample solution*
r_S = peak area of iodide from the *Standard solution*
C_S = concentration of iodide in the *Standard solution* (µg/mL)
C_U = nominal concentration of iodine in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of iodine (I)

• **IODIDE, *Method 2***

Analysis: Proceed as directed.

Acceptance criteria: 90.0%–160.0% of the labeled amount of iodine (I)

• **IRON**

Iron standard stock solution: Transfer 100 mg of iron powder to a 1000-mL volumetric flask. Dissolve in 6 N hydrochloric acid, and dilute with water to volume.

Standard solutions: To separate 100-mL volumetric flasks, transfer 2.0, 4.0, 5.0, 6.0, and 8.0 mL of *Iron standard stock solution*. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 2.0, 4.0, 5.0, 6.0, and 8.0 µg/mL of iron.

Sample solution: 6 µg/mL of iron from the Oral Solution in 0.125 N hydrochloric acid

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometer
Lamp: Iron hollow-cathode
Flame: Air–acetylene
Analytical wavelength: 248.3 nm
Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus concentration, in µg/mL, of iron, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of iron in the *Sample solution*.

Calculate the percentage of the labeled amount of iron (Fe) in the portion of Oral Solution taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of iron in the *Sample solution* from the graph (µg/mL)
C_U = nominal concentration of iron in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of iron (Fe)

• **MAGNESIUM**

Magnesium standard solution: Transfer 1.00 g of magnesium ribbon to a 1000-mL volumetric flask. Dissolve in 50 mL of 6 N hydrochloric acid, and dilute with water to volume.

Standard stock solution: 20 µg/mL of magnesium from *Magnesium standard solution* in 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 5.0, 7.5, 10.0, 12.5, and 15.0 mL of *Standard stock solution*. Dilute with 0.125 N hydrochloric acid to volume to obtain concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 µg/mL of magnesium.

Sample solution: 2.5 µg/mL of magnesium from the Oral Solution in 0.125 N hydrochloric acid

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometer
Analytical wavelength: 285.2 nm
Lamp: Magnesium hollow-cathode
Flame: Air–acetylene
Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus concentration, in µg/mL, of magnesium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of magnesium in the *Sample solution*.

Calculate the percentage of the labeled amount of magnesium (Mg) in the portion of Oral Solution taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of magnesium in the *Sample solution* from the graph (µg/mL)
C_U = nominal concentration of magnesium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of magnesium (Mg)

• **MANGANESE**

Manganese standard solution: Transfer 1.0 g of manganese to a 1000-mL volumetric flask. Dissolve in 20 mL of nitric acid, and dilute with 6 N hydrochloric acid to volume.

Standard stock solution: 50 µg/mL of manganese from the *Manganese standard stock solution* in 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Standard stock solution*. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions having known concentrations of 0.5, 0.75, 1.0, 1.5, and 2.0 µg/mL of manganese.

Sample solution: Dilute an accurately measured volume of the Oral Solution to obtain 1.5 µg/mL of manganese in 0.125 N hydrochloric acid.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometer
Analytical wavelength: 279.5 nm
Lamp: Manganese hollow-cathode
Flame: Air–acetylene
Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus concentration, in µg/mL, of manganese, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C, in µg/mL, of manganese in the *Sample solution*.

Calculate the percentage of the labeled amount of manganese (Mn) in the portion of Oral Solution taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of manganese in the *Sample solution* from the graph (µg/mL)
C_U = nominal concentration of manganese in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of manganese (Mn)

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• **MOLYBDENUM**

Molybdenum standard solution: Transfer 1.0 g of molybdenum wire to a 1000-mL volumetric flask, and dissolve in 50 mL of nitric acid, warming if necessary. Dilute with water to volume.

Standard stock solution: 100 µg/mL of molybdenum from the *Molybdenum standard solution* in water

Standard solutions: To separate 100-mL volumetric flasks transfer 0.5, 1.0, 1.5, 2.0, and 3.0 mL of the *Standard stock solution*. Add 0.125 N hydrochloric acid to volume, and mix to obtain the solutions having known concentrations of about 0.5, 1.0, 1.5, 2.0, and 3.0 µg/mL of molybdenum.

Sample solution: Dilute an accurately measured volume of the Oral Solution to obtain 1.5 µg/mL of molybdenum from the Oral Solution in 0.125 N hydrochloric acid.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometer

Analytical wavelength: 313.3 nm

Lamp: Molybdenum hollow-cathode

Flame: Nitrous oxide–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus concentration, in µg/mL, of molybdenum, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of molybdenum in the *Sample solution*.

Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Oral Solution taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of molybdenum in the *Sample solution* from the graph (µg/mL)

C_U = nominal concentration of molybdenum in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

• **ZINC**

Zinc standard solution: Transfer 311 mg of zinc oxide to a 250-mL volumetric flask, and add 80 mL of 6 N hydrochloric acid, warming if necessary to dissolve. Cool, dilute with water to volume, and mix to obtain a solution having a known concentration of 1000 µg/mL of zinc.

Standard stock solution: 50 µg/mL of zinc from the *Zinc standard solution* in 0.125 N hydrochloric acid

Standard solutions: Transfer 1.0, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of zinc.

Sample solution: Dilute an accurately measured volume of Oral Solution to obtain 1.5 µg/mL of zinc in 0.125 N hydrochloric acid.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometer

Analytical wavelength: 213.8 nm

Lamp: Zinc hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus concentration, in µg/mL, of zinc, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of zinc in the *Sample solution*.

Calculate the percentage of the labeled amount of zinc (Zn) in the portion of Oral Solution taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of zinc in the *Sample solution* from the graph (µg/mL)

C_U = nominal concentration of zinc in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of zinc (Zn)

OTHER COMPONENTS

• **ALCOHOL DETERMINATION,** *Method I* ⟨611⟩ (if present)**:** 90.0%–120.0% of the labeled amount of C_2H_5OH

CONTAMINANTS

• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 3×10^3 cfu/mL, and the combined molds and yeasts count does not exceed 3×10^2 cfu/mL.

• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, under an inert gas or with a minimum of headspace.

• **LABELING:**[1] The label states that the product is Oil-Soluble Vitamins with Minerals Oral Solution. The label states the quantity of each vitamin and mineral in a given volume of the Oral Solution and, where necessary, the chemical form in which a vitamin is present, and states also the salt form of the mineral used as the source of each element. Where the product contains vitamin E, the label indicates whether it is the *d-* or *dl-* form. Where more than one assay method is given for a particular mineral, the labeling states with which assay method the product complies only if *Method 1* is not used.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Alpha Tocopherol RS

USP Alpha Tocopheryl Acetate RS

USP Alpha Tocopheryl Acid Succinate RS

[1] USP Units of activity for vitamins, where such exist or formerly existed, are equivalent to the corresponding international units, where such formerly existed. The USP Unit for Vitamin E has been discontinued. International units (IU) for vitamins also have been discontinued. However, the use of IU on the labels of vitamin products continues. Where articles are labeled in terms of Units in addition to the required labeling, the relationship of the USP Units or IU to mass is as follows. One USP Vitamin A Unit = 0.3 µg of all-*trans*-retinol (vitamin A alcohol) or 0.344 µg of all-*trans*-retinyl acetate (vitamin A acetate) or 0.55 µg of all-*trans*-retinyl palmitate (vitamin A palmitate), and 1 µg of retinol (3.3 USP Vitamin A Units) = 1 retinol equivalent (RE); 1 IU of beta carotene = 0.6 µg of all-*trans*-beta carotene; 1 USP Vitamin D Unit = 0.025 µg of ergocalciferol or cholecalciferol; and 1 mg of *dl*-alpha tocopherol = 1.1 former USP Vitamin E Units, 1 mg of *dl*-alpha tocopheryl acetate = 1 former USP Vitamin E Unit, 1 mg of *dl*-alpha tocopheryl acid succinate = 0.89 former USP Vitamin E Unit, 1 mg of *d*-alpha tocopherol = 1.49 former USP Vitamin E Units, 1 mg of *d*-alpha tocopheryl acetate = 1.36 former USP Vitamin E Units, and 1 mg of *d*-alpha tocopheryl acid succinate = 1.21 former USP Vitamin E Units. In terms of *d*-alpha tocopherol equivalents, 1 mg of *d*-alpha tocopheryl acetate = 0.91, 1 mg of *d*-alpha tocopheryl acid succinate = 0.81, 1 mg of *dl*-alpha tocopherol = 0.74, 1 mg of *dl*-alpha tocopheryl acetate = 0.67, and 1 mg of *dl*-alpha tocopheryl acid succinate = 0.60.

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USP Beta Carotene RS
USP Beta Carotene System Suitability RS
USP Cholecalciferol RS
USP Ergocalciferol RS
USP Phytonadione RS
USP Retinyl Acetate RS
USP Retinyl Palmitate RS
USP Sodium Fluoride RS

Oil-Soluble Vitamins with Minerals Tablets

DEFINITION

Change to read:

Oil-Soluble Vitamins Tablets contain 2 or more of the following oil-soluble vitamins: Vitamin A ▲as retinyl acetate or retinyl palmitate,▲ (USP 1-May-2020) Vitamin D as Ergocalciferol (vitamin D_2) or Cholecalciferol (vitamin D_3), Vitamin E ▲as *RRR*- or *all-rac*-alpha-tocopherol, *RRR*- or *all-rac*-alpha-tocopheryl acetate, or *RRR*- or *all-rac*-alpha-tocopheryl-acid succinate,▲ (USP 1-May-2020) Phytonadione (vitamin K_1), and Beta Carotene; and one or more minerals derived from substances generally recognized as safe, furnishing one or more of the following elements in ionizable form: boron, calcium, chromium, copper, fluorine, iodine, iron, magnesium, manganese, molybdenum, nickel, phosphorus, potassium, selenium, tin, vanadium, and zinc. Tablets contain NLT 90.0% and NMT 165.0% of the labeled amount of vitamin A ▲as retinol▲ (USP 1-May-2020) ($C_{20}H_{30}O$), vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$), vitamin E ▲as 2*R*-alpha-tocopherol▲ (USP 1-May-2020) ($C_{29}H_{50}O_2$), phytonadione ($C_{31}H_{46}O_2$), and beta carotene ($C_{40}H_{56}$); NLT 90.0% and NMT 125.0% of the labeled amount of calcium (Ca), copper (Cu), iron (Fe), manganese (Mn), magnesium (Mg), phosphorus (P), potassium (K), and zinc (Zn); and NLT 90.0% and NMT 160.0% of the labeled amount of boron (B), chromium (Cr), fluorine (F), iodine (I), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), and vanadium (V).

Oil-Soluble Vitamins Tablets may contain other labeled added substances that are generally recognized as safe, in amounts that are unobjectionable.

IDENTIFICATION

Add the following:

▲• **A.** Characteristic absorption of each mineral as obtained in the tests in *Strength*▲ (USP 1-May-2020)

Add the following:

▲• **B.** The retention times of the vitamin peaks of the *Sample solutions* corresponds to those of the corresponding vitamin peaks of the *Standard solutions* as obtained in the tests for *Strength*.▲ (USP 1-May-2020)

STRENGTH

Add the following:

▲[NOTE—In the following assays, where more than one assay method is given for an individual ingredient, the requirements may be met by following any one of the specified methods, with the method used being stated in the labeling only if *Method 1* is not used.]▲ (USP 1-May-2020)

Change to read:

• **VITAMIN A,** ***Method 1***

▲Proceed as directed in *Vitamin A Assay* ⟨571⟩, *Assay, Chromatographic Methods, Procedure 1*, except for the *Sample stock solution* and *Sample solution*.▲ (USP 1-May-2020)

Sample stock solution: Finely powder NLT 20 Tablets. To a portion of the powder, equivalent to 5 Tablets, add 15 mL of water, and sonicate for 5 min. Add 15 mL of *n*-hexane, and shake for 15 min in a water bath maintained at 60°. Add 10 mL of dimethyl sulfoxide, and shake for an additional period of 30 min in a water bath maintained at 60°. Centrifuge at 3000 rpm for 10 min, and transfer the hexane layer by means of a pipet to a 100-mL volumetric flask. Add 15 mL of *n*-hexane to the dimethyl sulfoxide layer, shake thoroughly for 5 min, and transfer the hexane layer by means of a pipet to the 100-mL volumetric flask. Repeat this extraction with 3 additional 15-mL portions of *n*-hexane.

Sample solution: Dilute the *Sample stock solution* with *n*-hexane to obtain a solution with a concentration equivalent to 13 µg/mL of retinol ($C_{20}H_{30}O$).

▲▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$)

Change to read:

• **VITAMIN D (CHOLECALCIFEROL OR ERGOCALCIFEROL),** ***Method 1***

▲Proceed as directed in *Vitamin D Assay* ⟨581⟩, *Assay, Chromatographic Methods, Procedure 1*, except for the *Sample solution*.▲ (USP 1-May-2020)

Sample solution: Prepare as directed for the *Sample stock solution* in *Vitamin A, Method 1*. Transfer NLT 20 mL of this solution to a suitable container, and, if necessary, evaporate under vacuum at room temperature to obtain a concentration of 2 µg/mL of cholecalciferol or ergocalciferol.

▲▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

Change to read:

• **VITAMIN E,** ***Method 1***

▲Proceed as directed in *Vitamin E Assay* ⟨551⟩, *Assay, Procedure 1*, except for the *Sample solution* and *Analysis*.▲ (USP 1-May-2020)

Sample solution: Prepared as directed for the *Sample stock solution* in *Vitamin A, Method 1*. Transfer NLT 20 mL of this solution to a suitable container, and evaporate under vacuum at room temperature to dryness. Transfer the residue with the aid of methanol to a suitable volumetric flask, and dilute with methanol to volume to obtain a concentration of 2 mg/mL of alpha-tocopherol, alpha-tocopheryl acetate, or alpha-tocopheryl acid succinate.

▲▲ (USP 1-May-2020)

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas.

DS Monographs

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Calculate the percentage of the labeled amount of alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$) ▲as 2*R*-alpha-tocopherol ($C_{29}H_{50}O_2$)▲ (USP 1-May-2020) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \text{ ▲} \times F \text{▲ (USP 1-May-2020)} \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*

r_S = peak area of the relevant vitamin E form from the *Standard solution*

C_S = concentration of ▲*all-rac*-alpha-tocopherol from either USP Alpha Tocopherol RS or USP Alpha Tocopheryl Acetate RS or *RRR*-alpha tocopherol from USP Alpha Tocopheryl Acid Succinate RS▲ (USP 1-May-2020) in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding form of vitamin E ▲as 2*R*-alpha-tocopherol▲ (USP 1-May-2020) in the *Sample solution* (mg/mL)

▲F = conversion factor for the content of *all-rac* -alpha-tocopherol to 2*R*-alpha-tocopherol, 1/2 (for products labeled to contain *all-rac* vitamin E sources) and 1 (for products labeled to contain *RRR* vitamin E sources)▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–165.0% of the labeled amount of alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$) as ▲2*R*-alpha tocopherol▲ (USP 1-May-2020)

Change to read:

• **PHYTONADIONE,** ▲***Method 1***▲ (USP 1-May-2020)

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Methanol and water (19:1)

Standard stock solution: 200 µg/mL of USP Phytonadione RS in methanol. Dissolve with the aid of sonication if necessary.

Standard solution: 20 µg/mL of USP Phytonadione RS from the *Standard stock solution* diluted with methanol

System suitability solution: 0.65 mg/mL of USP Alpha Tocopheryl Acetate RS and 20 µg/mL of USP Phytonadione RS from the *Standard stock solution* diluted with methanol. [NOTE—Dissolve USP Alpha Tocopheryl Acetate RS in a portion of methanol, add the *Standard stock solution*, and then dilute with methanol to volume.]

Sample solution: Prepare as directed for the *Sample stock solution* in *Vitamin A, Method 1*. Transfer NLT 20 mL of this solution to a suitable container, and evaporate under vacuum at room temperature to dryness. Transfer the residue with the aid of methanol to a suitable volumetric flask, and dilute with methanol to volume to obtain a concentration of 20 µg/mL of phytonadione.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Flow rate: 1 mL/min

Injection volume: 50 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 5 between alpha tocopheryl acetate and phytonadione, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of phytonadione ($C_{31}H_{46}O_2$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Phytonadione RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of phytonadione in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of phytonadione ($C_{31}H_{46}O_2$)

Add the following:

▲• **VITAMIN A, VITAMIN D, VITAMIN E,** and **PHYTONADIONE,** ***Method 2***

[NOTE—Where vitamin A (retinyl acetate or retinyl palmitate), vitamin D (cholecalciferol or ergocalciferol), or vitamin E (alpha-tocopherol, alpha-tocopheryl acetate, or alpha-tocopheryl acid succinate) are specified in the following procedure, use the chemical form present in the formulation and the relevant Reference Standard. Use low-actinic glassware.]

Diluent: Methanol and water (90:10 v/v)

Solution A: 0.1% (v/v) trifluoroacetic acid in *Diluent*

Solution B: 0.1% (v/v) trifluoroacetic acid in methanol

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
19	0	100
27	0	100
28	100	0
30	100	0

Vitamin A standard stock solution: A solution containing the equivalent of 0.2 mg/mL of retinol from USP Retinyl Acetate RS or USP Retinyl Palmitate RS in isopropyl alcohol. Dissolve with the aid of sonication if necessary.

Vitamin D standard stock solution: 0.05 mg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in isopropyl alcohol. Dissolve with the aid of sonication if necessary.

Vitamin E standard stock solution: 3.0 mg/mL of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, or USP Alpha Tocopheryl Acid Succinate RS in isopropyl alcohol. Dissolve with the aid of sonication if necessary.

Phytonadione standard stock solution: 0.04 mg/mL of USP Phytonadione RS in isopropyl alcohol. Dissolve with the aid of sonication if necessary.

Sample solution 1: Finely powder NLT 20 Tablets. Transfer an accurately weighed portion of the powder into a 50-mL volumetric flask to obtain a solution having known nominal

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concentrations in the ranges of 0.05–0.1 mg/mL of vitamin A and 0.5–0.15 mg/mL of vitamin E. Add 10 mL of dimethyl sulfoxide, and sonicate for 30 min with vigorous intermittent shaking. Cool to room temperature, dilute with isopropyl alcohol to volume, and mix well. Pass a portion of the solution through a nylon filter of 0.45-µm pore size, and discard the first milliliter of the filtrate.

Sample solution 2: Transfer an accurately weighed portion of the powder from NLT 20 finely powdered Tablets into a 50-mL volumetric flask to obtain a solution having known nominal concentrations in the ranges of 1.0–3.5 µg/mL of vitamin D and 1.0–3.5 µg/mL of phytonadione. Add 5 mL of water and sonicate for 10 min with intermittent shaking. Add 10 mL of dimethyl sulfoxide and sonicate for 30 min with vigorous intermittent shaking. Cool to room temperature, dilute with isopropyl alcohol to volume, and mix well. Pass a portion of the solution through a nylon filter of 0.45-µm pore size, and discard the first milliliter of the filtrate.

Standard solution: Transfer calculated volumes of *Vitamin A standard stock solution*, Vitamin D standard stock solution, Vitamin E standard stock solution, and Phytonadione standard stock solution into a suitable volumetric flask, and dilute with isopropyl alcohol to volume to obtain a solution with final vitamin concentrations similar to those obtained for the corresponding vitamins in the *Sample solution 1* and *Sample solution 2*.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detectors

For *Sample solution 1* containing retinyl acetate: UV 325 nm for 7 min, then switch to 265 nm

For *Sample solution 1* containing retinyl palmitate: UV 265 nm for 18 min, then switch to 325 nm

For *Sample solution 2*: UV 265 nm

Column: 4.6-mm × 10-cm; 3-µm packing L1

Temperatures

Sample: 4°

Column: 35°

Flow rate: 1.5 mL/min

Injection volume: 20 µL

System suitability

Samples: *Sample solution 1*, *Sample solution 2*, and *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0% for each individual peak, *Standard solution*

Peak purity of vitamin D and vitamin A (as retinyl palmitate): Monitor the chromatograms of the *Sample solution 1* and *Sample solution 2* at 450 nm. The area of any peak at the retention time of retinyl palmitate from *Sample solution 1* and any peak at the retention time of vitamin D (as cholecalciferol or ergocalciferol) from *Sample solution 2* should be NMT 5% of the corresponding peak areas detected at 325 nm and 265 nm, respectively. [NOTE—This test is required due to possible interference with carotenoids (cryptoxanthin and lycopene) that may be present in the formulation. Use another procedure in the monograph for analysis of vitamin D and vitamin A (retinyl palmitate) if the system is not suitable.]

Analysis

Samples: *Sample solution 1*, *Sample solution 2*, and *Standard solution*

Calculate the percentage of the labeled amount of vitamin A activity, as retinol ($C_{20}H_{30}O$), in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the *all-trans*-retinyl ester from the *Sample solution*

r_S = peak area of the *all-trans*-retinyl ester from the *Standard solution*

C_S = concentration of retinol in the *Standard solution* (mg/mL)

C_U = nominal concentration of vitamin A, as retinol, in the *Sample solution* (mg/mL)

Calculate the percentage of the labeled amount of vitamin E, as 2*R*-alpha-tocopherol ($C_{29}H_{50}O_2$), in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*

r_S = peak area of the relevant vitamin E form from the *Standard solution*

C_S = concentration of *all-rac*-alpha-tocopherol from either USP Alpha Tocopherol RS or USP Alpha Tocopheryl Acetate RS or *RRR*-alpha tocopherol from USP Alpha Tocopheryl Acid Succinate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of vitamin E, as 2*R*-alpha tocopherol, in the *Sample solution* (mg/mL)

F = conversion factor for the content of *all-rac*-alpha tocopherol to *RRR*-alpha-tocopherol equivalent, 1/2 (for products labeled to contain *all-rac* vitamin E sources) and 1 (for products labeled to contain *RRR* vitamin E sources)

Calculate the percentage of the labeled amount of vitamin D, as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$), and phytonadione ($C_{31}H_{46}O_2$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the related vitamin from the *Sample solution*

r_S = peak area of the related vitamin from the *Standard solution*

C_S = concentration of related vitamin in the *Standard solution* (µg/mL)

C_U = nominal concentration of related vitamin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of each individual vitamin ▲ (USP 1-May-2020)

Change to read:

- **BETA CAROTENE**

[NOTE—Use low-actinic glassware.]

Mobile phase: Transfer 50 mg of butylated hydroxytoluene into a 1-L volumetric flask, and dissolve with 20 mL of 2-propanol. Add 0.2 mL of *N*-ethyldiisopropylamine, 25 mL of 0.2% ammonium acetate solution, 455 mL of acetonitrile, and about 450 mL of methanol. Allow the solution to reach room temperature, and dilute with methanol to volume.

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Diluent: 50 µg/mL of butylated hydroxytoluene in alcohol
System suitability solution: Transfer 20 mg of USP Beta Carotene System Suitability RS to a 50-mL volumetric flask. Add 1 mL of water, 4 mL of tetrahydrofuran, and sonicate for 5 min. Dilute with *Diluent* to volume, and sonicate for 5 min. Cool to room temperature, pass the suspension through a membrane filter of 0.45-µm pore size, and use the clear filtrate.
Standard stock solution: 60 µg/mL of USP Beta Carotene RS in tetrahydrofuran
Standard solution A: Transfer 5.0 mL of the *Standard stock solution* into a 100-mL volumetric flask, add 5.0 mL of tetrahydrofuran, and dilute with *Diluent* to volume.
Standard solution B: Transfer 5.0 mL of the *Standard stock solution* into a 100-mL volumetric flask, and dilute with cyclohexane to volume. Prepare in triplicate. Determine the concentration of *Standard solution A* from the concentration of *Standard solution B*.
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Analytical wavelength: 457 nm
Cell path: 1 cm
Blank: Cyclohexane
Analysis
Sample: *Standard solution B*
Calculate the concentration of total beta carotene (mg/mL) as *all-trans*-beta carotene ($C_{40}H_{56}$) in *Standard solution B*. [NOTE—The concentration of *Standard solution B* equals the concentration of *Standard solution A*.]

$$\text{Result} = A/F$$

A = average absorbance of the 3 preparations of *Standard solution B*
F = absorptivity of pure *all-trans*-beta carotene in cyclohexane, 250

Sample solution: Prepare as directed for the *Sample stock solution* in *Vitamin A*. Transfer NLT 20 mL of this solution to a suitable container, and evaporate under vacuum at room temperature to dryness. Dissolve the residue in a mixture of methylene chloride and *Diluent* (1:1), and dilute with the same mixture to obtain a concentration of 3 µg/mL of beta carotene. Pass through a membrane filter of 0.45-µm pore size, if necessary.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV-Vis 448 nm
Column: 4.6-mm × 25-cm; 5-µm packing L68
Column temperature: 30°
Flow rate: 0.6 mL/min
Injection volume: 20 µL
System suitability
Samples: *System suitability solution* and *Standard solution A*
The approximate relative retention times of the components in the *System suitability solution* are listed in ▲*Table 2*.▲ (USP 1-May-2020)

▲Table 2▲ (USP 1-May-2020)

Name	Relative Retention Time	Relative Response Factor
all-trans-Alpha carotene	0.93	1.1
all-trans-Beta carotene	1.00	1
9-*cis*-Beta carotene	1.07	1
13-*cis*-Beta carotene	1.17	1.2
15-*cis*-Beta carotene	1.21	1.4

Suitability requirements
Chromatogram similarity: The chromatogram from the *System suitability solution* is similar to the reference chromatogram provided with the USP Beta Carotene System Suitability RS being used.
Resolution: NLT 1.5 between beta carotene and alpha carotene and between beta carotene and 9-*cis*-beta carotene, *System suitability solution*
Tailing factor: NMT 2.0 for the beta carotene peak, *Standard solution A*
Relative standard deviation: NMT 2.0% for the beta carotene peak from replicate injections, *Standard solution A*
Analysis
Samples: *Standard solution A* and *Sample solution*
Calculate the percentage of *all-trans*-beta carotene in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of *all-trans*-beta carotene in the *Sample solution*
r_S = peak area of *all-trans*-beta carotene in *Standard solution A*
C_S = concentration of *all-trans*-beta carotene in *Standard solution A* as determined by spectrometric procedure
C_U = nominal concentration of beta carotene in the *Sample solution*

Acceptance criteria: 90.0%–165.0% of the labeled amount of beta carotene ($C_{40}H_{56}$)

• **CALCIUM, *Method 1***
Lanthanum chloride solution: 267 mg/mL of lanthanum chloride heptahydrate in 0.125 N hydrochloric acid
Calcium standard solution: 400 µg/mL of calcium. Dissolve 1.001 g of calcium carbonate, previously dried at 300° for 3 h and cooled in a desiccator for 2 h, in 25 mL of 1 N hydrochloric acid. Boil to expel carbon dioxide, and dilute with water to 1000 mL.
Standard stock solution: 100 µg/mL of calcium from the *Calcium standard solution* diluted with 0.125 N hydrochloric acid
Standard solutions: Into separate 100-mL volumetric flasks pipet 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, and dilute with 0.125 N hydrochloric acid to volume to obtain concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 µg/mL of calcium.
Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 5 Tablets, to a porcelain crucible. Heat the crucible in a muffle furnace maintained at 550° for 6–12 h, and cool. Add 60 mL of hydrochloric acid, and boil gently on a hot plate or steam bath for 30 min, intermittently rinsing the inner surface of the crucible with 6 N hydrochloric acid. Cool, and quantitatively transfer the contents of the crucible to a 100-mL volumetric flask. Rinse the crucible with small portions of 6 N hydrochloric acid, and add the rinsings to the flask. Dilute with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute this solution quantitatively, with 0.125 N hydrochloric acid, to obtain a

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concentration of 2 µg/mL of calcium, adding 1 mL of *Lanthanum chloride solution* per 100 mL of the final volume.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectroscopy

Analytical wavelength: Calcium emission line at 422.7 nm

Lamp: Calcium hollow-cathode

Flame: Nitrous oxide–acetylene

Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution* per 100 mL

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions, against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of calcium, and draw the straight line best fitting the 5 plotted points. From the graph, determine the concentration (C), in µg/mL, of calcium in the *Sample solution*.

Calculate the percentage of the labeled amount of calcium (Ca) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of calcium in the *Sample solution* (µg/mL)

C_U = nominal concentration of calcium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of calcium (Ca)

• **CHROMIUM, *Method 1***

Chromium standard solution: 1000 µg/mL of chromium from potassium dichromate, previously dried at 120° for 4 h in water. Store in a polyethylene bottle.

Standard stock solution: 10 µg/mL of chromium from the *Chromium standard solution* diluted with 6 N hydrochloric acid and water (1 in 20)

Standard solutions: Transfer 10.0 and 20.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks, and transfer 15.0 and 20.0 mL of the *Standard stock solution* to separate 50-mL volumetric flasks. Dilute the contents of each of the 4 flasks with 0.125 N hydrochloric acid to volume to obtain concentrations of 1.0, 2.0, 3.0, and 4.0 µg/mL of chromium.

Sample solution: Prepare as directed for *Calcium, Method 1*, except obtain a concentration of 2.5 µg/mL of chromium and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Chromium emission line at 357.9 nm

Lamp: Chromium hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of chromium, and draw the straight line best fitting the 4 plotted points. From the graph, determine the concentration (C), in µg/mL, of chromium in the *Sample solution*.

Calculate the percentage of the labeled amount of chromium (Cr) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of chromium in the *Sample solution* (µg/mL)

C_U = nominal concentration of chromium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of chromium (Cr)

• **COPPER, *Method 1***

Copper standard solution: Dissolve 1.00 g of copper foil in a minimum volume of a 50% solution of nitric acid, and dilute with a 1% solution of nitric acid to 1000 mL. This solution contains 1000 µg/mL of copper.

Standard stock solution: 100 µg/mL of copper from the *Copper standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 200-mL volumetric flasks transfer 1.0, 2.0, 4.0, 6.0, and 8.0 mL of the *Standard stock solution*. Dilute with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.5, 1.0, 2.0, 3.0, and 4.0 µg/mL of copper.

Sample solution: Prepare as directed for *Calcium, Method 1*, except obtain a concentration of 2 µg/mL of copper and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectroscopy

Analytical wavelength: Copper emission line at 324.7 nm

Lamp: Copper hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of copper, and draw the straight line best fitting the 5 plotted points. From the graph, determine the concentration (C), in µg/mL, of copper in the *Sample solution*.

Calculate the percentage of the labeled amount of copper (Cu) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of copper in the *Sample solution* (µg/mL)

C_U = nominal concentration of copper in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of copper (Cu)

• **FLUORIDE, *Method 1***

[NOTE—Store all solutions in plastic containers.]

3 M sodium acetate solution: Dissolve 408 g of sodium acetate in 600 mL of water contained in a 1000-mL volumetric flask. Allow the solution to equilibrate to room temperature, and dilute with water to volume. Adjust with a few drops of acetic acid to a pH of 7.0.

Sodium citrate solution: Dissolve 222 g of sodium citrate in 250 mL of water in a 1000-mL volumetric flask. Add 28 mL of perchloric acid, and dilute with water to volume.

Fluoride standard stock solution: 500 µg/mL of fluoride from a quantity of sodium fluoride in water, previously dried at 100° for 4 h and cooled in a desiccator

Intermediate stock solution A: 100 µg/mL of fluoride from the *Fluoride standard stock solution* diluted with water

Intermediate stock solution B: 10 µg/mL of fluoride from the *Fluoride standard stock solution* diluted with water

Standard solutions: To separate 100-mL volumetric flasks transfer 3.0, 5.0, and 10.0 mL of *Intermediate stock solution B* and 5.0 and 10.0 mL of *Intermediate stock solution A*. To

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each flask add 10.0 mL of 1 N hydrochloric acid, 25 mL of *3 M sodium acetate solution*, and 25.0 mL of *Sodium citrate solution*. Dilute the contents of each flask with water to volume to obtain concentrations of 0.3, 0.5, 1.0, 5.0, and 10.0 µg/mL of fluoride.

Sample solution: Transfer a quantity of the finely powdered Tablets, equivalent to 200 µg of fluoride, to a 100-mL volumetric flask. Add 10.0 mL of 1 N hydrochloric acid, 25.0 mL of *3 M sodium acetate solution*, and 25.0 mL of *Sodium citrate solution*, and dilute with water to volume.

Analysis

Samples: *Standard solutions* and *Sample solution*

To separate plastic beakers, each containing a plastic-coated stirring bar, transfer 50.0 mL each of the *Standard solutions* and the *Sample solution*. Measure the potentials (see *pH* ⟨791⟩), in mV, of the *Standard solutions* and the *Sample solution*, with a pH meter capable of a minimum reproducibility of ±0.2 mV and equipped with a fluoride-specific ion-indicating electrode and a calomel reference electrode. [NOTE—When taking measurements, immerse the electrodes in the solution, stir on a magnetic stirrer having an insulated top until equilibrium is attained (1–2 min), and record the potential. Rinse and dry the electrodes between measurements, taking care to avoid damaging the crystal of the specific-ion electrode.]

Plot the logarithms of fluoride concentrations, in µg/mL, of the *Standard solutions* versus potential, in mV. From the standard response curve and the measured potential of the *Sample solution*, determine the concentration (C), in µg/mL, of fluoride in the *Sample solution*.

Calculate the percentage of the labeled amount of fluorine (F) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of fluoride in the *Sample solution* (µg/mL)

C_U = nominal concentration of fluorine in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (F)

• **FLUORIDE, *Method 2***

[NOTE—Use plastic containers and deionized water throughout this procedure.]

pH 10.0 buffer: Add 214 mL of 0.1 N sodium hydroxide to 1000 mL of 0.05 M sodium bicarbonate.

Mobile phase: Alcohol, 0.1 N sulfuric acid, and water (20:5:175)

Standard stock solution: 220 µg/mL of USP Sodium Fluoride RS in water. This solution contains 100 µg/mL of fluoride.

Standard solution: [NOTE—Condition the solid-phase extraction column specified for use in the *Standard solution* and the *Sample solution* in the following manner. Using a vacuum at a pressure not exceeding 5 mm of mercury, wash the column with one column volume of methanol followed by one column volume of *pH 10.0 buffer*. Do not allow the column top to dry. If the top of the column becomes dry, recondition the column.] Transfer 10.0 mL of *Standard stock solution* to a 100-mL volumetric flask. Add 75 mL of water, and adjust with 0.1 N sodium hydroxide to a pH of 10.4 ± 0.1. Dilute with water to volume. Filter, discarding the first 15 mL of the filtrate. Transfer 25.0 mL of the filtrate to a 50-mL volumetric flask, add 15.0 mL of water, and adjust with 0.1 N sodium hydroxide to a pH of 10.0. Dilute with *pH 10.0 buffer* to volume. Elute a portion of this solution through a 3-mL solid-phase extraction column containing L1 packing that is connected through an adaptor to a second solid-phase extraction column containing sulfonylpropyl strong cation-exchange packing. Discard the first 3 mL of the eluate, and collect the rest of the eluate in a suitable flask for injection into the chromatograph.

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of powdered Tablets, equivalent to 1 mg of fluorine, in 15 mL of water, and shake vigorously. Rinse the sides of the flask with 15 mL of water, and allow to stand for 10 min. Dilute with water to 85 mL, adjust with 1 N sodium hydroxide to a pH of 10.4 ± 0.1, and dilute with water to 100 mL. Prepare as directed for the *Standard solution*, beginning with "Filter, discarding the first 15 mL of the filtrate."

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Conductivity

Column

Guard: 4.6-mm × 3-cm; packing L17

Analytical: 7.8-mm × 30-cm; packing L17

Flow rate: 0.5 mL/min

Injection volume: 100 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for fluoride.

Calculate the percentage of the labeled amount of fluorine (F) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of fluoride in the *Standard solution* (µg/mL)

C_U = nominal concentration of fluorine in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (F)

• **IODIDE, *Method 1***

Bromine water: To 20 mL of bromine in a glass-stoppered bottle add 100 mL of water. Insert the stopper into the bottle, and shake. Allow to stand for 30 min, and use the supernatant.

Analysis

Sample: Tablets

Transfer an amount of finely powdered Tablets, equivalent to 3 mg of iodide, to a nickel crucible. Add 5 g of sodium carbonate, 5 mL of 50% (w/v) sodium hydroxide solution, and 10 mL of alcohol, taking care that the entire specimen is moistened. Heat the crucible on a steam bath to evaporate the alcohol, then dry the crucible at 100° for 30 min to prevent spattering upon subsequent heating. Transfer the crucible with its contents to a furnace heated to 500°, and heat the crucible for 15 min. [NOTE—Heating at 500° is necessary to carbonize any organic matter present; a higher temperature may be used, if necessary, to ensure complete carbonization of all organic matter.]

Cool the crucible, add 25 mL of water, cover the crucible with a watchglass, and boil gently for 10 min. Filter the solution, and wash the crucible with boiling water, collecting the filtrate and washings in a beaker. Add phosphoric acid until the solution is neutral to methyl orange, then add 1 mL excess of phosphoric acid. Add excess of *Bromine water*, and boil the solution gently until

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colorless for 5 min longer. Add a few crystals of salicylic acid, and cool the solution to 20°. Add 1 mL of phosphoric acid and 0.5 g of potassium iodide, and titrate the liberated iodine with 0.005 N sodium thiosulfate VS, adding starch TS when the liberated iodine color has nearly disappeared.

Calculate the percentage of the labeled amount of iodine (I) in the portion of Tablets taken:

$$\text{Result} = V \times N_A \times F \times I_{me} \times (A_w/W) \times (100/L)$$

V = volume of sodium thiosulfate consumed (mL)
N_A = actual normality of the sodium thiosulfate solution used
F = correction factor to convert mg to µg, 1000 µg/mL
I_{me} = milliequivalent weight of iodine, 21.16 mg/meq
A_w = average weight of the Tablets
W = weight of the portion of Tablets taken
L = labeled amount of iodine (µg/Tablet)

Acceptance criteria: 90.0%–160.0% of the labeled amount of iodine (I)

• **IODIDE, *Method 2*:** Proceed as directed.

Acceptance criteria: 90.0%–160.0% of the labeled amount of iodine (I)

• **IRON, *Method 1***

Iron standard stock solution: Transfer 100 mg of iron powder to a 1000-mL volumetric flask. Dissolve in 25 mL of 6 N hydrochloric acid, dilute with water to volume, and mix.

Standard solutions: To separate 100-mL volumetric flasks transfer 2.0, 4.0, 5.0, 6.0, and 8.0 mL of the *Iron standard stock solution*. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 2.0, 4.0, 5.0, 6.0, and 8.0 µg/mL of iron.

Sample solution: Prepare as directed for *Calcium, Method 1*, except obtain a concentration of 5 µg/mL of iron and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectroscopy

Analytical wavelength: Iron emission line at 248.3 nm

Lamp: Iron hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of iron, and draw the straight line best fitting the 5 plotted points. From the graph, determine the concentration (C), in µg/mL, of iron in the *Sample solution*.

Calculate the percentage of the labeled amount of iron (Fe) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of iron in the *Sample solution* (µg/mL)
C_U = nominal concentration of iron in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of iron (Fe)

• **MAGNESIUM, *Method 1***

Lanthanum chloride solution: Prepare as directed in *Calcium, Method 1*.

Magnesium standard solution: Transfer 1.0 g of magnesium ribbon to a 1000-mL volumetric flask, dissolve in 50 mL of 6 N hydrochloric acid, dilute with water to volume, and mix to obtain a solution with a known concentration of 1000 µg/mL of magnesium.

Standard stock solution: 20 µg/mL of magnesium from the *Magnesium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, and dilute with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.2, 0.3, 0.4, 0.5, and 0.6 µg/mL of magnesium.

Sample solution: Prepare as directed for *Calcium, Method 1*, except obtain a concentration of 0.4 µg/mL of magnesium.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Magnesium emission line at 285.2 nm

Lamp: Magnesium hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution* per 100 mL

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of magnesium, and draw the straight line best fitting the 5 plotted points. From the graph, determine the concentration (C), in µg/mL, of magnesium in the *Sample solution*.

Calculate the percentage of the labeled amount of magnesium (Mg) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of magnesium in the *Sample solution* (µg/mL)
C_U = nominal concentration of magnesium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of magnesium (Mg)

• **MANGANESE, *Method 1***

Manganese standard stock solution: Transfer 1.00 g of manganese to a 1000-mL volumetric flask. Dissolve in 20 mL of nitric acid, dilute with 6 N hydrochloric acid to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of manganese.

Standard stock solution: 50 µg/mL of manganese from the *Manganese standard stock solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Standard stock solution*. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions with known concentrations of 0.5, 0.75, 1.0, 1.5, and 2.0 µg/mL of manganese.

Sample solution: Proceed as directed for *Calcium, Method 1*, except obtain a concentration of 1 µg/mL of manganese and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectroscopy

Analytical wavelength: Manganese emission line at 279.5 nm

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Lamp: Manganese hollow-cathode
Flame: Air–acetylene
Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of manganese, and draw the straight line best fitting the 5 plotted points. From the graph, determine the concentration (C), in µg/mL, of manganese in the *Sample solution*.

Calculate the percentage of the labeled amount of manganese (Mn) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of manganese in the *Sample solution* (µg/mL)
C_U = nominal concentration of manganese in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of manganese (Mn)

• **MOLYBDENUM, *Method 1***

Diluent: 20 mg/mL of ammonium chloride in water

Molybdenum standard solution: Transfer 1.0 g of molybdenum wire to a 1000-mL volumetric flask, and dissolve in 50 mL of nitric acid, warming if necessary. Dilute with water to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of molybdenum.

Standard stock solution: 100 µg/mL of molybdenum from the *Molybdenum standard solution* diluted with water

Standard solutions: To separate 100-mL volumetric flasks transfer 2.0, 10.0, and 25.0 mL of the *Standard stock solution*, and add 5.0 mL of perchloric acid to each flask. Gently boil the solution in each flask for 15 min, cool to room temperature, and dilute each with *Diluent* to volume to obtain concentrations of 5.0, 10.0, and 25.0 µg/mL of molybdenum.

Sample solution: Transfer a portion of the powder, equivalent to 1000 µg of molybdenum, to a suitable flask, and add 12 mL of nitric acid. [NOTE—The volume of nitric acid may be varied to ensure that the powder is uniformly dispersed.] Carefully swirl the flask to disperse the test specimen. Sonicate for 10 min, or until the test specimen is completely dissolved. Gently boil the solution for 15 min, and cool to room temperature. Carefully add 8 mL of perchloric acid, heat until perchloric acid fumes appear, and swirl the flask to dissipate the fumes. Repeat the heating and swirling until the fumes appear again. Cool to room temperature. Quantitatively transfer the contents of the flask to a 100-mL volumetric flask with the aid of the *Diluent*, and dilute with *Diluent* to volume.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectroscopy
Analytical wavelength: Molybdenum emission line at 313.3 nm
Lamp: Molybdenum hollow-cathode
Flame: Nitrous oxide–acetylene
Blank: *Diluent* and perchloric acid (20:1)

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of molybdenum, and draw the straight line best fitting the 3 plotted points. From the graph, determine the concentration (C), in µg/mL, of molybdenum in the *Sample solution*.

Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of molybdenum in the *Sample solution* (µg/mL)
C_U = nominal concentration of molybdenum in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

• **MOLYBDENUM, *Method 2***

Sodium fluoride solution: Add 200 mL of water to 10 g of sodium fluoride, stir until the solution is saturated, and filter. Store in a polyethylene bottle.

Ferrous sulfate solution: 4.98 mg/mL of ferrous sulfate in water

Potassium thiocyanate solution: 200 mg/mL of potassium thiocyanate in water

20% stannous chloride solution: Transfer 40 mg of stannous chloride to a beaker, add 20 mL of 6.5 N hydrochloric acid solution, and heat the solution until the stannous chloride is dissolved. Cool, and dilute with water to 100 mL.

Diluted stannous chloride solution: *20% stannous chloride solution* diluted with water (1 in 25). Prepare this solution fresh at the time of use.

Standard solution: 20 µg/mL of molybdenum in water

Sample: A portion of finely powdered Tablets, equivalent to 40 µg of molybdenum

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Mode: UV-Vis
Analytical wavelength: 465 nm
Cell: 1 cm
Blank: Amyl alcohol

Analysis

Samples: *Standard solution* and *Sample*

Transfer the *Sample* and 2.0 mL of the *Standard solution* to separate 200-mL beakers. Add 20 mL of nitric acid to each beaker. Cover each beaker with a watchglass, and boil slowly on a hot plate for 45 min. Cool to room temperature. Add 6 mL of perchloric acid, cover the beakers with a watchglass, and continue the heating until digestion is complete, as indicated when the liquid becomes colorless or pale yellow. Evaporate the solutions in the beakers to dryness. Rinse the sides of the beakers and the watchglasses with water, and add more water to complete 50 mL in each beaker. Gently boil the water solution for a few min. Cool to room temperature. Add 2 drops of methyl orange TS, and neutralize with ammonium hydroxide. Add 8.2 mL of hydrochloric acid. Quantitatively transfer the contents of the beakers to separate 100-mL volumetric flasks, rinse the beakers with water, transfer the rinsings to the corresponding volumetric flasks, and dilute with water to volume. Transfer 50.0 mL of each solution to separatory funnels. To each separatory funnel add 1.0 mL of *Sodium fluoride solution*, 0.5 mL of *Ferrous sulfate solution*, 4.0 mL of *Potassium thiocyanate solution*, 1.5 mL of *20% stannous chloride solution*, and 15.0 mL of amyl alcohol, and shake the separatory funnel for 1 min. Allow the layers to separate, and discard the aqueous layers. Add 25 mL of *Diluted stannous chloride solution* to each separatory funnel, and shake gently for 15 s. Allow the layers to separate, and discard the aqueous layers. Transfer the organic layer from each separatory funnel to a centrifuge tube, and centrifuge at 2000 rpm for 10 min. Determine

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the absorbances of the organic phases obtained from the *Standard solution* and the *Sample*, and correct with the *Blank*.

Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Tablets taken:

$$\text{Result} = (A_U/A_S) \times [(V \times C_S)/M_U] \times 100$$

A_U = absorbance of the *Sample*
A_S = absorbance of the *Standard solution*
V = volume of the *Standard solution* analyzed, 2.0 mL
C_S = concentration of molybdenum in the *Standard solution* (µg/mL)
M_U = nominal amount of molybdenum in the *Sample* (µg)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

• **PHOSPHORUS, *Method 1***

Sulfuric acid solution: Cautiously add sulfuric acid to water (37.5: 100), and mix.

Ammonium molybdate solution: 50 mg/mL of ammonium molybdate in *Sulfuric acid solution* and water (2:3). [NOTE—Dissolve in water first, and then dilute with *Sulfuric acid solution* to volume.]

Hydroquinone solution: 5 mg/mL of hydroquinone in water. Add one drop of sulfuric acid per 100 mL of solution.

Sodium bisulfite solution: 200 mg/mL of sodium bisulfite in water

Phosphorus standard stock solution: Weigh 4.395 g of monobasic potassium phosphate, previously dried at 105° for 2 h and stored in a desiccator, and transfer to a 1000-mL volumetric flask. Dissolve in water, add 6 mL of sulfuric acid as a preservative, dilute with water to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of phosphorus.

Standard solution: 20 µg/mL of phosphorus from the *Phosphorus standard stock solution* diluted with water

Sample solution: [NOTE—Finely powder and weigh a counted number of Tablets.] Transfer a portion of the powder, equivalent to 100 mg of phosphorus, to 25 mL of nitric acid, and digest on a hot plate for 30 min. Add 15 mL of hydrochloric acid, and continue the digestion to the cessation of brown fumes. Cool, and transfer the contents of the flask to a 500-mL volumetric flask with the aid of small portions of water. Dilute with water to volume. Transfer 10.0 mL of this solution to a 100-mL volumetric flask, and dilute with water to volume.

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Mode: Vis

Analytical wavelength: 650 nm

Cell: 1 cm

Analysis

Samples: *Standard solution* and *Sample solution*

To 3 separate 25-mL volumetric flasks transfer 5.0 mL each of the *Standard solution*, the *Sample solution*, and water to provide the blank. To each of the 3 flasks add 1.0 mL each of *Ammonium molybdate solution*, *Hydroquinone solution*, and *Sodium bisulfite solution*, and swirl to mix. Dilute the contents of each flask with water to volume, and allow the flasks to stand for 30 min. Determine the absorbances of the solutions against the blank.

Calculate the percentage of the labeled amount of phosphorus (P) in the portion of Tablets taken:

$$\text{Result} = (A_U/A_S) \times (C_S/C_U) \times 100$$

A_U = absorbance of the *Sample solution*
A_S = absorbance of the *Standard solution*
C_S = concentration of phosphorus in the *Standard solution* (µg/mL)
C_U = nominal concentration of phosphorus in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of phosphorus (P)

• **POTASSIUM**

Potassium standard solution: 100 µg/mL of potassium from potassium chloride, previously dried at 105° for 2 h, in water

Standard stock solution: 10 µg/mL of potassium from the *Potassium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Transfer 5.0, 10.0, 15.0, 20.0, and 25.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions containing 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of potassium.

Sample solution: Prepare as directed for *Calcium, Method 1*, except obtain a concentration of 1.5 µg/mL of potassium and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Potassium emission line at 766.5 nm

Lamp: Potassium hollow-cathode

Flame: Air–acetylene

Blank: Water

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of potassium, and draw the straight line best fitting the 5 plotted points. From the graph, determine the concentration (C), in µg/mL, of potassium in the *Sample solution*.

Calculate the percentage of the labeled amount of potassium (K) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of potassium in the *Sample solution* (µg/mL)
C_U = nominal concentration of potassium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of potassium (K)

• **SELENIUM, *Method 1***

Diluent: Prepare as directed in *Molybdenum, Method 1*.

Selenium standard solution: [**CAUTION**—Selenium is toxic; handle it with care.] Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness. Add 2 mL of water, and evaporate to dryness. Repeat the addition of water and the evaporation to dryness 3 times. Dissolve the residue in 3 N hydrochloric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume to obtain a concentration of 1000 µg/mL of selenium.

Standard stock solution: 100 µg/mL of selenium from the *Selenium standard solution* diluted with water

Standard solutions: To separate 100-mL volumetric flasks transfer 5.0, 10.0, and 25.0 mL of the *Standard stock solution*, and add 5.0 mL of perchloric acid to each flask. Gently boil the solutions for 15 min, cool to room temperature, and dilute each with *Diluent* to volume to

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obtain solutions with concentrations of 5.0, 10.0, and 25.0 µg/mL of selenium.

Sample solution: Transfer a portion of the powder, equivalent to 1000 µg of selenium, to a suitable flask, and add 12 mL of nitric acid. [NOTE—The volume of nitric acid may be varied to ensure that the powder is uniformly dispersed.] Carefully swirl the flask to disperse the test specimen. Sonicate for 10 min or until the test specimen is completely dissolved. Gently boil the solution for 15 min, and cool to room temperature. Carefully add 8 mL of perchloric acid to the flask, heat the flask until perchloric acid fumes appear, and swirl the flask to dissipate the fumes. Repeat the heating and swirling until the fumes appear again. Cool to room temperature. Transfer the contents of the flask to a 50-mL volumetric flask with the aid of the *Diluent*, and dilute with *Diluent* to volume.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Selenium emission line at 196.0 nm

Lamp: Selenium hollow-cathode

Flame: Air–acetylene

Blank: *Diluent* and perchloric acid (20:1)

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of selenium, and draw the straight line best fitting the 3 plotted points. From the graph, determine the concentration (C), in µg/mL, of selenium in the *Sample solution*.

Calculate the percentage of the labeled amount of selenium (Se) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of selenium in the *Sample solution* (µg/mL)

C_U = nominal concentration of selenium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of selenium (Se)

• **SELENIUM, *Method 2***

Hydrochloric acid solution: Hydrochloric acid diluted with water (1 in 10)

50% ammonium hydroxide solution: Ammonium hydroxide diluted with water (1 in 2)

Reagent A: 9 mg/mL of edetate disodium and 25 mg/mL of hydroxylamine hydrochloride in water. [NOTE—Dissolve edetate disodium in a portion of water first, then add hydroxylamine hydrochloride, and dilute with water to volume.]

Reagent B: Transfer 200 mg of 2,3-diaminonaphthalene to a 250-mL separatory funnel, and add 200 mL of 0.1 N hydrochloric acid. Wash the solution with three 40-mL portions of cyclohexane, and discard the cyclohexane layer. Filter the solution into a brown bottle, and cover the solution with a 1-cm layer of cyclohexane. This solution is stable for 1 week if stored in a refrigerator.

Standard stock solution: [**CAUTION**—Selenium is toxic; handle it with care.] Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness. Add 2 mL of water, and evaporate to dryness. Repeat the addition of water and evaporation to dryness 3 times. Dissolve the residue in 3 N hydrochloric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume to obtain a solution with a concentration of 1000 µg/mL of selenium. Dilute a volume of the solution with 0.125 N hydrochloric acid to obtain a concentration of 2.0 µg/mL of selenium.

Standard solution: Transfer 10 mL of the *Standard stock solution* to a glass-stoppered flask. Add 1 mL of perchloric acid and 1 mL of *Hydrochloric acid solution*, and dilute with water to 20 mL.

Sample solution: Transfer a portion of finely powdered Tablets, equivalent to 20 µg of selenium, to a suitable flask. Add 10 mL of nitric acid, and warm gently on a hot plate. Continue heating until the initial nitric acid reaction has subsided, then add 3 mL of perchloric acid. [**CAUTION**—Exercise care at this stage, because the perchloric acid reaction becomes vigorous.]

Continue heating on the hot plate until the appearance of white fumes of perchloric acid or until the digest begins to darken. Add 0.5 mL of nitric acid and resume heating, adding additional amounts of nitric acid if further darkening occurs. Digest for 10 min after the first appearance of perchloric acid fumes or until the digest becomes colorless. Cool the flask, add 2.5 mL of *Hydrochloric acid solution*, and return the flask to the hot plate to expel residual nitric acid. Heat the mixture for 3 min after it begins to boil. Cool the flask to room temperature, and dilute with water to 20 mL.

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Mode: UV

Analytical wavelength: 380 nm

Cell: 1 cm

Blank: 1 mL of perchloric acid and 1 mL of *Hydrochloric acid solution* diluted with water to 20 mL

Analysis

Samples: *Standard solution* and *Sample solution*

Treat the *Sample solution*, the *Standard solution*, and the *Blank* as follows. Add 5 mL of *Reagent A* to each flask, and swirl gently to mix. Adjust the solution in each flask with *50% ammonium hydroxide solution* to a pH of 1.1 ± 0.1. Add 5 mL of *Reagent B* to each flask, and swirl gently to mix. Place the flasks in a water bath maintained at 50°, and equilibrate for 30 min, taking care that the flasks are covered to protect them from light. Cool to room temperature, and transfer the contents of each flask to separate separatory funnels. Transfer 10.0 mL of cyclohexane to each separatory funnel, and extract vigorously for 1 min. Discard the aqueous layer. Transfer the cyclohexane layer to a centrifuge tube, and centrifuge at 1000 rpm for 1 min to remove any remaining water. Determine the absorbances of the solutions obtained from the *Samples* against the solution obtained from the *Blank*.

Calculate the percentage of the labeled amount of selenium (Se) in the portion of Tablets taken:

$$\text{Result} = (A_U/A_S) \times [(V \times C_S)/M_U] \times 100$$

A_U = absorbances of the cyclohexane layer from the *Sample solution*

A_S = absorbances of the cyclohexane layer from the *Standard solution*

V = volume of the *Standard stock solution* used to prepare the *Standard solution*, 10 mL

C_S = concentration of selenium in the *Standard stock solution* (µg/mL)

M_U = nominal amount of selenium in the *Sample solution* (µg)

Acceptance criteria: 90.0%–160.0% of the labeled amount of selenium (Se)

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• **ZINC, *Method 1***

Zinc standard stock solution: 1000 µg/mL of zinc from zinc oxide in 5 M hydrochloric acid (3.89 mg/mL) and diluted with water to final volume. [NOTE—Dissolve in 5 M hydrochloric acid by warming, if necessary, cool, and then dilute to final volume.]

Standard stock solution: 50 µg/mL of zinc from the *Zinc standard stock solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Transfer 1.0, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of zinc.

Sample solution: Prepare as directed for the *Sample solution* in *Calcium, Method 1*, except obtain a concentration of 2 µg/mL of zinc and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Zinc emission line at 213.8 nm

Lamp: Zinc hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of zinc, and draw the straight line best fitting the 5 plotted points. From the graph, determine the concentration (C), in µg/mL, of zinc in the *Sample solution*.

Calculate the percentage of the labeled amount of zinc (Zn) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of zinc in the *Sample solution* (µg/mL)

C_U = nominal concentration of zinc in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of zinc (Zn)

• **BORON, NICKEL, TIN,** and **VANADIUM, *Method 1*; CALCIUM, CHROMIUM, COPPER, IRON, MAGNESIUM, MANGANESE, PHOSPHORUS,** and **ZINC, *Method 2*; MOLYBDENUM** and **SELENIUM, *Method 3***

Stock aqua regia solution: Prepare a mixture of hydrochloric acid and nitric acid (3:1) by adding the nitric acid to the hydrochloric acid. [NOTE—Periodically vent the solution in an appropriate fume hood.]

Diluent: Prepare a mixture of *Stock aqua regia solution* and water (1:9) by adding 1 volume of *Stock aqua regia solution* to 2 volumes of water. Dilute with additional water to volume, and mix well.

System suitability solution: Prepare a mixture of 1000 mg/L of yttrium in 5% nitric acid solution, 1000 mg/L of scandium in 5% nitric acid solution, and *Diluent* (1:1:198), and mix.

Standard stock solution 1 (Ca, Cu, Fe, Mg, Mn, P, and Zn): [NOTE—It is only necessary to include the minerals of interest in the solution.] Using commercially available element standard (single- or multi-element) solutions in 5% nitric acid solution, pipet the appropriate amount of element standard solution into a volumetric flask, and dilute with 5% nitric acid solution to obtain a solution having final concentrations of about 1000 mg/L of calcium, 100 mg/L of copper, 250 mg/L of iron, 500 mg/L of magnesium, 100 mg/L of manganese, 800 mg/L of phosphorus, and 250 mg/L of zinc.

Standard stock solution 2 (B, Cr, Mo, Ni, Se, Sn, and V): [NOTE—It is only necessary to include the minerals of interest in the solution.] Using commercially available element standard (single- or multi-element) solutions in 20% hydrochloric acid solution, pipet the appropriate amount of element standard solution into a volumetric flask, and dilute with 20% hydrochloric acid solution to obtain a solution having final concentrations of about 200 mg/L of boron, and 100 mg/L each of chromium, molybdenum, nickel, selenium, tin, and vanadium.

Standard solutions: Prepare a mixture of *Standard stock solution 1* and *Standard stock solution 2*, as required, in *Diluent* to prepare a 6-point calibration curve to bracket the concentration range of each mineral of interest.

Sample solution 1 (for Tablets containing minerals in ***Standard stock solution 1*** and ***Standard stock solution 2***): Weigh and finely powder NLT 20 Tablets. Transfer a portion, equal to 3.5 times the average Tablet weight, to a 250-mL volumetric flask. Slowly add 25 mL of *Stock aqua regia solution* in 5-mL increments followed by mixing. [NOTE—If the sample contains a carbonate, bubbling will occur. Wait until bubbling ends to proceed.] Bring the solution to a boil on a hot plate. Continue to heat gently until fumes cease (about 1 h). [NOTE—If the sample contains selenium, digest for NMT 15 min.] Remove from heat, cool, and dilute with water to volume. Pass about 30 mL into a centrifuge tube using a nylon syringe filter of 5-µm pore size. If necessary, make any further dilutions using the *Diluent*.

Sample solution 2 (for Tablets containing minerals only in ***Standard stock solution 2***): Weigh and finely powder NLT 20 Tablets. Transfer a portion, equal to 3.5 times the average Tablet weight, to a 250-mL volumetric flask. Slowly add 25 mL of *Stock aqua regia solution* in 5-mL increments followed by mixing. [NOTE—If the sample contains a carbonate, bubbling will occur. Wait until bubbling ends to proceed.] Bring the solution to a boil on a hot plate. Continue to heat gently until fumes cease (about 1 h). [NOTE—If the sample contains selenium, digest for NMT 15 min.] Remove from heat, cool, and dilute with water to volume. Pass about 30 mL into a centrifuge tube using a nylon syringe filter of 5-µm pore size. If necessary, make any further dilutions using the *Diluent*.

Sample solution 3 (for Tablets containing minerals only in ***Standard stock solution 1***): Weigh and finely powder NLT 20 Tablets. Transfer a portion, equal to the average Tablet weight, to a 250-mL volumetric flask. Slowly add 25 mL of *Stock aqua regia solution* in 5-mL increments, followed by mixing. [NOTE—If the sample contains a carbonate, bubbling will occur. Wait until bubbling ends to proceed.] Bring the solution to a boil on a hot plate. Continue to heat gently (about 1 h) until fumes cease. Remove from heat, cool, and dilute with water to volume. Pass about 30 mL into a centrifuge tube using a nylon syringe filter of 5-µm pore size. If necessary, make any further dilutions using the *Diluent*.

Instrumental conditions

(See *Plasma Spectrochemistry* ⟨730⟩.)

Mode: Inductively coupled plasma spectrometry, using a spectrometer set to measure the emission of each mineral of interest at about the corresponding wavelength. [NOTE—The operating conditions may be developed and optimized based on the manufacturer's recommendation. The wavelengths selected should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision.]

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System suitability
Sample: *System suitability solution*
[NOTE—Analyze the *System suitability solution*, and obtain the response as directed in the *Analysis*.]
Suitability requirements
Relative standard deviation: NMT 2.0%
Analysis
Samples: *Standard solutions* and *Sample solution*
Determine the emission of each mineral of interest in the *Standard solutions* and *Sample solution* with an inductively coupled plasma system using the *Diluent* as the blank. Plot the emission of the *Standard solutions* versus the concentration, in mg/L, of the minerals of interest, and draw the straight line best fitting the plotted points. From the graph, determine the concentration (C), in mg/L, for each mineral of interest in the *Sample solution*.
Calculate the percentage of the labeled amount for each mineral taken:

$$\text{Result} = C \times (V/W) \times F \times (C_W/L) \times 100$$

C = measured concentration of the relevant element in the *Sample solution* (mg/L)
V = volume of the *Sample solution* (L)
W = sample weight (mg)
F = dilution factor of the *Sample solution*
C_W = average weight (mg/Tablet)
L = label claim (mg/Tablet)

Acceptance criteria: 90.0%–125.0% of the labeled amount of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), and zinc (Zn); and 90.0%–160.0% of the labeled amount of boron (B), chromium (Cr), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), and vanadium (V)

PERFORMANCE TESTS
- **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩, *Dissolution*: Meet the requirements
- **WEIGHT VARIATION** ⟨2091⟩: Meet the requirements

CONTAMINANTS
- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic microbial count does not exceed 3×10^3 cfu/g, and the combined molds and yeasts count does not exceed 3×10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species*: Meet the requirements

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

Change to read:

- **LABELING:**[1] The label states that the product is Oil-Soluble Vitamins with Minerals Tablets. The label also states the quantity of each vitamin and mineral ▲in mg/Tablet or µg/Tablet▲ (USP 1-May-2020) and where necessary the chemical form in which a vitamin is present and also states the salt form of the mineral used as the source of each element. Where the product contains vitamin E, the label indicates whether it is the ▲*RRR*▲ (USP 1-May-2020) or ▲*all-rac*▲ (USP 1-May-2020) form. Where more than one assay method is given for a particular mineral, the labeling states with which assay method the product complies only if *Method 1* is not used.

Change to read:

- **USP REFERENCE STANDARDS** ⟨11⟩
USP Alpha Tocopherol RS
USP Alpha Tocopheryl Acetate RS
USP Alpha Tocopheryl Acid Succinate RS
▲USP Beta Carotene RS
USP Beta Carotene System Suitability RS▲ (USP 1-May-2020)
USP Cholecalciferol RS
USP Ergocalciferol RS
USP Phytonadione RS
USP Retinyl Acetate RS
USP Retinyl Palmitate RS
USP Sodium Fluoride RS

Oil- and Water-Soluble Vitamins Capsules

DEFINITION
Oil- and Water-Soluble Vitamins Capsules contain one or more of the following oil-soluble vitamins: Vitamin A, vitamin D as Ergocalciferol (vitamin D_2) or Cholecalciferol (vitamin D_3), Vitamin E, Phytonadione (vitamin K_1), and Beta Carotene; and one or more of the following water-soluble vitamins: Ascorbic Acid or its equivalent as Calcium Ascorbate or Sodium Ascorbate, Biotin, Cyanocobalamin, Folic Acid, Niacin or Niacinamide, Dexpanthenol or Panthenol, pantothenic acid (as Calcium Pantothenate or Racemic Calcium Pantothenate), Pyridoxine Hydrochloride, Riboflavin, and Thiamine Hydrochloride or Thiamine Mononitrate. Capsules contain NLT 90.0% and NMT 165.0% of the labeled amounts of vitamin A ($C_{20}H_{30}O$) as retinol or esters of retinol in the form of retinyl acetate ($C_{22}H_{32}O_2$) or retinyl palmitate ($C_{36}H_{60}O_2$); vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$); vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$); phytonadione ($C_{31}H_{46}O_2$); and beta carotene ($C_{40}H_{56}$); and NLT 90.0% and NMT 150.0% of the labeled

[1] ▲(USP 1-May-2020) The USP Unit for Vitamin E has been discontinued. International units (IU) for vitamins also have been discontinued; however, the use of IU on the labels of vitamin products continues. Where articles are labeled in terms of Units in addition to the required labeling, the relationship of the USP Units or IU to mass is as follows. One USP Vitamin A Unit = 0.3 µg of all-*trans*-retinol (vitamin A alcohol) or 0.344 µg of all-*trans*-retinyl acetate (vitamin A acetate) or 0.55 µg of all-*trans*-retinyl palmitate (vitamin A palmitate), and 1 µg of retinol (3.3 USP Vitamin A Units) = 1 retinol equivalent (RE); 1 IU of beta carotene = 0.6 µg of all-*trans*-beta carotene; 1 USP Vitamin D Unit = 0.025 µg of ergocalciferol or cholecalciferol; and 1 mg of ▲*all-rac*▲ (USP 1-May-2020)-alpha tocopherol = 1.1 former USP Vitamin E Units; 1 mg of -alpha tocopheryl acetate = 1 former USP Vitamin E unit; 1 mg of ▲*all-rac*▲ (USP 1-May-2020)-alpha tocopheryl acid succinate = 0.89 former USP Vitamin E Units; 1 mg of ▲*RRR*▲ (USP 1-May-2020)-alpha tocopherol = 1.49 former USP Vitamin E Units; 1 mg of ▲*RRR*▲ (USP 1-May-2020)-alpha tocopheryl acetate = 1.36 former USP Vitamin E Units; and 1 mg of ▲*RRR*▲ (USP 1-May-2020)-alpha tocopheryl acid succinate = 1.21 former USP Vitamin E Units. In terms of ▲*RRR*▲ (USP 1-May-2020)-alpha tocopherol equivalents, 1 mg of ▲*RRR*▲ (USP 1-May-2020)-alpha tocopheryl acetate = 0.91; 1 mg of ▲*RRR*▲ (USP 1-May-2020)-alpha tocopheryl acid succinate = 0.81; 1 mg of ▲*all-rac*▲ (USP 1-May-2020)-alpha tocopherol = 0.74; 1 mg of ▲*all-rac*▲ (USP 1-May-2020)-alpha tocopheryl acetate = 0.67; and 1 mg of ▲*all-rac*▲ (USP 1-May-2020)-alpha tocopheryl acid succinate = 0.60. ▲Note that 1 mg of Institute of Medicine (IOM) alpha-tocopherol equivalent = 1 mg of *RRR*-alpha-tocopherol = 2 mg of *all-rac*-alpha-tocopherol.▲ (USP 1-May-2020)

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amounts of ascorbic acid ($C_6H_8O_6$) or its salts as calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$) or sodium ascorbate ($C_6H_7NaO_6$), biotin ($C_{10}H_{16}N_2O_3S$), cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$), folic acid ($C_{19}H_{19}N_7O_6$), niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), dexpanthenol ($C_9H_{19}NO_4$) or panthenol ($C_9H_{19}NO_4$), calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine ($C_{12}H_{17}ClN_4OS$) as thiamine hydrochloride or thiamine mononitrate.

They do not contain any minerals. They may contain other labeled added substances that are generally recognized as safe, in amounts that are unobjectionable.

STRENGTH

[NOTE—In the following assays, where more than one assay method is given for an individual ingredient, the requirements may be met by following any one of the specified methods, the method used being stated in the labeling only if *Method 1* is not used.]

• **VITAMIN A, *Method 1***

[NOTE—Where the use of a vitamin A ester (retinyl acetate or retinyl palmitate) is specified in the following procedure, use the chemical form present in the formulation. USP Vitamin A RS is retinyl acetate. It is to be used where USP Vitamin A RS is specified. Use low-actinic glassware throughout this procedure.]

Mobile phase: *n*-Hexane

Standard solution: 15 µg/mL of retinyl acetate from USP Vitamin A RS in *n*-hexane

System suitability stock solution: 15 µg/mL of retinyl palmitate in *n*-hexane

System suitability solution: Mix equal volumes of the *System suitability stock solution* and the *Standard solution* to obtain concentrations of 7.5 µg/mL each of retinyl acetate and retinyl palmitate.

Sample stock solution: Transfer the contents of NLT 20 Capsules to a suitable container, mix, and weigh. Transfer a portion of the mixture, equivalent to 5 Capsules, to a container with a polytef-lined screw cap. [NOTE—For hard gelatin Capsules, remove, as completely as possible, the contents of NLT 20 Capsules by cutting open the Capsule shells, transferring the shells and their contents to a suitable container, and triturating to a homogeneous mass. Transfer a portion of the mass, equivalent to 5 Capsules, to a container with a polytef-lined screw cap.] Add 10 mL of dimethyl sulfoxide and 15 mL of *n*-hexane, and shake for 45 min on a wrist-action shaker in a water bath maintained at 60°. [NOTE—Set up the wrist-action shaker to ensure that the contents of the container are mixed vigorously and thoroughly.] Centrifuge at 3000 rpm for 10 min, and transfer the hexane layer by means of a pipet to a 100-mL volumetric flask. Add 15 mL of *n*-hexane to the dimethyl sulfoxide layer, shake thoroughly for 5 min, and transfer the hexane layer by means of a pipet to the 100-mL volumetric flask. Repeat this extraction with three additional 15-mL portions of *n*-hexane. Dilute the extracts in the volumetric flask with *n*-hexane to volume.

Sample solution: Dilute a volume of the *Sample stock solution* with *n*-hexane to obtain a solution with a concentration of 15 µg/mL of vitamin A as retinol ($C_{20}H_{30}O$).

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 325 nm

Column: 4.6-mm × 15-cm; 3-µm packing L8

Flow rate: 1 mL/min

Injection volume: 40 µL

System suitability

Sample: *System suitability solution*

Suitability requirements

Resolution: NLT 10 between all-*trans*-retinyl acetate and all-*trans*-retinyl palmitate

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak area for all-*trans*-retinyl acetate from the *Standard solution* and the peak area for all-*trans*-retinyl acetate or all-*trans*-retinyl palmitate in the chromatogram of the *Sample solution*. For products containing vitamin A acetate or vitamin A palmitate, calculate the percentage of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$), in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of the all-*trans*-retinyl ester from the *Sample solution*

r_S = peak area of the all-*trans*-retinyl ester from the *Standard solution*

C_S = concentration of retinyl acetate ($C_{22}H_{32}O_2$) from USP Vitamin A RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of vitamin A, as retinol ($C_{20}H_{30}O$), in the *Sample solution* (µg/mL)

F = factor used to convert retinyl acetate, the ester form present in USP Vitamin A RS, to retinol, 0.872

[NOTE—The molar responses of retinyl acetate and retinyl palmitate are equivalent.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$)

• **VITAMIN A, *Method 2***

[NOTE—Where a vitamin A ester (retinyl acetate or retinyl palmitate) is indicated in the following procedure, use the chemical form present in the formulation. USP Vitamin A RS is retinyl acetate. It is to be used where USP Vitamin A RS is specified. Use low-actinic glassware throughout this procedure.]

3 N methanolic sulfuric acid solution: Cautiously add 9 mL of sulfuric acid to 80 mL of methanol in a 100-mL volumetric flask. Cool, and dilute with methanol to volume.

Sodium ascorbate–pyrogallol solution: Transfer 10 g of sodium ascorbate and 5 g of pyrogallol to a 100-mL volumetric flask, and add sufficient water to dissolve. Add 1.7 mL of sulfuric acid, and dilute with water to volume.

Lecithin solution: 5 mg/mL of lecithin in 2,2,4-trimethylpentane

Mobile phase: *n*-Hexane and ethyl acetate (99.7: 0.3)

Standard solution: 15 µg/mL of retinyl acetate from USP Vitamin A RS in 2,2,4-trimethylpentane

System suitability stock solution: 15 µg/mL of retinyl palmitate in 2,2,4-trimethylpentane

System suitability solution: Mix equal volumes of the *System suitability stock solution* and the *Standard solution* to obtain concentrations of 7.5 µg/mL each of retinyl acetate and retinyl palmitate.

Sample solution: [NOTE—This preparation is suitable for the determination of vitamin A, vitamin D, and vitamin E, when present in the formulation.] Weigh NLT 20 Capsules in a tared weighing bottle. Using a sharp blade if necessary, carefully open the Capsules, without loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and dry the

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Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to 30 µg of the labeled amount of cholecalciferol or ergocalciferol (vitamin D), to a container with a polytef-lined screw cap. If vitamin D is not present in the formulation, use a portion equivalent to 90 mg of the labeled amount of vitamin E. If vitamin E is not present in the formulation, use a portion equivalent to 2.5 mg of the labeled amount of vitamin A, as retinol. Add 0.5 g of sodium bicarbonate, 1.5 mL of *Lecithin solution*, and 12.5 mL of 2,2,4-trimethylpentane, and disperse on a vortex mixer. Add 6 mL of *Sodium ascorbate–pyrogallol solution*, shake slowly, and allow the solution to degas. Continue shaking until the evolution of gas has ceased, and then shake for an additional 12 min. Add 6 mL of dimethyl sulfoxide, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 6 mL of *3 N methanolic sulfuric acid solution*, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 12.5 mL of 2,2,4-trimethylpentane, mix on a vortex mixer to form a suspension, and shake for 10 min. Centrifuge for 10 min to break up the emulsion and to clarify the supernatant. [NOTE—The supernatant is used for the determination of vitamin A, and also vitamin D and vitamin E, if present in the formulation.] If necessary, quantitatively dilute a volume of the supernatant with 2,2,4-trimethylpentane to obtain a concentration close to that of the *Standard solution*.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 325 nm
Column: 4.6-mm × 25-cm; 5-µm packing L24
Flow rate: 1.5 mL/min
Injection volume: 40 µL

System suitability
Sample: *System suitability solution*
Suitability requirements
Resolution: NLT 8.0 between all-*trans*-retinyl acetate and all-*trans*-retinyl palmitate
Relative standard deviation: NMT 3.0%

Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak area for all-*trans*-retinyl acetate from the *Standard solution* and the peak area of all-*trans*-retinyl acetate or all-*trans*-retinyl palmitate from the *Sample solution*.
Calculate the percentage of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$), in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of the all-*trans*-retinyl ester from the *Sample solution*
r_S = peak area of the all-*trans*-retinyl ester from the *Standard solution*
C_S = concentration of retinyl acetate ($C_{22}H_{32}O_2$) from USP Vitamin A RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of vitamin A, as retinol ($C_{20}H_{30}O$), in the *Sample solution* (µg/mL)
F = factor used to convert retinyl acetate, the ester form present in USP Vitamin A RS, to retinol, 0.872

[NOTE—Account for the initial extraction volume of 26.5 mL of 2,2,4-trimethylpentane to calculate the nominal concentration. The molar responses of retinyl acetate and retinyl palmitate are equivalent.]
Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$)

• **VITAMIN A, *Method 3***
[NOTE—Where a vitamin A ester (retinyl acetate or retinyl palmitate) is indicated in the following procedure, use the chemical form present in the formulation. USP Vitamin A RS is retinyl acetate. It is to be used where USP Vitamin A RS is specified. Use low-actinic glassware throughout this procedure.]
Extraction solvent: *n*-Hexane and methylene chloride (3:1)
Potassium hydroxide solution: 800 mg/mL of potassium hydroxide in water. [NOTE—Cautiously add potassium hydroxide to the water. Mix, and cool.]
Diluent: 10 mg/mL of pyrogallol in alcohol
Mobile phase: *n*-Hexane and isopropyl alcohol (92:8)
Standard stock solution: 30 µg/mL of retinyl acetate from USP Vitamin A RS in *Diluent*. [NOTE—This solution may be stored in a refrigerator for 1 week.]
Standard solution: Dilute a volume of the *Standard stock solution* with *Diluent* to obtain a concentration of 1 µg/mL of retinyl acetate from USP Vitamin A RS. Transfer 10.0 mL of this solution to a stoppered 125-mL flask, and add 5 mL of water, 5 mL of *Diluent*, and 3 mL of *Potassium hydroxide solution*. Insert the stopper tightly, shake for 15 min over a water bath maintained at 60 ± 5°, and cool to room temperature. Add 7 mL of water and 25.0 mL of *Extraction solvent*. Insert the stopper tightly, and shake vigorously for 60 s. Rinse the sides of the flask with 60 mL of water, and allow to stand for 10 min until the layers separate. Withdraw a portion of the organic layer for injection into the chromatograph. This *Standard solution* contains 0.34 µg/mL of retinol.
Sample solution: Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to 1.5 mg of retinyl acetate, to a stoppered 125-mL flask. Add 5 mL of water, 15 mL of *Diluent*, and 3 mL of *Potassium hydroxide solution*. Insert the stopper tightly, shake for 15 min over a water bath maintained at 60 ± 5°, and cool to room temperature. Add 7 mL of water and 25.0 mL of *Extraction solvent*. Insert the stopper tightly, and shake vigorously for 60 s or longer, if necessary, for complete extraction. Rinse the sides of the flask with 60 mL of water, and allow to stand for 10 min until the layers separate. [NOTE—Do not shake, because an emulsion may form.] Withdraw a portion of the organic layer, and dilute quantitatively, and stepwise if necessary, with *Extraction solvent* to obtain a concentration of 0.34 µg/mL of retinol.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 335 nm
Column: 6.2-mm × 8-cm; packing L3
Column temperature: 40°
Flow rate: 4 mL/min
Injection volume: 50 µL
System suitability
Sample: *Standard solution*

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[NOTE—The relative retention times for 13-*cis*-retinol and all-*trans*-retinol are about 0.92 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for all-*trans*-retinol and 13-*cis*-retinol. Calculate the percentage of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$), in the portion of Capsules taken:

$$\text{Result} = (r_{T1}/r_{T2}) \times (C_S/C_U) \times F \times 100$$

r_{T1} = sum of the areas of the all-*trans*-retinol and 13-*cis*-retinol peaks from the *Sample solution*

r_{T2} = sum of the areas of all-*trans*-retinol and 13-*cis*-retinol peaks from the *Standard solution*

C_S = concentration of retinyl acetate ($C_{23}H_{32}O_2$) from USP Vitamin A RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of vitamin A, as retinol ($C_{20}H_{30}O$), in the *Sample solution* (µg/mL)

F = factor used to convert retinyl acetate, the ester form present in USP Vitamin A RS, to retinol, 0.872

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$)

• **CHOLECALCIFEROL or ERGOCALCIFEROL (VITAMIN D),** ***Method 1***

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Mobile phase: *n*-Hexane and isopropyl alcohol (99:1)

Standard solution: 2 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in *n*-hexane

System suitability solution: Heat a volume of the *Standard solution* at 60° for 1 h to partially isomerize vitamin D (cholecalciferol or ergocalciferol) to its corresponding precursor.

Sample solution: Proceed as directed for the *Sample stock solution* in *Vitamin A, Method 1*. Transfer NLT 20 mL of this solution to a suitable container, and evaporate, if necessary, under vacuum at room temperature to obtain a concentration of 2 µg/mL of cholecalciferol or ergocalciferol.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 15-cm; 3-µm packing L8

Flow rate: 1 mL/min

Injection volume: 100 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 10 between the vitamin D form present and its corresponding precursor, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for vitamin D. Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*

r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*

C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

F = correction factor to account for the average amount of previtamin D present in the *Sample solution*, 1.09

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

• **CHOLECALCIFEROL or ERGOCALCIFEROL (VITAMIN D),** ***Method 2***

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

3 N methanolic sulfuric acid solution, Sodium ascorbate–pyrogallol solution, Lecithin solution, and **Sample solution:** Proceed as directed in *Vitamin A, Method 2*.

Mobile phase: *n*-Hexane and tertiary butyl alcohol (98.75:1.25)

Standard solution: 1 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in 2,2,4-trimethylpentane

System suitability solution: Heat a volume of the *Standard solution* at 60° for 1 h to partially isomerize vitamin D (cholecalciferol or ergocalciferol) to its corresponding precursor.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 25-cm; 5-µm packing L24

Flow rate: 1 mL/min

Injection volume: 40 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 4.0 between the vitamin D form present and its corresponding precursor, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for vitamin D. Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*

r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*

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C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

• **CHOLECALCIFEROL** or **ERGOCALCIFEROL (VITAMIN D),** ***Method 3***

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Diluted acetic acid: Glacial acetic acid solution (1 in 10) in water

Phenolphthalein solution: 10 mg/mL of phenolphthalein in alcohol

Potassium hydroxide solution: Slowly dissolve 14 g of potassium hydroxide in a mixture of 31 mL of dehydrated alcohol and 5 mL of water. Prepare fresh daily.

Extraction solvent: Methylene chloride and isopropyl alcohol (99.8: 0.2)

Mobile phase: Acetonitrile and methanol (91:9)

Standard stock solution: 0.2 mg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in dehydrated alcohol. [NOTE—Prepare fresh every 4 weeks. Store in a freezer.]

Standard solution: [NOTE—Condition the solid-phase extraction column specified for use in the *Standard solution* and the *Sample solution* by initially washing the column with 4.0 mL of a mixture of methylene chloride and isopropyl alcohol (4:1), followed by 5.0 mL of *Extraction solvent*. Do not allow the column to dry.] Dilute a volume of the *Standard stock solution* with dehydrated alcohol to obtain a concentration of 5 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS. Prepare this solution fresh daily. Transfer 2.0 mL of this solution to a stoppered 125-mL flask. Add 15.0 mL of water and 15.0 mL of *Potassium hydroxide solution*, insert the stopper, and shake for 30 min in a water bath maintained at 60°. Allow to cool to room temperature, and transfer the contents of the flask to a 250-mL separatory funnel. Add 15.0 mL of water to the flask, insert the stopper, shake vigorously, and transfer this solution to the separatory funnel. Rinse the flask with 60 mL of *n*-hexane, and transfer the rinsing to the separatory funnel. Insert the stopper, shake vigorously for 90 s, and allow to stand for 15 min until the layers separate. Drain and discard the aqueous layer. Add 15.0 mL of water to the hexane layer in the separatory funnel, insert the stopper, and shake vigorously. Allow to stand for 10 min until the layers separate, and discard the aqueous layer. Add 1 drop of *Phenolphthalein solution* and 15.0 mL of water to the separatory funnel. Add *Diluted acetic acid* dropwise, with shaking, until the washing is neutral. Allow to stand for 10 min until the layers separate. Drain and discard the aqueous layer. Filter the hexane layer through anhydrous sodium sulfate supported by a small pledget of cotton into a 100-mL round-bottom flask. Rinse the funnel and sodium sulfate with a few mL of *n*-hexane, and collect the rinsings in the same flask. Evaporate the hexane in the flask on a rotary evaporator at 50° to dryness. Immediately add 2.0 mL of *Extraction solvent* to dissolve the residue. Transfer this solution to a freshly conditioned solid-phase extraction column containing silica packing with a sorbent mass-to-column volume ratio of 500 mg to 2.8 mL or equivalent, rinse the round-bottom flask with 1.0 mL of *Extraction solvent*, and transfer to the column. Elute the column with 2.0 mL of *Extraction solvent*, and discard this fraction. Elute the column with 7.0 mL of *Extraction solvent*, and collect the eluate in a suitable flask. Place the flask in a warm water bath maintained at 42°, and evaporate the solvent with the aid of a stream of nitrogen. Immediately add 2.0 mL of acetonitrile to the residue, and use the solution for injection into the chromatograph.

Sample solution: Proceed as directed in *Vitamin A, Method 3*, through "calculate the net weight of the Capsule contents." Transfer a portion of the Capsule contents, equivalent to 10 µg of cholecalciferol or ergocalciferol, to a stoppered 125-mL flask, and proceed as directed for the *Standard solution*, beginning with "Add 15.0 mL of water and 15.0 mL of *Potassium hydroxide solution*".

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 27°

Flow rate: 0.7 mL/min

Injection volume: 15 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 4.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for vitamin D. Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*

r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*

C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

• **VITAMIN E,** ***Method 1***

[NOTE—Where vitamin E (alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Solution A: Phosphoric acid solution (1 in 100) in water

Mobile phase: Methanol and *Solution A* (19:1)

System suitability solution: Prepare a 0.65-mg/mL solution of USP Ergocalciferol RS in methanol. Transfer 1.0 mL of this solution to a 100-mL volumetric flask containing 100 mg of USP Alpha Tocopheryl Acetate RS. Dissolve in 30 mL of methanol, with the aid of sonication if necessary, and dilute with methanol to volume. Store this solution in a refrigerator.

Standard solution: 2 mg/mL of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, or USP Alpha Tocopheryl Acid Succinate RS in methanol

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Sample solution: Proceed as directed for the *Sample stock solution* in *Vitamin A, Method 1*. Transfer NLT 20 mL of this solution to a suitable container, and evaporate if necessary, under vacuum at room temperature to dryness. Transfer the contents of the flask to a suitable volumetric flask with the aid of methanol, and dilute with methanol to volume to obtain a concentration of 2 mg/mL of alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 8-mm × 10-cm; 5-µm packing L1
Flow rate: 2 mL/min
Injection volume: 100 µL

System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The relative retention times for ergocalciferol and alpha tocopheryl acetate are about 0.5 and 1.0, respectively.]
Suitability requirements
Resolution: NLT 12 between ergocalciferol and alpha tocopheryl acetate, *System suitability solution*
Tailing factor: Between 0.8 and 1.2, *System suitability solution*
Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas. Calculate the percentage of the labeled amount of alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*
r_S = peak area of the relevant vitamin E form from the *Standard solution*
C_S = concentration of the corresponding USP Reference Standard in the *Standard solution* (mg/mL)
C_U = nominal concentration of the corresponding form of vitamin E in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$)

• **VITAMIN E, *Method 2***
[NOTE—Where vitamin E (alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Mobile phase: Mix 240 mL of methanol with 10 mL of water, followed by 0.5 mL of 50% phosphoric acid, and dilute with acetonitrile to 1000 mL.
System suitability solution: 2 mg/mL each of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, and USP Alpha Tocopheryl Acid Succinate RS in methanol
Standard solution: 2 mg/mL of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, or USP Alpha Tocopheryl Acid Succinate RS in methanol
3 N methanolic sulfuric acid solution: Cautiously mix sulfuric acid and methanol (9 in 100) in a 100-mL volumetric flask. [NOTE—Dissolve in a portion of methanol, cool, and then dilute to final volume.]
Sodium ascorbate–pyrogallol solution: Transfer 10 g of sodium ascorbate and 5 g of pyrogallol to a 100-mL volumetric flask. Add sufficient water to dissolve. Add 1.7 mL of sulfuric acid, and dilute with water to volume.
Lecithin solution: 5 mg/mL of lecithin in 2,2,4-trimethylpentane
Sample solution: Proceed as directed for the *Sample solution* in *Vitamin A, Method 2*, through "calculate the net weight of the Capsule contents." Transfer a portion of the Capsule contents, equivalent to 55 mg of vitamin E, to a container with a polytef-lined screw cap. Add 0.5 g of sodium bicarbonate, 1.5 mL of *Lecithin solution*, and 12.5 mL of 2,2,4-trimethylpentane, and disperse on a vortex mixer. Add 6 mL of *Sodium ascorbate–pyrogallol solution*, shake slowly, and allow the solution to degas. Continue shaking until the evolution of gas has ceased, and then shake for an additional 12 min. Add 6 mL of dimethyl sulfoxide, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 6 mL of *3 N methanolic sulfuric acid solution*, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 12.5 mL of 2,2,4-trimethylpentane, mix on a vortex mixer to form a suspension, and shake for 10 min. Centrifuge for 10 min to break up the emulsion and to clarify the supernatant layer. Transfer a volume of the supernatant 2,2,4-trimethylpentane layer to a suitable volumetric flask, the volume of the specimen withdrawn from the 2,2,4-trimethylpentane layer and the size of the volumetric flask being such that the final concentration of the *Sample solution* is equivalent to that of the *Standard solution*. Evaporate nearly to dryness, add several mL of methanol, and evaporate the remaining 2,2,4-trimethylpentane. Dilute with methanol to volume.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 280 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Flow rate: 1.5 mL/min
Injection volume: 25 µL

System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The relative retention times for alpha tocopheryl acid succinate, alpha tocopherol, and alpha tocopheryl acetate are about 0.6, 0.8, and 1.0, respectively.]
Suitability requirements
Resolution: NLT 4.0 between alpha tocopheryl acid succinate and alpha tocopherol and NLT 3.0 between alpha tocopherol and alpha tocopheryl acetate, *System suitability solution*
Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas. Calculate the percentage of the labeled amount of alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*
r_S = peak area of the relevant vitamin E form from the *Standard solution*

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C_S = concentration of the corresponding USP Reference Standard in the *Standard solution* (mg/mL)
C_U = nominal concentration of the corresponding form of vitamin E in the *Sample solution* (mg/mL)

[NOTE—Account for the initial extraction volume of 26.5 mL of 2,2,4-trimethylpentane and the dilution factor to exchange the solvent from 2,2,4-trimethylpentane to methanol to calculate the nominal concentration.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$)

• **VITAMIN E, *Method 3***

Diluent: Acetonitrile and ethyl acetate (1:1)

Mobile phase: Methanol, acetonitrile, and *n*-hexane (46.5:46.5:7.0)

Standard solution: 0.3 mg/mL of USP Alpha Tocopherol RS in methanol

Sample solution: Proceed as directed in *Vitamin A, Method 3*, through "calculate the net weight of the Capsule contents." Transfer a portion of the Capsule contents, equivalent to 8.0 mg of alpha tocopherol, to a glass-stoppered conical flask. Add 25.0 mL of water, 25.0 mL of dehydrated alcohol, and 3.5 g of potassium hydroxide pellets. Shake for 1 h in a water bath maintained at 55°. Cool, and transfer with the aid of a minimum volume of water to a 125-mL separatory funnel. Rinse the flask with 50 mL of *n*-hexane, and add the rinsing to the separatory funnel. Insert the stopper, shake vigorously for 60 s, and allow the layers to separate. Drain the aqueous layer into a second 250-mL separatory funnel, and repeat the extraction with 50 mL of *n*-hexane. Discard the aqueous layer, and combine the hexane extracts. Wash the combined extracts with 25 mL of water, allow the layers to separate, and discard the aqueous layer. Add 3 drops of glacial acetic acid, and repeat the washing procedure two more times. Filter the washed hexane layer through anhydrous sodium sulfate into a 250-mL round-bottom flask. Rinse the funnel and sodium sulfate with a few mL of *n*-hexane, and add the rinsing to the hexane solution in the flask. Place the flask in a water bath maintained at 50°, and evaporate the hexane solution with the aid of a rotary evaporator to dryness. Immediately add 25.0 mL of *Diluent*, and swirl to dissolve the residue.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 291 nm

Column: 4.6-mm × 25-cm; packing L1

Column temperature: 40°

Flow rate: 3 mL/min

Injection volume: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas. Calculate the percentage of the labeled amount of vitamin E, as alpha tocopherol ($C_{29}H_{50}O_2$), in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of alpha tocopherol from the *Sample solution*
r_S = peak area of alpha tocopherol from the *Standard solution*
C_S = concentration of alpha tocopherol in the *Standard solution* (mg/mL)
C_U = nominal concentration of vitamin E, as alpha tocopherol, in the *Sample solution* (mg/mL)

[NOTE—Calculate the nominal amount of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$) by multiplying the content of alpha tocopheryl acetate ($C_{31}H_{52}O_3$) or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$), in mg/Capsule by the factor 0.91 or 0.81, respectively.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$)

• **PHYTONADIONE**

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Methanol and water (19:1)

Standard stock solution: 200 µg/mL of USP Phytonadione RS in methanol. Dissolve with the aid of sonication if necessary.

System suitability solution: 0.65 mg/mL of USP Alpha Tocopheryl Acetate RS and 20 µg/mL of USP Phytonadione RS from the *Standard stock solution* diluted with methanol. [NOTE—Dissolve USP Alpha Tocopheryl Acetate RS in a portion of methanol, add the *Standard stock solution*, and then dilute with methanol to volume.]

Standard solution: 20 µg/mL of USP Phytonadione RS from the *Standard stock solution* diluted with methanol

Sample solution: Transfer NLT 20 mL of the solution retained as specified in the directions for the *Sample stock solution* in *Vitamin A, Method 1* to a suitable container, and evaporate, if necessary, under vacuum at room temperature to dryness. Transfer the contents of the flask to a suitable volumetric flask with the aid of methanol, and dilute with methanol to volume to obtain a concentration of 20 µg/mL of phytonadione.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 8-mm × 10-cm; 5-µm packing L1

Flow rate: 2 mL/min

Injection volume: 100 µL

System suitability

Samples: *System suitability solution* and *Standard solution*

[NOTE—The relative retention times for alpha tocopheryl acetate and phytonadione are about 0.68 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 5 between alpha tocopheryl acetate and phytonadione, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas. Calculate the percentage of the labeled amount of phytonadione ($C_{31}H_{46}O_2$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area for phytonadione from the *Sample solution*
r_S = peak area for phytonadione from the *Standard solution*

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C_S = concentration of USP Phytonadione RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of phytonadione in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of phytonadione ($C_{31}H_{46}O_2$)

• **BETA CAROTENE**

[NOTE—Use low-actinic glassware throughout this procedure.]

Potassium hydroxide solution: Dissolve 58.8 g of potassium hydroxide in 50 mL of water.

Iodine solution: Transfer 10 mg of iodine to a 100-mL volumetric flask. Dissolve in cyclohexane, and dilute with cyclohexane to volume. Dilute 10 mL of this solution with cyclohexane to 100 mL. [NOTE—Prepare this solution fresh daily.]

Sample solution A (for preparations containing beta carotene in oil solutions): Proceed as directed in *Vitamin A, Method 1*, except use cyclohexane instead of *n*-hexane as the extraction solvent, and dilute the filtered extracts with cyclohexane, to obtain a concentration of 2 µg/mL of beta carotene.

Sample solution B (for preparations containing beta carotene in dry powder): Remove the contents of NLT 20 Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the Capsule contents, equivalent to 2 mg of beta carotene, to a 500-mL saponification flask. Add 100 mL of alcohol, 6 mL of *Potassium hydroxide solution*, and a magnetic stirring bar. Attach an air condenser to the flask, and heat under reflux for 45 min with constant stirring. Cool to room temperature. Add 170 mL of solvent hexane, and stir for 30 min. Quantitatively transfer the contents of the flask to a 500-mL separatory funnel with portions of solvent hexane. Allow the layers to separate for 5–10 min, and transfer the upper organic layer to a 500-mL volumetric flask. Transfer the lower aqueous layer into the saponification flask. Add 170 mL of solvent hexane, and stir for an additional 20 min. Quantitatively transfer the contents of the saponification flask to the separatory funnel with the aid of portions of solvent hexane. Allow the layers to separate for 10 min. Drain the lower aqueous layer, and discard. Transfer the organic layer to the volumetric flask containing the previously collected organic layer. Rinse the separatory funnel with small portions of solvent hexane, and transfer the washings to the volumetric flask. Dilute the hexane extracts with solvent hexane to volume. Add 3 g of anhydrous sodium sulfate, shake, and allow to settle. Quantitatively transfer a volume of this solution, equivalent to 100 µg of beta carotene, to a 50-mL volumetric flask. Evaporate under a stream of nitrogen to dryness, and immediately add cyclohexane. Add 2 mL of *Iodine solution*, and heat for 15 min in a water bath maintained at 65°. Cool rapidly, and dilute with cyclohexane to volume.

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Mode: Vis

Analytical wavelength: 452 nm

Blank: Cyclohexane

Analysis

Samples: *Sample solution A* or *Sample solution B*

Determine the absorbance against the *Blank*. Calculate the percentage of the labeled amount of beta carotene ($C_{40}H_{56}$) in the portion of Capsules taken:

$$\text{Result} = (A_U/F) \times (100/C_U)$$

A_U = absorbance of *Sample solution A* or *Sample solution B*
F = absorptivity of beta carotene at 452 nm, 223
C_U = nominal concentration of beta carotene in *Sample solution A* or *Sample solution B* (mg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of beta carotene ($C_{40}H_{56}$)

• **ASCORBIC ACID, CALCIUM ASCORBATE,** and **SODIUM ASCORBATE**

(See *Vitamin C Assay* ⟨580⟩.)

[NOTE—For labeling purposes, consider *Method I—Titrimetric Method* as *Method 1*.]

Acceptance criteria: 90.0%–150.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$) or its salts as calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$) or sodium ascorbate ($C_6H_7NaO_6$)

• **BIOTIN, *Method 1***

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Mix 85 mL of acetonitrile, 1 g of sodium perchlorate, and 1 mL of phosphoric acid, and dilute with water to 1000 mL.

Standard stock solution: 0.333 mg/mL of USP Biotin RS in dimethyl sulfoxide

Standard solution: 5 µg/mL of USP Biotin RS prepared by diluting the *Standard stock solution* in water

Sample solution: Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without the loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing, if necessary, with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the average net weight per Capsule. Proceed as directed in *Ascorbic Acid* through "calculate the average net weight per Capsule." Transfer a portion of the Capsule contents, equivalent to 1 mg of biotin, to a 200-mL volumetric flask. Add 3 mL of dimethyl sulfoxide, and swirl to wet the contents. Place the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with water to volume, and filter.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 200 nm

Column: 4.6-mm × 15-cm; 3-µm packing L7

Flow rate: 1.2 mL/min

Injection volume: 100 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of biotin. Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of biotin from the *Sample solution*
r_S = peak area of biotin from the *Standard solution*
C_S = concentration of USP Biotin RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of biotin in the *Sample solution* (µg/mL)

DS Monographs

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Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

• **BIOTIN, *Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Dehydrated mixtures yielding formulations similar to the media described herein may be used provided that, when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described herein.

Standard stock solution: 50 µg/mL of USP Biotin RS in 50% alcohol. Store this solution in a refrigerator.

Standard solution: 0.1 ng/mL of USP Biotin RS in water, prepared by dilution of the *Standard stock solution* with water on the day of the assay

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." Transfer a portion of the Capsule contents, equivalent to 100 µg of biotin, to a 200-mL volumetric flask. Add 3 mL of 50% alcohol, and swirl to wet the contents. Heat the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with 50% alcohol to volume, and filter. Dilute a volume of the filtrate quantitatively, and stepwise if necessary, with water to obtain a solution having a concentration of 0.1 ng/mL.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and dilute with water to 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature NLT 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water. Heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and dilute with water to 1000 mL. Store under toluene in a cool place at a temperature NLT 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and dilute with water to 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Calcium pantothenate solution: 10 µg/mL of calcium pantothenate in 50% alcohol. Store in a refrigerator.

Riboflavin–thiamine hydrochloride solution: 20 µg/mL of riboflavin and 10 µg/mL of thiamine hydrochloride in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

***p*-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution:** 10 µg/mL of *p*-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid, and mix. Store under toluene.

Basal medium stock solution: Dissolve the *anhydrous Dextrose* and *anhydrous Sodium acetate* in the solutions previously mixed according to *Table 1*, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Table 1

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Calcium pantothenate solution	5 mL
Riboflavin–thiamine hydrochloride solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water. Add 500 mg of *anhydrous Dextrose*, 500 mg of *anhydrous Sodium acetate*, and 1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to test tubes, close or cover the tubes, sterilize in an autoclave at 121° for 15 min, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*,[1] incubating for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for Inoculum if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution* add 5.0 mL of water containing 0.5 ng of biotin. Plug the tubes with cotton, sterilize in an autoclave at 121° for 15 min, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Transfer cells from the *Stock culture of Lactobacillus plantarum* to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. The cell suspension so obtained is the *Inoculum*.

Analysis

Samples: *Standard solution* and *Sample solution*

To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to four similar empty tubes add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL.

To similar test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate

[1] ATCC No. 8014 is suitable. This strain was formerly known as Lactobacillus arabinosus 17-5.

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set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool. Add 1 drop of *Inoculum* to each tube, except two of the four tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37° held constant to within ±0.5° until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of Standard during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to a spectrophotometer cell. Place the cell in a spectrophotometer that has been set at a specific wavelength of 540–660 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittance (Σ_S) as the difference, $y = 2.00 - \Sigma_S$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_U$, adding together the two transmittances (Σ_U) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X, for each dosage level. Average the values of X for each of three or more dosage levels to obtain $\overline{X}$, which equals the log-relative potency, M', of the *Sample solution*.

Determine the quantity, in µg, of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Capsules taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of µg of biotin assumed to be present in the portion of Capsules taken

Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Capsules taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of biotin in the portion of Capsules taken (µg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, $\overline{M}$, is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* ⟨111⟩, *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

• **CYANOCOBALAMIN, *Method 1***

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Methanol and water (7:13)

Standard stock solution: 10 µg/mL of USP Cyanocobalamin (Crystalline) RS in water. [NOTE—Store this stock solution in a dark place, and discard after 1 week.]

Standard solution: 1 µg/mL of USP Cyanocobalamin (Crystalline) RS from the *Standard stock solution* diluted with water

Sample solution: Weigh NLT 30 Capsules in a tared weighing bottle. Open the Capsules, without the loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing, if necessary, with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the average net weight per Capsule. Transfer a portion of the Capsule contents, equivalent to 100 µg of cyanocobalamin, to a 250-mL flask. Quantitatively add 100.0 mL of water, and carefully extract for 2 min. Filter 10 mL of the extract, and use the clear filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: 550 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Flow rate: 0.5 mL/min

Injection volume: 200 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of cyanocobalamin. Calculate the percentage of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of cyanocobalamin from the *Sample solution*

r_S = peak area of cyanocobalamin from the *Standard solution*

C_S = concentration of USP Cyanocobalamin (Crystalline) RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cyanocobalamin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$)

• **CYANOCOBALAMIN, *Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Standard stock solution: 1.0 µg/mL of USP Cyanocobalamin (Crystalline) RS in 25% alcohol. Store in a refrigerator.

Standard solution: Dilute a suitable volume of *Standard stock solution* with water to a measured volume such that

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after the incubation period as described in the *Analysis*, the difference in transmittance between the inoculated blank and the 5.0-mL level of the *Standard solution* is NLT that which corresponds to a difference of 1.25 mg in dried cell weight. This concentration usually falls between 0.01 and 0.04 ng/mL of the *Standard solution*. Prepare this solution fresh for each assay.

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." Transfer a portion of the Capsule contents, equivalent to 1.0 µg of cyanocobalamin, to an appropriate vessel containing, for each g of Capsule contents taken, 25 mL of an aqueous extracting solution prepared just before use to contain 12.9 mg/mL of dibasic sodium phosphate, 11.0 mg/mL of anhydrous citric acid, and 10 mg/mL of sodium metabisulfite. Autoclave the mixture at 121° for 10 min. Allow any undissolved particles of the extract to settle, and filter or centrifuge, if necessary. Dilute an aliquot of the clear solution with water to obtain a final solution containing vitamin B_{12} activity approximately equivalent to that of the *Standard solution*.

Acid-hydrolyzed casein solution: Prepare as directed in *Calcium Pantothenate, Method 2*.

Asparagine solution: Dissolve 2.0 g of L-asparagine in water to make 200 mL. Store under toluene in a refrigerator.

Adenine–guanine–uracil solution: Prepare as directed in *Calcium Pantothenate, Method 2*.

Xanthine solution: Suspend 0.20 g of xanthine in 30–40 mL of water, heat to 70°, add 6.0 mL of 6 N ammonium hydroxide, and stir until the solid is dissolved. Cool, and dilute with water to 200 mL. Store under toluene in a refrigerator.

Salt solution A: Dissolve 10 g of monobasic potassium phosphate and 10 g of dibasic potassium phosphate in water to make 200 mL, and add 2 drops of hydrochloric acid. Store this solution under toluene.

Salt solution B: Dissolve 4.0 g of magnesium sulfate, 0.20 g of sodium chloride, 0.20 g of ferrous sulfate, and 0.20 g of manganese sulfate in water to make 200 mL. Add 2 drops of hydrochloric acid. Store this solution under toluene.

Polysorbate 80 solution: Dissolve 20 g of polysorbate 80 in alcohol to make 200 mL. Store in a refrigerator.

Vitamin solution A: Dissolve 10 mg of riboflavin, 10 mg of thiamine hydrochloride, 100 µg of biotin, and 20 mg of niacin in 0.02 N acetic acid to make 400 mL. Store under toluene, protected from light, in a refrigerator.

Vitamin solution B: Dissolve 20 mg of *p*-aminobenzoic acid, 10 mg of calcium pantothenate, 40 mg of pyridoxine hydrochloride, 40 mg of pyridoxal hydrochloride, 8 mg of pyridoxamine dihydrochloride, and 2 mg of folic acid in a mixture of water and neutralized alcohol (3:1) to make 400 mL. Store, protected from light, in a refrigerator.

Basal medium stock solution: Prepare the medium according to the following formula and directions. A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium comparable to that obtained from the formula given herein.

Add the ingredients in the order listed in *Table 2*, carefully dissolving *Cystine* and *Tryptophan* in the *hydrochloric acid* before adding the next eight solutions to the resulting solution. Add 100 mL of water, and dissolve the *Dextrose*, *Sodium acetate*, and *Ascorbic acid*. Filter, if necessary. Add the *Polysorbate 80 solution*, adjust with 1 N sodium hydroxide to a pH of 5.5–6.0, and dilute with Purified Water to 250 mL.

Table 2

L-Cystine	0.1 g
L-Tryptophan	0.05 g
1 N hydrochloric acid	10 mL
Adenine–guanine–uracil solution	5 mL
Xanthine solution	5 mL
Vitamin solution A	10 mL
Vitamin solution B	10 mL
Salt solution A	5 mL
Salt solution B	5 mL
Asparagine solution	5 mL
Acid-hydrolyzed casein solution	25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Ascorbic acid	1 g
Polysorbate 80 solution	5 mL

Tomato juice preparation: Centrifuge commercially canned tomato juice so that most of the pulp is removed. Suspend 5 g/L of analytical filter aid in the supernatant, and pass, with the aid of reduced pressure, through a layer of the filter aid. Repeat, if necessary, until a clear, straw-colored filtrate is obtained. Store under toluene in a refrigerator.

Culture medium: [NOTE—A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium equivalent to that obtained from the formula given herein.] Dissolve 0.75 g of yeast extract, 0.75 g of dried peptone, 1.0 g of anhydrous dextrose, and 0.20 g of monobasic potassium phosphate in 60–70 mL of water. Add 10 mL of *Tomato juice preparation* and 1 mL of *Polysorbate 80 solution*. Adjust with 1 N sodium hydroxide to a pH of 6.8, and dilute with water to 100 mL. Place 10-mL portions of the solution in test tubes, and plug with cotton. Sterilize the tubes and contents in an autoclave at 121° for 15 min. Cool as rapidly as possible to avoid color formation resulting from overheating the medium.

Suspension medium: Dilute a measured volume of the *Basal medium stock solution* with an equal volume of water. Place 10-mL portions of the diluted medium in test tubes. Sterilize, and cool as directed for *Culture medium*.

Stock culture of *Lactobacillus leichmannii*: To 100 mL of *Culture medium* add 1.0–1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Place 10-mL portions of the hot solution in test tubes, cover the tubes, sterilize at 121° for 15 min in an autoclave, and allow the tubes to cool in an upright position. Inoculate three or more of the tubes by stab transfer of a pure culture of *Lactobacillus leichmannii*.[2] [NOTE—Before first using a fresh culture in this assay, make NLT 10 successive transfers of the culture in a 2-week period.] Incubate for 16–24 h at a temperature between 30° and 40° held constant to within ±0.5°. Store in a refrigerator.

Prepare fresh stab cultures at least three times each week, and do not use them for preparing the *Inoculum* if more than 4 days old. The activity of the microorganism can be increased by daily or twice-daily transfer of the stab culture, to the point where definite turbidity in the liquid *Inoculum* can be observed 2–4 h after inoculation. A

[2] Pure cultures of *Lactobacillus leichmannii* (listed as *Lactobacillus delbruekii*) may be obtained as No. 7830 from ATCC, 10801 University Blvd., Manassas, VA 20110-2209 (www.atcc.org).

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slow-growing culture seldom gives a suitable response curve and may lead to erratic results.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus leichmannii* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Transfer cells from the *Stock culture of Lactobacillus leichmannii* to two sterile tubes containing 10 mL each of the *Culture medium*. Incubate these cultures for 16–24 h at a temperature between 30° and 40° held constant to within ±0.5°. Under aseptic conditions, centrifuge the cultures, and decant the supernatant. Suspend the cells from the culture in 5 mL of sterile *Suspension medium*, and combine. Using sterile *Suspension medium*, adjust the volume so that a 1-in-20 dilution in saline TS produces 70% transmittance when read on a suitable spectrophotometer that has been set at a wavelength of 530 nm, equipped with a 10-mm cell, and read against saline TS set at 100% transmittance. Prepare a 1-in-400 dilution of the adjusted suspension using sterile *Basal medium stock solution*. [NOTE—This dilution may be altered, when necessary, to obtain the desired test response.] The cell suspension so obtained is the *Inoculum*.

Calibration of spectrophotometer: Check the wavelength of the spectrophotometer periodically, using a standard wavelength cell or other suitable device. Before reading any tests, calibrate the spectrophotometer for 0% and 100% transmittance, using water, and with the wavelength set at 530 nm.

Analysis

Samples: *Standard solution* and *Sample solution*

Because of the high sensitivity of the test organism to minute amounts of vitamin B_{12} activity and to traces of many cleansing agents, cleanse meticulously by suitable means, followed preferably by heating at 250° for 2 h, using hard-glass 20-mm × 150-mm test tubes and other necessary glassware.

To separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each of these tubes and to four similar empty tubes add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes to prevent bacterial contamination, and sterilize in an autoclave at 121° for 5 min, arranging to reach this temperature in NMT 10 min by preheating the autoclave if necessary. Cool as rapidly as possible to avoid color formation resulting from overheating the medium. Take precautions to maintain uniformity of sterilizing and cooling conditions throughout the assay, because packing the tubes too closely in the autoclave or overloading it may cause variation in the heating rate.

Aseptically add 0.5 mL of *Inoculum* to each tube so prepared, except two of the four containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 40°, held constant to within ±0.5°, for 16–24 h.

Terminate growth by heating to a temperature NLT 80° for 5 min. Cool to room temperature. After agitating contents, read the transmittance at 530 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of the inoculated blank. If the difference is greater than 5%, or if there is evidence of contamination with a foreign microorganism, disregard the results of the assay.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of each of the remaining tubes. Disregard the results of the assay if the slope of the standard curve indicates a problem with sensitivity.

Calculation: Prepare a standard concentration-response curve by the following procedure. Test for and replace any aberrant individual transmittances. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittances (Σ_S) as the difference, $y = 2.00 - \Sigma_S$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_U$, adding together the two transmittances (Σ_U) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X, for each dosage level. Average the values of X for each of three or more dosage levels to obtain $\bar{X}$, which equals the log-relative potency, M', of the *Sample solution*.

Determine the quantity, in µg, of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$) in the portion of Capsules taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of µg of cyanocobalamin assumed to be present in the portion of Capsules taken

Calculate the percentage of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$) in the portion of Capsules taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of cyanocobalamin in the portion of Capsules taken (µg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, $\bar{M}$, is the assayed log-potency of the test material (see *Vitamin B_{12} Activity* in *Design and Analysis of Biological Assays* ⟨111⟩, *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$)

• **FOLIC ACID, *Method 1***

[NOTE—Use low-actinic glassware throughout this procedure.]

Reagent A: 25% solution of tetrabutylammonium hydroxide in methanol

Reagent B: Transfer 5.0 g of pentetic acid to a 50-mL volumetric flask. Using sonication if necessary, dissolve in and dilute with 1 N sodium hydroxide to volume.

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Mobile phase: 2 g of monobasic potassium phosphate in 650 mL of water. Add 12.0 mL of *Reagent A*, 7.0 mL of 3 N phosphoric acid, and 240 mL of methanol. Cool to room temperature, adjust with phosphoric acid or ammonia TS to a pH of 7.0, dilute with water to 1000 mL, and filter. Recheck the pH before use by adding water or methanol to the prepared *Mobile phase* to obtain baseline separation of folic acid and the internal standard. The pH may be increased up to 7.15 to obtain better separation. [NOTE—The methanol and water content may be varied (between 1% and 3%).]

Internal standard solution: Transfer 40 mg of methylparaben to a 1000-mL volumetric flask, and add 220 mL of methanol to dissolve. Dissolve 2.0 g of monobasic potassium phosphate in 300 mL of water in a separate beaker, quantitatively transfer this solution to the flask containing the methylparaben solution, and add an additional 300 mL of water. Add 19 mL of *Reagent A*, 7 mL of 3 N phosphoric acid, and 30 mL of *Reagent B*. Adjust with ammonia TS to a pH of 9.8, bubble nitrogen through the solution for 30 min, dilute with water to volume, and mix.

Standard solution: 0.016 mg/mL of USP Folic Acid RS in *Internal standard solution*

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." Transfer an amount of Capsule contents to a suitable centrifuge tube, and add a volume of the *Internal standard solution* to obtain a nominal concentration of 0.016 mg/mL of folic acid. Shake by mechanical means for 10 min, and centrifuge. Filter a portion of the clear supernatant, and use the filtrate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 280 nm
Column: 3.9-mm × 30-cm; packing L1
Flow rate: 1 mL/min
Injection volume: 15 µL

System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for folic acid and methylparaben are about 0.8 and 1.0, respectively.]
Suitability requirements
Relative standard deviation: NMT 3.0%

Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas for folic acid and methylparaben. Calculate the percentage of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$) in the portion of Capsules taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak area ratio of folic acid to methylparaben from the *Sample solution*
R_S = peak area ratio of folic acid to methylparaben from the *Standard solution*
C_S = concentration of USP Folic Acid RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of folic acid in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$)

• **FOLIC ACID, *Method 2***
[NOTE—Use low-actinic glassware throughout this procedure.]

Diluent: 60 µg/mL of ammonium hydroxide

Mobile phase: Transfer 0.4 mL of triethylamine, 15.0 mL of glacial acetic acid, and 350 mL of methanol to a 2000-mL volumetric flask, and dilute with 0.008 M sodium 1-hexanesulfonate to volume.

Standard stock solution: 60 µg/mL of USP Folic Acid RS in *Diluent*. Prepare this solution fresh daily.

Standard solution: Mix 5.0 mL of the *Standard stock solution* with 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." Transfer a portion of the Capsule contents, equivalent to 0.3 mg of folic acid, to a 125-mL stoppered flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 270 nm
Column: 4.6-mm × 25-cm; packing L7
Column temperature: 50°
Flow rate: 2 mL/min
Injection volume: 5 µL

System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 2.0%

Analysis
Samples: *Standard solution* and *Sample solution*
Measure the areas of the major peaks. Calculate the percentage of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of folic acid from the *Sample solution*
r_S = peak area of folic acid from the *Standard solution*
C_S = concentration of USP Folic Acid RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of folic acid in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$)

• **DEXPANTHENOL OR PANTHENOL**
[NOTE—The following procedure is applicable also to the determination of the dextrorotatory component of racemic panthenol in preparations containing panthenol.]

Dehydrated mixtures yielding formulations similar to the media described herein may be used provided that, when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described herein.

Standard stock solution: 800 µg/mL of USP Dexpanthenol RS, or 1600 µg/mL of USP Racemic Panthenol RS in water. Store in a refrigerator, protected from light, and use within 30 days.

Standard solution: On the day of the assay, prepare a dilution of 1.2 µg/mL of dexpanthenol or 2.4 µg/mL of panthenol from the *Standard stock solution* diluted with water.

Sample solution: Weigh NLT 30 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents as completely as

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possible to a beaker. Remove any contents adhering to the empty Capsule shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the average net weight per Capsule. Dissolve a portion of the Capsule contents, nominally equivalent to 1.2 mg of dexpanthenol or 2.4 mg of panthenol, in 100.0 mL of water. Quantitatively dilute a portion of this solution with water to obtain a concentration of 1.2 µg/mL of dexpanthenol or 2.4 µg/mL of panthenol.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in about 500 mL of water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and dilute with water to 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature NLT 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to 75 ± 5°, and add hydrochloric acid solution (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and dilute with water to 1000 mL. Store under toluene in a cool place at a temperature NLT 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and dilute with water to 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin–thiamine hydrochloride–biotin solution: 20 µg/mL of riboflavin, 10 µg/mL of thiamine hydrochloride, and 0.04 µg/mL of biotin in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

***p*-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution:** 10 µg/mL of *p*-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in neutral 25% alcohol. Store in a refrigerator.

Salt solution A: 50 mg/mL of monobasic potassium phosphate and 50 mg/mL of dibasic potassium phosphate in water. Add 10 drops of hydrochloric acid per L of solution. Store under toluene.

Salt solution B: 20 mg/mL of magnesium sulfate, 1 mg/mL of sodium chloride, 1 mg/mL of ferrous sulfate, and 1 mg/mL of manganese sulfate in water. Add 10 drops of hydrochloric acid per L of the solution. Store under toluene.

Pyridoxal–calcium pantothenate solution: 200 µg/mL of pyridoxal hydrochloride and 1.875 µg/mL of calcium pantothenate in 10% alcohol. Store in a refrigerator, and use within 30 days.

Polysorbate 40–oleic acid solution: 50 mg/mL of polysorbate 40 and 0.5 mg/mL of oleic acid in 20% alcohol. Store in a refrigerator, and use within 30 days.

Modified pantothenate medium: Dissolve *anhydrous Dextrose* and *Sodium acetate* in the solutions previously mixed according to *Table 3*, and adjust with 1 N sodium hydroxide to a pH of 6.8. Finally, dilute with water to 250 mL.

Table 3

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Riboflavin–thiamine hydrochloride–biotin solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL
Pyridoxal–calcium pantothenate solution	5 mL
Polysorbate 40–oleic acid solution	5 mL

Double-strength modified pantothenate medium: Prepare as directed in *Modified pantothenate medium*, but make the final dilution to 125 mL instead of 250 mL. Prepare fresh.

Stock culture of *Pediococcus acidilactici*: Dissolve in 800 mL of water, with the aid of heat, 6.0 g of peptone, 4.0 g of pancreatic digest of casein, 3.0 g of yeast extract, 1.5 g of beef extract, 1.0 g of dextrose, and 15.0 g of agar. Adjust with 0.1 N sodium hydroxide or 0.1 N hydrochloric acid to a pH of 6.5–6.6, and dilute with water to 1000 mL. Add 10-mL portions of the solution to culture tubes, place caps on the tubes, and sterilize in an autoclave at 121° for 15 min. Cool on a slant, and store in a refrigerator. Prepare a stock culture of *Pediococcus acidilactici*[3] on a slant of this medium. Incubate at 35° for 20–24 h, and store in a refrigerator. Maintain the stock culture by monthly transfer onto fresh slants.

Inoculum: Inoculate three 250-mL portions of sterile *Modified pantothenate medium* from a stock culture slant, and incubate at 35° for 20–24 h. Centrifuge the suspension from the combined portions, and wash the cells with sterile *Modified pantothenate medium*. Resuspend the cells in sufficient *Modified pantothenate medium* so that a 1-in-50 dilution, when tested in a 13-mm diameter test tube, gives 80% light transmission at 530 nm. Transfer 1.2-mL portions of this stock suspension to sterile glass ampuls, seal, freeze in liquid nitrogen, and store in a freezer. On the day of the assay, allow the ampuls to reach room temperature, mix the contents, and dilute 1 mL of thawed culture with sterile saline TS to 150 mL. [NOTE—This dilution may be altered when necessary to obtain the desired test response.]

Analysis: Prepare in triplicate a series of eight culture tubes by adding the following quantities of water to the tubes within a set: 5.0, 4.5, 4.0, 3.5, 3.0, 2.0, 1.0, and 0.0 mL. To these same tubes and in the same order add 0.0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. Prepare in duplicate a series of five culture tubes by adding the following quantities of water to the tubes within a set: 4.0, 3.5, 3.0, 2.0, and 1.0 mL. To these same tubes and in the same order add 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*.

Add 5.0 mL of *Double-strength modified pantothenate medium* to each tube. Cover the tubes with metal caps, and sterilize in an autoclave at 121° for 5 min. Cool to room temperature in a chilled water bath, and inoculate each tube with 0.5 mL of the *Inoculum*. Allow to incubate at 37° for 16 h. Terminate growth by heating to a temperature NLT 80°, such as by steaming at atmospheric pressure in a sterilizer for 5–10 min. Cool, and determine the percentage transmittance of the suspensions, in cells

[3] ATCC No. 8042 is suitable.

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of equal pathlength, on a suitable spectrophotometer, at a wavelength of 530 nm.

Calculation: Draw a dose-response curve on arithmetic graph paper by plotting the average response, in percentage of transmittance, for each set of tubes of the standard curve against the standard level concentrations. The curve is drawn by connecting each adjacent pair of points with a straight line. From this standard curve, determine by interpolation the potency of each tube containing portions of the *Sample solution*. To obtain the individual responses divide the potency of each tube by the amount of the *Sample solution* added to it. Calculate the mean response by averaging the individual responses that vary from their mean by NMT 15%, using NLT half the total number of tubes. Calculate the potency of the portion of the material taken for the assay, by multiplying the mean response by the appropriate dilution factor.

Calculate the percentage of the labeled amount of dexpanthenol or panthenol ($C_9H_{19}NO_4$) in the portion of Capsules taken:

$$\text{Result} = (P/N) \times 100$$

P = potency of dexpanthenol or panthenol in the portion taken (mg)

N = nominal amount of dexpanthenol or panthenol in the portion taken (mg)

Acceptance criteria: 90.0%–150.0% of the labeled amount of dexpanthenol or panthenol ($C_9H_{19}NO_4$)

• **CALCIUM PANTOTHENATE, *Method 1***

Mobile phase: Phosphoric acid and water (1:1000)

Internal standard solution: 80 mg of *p*-hydroxybenzoic acid in 3 mL of alcohol. Add 50 mL of water and 7.1 g of dibasic sodium phosphate, and dilute with water to 1000 mL. Adjust with phosphoric acid to a pH of 6.7.

Standard solution: 0.6 mg/mL of USP Calcium Pantothenate RS in the *Internal standard solution*

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." To a centrifuge tube transfer an amount of mixed Capsule contents and a volume of *Internal standard solution* to obtain a concentration of 0.6 mg/mL in the *Sample solution*.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 210 nm

Column: 3.9-mm × 15-cm; packing L1

Flow rate: 1.5 mL/min

Injection volume: 10 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for calcium pantothenate and *p*-hydroxybenzoic acid are about 0.5 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of calcium pantothenate and the internal standard. Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Capsules taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak area ratio of calcium pantothenate to *p*-hydroxybenzoic acid from the *Sample solution*

R_S = peak area ratio of calcium pantothenate to *p*-hydroxybenzoic acid from the *Standard solution*

C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• **CALCIUM PANTOTHENATE, *Method 2***

Standard stock solution: Dissolve 50 mg of USP Calcium Pantothenate RS, previously dried and stored in the dark over phosphorus pentoxide and protected from absorption of moisture while weighing, in 500 mL of water in a 1000-mL volumetric flask. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), and dilute with water to volume, to obtain a concentration of 50 µg/mL of USP Calcium Pantothenate RS. Store under toluene in a refrigerator.

Standard solution: On the day of the assay, dilute a volume of *Standard stock solution* with water to obtain a concentration of 0.01–0.04 µg/mL of calcium pantothenate, the exact concentration being such that the responses obtained as directed in the *Analysis*, 2.0 and 4.0 mL of the *Standard solution* being used, are within the linear portion of the log-concentration response curve.

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." Transfer a portion of the Capsule contents, equivalent to 50 mg of calcium pantothenate, to a 1000-mL volumetric flask containing 500 mL of water. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (16.66 mg/mL), dilute with water to volume, and filter. Dilute a volume of this solution to obtain a solution with approximately the same concentration as that of the *Standard solution*.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and dilute with water to 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature NLT 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and dilute with water to 1000 mL. Store under toluene in a cool place at a temperature NLT 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and dilute with water to 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin–thiamine hydrochloride–biotin solution: 20 µg/mL of riboflavin, 10 µg/mL of thiamine hydrochloride, and 0.04 µg/mL of biotin in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

***p*-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution:** 10 µg/mL of *p*-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a

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mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Basal medium stock solution: Dissolve the *anhydrous Dextrose* and *anhydrous Sodium acetate* in the solutions previously mixed according to *Table 4*, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Table 4

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Riboflavin–thiamine hydrochloride–biotin solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water. Add 500 mg of *anhydrous Dextrose*, 500 mg of *anhydrous Sodium acetate*, and 1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to the test tubes, close or cover the tubes, sterilize in an autoclave at 121° for 15 min, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*[1] incubating for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution* add 5.0 mL of water containing 0.2 µg of calcium pantothenate. Plug the tubes with cotton, sterilize in an autoclave at 121° for 15 min, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Transfer cells from the *Stock culture of Lactobacillus plantarum* to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. The cell suspension so obtained is the *Inoculum*.

Analysis

Samples: *Standard solution* and *Sample solution*

To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool, and add 1 drop of *Inoculum* to each tube, except two of the four tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37°, held constant to within ±0.5° until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of Standard during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to an optical container if necessary. Read the transmittance between 540 and 660 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittance (Σ_S) as the difference, $y = 2.00 - \Sigma_S$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_U$, adding together the two transmittances (Σ_U) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X, for each dosage level. Average the values of X for each of three or more dosage levels to obtain $\overline{X}$, which equals the log-relative potency, M', of the *Sample solution*.

Determine the quantity, in mg, of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Capsules taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of mg of calcium pantothenate assumed to be present in the portion of Capsules taken

Calculate the percentage of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Capsules taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of calcium pantothenate in the portion of Capsules taken (mg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference

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between the two log-potencies M is NMT 0.08, their mean, $\overline{M}$, is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* ⟨111⟩, *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• **CALCIUM PANTOTHENATE, *Method 3***

Buffer solution: Dissolve 10.0 g of monobasic potassium phosphate in 2000 mL of water, and adjust with phosphoric acid to a pH of 3.5.

Mobile phase: Methanol and *Buffer solution* (1:9)

Standard stock solution: 0.25 mg/mL of USP Calcium Pantothenate RS in water. Prepare fresh every 4 weeks. Store in a refrigerator.

Standard solution: 40 µg/mL of USP Calcium Pantothenate RS from the *Standard stock solution* diluted with water

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the net weight of the Capsule contents." Transfer a portion of the Capsule contents, equivalent to a nominal amount of 10 mg of calcium pantothenate, to a 250-mL volumetric flask. Add 10 mL of methanol, and swirl the flask to disperse the Capsules contents. Dilute with water to volume, mix, and filter.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 205 nm
Column: 3.9-mm × 30-cm; 5-µm packing L1
Column temperature: 50°
Flow rate: 2 mL/min
Injection volume: 25 µL

System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 3.0%

Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas for calcium pantothenate. Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of calcium pantothenate from the *Sample solution*
r_S = peak area of calcium pantothenate from the *Standard solution*
C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• **NIACIN OR NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, RIBOFLAVIN,** and **THIAMINE, *Method 1***

[NOTE—Use low-actinic glassware throughout this procedure.]

Diluent: Acetonitrile, glacial acetic acid, and water (5:1:94)

Mobile phase: A mixture of methanol, glacial acetic acid, and water (27:1:73) containing 140 mg of sodium 1-hexanesulfonate per 100 mL

Standard solution: [NOTE—Use USP Niacin RS in place of USP Niacinamide RS for formulations containing niacin.] Transfer 80 mg of USP Niacinamide RS, 20 mg of USP Pyridoxine Hydrochloride RS, 20 mg of USP Riboflavin RS, and 20 mg of USP Thiamine Hydrochloride RS to a 200-mL volumetric flask, and add 180 mL of *Diluent*. Immerse the flask in a hot water bath maintained at 65°–70° for 10 min with regular shaking or using a vortex mixer, until all the solid materials are dissolved. Chill rapidly in a cold water bath for 10 min to room temperature, and dilute with *Diluent* to volume.

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." Transfer a portion of the Capsule contents, equivalent to 10 mg of niacinamide and 2.5 mg each of pyridoxine hydrochloride, riboflavin, and thiamine hydrochloride, to a 50-mL centrifuge tube. Add 25.0 mL of *Diluent*, and mix using a vortex mixer for 30 s to completely suspend the powder. Immerse the centrifuge tube in a hot water bath maintained at 65°–70°, heat for 5 min, and mix on a vortex mixer for 30 s. Return the tube to the hot water bath, heat for another 5 min, and mix on a vortex mixer for 30 s. Filter a portion of the solution, cool to room temperature, and use the clear filtrate. [NOTE—Use the filtrate within 3 h of filtration.]

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 280 nm
Column: 3.9-mm × 30-cm; packing L1
Flow rate: 1 mL/min
Injection volume: 10 µL

System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for niacinamide, pyridoxine, riboflavin, and thiamine are about 0.3, 0.5, 0.8, and 1.0, respectively.]
Suitability requirements
Relative standard deviation: NMT 3.0%

Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas for niacin or niacinamide, pyridoxine, riboflavin, and thiamine. Calculate the percentage of the labeled amount of niacinamide ($C_6H_6N_2O$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacinamide from the *Sample solution*
r_S = peak area of niacinamide from the *Standard solution*
C_S = concentration of USP Niacinamide RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of niacinamide in the *Sample solution* (mg/mL)

For formulations containing niacin:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacin from the *Sample solution*
r_S = peak area of niacin from the *Standard solution*
C_S = concentration of USP Niacin RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of niacin in the *Sample solution* (mg/mL)

Separately calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin

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($C_{17}H_{20}N_4O_6$), and thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the corresponding vitamin from the *Sample solution*
r_S = peak area of the corresponding vitamin from the *Standard solution*
C_S = concentration of the relevant USP Reference Standard in the *Standard solution* (mg/mL)
C_U = nominal concentration of the corresponding vitamin in the *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of thiamine from the *Sample solution*
r_S = peak area of thiamine from the *Standard solution*
C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL)
M_{r1} = molecular weight of thiamine mononitrate, 327.36
M_{r2} = molecular weight of thiamine hydrochloride, 337.27

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine as thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$)

• **NIACIN, *Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A: Transfer 1 mL of glacial acetic acid and 2.5 g of edetate disodium to a 100-mL volumetric flask. Dissolve in and dilute with water to volume.

Extraction solvent: *Solution A* and methanol (3:1)

Mobile phase: 0.1 M sodium acetate solution (13.6 mg/mL of sodium acetate in water). Adjust with acetic acid to a pH of 5.4. [NOTE—A small amount of methanol (up to 1%) may be added to the *Mobile phase* to improve resolution.]

Standard stock solution: 1 mg/mL of USP Niacin RS in *Extraction solvent*

Standard solution: Transfer 5.0 mL of the *Standard stock solution* to a 25-mL volumetric flask, and dilute with *Extraction solvent* to volume.

Sample solution: [NOTE—This preparation is suitable for the determination of niacin or niacinamide, pyridoxine, and riboflavin, when present in the formulation.] Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents to a beaker. Remove any contents adhering to the shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to 2 mg of riboflavin, to a 200-mL volumetric flask. If riboflavin is not present in the formulation, use a portion equivalent to 2 mg of pyridoxine. If pyridoxine is not present in the formulation, use a portion equivalent to 20 mg of niacin or niacinamide. Add 100.0 mL of *Extraction solvent*, and mix for 20 min, using a wrist-action shaker. Immerse the flask in a water bath maintained at 70°–75°, and heat for 20 min. Mix on a vortex mixer for 30 s, cool to room temperature, and filter. Use the clear filtrate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; packing L1
Flow rate: 1 mL/min
Injection volume: 20 µL

System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 3.0%
[NOTE—If necessary, flush the column with methanol between injections.]

Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas of niacin. Calculate the percentage of the labeled amount of niacin ($C_6H_5NO_2$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacin from the *Sample solution*
r_S = peak area of niacin from the *Standard solution*
C_S = concentration of USP Niacin RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of niacin in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacin ($C_6H_5NO_2$)

• **NIACINAMIDE, *Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Extraction solvent, Mobile phase, Standard stock solution, Standard solution, Sample solution, and **Chromatographic system:** Using USP Niacinamide RS in place of USP Niacin RS, proceed as directed in *Niacin, Method 2*.

Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas of niacinamide. Calculate the percentage of the labeled amount of niacinamide ($C_6H_6N_2O$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacinamide from the *Sample solution*
r_S = peak area of niacinamide from the *Standard solution*
C_S = concentration of USP Niacinamide RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of niacinamide in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacinamide ($C_6H_6N_2O$)

• **PYRIDOXINE HYDROCHLORIDE, *Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Extraction solvent, Mobile phase, and **Sample solution:** Prepare as directed in *Niacin, Method 2*.

Standard stock solution: 0.1 mg/mL of USP Pyridoxine Hydrochloride RS in *Extraction solvent*

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Standard solution: Transfer 20 µg/mL of USP Pyridoxine Hydrochloride RS from the *Standard stock solution*, and dilute with *Extraction solvent* to volume.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; packing L1
Flow rate: 1 mL/min
Injection volume: 20 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 3.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas of pyridoxine. Calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of pyridoxine from the *Sample solution*
r_S = peak area of pyridoxine from the *Standard solution*
C_S = concentration of USP Pyridoxine Hydrochloride RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of pyridoxine hydrochloride in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$)

• **RIBOFLAVIN, *Method 2***
[NOTE—Use low-actinic glassware throughout this procedure.]
Extraction solvent and **Sample solution:** Prepare as directed in *Niacin, Method 2*.
Solution A: 6.8 g/L of sodium acetate in water
Mobile phase: Prepare a mixture of *Solution A* and methanol (13:7). Add 2 mL of triethylamine per L of the mixture, and adjust with glacial acetic acid to a pH of 5.2.
Standard stock solution: Transfer 20 mg of USP Riboflavin RS to a 200-mL volumetric flask, and add 180 mL of *Extraction solvent*. Immerse the flask for 5 min in a water bath maintained at 65°–75°. Mix well, and repeat if necessary until dissolved. Chill rapidly in a cold water bath to room temperature, and dilute with *Extraction solvent* to volume.
Standard solution: Dilute 5.0 mL of the *Standard stock solution* with *Extraction solvent* to 25.0 mL.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; packing L1
Flow rate: 1 mL/min
Injection volume: 20 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 3.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas of riboflavin. Calculate the percentage of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of riboflavin from the *Sample solution*
r_S = peak area of riboflavin from the *Standard solution*
C_S = concentration of USP Riboflavin RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of riboflavin in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$)

• **THIAMINE, *Method 2***
[NOTE—Use low-actinic glassware throughout this procedure.]
Solution A: 1.88 g/L of sodium 1-hexanesulfonate in 0.1% phosphoric acid
Mobile phase: *Solution A* and acetonitrile (46:9)
Standard stock solution: 0.1 mg/mL of USP Thiamine Hydrochloride RS in 0.2 N hydrochloric acid
Standard solution: 0.02 mg/mL of USP Thiamine Hydrochloride RS from the *Standard stock solution* diluted with 0.2 N hydrochloric acid
Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the net weight of the Capsule contents." Mix a portion of the Capsule contents with a volume of 0.2 N hydrochloric acid to obtain a concentration of 0.02 mg/mL of thiamine. Shake for 15 min with a wrist-action shaker, and heat to boiling for 30 min. Cool to room temperature, and filter. Use the clear filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; packing L1
Flow rate: 2 mL/min
Injection volume: 20 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 3.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas for the major peaks. For products containing thiamine hydrochloride, calculate the percentage of the labeled amount of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of thiamine from the *Sample solution*
r_S = peak area of thiamine from the *Standard solution*
C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of thiamine hydrochloride in the *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of thiamine from the *Sample solution*
r_S = peak area of thiamine from the *Standard solution*
C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL)

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M_{r1} = molecular weight of thiamine mononitrate, 327.36
M_{r2} = molecular weight of thiamine hydrochloride, 337.27

Acceptance criteria: 90.0%–150.0% of the labeled amount of thiamine as thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$)

- **NIACIN OR NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, RIBOFLAVIN,** and **THIAMINE,** ***Method 3***

[NOTE—Use low-actinic glassware throughout this procedure.]

Reagent: 25 mg/mL of edetate disodium in water

Mobile phase: Transfer 0.4 mL of triethylamine, 15.0 mL of glacial acetic acid, and 350 mL of methanol to a 2000-mL volumetric flask. Dilute with 0.008 M sodium 1-hexanesulfonate to volume.

Standard stock solution: 1.5 mg/mL of USP Niacin RS or USP Niacinamide RS, 0.24 mg/mL of USP Pyridoxine Hydrochloride RS, 0.08 mg/mL of USP Riboflavin RS, and 0.24 mg/mL of USP Thiamine Hydrochloride RS in the *Reagent*, with heating if necessary

Standard solution: Transfer 5.0 mL of the *Standard stock solution* to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." Transfer a portion of the Capsule contents, equivalent to 7.5 mg of niacin or niacinamide, 1.2 mg of pyridoxine hydrochloride, 0.4 mg of riboflavin, and 1.2 mg of thiamine hydrochloride, to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1), and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 270 nm
Column: 4.6-mm × 25-cm; packing L7
Column temperature: 50°
Flow rate: 2 mL/min
Injection volume: 5 µL

System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 2.0%

Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas of niacin or niacinamide. Calculate the percentage of the labeled amount of niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacin or niacinamide from the *Sample solution*
r_S = peak area of niacin or niacinamide from the *Standard solution*
C_S = concentration of USP Niacin RS or USP Niacinamide RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of niacin or niacinamide in the *Sample solution* (mg/mL)

Separately calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) (for products containing thiamine hydrochloride) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the corresponding vitamin from the *Sample solution*
r_S = peak area of the corresponding vitamin from the *Standard solution*
C_S = concentration of the relevant USP Reference Standard in the *Standard solution* (mg/mL)
C_U = nominal concentration of the corresponding vitamin in the *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of thiamine from the *Sample solution*
r_S = peak area of thiamine from the *Standard solution*
C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL)
M_{r1} = molecular weight of thiamine mononitrate, 327.36
M_{r2} = molecular weight of thiamine hydrochloride, 337.27

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine as thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$)

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** ⟨2040⟩**:** Meet the requirements for *Dissolution*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩**:** Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 3×10^3 cfu/g, and the combined molds and yeasts count does not exceed 3×10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meet the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

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- **LABELING:**[4] The label states that the product is Oil- and Water-Soluble Vitamins Capsules. The label also states the quantity of each vitamin per dosage unit and, where necessary, the chemical form in which it is present. Where the product contains vitamin E, the label indicates whether it is the *d*- or *dl*- form. Where more than one assay method is given for a particular vitamin, the labeling states with which assay method the product complies only if *Method 1* is not used.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Alpha Tocopherol RS
USP Alpha Tocopheryl Acetate RS
USP Alpha Tocopheryl Acid Succinate RS
USP Biotin RS
1*H*-Thieno[3,4-*d*]imidazole-4-pentanoic acid, hexahydro-2-oxo-, 3a*S*-(3aα,4β,6aα)-;
(3a*S*,4*S*,6a*R*)-Hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazole-4-valeric acid.
$C_{10}H_{16}N_2O_3S$ 244.31
USP Calcium Pantothenate RS
β-Alanine, *N*-(2,4-dihydroxy-3,3-dimethyl-1-oxobutyl)-, calcium salt (2:1), (*R*)-;
Calcium D-pantothenate (1:2).
$C_{18}H_{32}CaN_2O_{10}$ 476.53
USP Cholecalciferol RS
9,10-Secocholesta-5,7,10(19)-trien-3-ol, (3β,5*Z*,7*E*)-;
Cholecalciferol.
$C_{27}H_{44}O$ 384.64
USP Cyanocobalamin (Crystalline) RS
Vitamin B_{12}.
$C_{63}H_{88}CoN_{14}O_{14}P$ 1355.37
USP Dexpanthenol RS
Butanamide, 2,4-dihydroxy-*N*-(3-hydroxypropyl)-3,3-dimethyl-, (*R*)-;
D-(+)-2,4-Dihydroxy-*N*-(3-hydroxypropyl)-3,3-dimethylbutyramide.
$C_9H_{19}NO_4$ 205.25
USP Ergocalciferol RS
9,10-Secoergosta-5,7,10(19),22-tetraen-3-ol, (3β,5*Z*,7*E*,22*E*)-;
Ergocalciferol.
$C_{28}H_{44}O$ 396.65
USP Folic Acid RS
L-Glutamic acid, *N*-[4-[[(2-amino-1,4-dihydro-4-oxo-6-pteridinyl)methyl]amino]benzoyl]-;
Folic acid;
N-[*p*-[[(2-Amino-4-hydroxy-6-pteridinyl)methyl]amino]-benzoyl]-L-glutamic acid.
$C_{19}H_{19}N_7O_6$ 441.40
USP Niacin RS
3-Pyridinecarboxylic acid;
Nicotinic acid.
$C_6H_5NO_2$ 123.11
USP Niacinamide RS
3-Pyridinecarboxamide;
Nicotinamide.
$C_6H_6N_2O$ 122.12
USP Phytonadione RS
1,4-Naphthalenedione, 2-methyl-3-(3,7,11,15-tetramethyl-2-hexadecenyl)-, [*R*-[*R**,*R**-(*E*)]]-;
Phylloquinone.
$C_{31}H_{46}O_2$ 450.70
USP Pyridoxine Hydrochloride RS
3,4-Pyridinedimethanol, 5-hydroxy-6-methyl-, hydrochloride;
Pyridoxol hydrochloride.
$C_8H_{11}NO_3 \cdot HCl$ 205.64
USP Racemic Panthenol RS
USP Riboflavin RS
Riboflavine.
$C_{17}H_{20}N_4O_6$ 376.36
USP Thiamine Hydrochloride RS
Thiazolium, 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-5-(2-hydroxyethyl)-4-methyl-, chloride, monohydrochloride;
Thiamine monohydrochloride.
$C_{12}H_{17}ClN_4OS \cdot HCl$ 337.27
USP Vitamin A RS

[4] USP Units of activity for vitamins, where such exist or formerly existed, are equivalent to the corresponding international units (IU), where such formerly existed. The USP Unit for Vitamin E has been discontinued. International units (IU) for vitamins also have been discontinued; however, the use of IU on the labels of vitamin products continues. Where articles are labeled in terms of Units in addition to the required labeling, the relationship of the USP Units or IU to mass is as follows. One USP Vitamin A Unit = 0.3 µg of all-*trans*-retinol (vitamin A alcohol) or 0.344 µg of all-*trans*-retinyl acetate (vitamin A acetate) or 0.55 µg of all-*trans*-retinyl palmitate (vitamin A palmitate), and 1 µg of retinol (3.3 USP Vitamin A Units) = 1 retinol equivalent (RE); 1 IU of beta carotene = 0.6 µg of all-*trans*-beta carotene; 1 USP Vitamin D Unit = 0.025 µg of cholecalciferol or ergocalciferol; and 1 mg of *dl*-alpha tocopherol = 1.1 former USP Vitamin E Units, 1 mg of *dl*-alpha tocopheryl acetate = 1 former USP Vitamin E Unit, 1 mg of *dl*-alpha tocopheryl acid succinate = 0.89 former USP Vitamin E Unit, 1 mg of *d*-alpha tocopherol = 1.49 former USP Vitamin E Units, and 1 mg of *d*-alpha tocopheryl acetate = 1.36 former USP Vitamin E Units, and 1 mg of *d*-alpha tocopheryl acid succinate = 1.21 former USP Vitamin E Units. In terms of *d*-alpha tocopherol equivalents, 1 mg of *d*-alpha tocopheryl acetate = 0.91, 1 mg of *d*-alpha tocopheryl acid succinate = 0.81, 1 mg of *dl*-alpha tocopherol = 0.74, 1 mg of *dl*-alpha tocopheryl acetate = 0.67, and 1 mg of *dl*-alpha tocopheryl acid succinate = 0.60.

Oil- and Water-Soluble Vitamins Oral Solution

DEFINITION

Oil- and Water-Soluble Vitamins Oral Solution contains one or more of the following oil-soluble vitamins: Vitamin A, vitamin D as Ergocalciferol (vitamin D_2) or Cholecalciferol (vitamin D_3), and Vitamin E; one or more of the following water-soluble vitamins: Ascorbic Acid or its equivalent as Calcium Ascorbate or Sodium Ascorbate, Cyanocobalamin, Niacin or Niacinamide, Dexpanthenol or Panthenol, pantothenic acid (as Calcium Pantothenate or Racemic Calcium Pantothenate), Pyridoxine Hydrochloride, Riboflavin or Riboflavin-5′-Phosphate Sodium, and Thiamine Hydrochloride or Thiamine Mononitrate. It contains NLT 90.0% and NMT 250.0% of the labeled amounts of vitamin A ($C_{20}H_{30}O$) as retinol or esters of retinol in the form of retinyl acetate ($C_{22}H_{32}O_2$) or retinyl palmitate ($C_{36}H_{60}O_2$), vitamin D as ergocalciferol ($C_{28}H_{44}O$) or cholecalciferol ($C_{27}H_{44}O$), vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$) or alpha tocopheryl acetate ($C_{31}H_{52}O_3$) or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$), ascorbic acid ($C_6H_8O_6$) or its salts as calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$) or sodium ascorbate ($C_6H_7NaO_6$), and thiamine ($C_{12}H_{17}ClN_4OS$) as thiamine hydrochloride or thiamine mononitrate; NLT 90.0% and NMT 150.0% of the labeled amounts of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), dexpanthenol ($C_9H_{19}NO_4$) or panthenol ($C_9H_{19}NO_4$), niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), and riboflavin ($C_{17}H_{20}N_4O_6$) or riboflavin-5′-phosphate sodium ($C_{17}H_{20}N_4NaO_9P$); and NLT 90.0% and NMT 450.0% of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$).

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STRENGTH

[NOTE—In the following assays, where more than one assay method is given for an individual ingredient the requirements may be met by following any one of the specified methods, the method used being stated in the labeling only if *Method* 1 is not used.]

• **VITAMIN A**

[NOTE—Use low-actinic glassware throughout this procedure.]

Diluent: Tetrahydrofuran and acetonitrile (1:1)

Mobile phase: Methanol, acetonitrile, and *n*-hexane (46.5: 46.5: 7.0)

Standard solution: 0.33 mg/mL of retinol ($C_{20}H_{30}O$) from USP Vitamin A RS in *Diluent*. [NOTE—USP Vitamin A RS is retinyl acetate. Use it to analyze Oral Solution that contains vitamin A as retinol, retinyl acetate, or retinyl palmitate.]

Sample solution: Transfer an accurately measured volume of Oral Solution, equivalent to 3.3 mg of retinol, to a 500-mL separatory funnel containing 10 mL of water and 20 mL of dehydrated alcohol. Add 150 mL of solvent hexane, insert the stopper, and shake for 1 min. Add another 150 mL of solvent hexane, insert the stopper, shake, and allow the layers to separate. Discard the aqueous layer, and filter the solvent hexane extract through anhydrous sodium sulfate into a 500-mL round-bottom flask. Evaporate the solution to dryness with the aid of a rotary evaporator over a water bath maintained at about 65°. Immediately add 10.0 mL of *Diluent*, swirl to dissolve the residue, and filter.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 50-cm (prepared from two concatenated 4.6-mm × 25-cm columns); packing L1

Column temperature: 40°

Flow rate: 1.5 mL/min

Injection volume: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of vitamin A as retinol ($C_{20}H_{30}O$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of retinol or retinyl ester from the *Sample solution*

r_S = peak area of retinyl acetate from the *Standard solution*

C_S = concentration of retinyl acetate ($C_{22}H_{32}O_2$) in the *Standard solution* (µg/mL)

C_U = nominal concentration of vitamin A as retinol ($C_{20}H_{30}O$) in the *Sample solution* (µg/mL)

F = factor used to convert retinyl acetate, the ester form present in USP Vitamin A RS, to retinol, 0.872

Acceptance criteria: 90.0%–250.0% of the labeled amount of vitamin A as retinol ($C_{20}H_{30}O$)

• **CHOLECALCIFEROL OR ERGOCALCIFEROL (VITAMIN D)**

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Diluent and **Mobile phase:** Prepare as directed in *Vitamin A*.

Standard solution: 5 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in *Diluent*

Sample solution: Transfer an accurately measured volume of Oral Solution, equivalent to 50 µg of cholecalciferol or ergocalciferol, to a 500-mL separatory funnel containing 10 mL of water and 20 mL of dehydrated alcohol. Add 150 mL of solvent hexane, insert the stopper, and shake for 1 min. Add another 150 mL of solvent hexane, insert the stopper, shake, and allow the layers to separate. Discard the aqueous layer. Drain the solvent hexane extract through anhydrous sodium sulfate into a 500-mL round-bottom flask. Evaporate the solution to dryness with the aid of a rotary evaporator over a water bath maintained at about 65°. Immediately add 10.0 mL of *Diluent*, swirl to dissolve the residue, and filter.

Chromatographic system: Prepare as directed in *Vitamin A*.

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of vitamin D. Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak areas of cholecalciferol or ergocalciferol from the *Sample solution*

r_S = peak areas of cholecalciferol or ergocalciferol from the *Standard solution*

C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

F = correction factor to account for the average amount of previtamin D present in the formulation, 1.09

Acceptance criteria: 90.0%–250.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

• **VITAMIN E**

[NOTE—Use low-actinic glassware throughout this procedure.]

Diluent: Acetonitrile and ethyl acetate (1:1)

Potassium hydroxide solution: Transfer 90 g of potassium hydroxide pellets to a 100-mL volumetric flask containing 60 mL of water. Mix to dissolve, cool, and dilute with water to volume.

Mobile phase: Methanol, acetonitrile, and *n*-hexane (46.5: 46.5: 7.0)

Standard solution: 0.3 mg/mL of USP Alpha Tocopherol RS in *Diluent*

Sample solution: Transfer an amount of Oral Solution, equivalent to 1.5 mg of alpha tocopherol, to a 125-mL conical flask fitted with a ground-glass joint, and add 25.0 mL of dehydrated alcohol. Attach a reflux condenser, and reflux in a boiling water bath for 1 min. Cautiously add 3 mL of the *Potassium hydroxide solution* through the condenser, and continue to reflux for 30 min. Remove the flask from the bath, and rinse the condenser with about 15 mL of water. Cool, and transfer with a minimum volume

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of water to a 250-mL separatory funnel. Rinse the flask with 50 mL of *n*-hexane, and add the rinsings to the separatory funnel. Insert the stopper, shake vigorously for 1 min, and allow the layers to separate. Drain the aqueous layer into a second 250-mL separatory funnel, and repeat the extraction with 50 mL of *n*-hexane. Discard the aqueous layer, and combine the hexane extracts. Wash the combined extracts with 25 mL of water, allow the layers to separate, and discard the aqueous layer. Add 3 drops of glacial acetic acid, and repeat the washing procedure two more times. Filter the washed hexane layer through anhydrous sodium sulfate into a 250-mL round-bottom flask. Rinse the funnel and sodium sulfate with *n*-hexane, and add the rinsing to the hexane solution in the flask. Evaporate the hexane solution to dryness with the aid of a rotary evaporator over a water bath maintained at about 50°. Immediately add 5.0 mL of *Diluent*, and swirl to dissolve the residue.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 291 nm
Column: 4.6-mm × 25-cm; packing L1
Column temperature: 40°
Flow rate: 3.0 mL/min
Injection volume: 20 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 5.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas. Calculate the percentage of the labeled amount of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of alpha tocopherol from the *Sample solution*
r_S = peak area of alpha tocopherol from the *Standard solution*
C_S = concentration of alpha tocopherol in the *Standard solution* (mg/mL)
C_U = nominal concentration of vitamin E, as alpha tocopherol, in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–250.0% of the labeled amount of vitamin E

• **ASCORBIC ACID, CALCIUM ASCORBATE,** and **SODIUM ASCORBATE**
(See *Vitamin C Assay* ⟨580⟩.)
[NOTE—For labeling purposes, consider *Method I—Titrimetric Method* as *Method 1*.]
Acceptance criteria: 90.0%–250.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$), calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$) or sodium ascorbate ($C_6H_7NaO_6$)

• **CYANOCOBALAMIN**
[NOTE—Use low-actinic glassware throughout this procedure.]
Standard stock solution: 1.0 µg/mL of cyanocobalamin from USP Cyanocobalamin (Crystalline) RS in 25% alcohol. Store in a refrigerator.
Standard solution: Dilute a suitable volume of *Standard stock solution* with water to a measured volume such that after the incubation period as described in *Analysis*, the difference in transmittance between the inoculated blank and the 5.0-mL level of the *Standard solution* is NLT that which corresponds to a difference of 1.25 mg in dried cell weight. This concentration usually falls between 0.01 and 0.04 ng/mL of the *Standard solution*. Prepare this solution fresh for each assay.
Sample solution: Transfer an accurately measured volume of Oral Solution, assumed to contain 1.0 µg of cyanocobalamin, to an appropriate vessel containing, for each mL of the Oral Solution taken, 25 mL of an aqueous extracting solution prepared just before use to contain, in each 100 mL, 1.29 g of dibasic sodium phosphate, 1.1 g of anhydrous citric acid, and 1.0 g of sodium metabisulfite. Autoclave the mixture at 121° for 10 min. Allow any undissolved particles of the extract to settle, and filter or centrifuge if necessary. Dilute an aliquot of the clear solution with water to obtain a final solution containing vitamin B_{12} activity approximately equivalent to that of the *Standard solution*.
Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and dilute with water to 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature NLT 10°. Filter the solution if a precipitate forms during storage.
Asparagine solution: Dissolve 2.0 g of L-asparagine in water to make 200 mL. Store under toluene in a refrigerator.
Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and dilute with water to 200 mL. Store under toluene in a refrigerator.
Xanthine solution: Suspend 0.20 g of xanthine in 30–40 mL of water, heat to 70°, add 6.0 mL of 6 N ammonium hydroxide, and stir until the solid is dissolved. Cool, and dilute with water to 200 mL. Store under toluene in a refrigerator.
Salt solution A: Dissolve 10 g of monobasic potassium phosphate and 10 g of dibasic potassium phosphate in water to make 200 mL, and add 2 drops of hydrochloric acid. Store this solution under toluene.
Salt solution B: Dissolve 4.0 g of magnesium sulfate, 0.20 g of sodium chloride, 0.20 g of ferrous sulfate, and 0.20 g of manganese sulfate in water to make 200 mL, and add 2 drops of hydrochloric acid. Store this solution under toluene.
Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol. Store in a refrigerator.
Vitamin solution A: 10 mg of riboflavin, 10 mg of thiamine hydrochloride, 100 µg of biotin, and 20 mg of niacin in 0.02 N acetic acid to make 400 mL. Store under toluene protected from light in a refrigerator.
Vitamin solution B: 20 mg of *p*-aminobenzoic acid, 10 mg of calcium pantothenate, 40 mg of pyridoxine hydrochloride, 40 mg of pyridoxal hydrochloride, 8 mg of pyridoxamine dihydrochloride, and 2 mg of folic acid in a mixture of water and neutralized alcohol (3:1) to make 400 mL. Store, protected from light, in a refrigerator.
Basal medium stock solution: Prepare the medium according to the following formula and directions. A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium comparable to that obtained from the formula given herein.
Add the ingredients in the order listed in *Table 1*, carefully dissolving *Cystine* and *Tryptophan* in the *hydrochloric acid* before adding the next eight solutions in the resulting

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solution. In the resulting solution, add 100 mL of water, and dissolve *Dextrose*, *Sodium acetate*, and *Ascorbic acid*. Filter if necessary. Add the *Polysorbate 80 solution*, adjust with 1 N sodium hydroxide to a pH of 5.5–6.0, and dilute with Purified Water to 250 mL.

Table 1

L-Cystine	0.1 g
L-Tryptophan	0.05 g
1 N hydrochloric acid	10 mL
Adenine–guanine–uracil solution	5 mL
Xanthine solution	5 mL
Vitamin solution A	10 mL
Vitamin solution B	10 mL
Salt solution A	5 mL
Salt solution B	5 mL
Asparagine solution	5 mL
Acid-hydrolyzed casein solution	25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Ascorbic acid	1 g
Polysorbate 80 solution	5 mL

Tomato juice preparation: Centrifuge commercially canned tomato juice so that most of the pulp is removed. Suspend 5 g/L of analytical filter aid in the supernatant, and filter with the aid of reduced pressure, through a layer of the filter aid. Repeat, if necessary, until a clear, straw-colored filtrate is obtained. Store under toluene in a refrigerator.

Culture medium: [NOTE—A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium equivalent to that obtained from the formula given herein.] Dissolve 0.75 g of yeast extract, 0.75 g of dried peptone, 1.0 g of *anhydrous Dextrose*, and 0.20 g of monobasic potassium phosphate in 60–70 mL of water. Add 10 mL of *Tomato juice preparation* and 1 mL of *Polysorbate 80 solution*. Adjust with 1 N sodium hydroxide to a pH of 6.8, and dilute with water to 100 mL. Place 10-mL portions of the solution in test tubes, and plug with cotton. Sterilize the tubes and contents in an autoclave at 121° for 15 min. Cool as rapidly as possible to avoid color formation resulting from overheating the medium.

Suspension medium: Dilute a measured volume of the *Basal medium stock solution* with an equal volume of water. Place 10-mL portions of the diluted medium in test tubes. Sterilize, and cool as directed in *Culture medium*.

Stock culture of *Lactobacillus leichmannii*: To 100 mL of the *Culture medium* add 1.0–1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Place 10-mL portions of the hot solution in test tubes, cover the tubes, sterilize at 121° for 15 min in an autoclave, and allow the tubes to cool in an upright position. Inoculate three or more of the tubes by stab transfer of a pure culture of *Lactobacillus leichmannii*.[1] [NOTE—Before first using a fresh culture in this assay, make NLT 10 successive transfers of the culture in a 2-week period.] Incubate for 16–24 h at a temperature between 30° and 40° held constant to within ±0.5°. Store in a refrigerator. Prepare fresh stab cultures at least three times each week, and do not use them for preparing the *Inoculum* if more than 4 days old. The activity of the microorganism can be increased by daily or twice-daily transfer of the stab culture to the point where definite turbidity in the liquid *Inoculum* can be observed 2–4 h after inoculation. A slow-growing culture seldom gives a suitable response curve and may lead to erratic results.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus leichmannii* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Transfer cells from the *Stock culture of Lactobacillus leichmannii* to two sterile tubes each containing 10 mL of the *Culture medium*. Incubate these cultures for 16–24 h at a temperature between 30° and 40° held constant to within ± 0.5°. Under aseptic conditions centrifuge the cultures, and decant the supernatant. Suspend the cells from the culture in 5 mL of sterile *Suspension medium*, and combine. Using sterile *Suspension medium*, adjust the volume so that a 1-in-20 dilution in saline TS produces 70% transmittance when read on a suitable spectrophotometer that has been set at a wavelength of 530 nm, equipped with a 10-mm cell, and read against saline TS set at 100% transmittance. Prepare a 1-in-400 dilution of the adjusted suspension using sterile *Basal medium stock solution*. [NOTE—This dilution may be altered, when necessary, to obtain the desired test response.] The cell suspension so obtained is the *Inoculum*.

Calibration of spectrophotometer: Check the wavelength of the spectrophotometer periodically using a standard wavelength cell or other suitable device. Before reading any tests calibrate the spectrophotometer for 0% and 100% transmittance using water and with the wavelength set at 530 nm.

Analysis

Samples: *Standard solution* and *Sample solution*

Because of the high sensitivity of the test organism to minimum amounts of vitamin B_{12} activity and to traces of many cleansing agents, cleanse meticulously by suitable means, followed preferably by heating at 250° for 2 h, using hard-glass 20-mm × 150-mm test tubes and other necessary glassware.

To separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each of these tubes and to four similar empty tubes add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes to prevent bacterial contamination, and sterilize in an autoclave at 121° for 5 min arranging to reach this temperature in NMT 10 min by preheating the autoclave if necessary. Cool as rapidly as possible to avoid color formation resulting from overheating the medium. Take precautions to maintain uniformity of sterilizing and cooling conditions throughout the assay, because packing the tubes too closely in the autoclave or overloading it may cause variation in the heating rate.

Aseptically add 0.5 mL of the *Inoculum* to each tube so prepared, except two of the four containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 40°, held constant to within ±0.5°, for 16–24 h.

[1] Pure cultures of *Lactobacillus leichmannii* (listed as *Lactobacillus delbrueckii*) may be obtained as No. 7830 from ATCC, 10801 University Blvd., Manassas, VA 20110-2209 (www.atcc.org).

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Terminate growth by heating to a temperature NLT 80° for 5 min. Cool to room temperature. After agitating the contents, read the transmittance at 530 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of the inoculated blank. If the difference is greater than 5% or if there is evidence of contamination with a foreign microorganism, disregard the results of the assay.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of each of the remaining tubes. Disregard the results of the assay if the slope of the standard curve indicates a problem with sensitivity.

Calculation: Prepare a standard concentration-response curve by the following procedure. Test for and replace any aberrant individual transmittances. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittances (Σ_S) as the difference, $y = 2.00 - \Sigma_S$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of the *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_U$, adding together the two transmittances (Σ_U) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X, for each dosage level. Average the values of X for each of three or more dosage levels to obtain $\overline{X}$, which equals the log-relative potency, M', of the *Sample solution*.

Determine the quantity, in µg, of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$) in the portion of Oral Solution taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = µg of cyanocobalamin assumed to be present in the portion of Oral Solution taken

Calculate the percentage of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$) in the portion of Oral Solution taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of cyanocobalamin in the portion of Oral Solution taken

Replication: Repeat the entire determination at least once using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08 their mean, $\overline{M}$, is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* ⟨111⟩, *The Confidence Interval and Limits of Potency, Vitamin B_{12} Activity*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–450.0% of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$)

• **CALCIUM PANTOTHENATE,** ***Method 1***

Mobile phase: 0.2 M monobasic sodium phosphate and methanol (97:3). Adjust with 1.7 M phosphoric acid to a pH of 3.2 ± 0.1.

Standard solution: 80 µg/mL of USP Calcium Pantothenate RS in *Mobile phase*

System suitability solution: 80 µg/mL of USP Racemic Panthenol RS in *Mobile phase*. Mix the resulting solution and the *Standard solution* (1:1).

Sample solution: Equivalent to 80 µg/mL of calcium pantothenate from Oral Solution in *Mobile phase*

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 210 nm

Column: 4.0-mm × 10-cm; packing L1

Flow rate: 1 mL/min

Injection volume: 20 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 1.5 between panthenol and calcium pantothenate, *System suitability solution*

Tailing factor: NMT 2.0 for both the calcium pantothenate and the panthenol peaks, *Standard solution*

Relative standard deviation: NMT 2.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for calcium pantothenate. Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of calcium pantothenate from the *Sample solution*

r_S = peak area of calcium pantothenate from the *Standard solution*

C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• **CALCIUM PANTOTHENATE,** ***Method 2***

Standard stock solution: Dissolve 50 mg of USP Calcium Pantothenate RS, previously dried and stored in the dark over phosphorus pentoxide and protected from absorption of moisture while weighing, in 500 mL of water in a 1000-mL volumetric flask. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), and dilute with water to volume to obtain a concentration of 50 µg/mL of USP Calcium Pantothenate RS. Store under toluene in a refrigerator.

Standard solution: On the day of the assay, dilute a volume of the *Standard stock solution* with water to obtain a concentration of 0.01–0.04 µg/mL of calcium pantothenate, the exact concentration being such that the responses obtained as directed in *Analysis*, 2.0 and 4.0 mL of the *Standard solution* being used, are within the linear portion of the log-concentration response curve.

Sample solution: Transfer an accurately measured volume of Oral Solution, equivalent to 50 mg of calcium

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pantothenate, to a 1000-mL volumetric flask containing 500 mL of water. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), dilute with water to volume, and filter. Dilute a measured volume of this solution quantitatively, and stepwise if necessary, with water to obtain a solution with about the same concentration as that of the *Standard solution*.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and dilute with water to 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature NLT 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and dilute with water to 1000 mL. Store under toluene in a cool place at a temperature NLT 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat in 10 mL of 4 N hydrochloric acid. Cool, and dilute with water to 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin–thiamine hydrochloride–biotin solution: 20 µg/mL of riboflavin, 10 µg/mL of thiamine hydrochloride, and 0.04 µg/mL of biotin in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

***p*-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution:** 10 µg/mL of *p*-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Basal medium stock solution: Dissolve the *anhydrous Dextrose* and *anhydrous Sodium acetate* in the solutions previously mixed according to *Table 2*, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Table 2

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Riboflavin–thiamine hydrochloride–biotin solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water. Add 500 mg of *anhydrous Dextrose*, 500 mg of *anhydrous Sodium acetate*, and 1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to the test tubes, close or cover the tubes, sterilize in an autoclave at 121° for 15 min, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*,[2] incubating for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for the *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of the *Basal medium stock solution* add 5.0 mL of water containing 0.2 µg of calcium pantothenate. Plug the tubes with cotton, sterilize in an autoclave at 121° for 15 min, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Transfer cells from the *Stock culture of Lactobacillus plantarum*to a sterile tube containing 10 mL of the *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. The cell suspension so obtained is the *Inoculum*.

Analysis

Samples: *Standard solution* and *Sample solution*

To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to four similar empty tubes add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool. Add 1 drop of the *Inoculum* to each tube, except two of the four tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37°, held constant to within ±0.5° until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of Standard during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to an optical container if necessary. Read the transmittance between 540 and 660 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

[2] ATCC No. 8014 is suitable. This strain was formerly known as *Lactobacillus arabinosus* 17-5.

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With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittance (Σ_S) as the difference, $y = 2.00 - \Sigma_S$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of the *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_U$, adding together the two transmittances (Σ_U) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X, for each dosage level. Average the values of X for each of three or more dosage levels to obtain $\overline{X}$, which equals the log-relative potency, M', of the *Sample solution*.

Determine the quantity, in mg, of USP Calcium Pantothenate RS corresponding to calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Oral Solution taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of mg of calcium pantothenate assumed to be present in the portion of Oral Solution taken

Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Oral Solution taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of calcium pantothenate in the portion of Oral Solution (mg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, $\overline{M}$, is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* ⟨111⟩, *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• **DEXPANTHENOL OR PANTHENOL, *Method 1***

Mobile phase and **Chromatographic system:** Proceed as directed in the assay for *Calcium Pantothenate, Method 1*.

Standard solution: 80 µg/mL of USP Dexpanthenol RS or USP Racemic Panthenol RS in the *Mobile phase*. [NOTE—Use USP Dexpanthenol RS to analyze Oral Solution that contains dexpanthenol and USP Racemic Panthenol RS to analyze Oral Solution that contains panthenol.]

System suitability solution: 80 µg/mL of USP Calcium Pantothenate RS in *Mobile phase*. Mix the resulting solution and *Standard solution* (1:1).

Sample solution: Equivalent to 80 µg/mL of dexpanthenol or panthenol from the Oral Solution in the *Mobile phase*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the areas for panthenol. Calculate the percentage of the labeled amount of dexpanthenol or panthenol ($C_9H_{19}NO_4$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of dexpanthenol or panthenol from the *Sample solution*

r_S = peak area of dexpanthenol or panthenol from the *Standard solution*

C_S = concentration of USP Dexpanthenol RS or USP Racemic Panthenol RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of dexpanthenol or panthenol in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of dexpanthenol or panthenol ($C_9H_{19}NO_4$)

• **DEXPANTHENOL OR PANTHENOL, *Method 2***

[NOTE—The following procedure is applicable also to the determination of the dextrorotatory component of racemic panthenol in preparations containing panthenol.]

Dehydrated mixtures yielding formulations similar to the media described herein may be used provided that, when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described herein.

Standard stock solution: 800 µg/mL of USP Dexpanthenol RS or 1600 µg/mL of USP Racemic Panthenol RS in water. Store in a refrigerator, protected from light, and use within 30 days. [NOTE—Use USP Dexpanthenol RS to analyze Oral Solution that contains dexpanthenol and USP Racemic Panthenol RS to analyze Oral Solution that contains panthenol.]

Standard solution: On the day of the assay, prepare 1.2 µg/mL of dexpanthenol or 2.4 µg/mL of racemic panthenol from the *Standard stock solution* with water.

Sample solution: 1.2 µg/mL of dexpanthenol or 2.4 µg/mL of panthenol from Oral Solution in water

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in about 500 mL of water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and dilute with water to 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature NLT 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to 75 ± 5°, and add 6 M hydrochloric acid solution dropwise, with stirring, until the solids are dissolved. Cool, dilute with water to 1000 mL, and mix. Store under toluene in a cool place at a temperature NLT 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool,

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dilute with water to 200 mL, and mix. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin–thiamine hydrochloride–biotin solution: Prepare a solution of riboflavin, thiamine hydrochloride, and biotin in 0.02 N acetic acid containing 20 µg/mL of riboflavin, 10 µg/mL of thiamine hydrochloride, and 0.04 µg/mL of biotin. Store under toluene, protected from light, in a refrigerator.

***p*-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution:** Prepare a solution in neutral 25% alcohol containing 10 µg/mL of *p*-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride. Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid, and mix. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid, and mix. Store under toluene.

Pyridoxal–calcium pantothenate solution: Dissolve 40 mg of pyridoxal hydrochloride and 375 µg of calcium pantothenate in 10% alcohol to make 200 mL, and mix. Store in a refrigerator, and use within 30 days.

Polysorbate 40–oleic acid solution: Dissolve 25 g of polysorbate 40 and 0.25 g of oleic acid in 20% alcohol to make 500 mL, and mix. Store in a refrigerator, and use within 30 days.

Modified pantothenate medium: Dissolve *anhydrous Dextrose* and *Sodium acetate* in the solutions previously mixed according to *Table 3*, adjust with 1 N sodium hydroxide to a pH of 6.8, dilute with water to 250 mL, and mix.

Table 3

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Riboflavin–thiamine hydrochloride–biotin solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL
Pyridoxal–calcium pantothenate solution	5 mL
Polysorbate 40–oleic acid solution	5 mL

Double-strength modified pantothenate medium: Prepare as directed in *Modified pantothenate medium*, but make the final dilution to 125 mL instead of 250 mL. Prepare fresh.

Stock culture of *Pediococcus acidilactici*: Dissolve in 800 mL of water, with the aid of heat, 6.0 g of peptone, 4.0 g of pancreatic digest of casein, 3.0 g of yeast extract, 1.5 g of beef extract, 1.0 g of dextrose, and 15.0 g of agar. Adjust with 0.1 N sodium hydroxide or 0.1 N hydrochloric acid to a pH between 6.5 and 6.6, dilute with water to 1000 mL, and mix. Add 10-mL portions of the solution to culture tubes, place caps on the tubes, and sterilize in an autoclave at 121° for 15 min. Cool on a slant, and store in a refrigerator. Prepare a stock culture of *Pediococcus acidilactici*[3] on a slant of this medium. Incubate at 35° for 20–24 h, and store in a refrigerator. Maintain the stock culture by monthly transfer onto fresh slants.

Inoculum: Inoculate three 250-mL portions of sterile *Modified pantothenate medium* from a stock culture slant, and incubate at 35° for 20–24 h. Centrifuge the suspension from the combined portions, and wash the cells with sterile *Modified pantothenate medium*. Resuspend the cells in sufficient *Modified pantothenate medium* so that a 1-in-50 dilution, when tested in a 13-mm diameter test tube, gives 80% light transmission at 530 nm. Transfer 1.2-mL portions of this stock suspension to glass ampuls, seal, freeze in liquid nitrogen, and store in a freezer. On the day of the assay, allow the ampuls to reach room temperature, mix the contents, and dilute 1 mL of thawed culture with sterile saline TS to 150 mL. [NOTE—This dilution may be altered when necessary to obtain the desired test response.]

Analysis: Prepare in triplicate a series of eight culture tubes by adding the following quantities of water to the tubes within a set: 5.0, 4.5, 4.0, 3.5, 3.0, 2.0, 1.0, and 0.0 mL. To these same tubes, and in the same order, add 0.0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. Prepare in duplicate a series of five culture tubes by adding the following quantities of water to the tubes within a set: 4.0, 3.5, 3.0, 2.0, and 1.0 mL. To these same tubes, and in the same order, add 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*.

Add 5.0 mL of *Double-strength modified pantothenate medium* to each tube, and mix. Cover the tubes with metal caps, and sterilize in an autoclave at 121° for 5 min. Cool to room temperature in a chilled water bath, and inoculate each tube with 0.5 mL of the *Inoculum*. Allow to incubate at 37° for 16 h. Terminate growth by heating to a temperature NLT 80°, such as by steaming at atmospheric pressure in a sterilizer for 5–10 min. Cool, and concomitantly determine the percentage transmittance of the suspensions, in cells of equal pathlength, on a suitable spectrophotometer at a wavelength of 530 nm.

Calculation: Draw a dose-response curve on arithmetic graph paper by plotting the average response, in percent transmittance, for each set of tubes of the standard curve against the standard level concentrations. The curve is drawn by connecting each adjacent pair of points with a straight line. From this standard curve, determine by interpolation the potency, in terms of dexpanthenol, of each tube containing portions of the *Sample solution*. Divide the potency of each tube by the amount of the *Sample solution* added to it to obtain the individual responses. Calculate the mean response by averaging the individual responses that vary from their mean by NMT 15%, using NLT half the total number of tubes.

Calculate the potency of the portion of the material taken for assay by multiplying the mean response by the appropriate dilution factor. Calculate the percentage of the labeled amount of dexpanthenol or panthenol in the portion of Oral Solution taken:

$$\text{Result} = (P/N) \times 100$$

P = calculated potency of dexpanthenol or panthenol in the portion of Oral Solution taken (mg)

N = nominal amount of dexpanthenol or panthenol in the portion of Oral Solution taken (mg)

[3] ATCC No. 8042 is suitable. This strain was formerly known as *Lactobacillus arabinosus* 17-5.

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Acceptance criteria: 90.0%–150.0% of the labeled amount of dexpanthenol or panthenol ($C_9H_{19}NO_4$)

• **NIACIN OR NIACINAMIDE**

[NOTE—Use low-actinic glassware throughout this procedure.]

Diluent: 25 mg/mL of edetate disodium in water

Mobile phase: Mix 0.4 mL of triethylamine, 15.0 mL of glacial acetic acid, and 350 mL of methanol, and dilute with 0.008 M sodium 1-hexanesulfonate to 2000 mL.

Standard solution: 0.10 mg/mL of USP Niacin RS or USP Niacinamide RS in *Diluent*. [NOTE—Use USP Niacin RS for Oral Solution that contains niacin, and USP Niacinamide RS for Oral Solution that contains niacinamide.]

Sample solution: Dilute an accurately measured volume of Oral Solution with *Diluent* to obtain a solution with a concentration of 0.1 mg/mL of niacin or niacinamide.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 270 nm

Column: 4.6-mm × 25-cm; packing L7

Flow rate: 2 mL/min

Injection volume: 5 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacin or niacinamide from the *Sample solution*

r_S = peak area of niacin or niacinamide from the *Standard solution*

C_S = concentration of USP Niacin RS or USP Niacinamide RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacin or niacinamide in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$)

• **PYRIDOXINE HYDROCHLORIDE**

Diluent, Mobile phase, and **Chromatographic system:** Proceed as directed in *Niacin or Niacinamide*.

Standard solution: Equivalent to 24 µg/mL of USP Pyridoxine Hydrochloride RS in *Diluent*

Sample solution: Equivalent to 24 µg/mL of Pyridoxine Hydrochloride from Oral Solution in *Diluent*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of pyridoxine hydrochloride from the *Sample solution*

r_S = peak area of pyridoxine hydrochloride from the *Standard solution*

C_S = concentration of USP Pyridoxine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of pyridoxine hydrochloride in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$)

• **RIBOFLAVIN-5′-PHOSPHATE SODIUM, *Method 1***

[NOTE—Riboflavin-5′-phosphate sodium is quantitated against USP Riboflavin RS in this procedure. In the chromatogram of the *Sample solution*, the riboflavin-5′-phosphate peak is the only peak measured for calculation.]

Diluent, Mobile phase, and **Chromatographic system:** Proceed as directed in *Niacin or Niacinamide*.

Standard solution: Equivalent to 8 µg/mL of USP Riboflavin RS in *Diluent*, by heating if necessary

Sample solution: Equivalent to 8 µg/mL of riboflavin from Oral Solution in *Diluent*

Analysis

Samples: *Standard solution* and *Sample solution*

[NOTE—The relative retention times for riboflavin-5′-phosphate and riboflavin are about 0.18 and 1.0, respectively.]

Calculate the percentage of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of riboflavin-5′-phosphate from the *Sample solution*

r_S = peak area of riboflavin from the *Standard solution*

C_S = concentration of USP Riboflavin RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of riboflavin in the *Sample solution* (mg/mL)

F = factor for converting the response obtained for riboflavin-5′-phosphate to riboflavin, 1.493. [NOTE—Riboflavin phosphate sodium is a mixture of isomeric monophosphates and diphosphates containing an average amount of 67% of riboflavin-5′-monophosphate, which separates in this chromatographic system. The factor 1.493 assumes 67% of riboflavin-5′-monophosphate.]

Acceptance criteria: 90.0%–150.0% of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$)

• **RIBOFLAVIN OR RIBOFLAVIN-5′-PHOSPHATE SODIUM, *Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Solvent blank: 1 N hydrochloric acid, 2.5 M sodium acetate, and water (1:2:97)

Standard stock solution: Transfer 16 mg of USP Riboflavin RS to a 100-mL volumetric flask, dissolve in 1.0 mL of 1 N hydrochloric acid and 2.0 mL of 2.5 M sodium acetate, and dilute with water to volume. Transfer 10.0 mL of this solution to a 100-mL volumetric flask, and dilute with water to volume.

Standard solution: Transfer 5.0 mL of the *Standard stock solution* to a 500-mL volumetric flask, add 5.0 mL of 1 N hydrochloric acid and 10.0 mL of 2.5 M sodium acetate, and dilute with water to volume.

Sample solution: Transfer an accurately measured volume of Oral Solution, equivalent to 0.8 mg of riboflavin, to a 100-mL volumetric flask, and dilute with water to volume. Transfer 10.0 mL of the resulting solution to a 500-mL volumetric flask, add 5.0 mL of 1 N hydrochloric acid and 10.0 mL of 2.5 M sodium acetate, and dilute with water to volume.

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Instrumental conditions
(See *Fluorescence Spectroscopy* ⟨853⟩.)
Mode: Fluorescence
Analytical wavelengths
Excitation: 440 nm
Emission: 530 nm
Blank: Solvent
Analysis
Samples: *Standard solution* and *Sample solution*
Determine the maximum fluorescence intensities, I_S and I_U, of the *Standard solution* and the *Sample solution*, respectively. Calculate the percentage of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$) in the portion of Oral Solution taken:

$$\text{Result} = (I_U/I_S) \times (C_S/C_U) \times 100$$

I_U = fluorescence intensity from the *Sample solution*
I_S = fluorescence intensity from the *Standard solution*
C_S = concentration of USP Riboflavin RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of riboflavin in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$)

- **THIAMINE**

Diluent, Mobile phase, and **Chromatographic system:** Proceed as directed in *Niacin or Niacinamide*.
Standard solution: Equivalent to 24 µg/mL of USP Thiamine Hydrochloride RS in *Diluent*
Sample solution: Equivalent to 24 µg/mL of thiamine hydrochloride from Oral Solution in *Diluent*
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the areas for the major peaks. Calculate the percentage of the labeled amount of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of thiamine from the *Sample solution*
r_S = peak area of thiamine from the *Standard solution*
C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of thiamine hydrochloride or thiamine mononitrate in the *Sample solution* (mg/mL)
F = 1 (for products containing thiamine hydrochloride) and 0.97 (for products containing thiamine mononitrate)

Acceptance criteria: 90.0%–250.0% of the labeled amount of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$)

OTHER COMPONENTS

- **ALCOHOL DETERMINATION,** *Method I* ⟨611⟩ (if present)**:** 90.0%–120.0% of the labeled amount of C_2H_5OH

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 3×10^3 cfu/mL, and the combined molds and yeasts count does not exceed 3×10^2 cfu/mL.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers under an inert gas or with a minimum of headspace.
- **LABELING:**[4] The label states that the product is Oil- and Water-Soluble Vitamins Oral Solution. The label states the quantity of each vitamin present in a given volume of Oral Solution and, where necessary, the chemical form in which a vitamin is present. Where the product contains vitamin E, the label indicates whether it is the *d*- or *dl*- form. Where the product is labeled to contain panthenol, the label states the equivalent content of dexpanthenol. Where more than one assay method is given for a particular vitamin, the labeling states with which assay method the product complies only if *Method 1* is not used.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Alpha Tocopherol RS
USP Calcium Pantothenate RS
β-Alanine, *N*-(2,4-dihydroxy-3,3-dimethyl-1-oxobutyl)-, calcium salt (2:1), (*R*)-.
$C_{18}H_{32}CaN_2O_{10}$ 476.53
USP Cholecalciferol RS
9,10-Secocholesta-5,7,10(19)-trien-3-ol, (3β,5*Z*,7*E*)-.
$C_{27}H_{44}O$ 384.64
USP Cyanocobalamin (Crystalline) RS
Vitamin B_{12}.
$C_{63}H_{88}CoN_{14}O_{14}P$ 1355.37
USP Dexpanthenol RS
D-(+)-2,4-Dihydroxy-*N*-(3-hydroxypropyl)-3-3-dimethylbutyramide.
$C_9H_{19}NO_4$ 205.25
USP Ergocalciferol RS
9,10-Secoergosta-5,7,10(19),22-tetraen-3-ol, (3β,5*Z*,7*E*,22*E*)-.
$C_{28}H_{44}O$ 396.65
USP Niacin RS
3-Pyridinecarboxylic acid.
$C_6H_5NO_2$ 123.11
USP Niacinamide RS
3-Pyridinecarboxamide.
$C_6H_6N_2O$ 122.12
USP Pyridoxine Hydrochloride RS
3,4-Pyridinedimethanol, 5-hydroxy-6-methyl-, hydrochloride.
$C_8H_{11}NO_3 \cdot HCl$ 205.64

[4] USP Units of activity for vitamins, where such exist or formerly existed, are equivalent to the corresponding international units (IU), where such formerly existed. The USP Unit for Vitamin E has been discontinued. IU for vitamins also have been discontinued; however, the use of IU on the labels of vitamin products continues. Where articles are labeled in terms of Units in addition to the required labeling, the relationship of the USP Units or IU to mass is as follows. One USP Vitamin A Unit = 0.3 µg of all-*trans*-retinol (vitamin A alcohol) or 0.344 µg of all-*trans*-retinyl acetate (vitamin A acetate) or 0.55 µg of all-*trans*-retinyl palmitate (vitamin A palmitate), and 1 µg of retinol (3.3 USP Vitamin A Units) = 1 retinol equivalent (RE); 1 IU of beta carotene = 0.6 µg of all-*trans*-beta carotene; 1 USP Vitamin D Unit = 0.025 µg of ergocalciferol or cholecalciferol; and 1 mg of *dl*-alpha tocopherol = 1.1 former USP Vitamin E Units, 1 mg of *dl*-alpha tocopheryl acetate = 1 former USP Vitamin E Unit, 1 mg of *dl*-alpha tocopheryl acid succinate = 0.89 former USP Vitamin E Unit, 1 mg of *d*-alpha tocopherol = 1.49 former USP Vitamin E Units, 1 mg of *d*-alpha tocopheryl acetate = 1.36 former USP Vitamin E Units; and 1 mg of *d*-alpha tocopheryl acid succinate = 1.21 former USP Vitamin E Units. In terms of *d*-alpha tocopherol equivalents, 1 mg of *d*-alpha tocopheryl acetate = 0.91, 1 mg of *d*-alpha tocopheryl acid succinate = 0.81, 1 mg of *dl*-alpha tocopherol = 0.74, 1 mg of *dl*-alpha tocopheryl acetate = 0.67, and 1 mg of *dl*-alpha tocopheryl acid succinate = 0.60.

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USP Racemic Panthenol RS
USP Riboflavin RS
Riboflavine.
$C_{17}H_{20}N_4O_6$ 376.36
USP Thiamine Hydrochloride RS
Thiazolium, 3-[(4-amino-2-methyl-5-pyrimidinyl) methyl]-5-(2-hydroxyethyl)-4-methyl-, chloride, monohydrochloride.
$C_{12}H_{17}ClN_4OS \cdot HCl$ 337.27
USP Vitamin A RS
3,7-Dimethyl-9-(2,6,6-trimethyl-1- cyclohexen-1-yl) 2,4,6,8-nonatetraen-1-ol acetate (vitamin A acetate).

Oil- and Water-Soluble Vitamins Tablets

DEFINITION

Change to read:

Oil- and Water-Soluble Vitamins Tablets contain one or more of the following oil-soluble vitamins: Vitamin A ▲as retinyl acetate or retinyl palmitate,▲ (USP 1-May-2020) vitamin D as Ergocalciferol (vitamin D_2) or Cholecalciferol (vitamin D_3), Vitamin E ▲as *RRR*- or *all-rac*-alpha-tocopherol, *RRR*- or *all-rac*-alpha-tocopheryl acetate, or *RRR*- or *all-rac*-alpha-tocopheryl acid succinate,▲ (USP 1-May-2020) Phytonadione (vitamin K_1), and Beta Carotene; and one or more of the following water-soluble vitamins: Ascorbic Acid or its equivalent as Calcium Ascorbate or Sodium Ascorbate, Biotin, Cyanocobalamin, Folic Acid, Niacin or Niacinamide, pantothenic acid (as Calcium Pantothenate or Racemic Calcium Pantothenate), Pyridoxine Hydrochloride, Riboflavin, and Thiamine Hydrochloride or Thiamine Mononitrate. Tablets contain NLT 90.0% and NMT 165.0% of the labeled amount of vitamin A ▲as retinol▲ (USP 1-May-2020) ($C_{20}H_{30}O$); vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$); vitamin E ▲as *2R*-alpha-tocopherol▲ (USP 1-May-2020) ($C_{29}H_{50}O_2$); phytonadione ($C_{31}H_{46}O_2$); and beta carotene ($C_{40}H_{56}$); and NLT 90.0% and NMT 150.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$); ▲▲ (USP 1-May-2020) biotin ($C_{10}H_{16}N_2O_3S$), cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$), folic acid ($C_{19}H_{19}N_7O_6$), niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), pyridoxine ▲▲ (USP 1-May-2020) ($C_8H_{11}NO_3$ ▲▲ (USP 1-May-2020)), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine ▲as thiamine ion ($C_{12}H_{17}N_4OS+$)▲ (USP 1-May-2020)

They do not contain any minerals. They may contain other labeled added substances that are generally recognized as safe, in amounts that are unobjectionable.

IDENTIFICATION

Add the following:

▲• **A.** The retention times of the vitamin peaks of the *Sample solutions* correspond to those of the corresponding vitamin peaks of the *Standard solutions* as obtained in the tests for *Strength*.▲ (USP 1-May-2020)

STRENGTH

[NOTE—Where more than one assay method is given for an individual ingredient, the requirements may be met by following any one of the specified methods, with the method used being stated in the labeling only if *Method 1* is not used.]

Change to read:

• **VITAMIN A,** ***Method 1***

▲Proceed as directed in *Vitamin A Assay* ⟨571⟩, *Assay, Chromatographic Methods, Procedure 1* except for the *Sample stock solution* and *Sample solution*.▲ (USP 1-May-2020)

Sample stock solution: Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 5 Tablets, to a container having a polytef-lined screw-cap. Add 10 mL of dimethyl sulfoxide and 15 mL of *n*-hexane, and shake for 45 min on a wrist-action shaker in a water bath maintained at 60°. [NOTE—Set up the wrist-action shaker to ensure that the contents of the container are mixed vigorously and thoroughly.] Centrifuge at 3000 rpm for 10 min, and transfer the hexane layer by means of a pipet to a 100-mL volumetric flask. Add 15 mL of *n*-hexane to the dimethyl sulfoxide layer, shake thoroughly for 5 min, and transfer the hexane layer by means of a pipet to the 100-mL volumetric flask. Repeat this extraction with 3 additional 15-mL portions of *n*-hexane. Dilute the extracts in the volumetric flask with *n*-hexane to volume.

Sample solution: Dilute a 10-mL volume of the *Sample stock solution* with *n*-hexane to obtain a solution with a concentration of 15 µg/mL of vitamin A as retinol ($C_{20}H_{30}O$).

▲▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A as retinol ($C_{20}H_{30}O$)

Change to read:

• **VITAMIN A,** ***Method 2***

▲Proceed as directed in *Vitamin A Assay* ⟨571⟩, *Assay, Chromatographic Methods, Procedure 2*.▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A as retinol ($C_{20}H_{30}O$)

Change to read:

• **VITAMIN A,** ***Method 3***

▲Proceed as directed in *Vitamin A Assay* ⟨571⟩, *Assay, Chromatographic Methods, Procedure 3*.▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A as retinol ($C_{20}H_{30}O$)

Change to read:

• **VITAMIN D (CHOLECALCIFEROL OR ERGOCALCIFEROL),** ***Method 1***

▲Proceed as directed in *Vitamin D Assay* ⟨581⟩, *Assay, Chromatographic Methods, Procedure 1*, except for the *Sample solution*.▲ (USP 1-May-2020)

Sample solution: Proceed as directed for the *Sample stock solution* in *Vitamin A, Method 1*. Transfer NLT 20 mL of this solution to a suitable container, and, if necessary, evaporate under vacuum at room temperature to obtain a concentration of 2 µg/mL of cholecalciferol or ergocalciferol.

▲▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

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Change to read:

- **VITAMIN D (CHOLECALCIFEROL OR ERGOCALCIFEROL), *Method 2***

▲Proceed as directed in *Vitamin D Assay* ⟨581⟩, *Assay, Chromatographic Methods, Procedure 2*.▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

Change to read:

- **VITAMIN D (CHOLECALCIFEROL OR ERGOCALCIFEROL), *Method 3***

▲Proceed as directed in *Vitamin D Assay* ⟨581⟩, *Assay, Chromatographic Methods, Procedure 3*, except for the *Analysis*.▲ (USP 1-May-2020)

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for vitamin D.

Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*

r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*

C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

Change to read:

- **VITAMIN E, *Method 1***

▲Proceed as directed in *Vitamin E Assay* ⟨551⟩, *Assay, Procedure 1*, except for the *Sample solution* and *Analysis*.▲ (USP 1-May-2020)

Sample solution: Prepare as directed for the *Sample stock solution* in *Vitamin A, Method 1*. Transfer NLT 20 mL of this solution to a suitable container and, if necessary, evaporate under vacuum at room temperature to dryness. Transfer the residue to a suitable volumetric flask with the aid of methanol, and dilute with methanol to volume to obtain a concentration of 2 mg/mL of alpha-tocopherol, alpha-tocopheryl acetate, or alpha-tocopheryl acid succinate.

▲▲ (USP 1-May-2020)

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas.

Calculate the percentage of the labeled amount of alpha-tocopherol ($C_{29}H_{50}O_2$), alpha-tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha-tocopheryl acid succinate ($C_{33}H_{54}O_5$) ▲as 2*R*-alpha-tocopherol ($C_{29}H_{50}O_2$)▲ (USP 1-May-2020) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \text{ ▲}\times F\text{▲ (USP 1-May-2020)} \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*

r_S = peak area of the relevant vitamin E form from the *Standard solution*

C_S = concentration of ▲*all-rac*-alpha-tocopherol from either USP Alpha Tocopherol RS or USP Alpha Tocopheryl Acetate RS or *RRR*-alpha-tocopherol from USP Alpha Tocopheryl Acid Succinate RS▲ (USP 1-May-2020) in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding form of vitamin E ▲as 2*R*-alpha-tocopherol▲ (USP 1-May-2020) in the *Sample solution* (mg/mL)

▲F = conversion factor for the content of *all-rac*-alpha-tocopherol to 2*R*-alpha-tocopherol, 1/2 (for products labeled to contain *all-rac* vitamin E sources) and 1 (for products labeled to contain *RRR* vitamin E sources)▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–165.0% of the labeled amount of alpha-tocopherol ($C_{29}H_{50}O_2$), alpha-tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha-tocopheryl acid succinate ($C_{33}H_{54}O_5$) ▲as 2*R*-alpha-tocopherol▲ (USP 1-May-2020)

Change to read:

- **VITAMIN E, *Method 2***

▲Proceed as directed in *Vitamin E Assay* ⟨551⟩, *Assay, Procedure 2*, except for *Analysis*.

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of alpha-tocopherol ($C_{29}H_{50}O_2$), alpha-tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha-tocopheryl acid succinate ($C_{33}H_{54}O_5$) as 2*R*-alpha-tocopherol ($C_{29}H_{50}O_2$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*

r_S = peak area of the relevant vitamin E form from the *Standard solution*

C_S = concentration of *all-rac*-alpha-tocopherol from either USP Alpha Tocopherol RS or USP Alpha Tocopheryl Acetate RS or *RRR*-alpha-tocopherol from USP Alpha Tocopheryl Acid Succinate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding form of vitamin E as 2*R*-alpha-tocopherol in the *Sample solution* (mg/mL)

F = conversion factor for the content of *all-rac*-alpha-tocopherol to 2*R*-alpha-tocopherol, 1/2 for products labeled to contain *all-rac*. vitamin E sources) and 1 (for products labeled to contain *RRR* vitamin E sources)▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–165.0% of the labeled amount of alpha-tocopherol ($C_{29}H_{50}O_2$), alpha-tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha-tocopheryl acid succinate ($C_{33}H_{54}O_5$) ▲as 2*R*-alpha-tocopherol▲ (USP 1-May-2020)

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Change to read:

- **VITAMIN E, *Method 3***

▲Proceed as directed in *Vitamin E Assay* ⟨551⟩, *Assay, Procedure 3*, except for the *Analysis*.

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of alpha-tocopherol ($C_{29}H_{50}O_2$), alpha-tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha-tocopheryl acid succinate ($C_{33}H_{54}O_5$) as 2*R*-alpha-tocopherol ($C_{29}H_{50}O_2$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*

r_S = peak area of the relevant vitamin E form from the *Standard solution*

C_S = concentration of *all-rac*-alpha-tocopherol from either USP Alpha Tocopherol RS or USP Alpha Tocopheryl Acetate RS or *RRR*-alpha-tocopherol from USP Alpha Tocopheryl Acid Succinate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding form of vitamin E as 2*R*-alpha-tocopherol in the *Sample solution* (mg/mL)

F = conversion factor for the content of *all-rac*-alpha-tocopherol to 2*R*-alpha-tocopherol, 1/2 (for products labeled to contain *all-rac* vitamin E sources) and 1 (for products labeled to contain *RRR* vitamin E sources)▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–165.0% of the labeled amount of alpha-tocopherol ($C_{29}H_{50}O_2$), alpha-tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha-tocopheryl acid succinate ($C_{33}H_{54}O_5$) ▲as 2*R*-alpha-tocopherol▲ (USP 1-May-2020)

- **PHYTONADIONE, *Method 1***

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Methanol and water (19:1)

Standard stock solution: 200 µg/mL of USP Phytonadione RS in methanol. Dissolve with the aid of sonication if necessary.

System suitability solution: 0.65 mg/mL of USP Alpha Tocopheryl Acetate RS and 20 µg/mL of USP Phytonadione RS from the *Standard stock solution* diluted with methanol. [NOTE—Dissolve USP Alpha Tocopheryl Acetate RS in a portion of methanol, add the *Standard stock solution*, and then dilute with methanol to volume.]

Standard solution: 20 µg/mL of USP Phytonadione RS from the *Standard stock solution* diluted with methanol

Sample solution: Transfer NLT 20 mL of the solution retained as directed for the *Sample stock solution* in *Vitamin A, Method 1* to a suitable container, and, if necessary, evaporate under vacuum at room temperature to dryness. Transfer the residue to a suitable volumetric flask with the aid of methanol, and dilute with methanol to volume to obtain a concentration of 20 µg/mL of phytonadione.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 8-mm × 10-cm; 5-µm packing L1

Flow rate: 2 mL/min

Injection volume: 100 µL

System suitability

Samples: *System suitability solution* and *Standard solution*

[NOTE—The relative retention times for alpha-tocopheryl acetate and phytonadione are 0.68 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 5 between alpha-tocopheryl acetate and phytonadione, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas. Calculate the percentage of the labeled amount of phytonadione ($C_{31}H_{46}O_2$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of phytonadione from the *Sample solution*

r_S = peak area of phytonadione from the *Standard solution*

C_S = concentration of USP Phytonadione RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of phytonadione in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of phytonadione ($C_{31}H_{46}O_2$)

- **PHYTONADIONE, *Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Solvent: Methanol and isopropyl alcohol (19:1)

Mobile phase: Mix 800 mL of methanol, 200 mL of methylene chloride, 0.1 mL of glacial acetic acid, 1.36 g of zinc chloride, and 0.41 g of sodium acetate.

Internal standard solution: 5 µg/mL of menaquinone 4 (vitamin K_2) in *Solvent*. [NOTE—A concentrated stock solution of menaquinone 4 (100 µg/mL) can be stored for 2 months in a refrigerator.]

Standard stock solution: 5 µg/mL of USP Phytonadione RS, prepared by dissolving in methylene chloride with the aid of sonication, and diluting with *Solvent* to final volume.

Standard solution: Transfer 1.0 mL of the *Standard stock solution* and 1.0 mL of the *Internal standard solution* to a suitable flask, and dilute with *Solvent* to 5 mL. Pass through a membrane filter of 0.45-µm or finer pore size.

Sample solution: Finely powder NLT 20 Tablets. To a centrifuge tube fitted with a cap transfer an amount of powder not exceeding 800 mg and equivalent to an amount of phytonadione not exceeding 50 µg. Add 4 mL of water. Insert the stopper, and mix using a vortex mixer until the sample is dispersed. Place the tube in a water bath at 60° for 5 min. Remove from the bath, and again shake or mix using a vortex mixer for 1 min while the preparation is still hot. Add 8 mL of alcohol, and swirl the contents to mix. Place the tube in a water bath at 60° for 5 min. Remove from the bath, and again shake or mix using a vortex mixer for 2 min while the preparation is still hot. Cool to room temperature. Add a volume of the *Internal standard solution*, equivalent to 1.0 mL for each 5 µg of the expected amount of phytonadione in the aliquot taken. Add 20.0 mL of petroleum ether, and cap the tube tightly. Shake or mix using a vortex mixer for 15 min to thoroughly mix the contents. Centrifuge to separate the 2 layers. Transfer a volume of the top layer of petroleum ether, equivalent to 5–50 µg of the nominal amount of phytonadione, to an appropriate flask. Place the flask in a water bath at 35°–45°, and evaporate the solvent under a stream of nitrogen until an oily residue is left. Dissolve the residue in a volume of

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Solvent to obtain a concentration of 1 µg/mL of phytonadione.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Fluorometric detector
Excitation wavelength: 320 nm
Emission wavelength: 420 nm
Column: 4.6-mm × 25-cm; 5-µm, end-capped packing L1, and a postcolumn reactor constituted with a 4.6-mm × 3-cm PEEK column tightly packed with zinc powder. [NOTE—Prepare the postcolumn reactor daily, or as necessary, to meet the *System suitability* requirements.]
Flow rate: 1 mL/min
Injection volume: 25 µL

System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for the internal standard and phytonadione are 1.0 and 1.4, respectively.]
Suitability requirements
Column efficiency: NLT 2500 theoretical plates for the phytonadione peak
Tailing factor: NMT 1.5 for the phytonadione peak
Relative standard deviation: NMT 3.0%

Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of phytonadione ($C_{31}H_{46}O_2$) in the portion of Tablets taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of phytonadione to that of the internal standard from the *Sample solution*
R_S = peak response ratio of phytonadione to that of the internal standard from the *Standard solution*
C_S = concentration of USP Phytonadione RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of phytonadione in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of labeled amount of phytonadione ($C_{31}H_{46}O_2$)

Add the following:

▲• **VITAMIN A, VITAMIN D (CHOLECALCIFEROL OR ERGOCALCIFEROL), VITAMIN E, *Method 4*; PHYTONADIONE, *Method 3***
[NOTE—Where vitamin A (retinyl acetate or retinyl palmitate), vitamin D (cholecalciferol or ergocalciferol), or vitamin E (alpha-tocopherol, alpha-tocopheryl acetate, or alpha-tocopheryl acid succinate) are specified in the following procedure, use the chemical form present in the formulation and the relevant Reference Standard. Use low-actinic glassware.]
Diluent: Methanol and water (90:10, v/v)
Solution A: 0.1% (v/v) trifluoroacetic acid in *Diluent*
Solution B: 0.1% (v/v) trifluoroacetic acid in methanol
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
19	0	100
27	0	100
28	100	0
30	100	0

Vitamin A standard stock solution: A solution containing the equivalent of 0.2 mg/mL of retinol from USP Retinyl Acetate RS or from USP Retinyl Palmitate RS in isopropyl alcohol. Dissolve with the aid of sonication if necessary.
Vitamin D standard stock solution: 0.05 mg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in isopropyl alcohol. Dissolve with the aid of sonication if necessary.
Vitamin E standard stock solution: 3.0 mg/mL of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, or USP Alpha Tocopheryl Acid Succinate RS in isopropyl alcohol. Dissolve with the aid of sonication if necessary.
Phytonadione standard stock solution: 0.04 mg/mL of USP Phytonadione RS in isopropyl alcohol. Dissolve with the aid of sonication if necessary.
Sample solution 1: Finely powder NLT 20 Tablets. Transfer an accurately weighed portion of the powder to a 50-mL volumetric flask to obtain a solution containing known nominal concentrations in the ranges of 0.05–0.1 mg/mL of vitamin A and 0.5–0.15 mg/mL of vitamin E. Add 10 mL of dimethyl sulfoxide and sonicated for 30 min with vigorous intermittent shaking. Cool to room temperature, dilute to volume with isopropyl alcohol and mixed well. Pass a portion of the solution through a 0.45-µm pore size nylon filter and discard the first milliliter of the filtrate.
Sample solution 2: Transfer an accurately weighed portion of the powder from NLT 20 finely powdered Tablets to a 50-mL volumetric flask to obtain a solution containing known nominal concentrations in the ranges of 1.0–3.5 µg/mL of vitamin D and 1.0–3.5 µg/mL of phytonadione. Add 5 mL of water and sonicate for 10 min with intermittent shaking. Add 10 mL of dimethyl sulfoxide and sonicate for 30 min with vigorous intermittent shaking. Cool to room temperature, dilute with isopropyl alcohol to volume, and mix well. Pass a portion of the solution through a nylon filter of 0.45-µm pore size, and discard the first milliliter of the filtrate.
Standard solution: Transfer calculated volumes of *Vitamin A standard stock solution*, *Vitamin D standard stock solution*, *Vitamin E standard stock solution*, and *Phytonadione standard stock solution* to a suitable volumetric flask, and dilute with isopropyl alcohol to volume to obtain a solution with final vitamin concentrations similar to those obtained for the corresponding vitamins in *Sample solution 1* and *Sample solution 2*.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detectors
For *Sample solution 1* containing retinyl acetate: UV 325 nm for 7 min, then switch to 265 nm
For *Sample solution 1* containing retinyl palmitate: UV 265 nm for 18 min, then switch to 325 nm
For *Sample solution 2*: UV 265 nm
Column: 4.6-mm × 10-cm; 3-µm packing L1
Temperatures
Sample: 4°
Column: 35°
Flow rate: 1.5 mL/min

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Injection volume: 20 µL
System suitability
Samples: *Sample solution 1*, *Sample solution 2*, and *Standard solution*
Suitability requirements
Relative standard deviation: NMT 2.0% for each individual peak, *Standard solution*
Peak purity of vitamin D and vitamin A (as retinyl palmitate): Monitor the chromatograms of *Sample solution 1* and *Sample solution 2* at 450 nm. The area of any peak at the retention time of retinyl palmitate from *Sample solution 1* and any peak at the retention time of vitamin D (as cholecalciferol or ergocalciferol) from *Sample solution 2* should be NMT 5% of the corresponding peak areas detected at 325 nm and 265 nm, respectively. [NOTE—This test is required because of possible interference with carotenoids (cryptoxanthin and lycopene) that may be present in the formulation. Use another procedure in the monograph for analysis of vitamin D and vitamin A (as retinyl palmitate) if the system is not suitable.]
Analysis
Samples: *Sample solution 1*, *Sample solution 2*, and *Standard solution*
Calculate the percentage of the labeled amount of vitamin A activity, as retinol ($C_{20}H_{30}O$), in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the *all-trans*-retinyl ester from the *Sample solution*
r_S = peak area of the *all-trans*-retinyl ester from the *Standard solution*
C_S = concentration of retinol in the *Standard solution* (mg/mL)
C_U = nominal concentration of vitamin A, as retinol, in the *Sample solution* (mg/mL)

Calculate the percentage of the labeled amount of vitamin E, as 2*R*-alpha-tocopherol ($C_{29}H_{50}O_2$), in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*
r_S = peak area of the relevant vitamin E form from the *Standard solution*
C_S = concentration of *all-rac*-alpha-tocopherol from either USP Alpha Tocopherol RS or USP Alpha Tocopheryl Acetate RS or *RRR*-alpha-tocopherol from USP Alpha Tocopheryl Acid Succinate RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of vitamin E, as 2*R*-alpha-tocopherol, in the *Sample solution* (mg/mL)
F = conversion factor for the content of *all-rac*-alpha-tocopherol to *RRR*-alpha-tocopherol equivalent, 1/2 for products labeled to contain *all-rac* vitamin E sources) and 1 (for products labeled to contain *RRR* vitamin E) sources

Calculate the percentage of the labeled amount of vitamin D, as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$), and phytonadione ($C_{31}H_{46}O_2$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the related vitamin from the *Sample solution*
r_S = peak area of the related vitamin from the *Standard solution*
C_S = concentration of the related vitamin in the *Standard solution* (µg/mL)
C_U = nominal concentration of the related vitamin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of each individual vitamin ▲(USP 1-May-2020)

• **BETA CAROTENE**
[NOTE—Use low-actinic glassware throughout this procedure.]
Potassium hydroxide solution: Dissolve 58.8 g of potassium hydroxide in 50 mL of water.
Iodine solution: 0.01 mg/mL of iodine in cyclohexane. [NOTE—Prepare this solution fresh daily.]
Sample solution: Weigh NLT 20 Tablets. Grind the Tablets to a fine powder, and transfer a quantity of the powder, equivalent to 2 mg of beta carotene, to a 500-mL saponification flask. Add 100 mL of alcohol, 6 mL of *Potassium hydroxide solution*, and a magnetic stirring bar. Attach an air condenser to the flask, and heat under reflux for 45 min with constant stirring. Cool to room temperature. Add 170 mL of solvent hexane, and stir for 30 min. Quantitatively transfer the contents of the flask to a 500-mL separatory funnel with portions of solvent hexane. Allow the layers to separate for 5–10 min, and transfer the upper organic layer to a 500-mL volumetric flask. Transfer the lower aqueous layer into the saponification flask. Add 170 mL of solvent hexane, and stir for an additional 20 min. Quantitatively transfer the contents of the saponification flask to the separatory funnel with the aid of portions of solvent hexane. Allow the layers to separate for 10 min. Drain the lower aqueous layer, and discard. Transfer the organic layer to the volumetric flask containing the previously collected organic layer. Rinse the separatory funnel with small portions of solvent hexane, and transfer the washings to the volumetric flask. Dilute the hexane extracts with solvent hexane to volume. Add 3 g of anhydrous sodium sulfate, shake, and allow to settle. Quantitatively transfer a volume of this solution, equivalent to 100 µg of beta carotene, to a 50-mL volumetric flask. Evaporate under a stream of nitrogen to dryness, and immediately add cyclohexane. Add 2 mL of *Iodine solution*, and heat for 15 min in a water bath maintained at 65°. Cool rapidly, and dilute with cyclohexane to volume.
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: Vis
Analytical wavelength: 452 nm
Blank: Cyclohexane
Analysis
Sample: *Sample solution*
Determine the absorbance against the *Blank*. Calculate the percentage of the labeled amount of beta carotene ($C_{40}H_{56}$) in the portion of Tablets taken:

$$\text{Result} = (A_U/F) \times (100/C_U)$$

A_U = absorbance of the *Sample solution*
F = absorptivity of beta carotene at 452 nm, 223

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C_U = nominal concentration of beta carotene in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of beta carotene ($C_{40}H_{56}$)

Change to read:

- **ASCORBIC ACID, CALCIUM ASCORBATE,** and **SODIUM ASCORBATE**

(See *Vitamin C Assay* ⟨580⟩.)

[NOTE—For labeling purposes, consider ⟨580⟩, *Method I—Titrimetric Method* as *Method 1*; ▲⟨580⟩, *Method II—Chromatographic Method, Procedure 1* as *Method 2*; and ⟨580⟩, *Method II—Chromatographic Method, Procedure 2* as *Method 3*.▲ (USP 1-May-2020)]

Acceptance criteria: 90.0%–150.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$), calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$), or sodium ascorbate ($C_6H_7NaO_6$)

- **BIOTIN,** ***Method 1***

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Mix 85 mL of acetonitrile, 1 g of sodium perchlorate, 1 mL of phosphoric acid, and dilute with water to 1000 mL.

Standard stock solution: 0.333 mg/mL of USP Biotin RS in dimethyl sulfoxide

Standard solution: 5 µg/mL of USP Biotin RS prepared by diluting the *Standard stock solution* in water

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 1 mg of biotin, to a 200-mL volumetric flask. Add 3 mL of dimethyl sulfoxide, and swirl to wet the contents. Place the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with water to volume, and filter.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 200 nm

Column: 4.6-mm × 15-cm; 3-µm packing L7

Flow rate: 1.2 mL/min

Injection volume: 100 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of biotin. Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of biotin from the *Sample solution*

r_S = peak area of biotin from the *Standard solution*

C_S = concentration of USP Biotin RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of biotin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

Change to read:

- **BIOTIN,** ***Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Dehydrated mixtures yielding formulations similar to the media described herein may be used provided that, when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described herein.

Standard stock solution: 50 µg/mL of USP Biotin RS in 50% alcohol. Store this solution in a refrigerator.

Standard solution: 0.1 ng/mL of USP Biotin RS in water, prepared by diluting the *Standard stock solution* with water on the day of the assay

Sample solution: Finely powder NLT 30 Tablets. Transfer a portion of the powder, equivalent to 100 µg of biotin, to a 200-mL volumetric flask. Add 3 mL of 50% alcohol, and swirl to wet the contents. Heat the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with 50% alcohol to volume, and filter. Dilute a volume of the filtrate quantitatively, and stepwise if necessary, with water to obtain a solution with a concentration of 0.1 ng/mL.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and dilute with water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water. Heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and dilute with water to make 1000 mL. Store under toluene in a cool place at a temperature NLT 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Calcium pantothenate solution: 10 µg/mL of calcium pantothenate in 50% alcohol. Store in a refrigerator.

Riboflavin–thiamine hydrochloride solution: 20 µg/mL of riboflavin and 10 µg/mL of thiamine hydrochloride in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

***p*-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution:** 10 µg/mL of *p*-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid, and mix. Store under toluene.

Basal medium stock solution: Dissolve the anhydrous dextrose and anhydrous Sodium acetate in the solutions previously mixed according to ▲*Table 2*,▲ (USP 1-May-2020) and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

DS Monographs

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▲Table 2▲ (USP 1-May-2020)

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Calcium pantothenate solution	5 mL
Riboflavin–thiamine hydrochloride solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water. Add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to test tubes, close or cover the tubes, sterilize in an autoclave at 121° for 15 min, and allow the tubes to cool in an upright position. Prepare stab cultures in 3 or more of the tubes, using a pure culture of *Lactobacillus plantarum*,[1] incubating for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution* add 5.0 mL of water containing 0.5 ng of biotin. Plug the tubes with cotton, sterilize in an autoclave at 121° for 15 min, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an inoculum comparable to a fresh culture.]

Transfer cells from the *Stock culture of Lactobacillus plantarum* to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. The cell suspension is the *Inoculum*.

Analysis

Samples: *Standard solution* and *Sample solution*

To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to 4 similar but empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool. Add 1 drop of *Inoculum* to each tube, except 2 of the 4 tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature of between 30° and 37° held constant to within ±0.5° until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of Standard during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to a spectrophotometer cell. Place the cell in a spectrophotometer that has been set at a specific wavelength of 540–660 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittance (Σ_S) as the difference, $y = 2.00 - \Sigma_S$. Plot this response on the ordinate of cross-section paper against the logarithm of the milliliter of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_U$, adding together the 2 transmittances (Σ_U) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X, for each dosage level. Average the values of X for each of three or more dosage levels to obtain $\bar{X}$, which equals the log-relative potency, M', of the *Sample solution*. Determine the quantity, in µg, of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Tablets taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = amount of biotin assumed to be present in the portion of Tablets taken (µg)

Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of the Tablets taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of biotin in the portion of the Tablets taken (µg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, $\bar{M}$, is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* ⟨111⟩, *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

[1] ATCC #8014 is suitable. This strain was formerly known as *Lactobacillus arabinosus* 17-5.

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• **BIOTIN, *Method 3***

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A: Transfer 800 mL of water and 100 mL of triethylamine to a 1000-mL volumetric flask. Add 80 mL of 85% phosphoric acid, and dilute with water to volume.

Mobile phase: Transfer 80 mL of acetonitrile and 10 mL of *Solution A* to a 1000-mL volumetric flask. Dilute with water to volume.

Standard solution: 0.6 µg/mL of USP Biotin RS in water. [NOTE—A portion of the *Standard solution* will be used to determine the percent recovery of biotin from the *Solid-phase extraction* procedure.]

Sample solution: Finely powder NLT 20 Tablets. Transfer an amount of powdered Tablets to a volumetric flask to obtain a nominal concentration of 0.6 µg/mL of biotin. Add water up to 80% of the flask capacity, and sonicate for 30–40 min, with occasional mixing, to dissolve. Dilute with water to volume, and filter. Adjust the pH of the solution with either dilute acetic acid or 0.1 N sodium hydroxide to a pH of 6.0–7.0.

Solid-phase extraction: [NOTE—Condition the extraction column specified in this procedure in the following manner. Wash the column with a 2-mL portion of methanol. Equilibrate with a 2-mL portion of water.] Separately pipet 5.0 mL each of the *Sample solution* and *Standard solution* into freshly conditioned solid-phase extraction columns consisting of a mixed-mode packing with a sorbent-mass of 60 mg. [NOTE—The mixed-mode packing consists of anion-exchange and reversed-phase sorbents. The reverse-phase component is a copolymer of *N*-vinylpyrrolidone and divinylbenzene. The anion exchange moiety is a trialkylamino group.[2]]

Wash the column with 10 mL of 30% (v/v) methanol in water. Apply an appropriate volume (4.9 mL) of 30% (v/v) methanol in 0.1 N hydrochloric acid to the column. Collect the eluate in a 5-mL volumetric flask, containing 100 µL of 40% (w/v) sodium acetate in water, and dilute with 30% (v/v) methanol in 0.1 N hydrochloric acid to volume.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 200 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 2 mL/min

Injection volume: 100 µL

System suitability

Samples: *Standard solution* and a portion of the *Standard solution* that has undergone *Solid-phase extraction*

Suitability requirements

Tailing factor: NMT 1.5, *Standard solution*

Relative standard deviation: NMT 2.0%, *Standard solution* and *Standard solution* that has undergone *Solid-phase extraction*

Recovery: 95%–100%, *Standard solution* that has undergone *Solid-phase extraction*

Analysis

Samples: *Standard solution* and *Sample solution* that have both undergone *Solid-phase extraction*

Measure the peak area of biotin. Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of biotin from the *Sample solution*

r_S = peak area of biotin from the *Standard solution*

C_S = concentration of USP Biotin RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of biotin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

• **CYANOCOBALAMIN, *Method 1***

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Methanol and water (7:13)

Standard stock solution: 10 µg/mL of USP Cyanocobalamin (Crystalline) RS in water. [NOTE—Store in a dark place, and discard after 1 week.]

Standard solution: 1 µg/mL of USP Cyanocobalamin (Crystalline) RS from the *Standard stock solution* diluted with water

Sample solution: Finely powder NLT 30 Tablets. Transfer a portion of the powder, equivalent to 100 µg of cyanocobalamin, to a 250-mL flask. Quantitatively add 100.0 mL of water, and carefully extract for 2 min. Filter 10 mL of the extract, and use the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: 550 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Flow rate: 0.5 mL/min

Injection volume: 200 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak area of cyanocobalamin. Calculate the percentage of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of cyanocobalamin from the *Sample solution*

r_S = peak area of cyanocobalamin from the *Standard solution*

C_S = concentration of USP Cyanocobalamin (Crystalline) RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cyanocobalamin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$)

Change to read:

• **CYANOCOBALAMIN, *Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Standard stock solution: 1.0 µg/mL of USP Cyanocobalamin (Crystalline) RS in 25% alcohol. Store in a refrigerator.

Standard solution: Dilute a suitable volume of the *Standard stock solution* with water to a measured volume such that after the incubation period as described in the *Analysis*, the difference in transmittance between the inoculated blank and the 5.0-mL level of the *Standard solution* is NLT that which corresponds to a difference of 1.25 mg in dried cell

[2] A suitable cartridge is the Waters Oasis MAX Vac RC, particle size 30 µm, part #186000371.

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weight. This concentration usually falls between 0.01 and 0.04 ng/mL of *Standard solution*. Prepare this solution fresh for each assay.

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powdered Tablets, equivalent to 1.0 µg of cyanocobalamin, to an appropriate vessel containing, for each gram of powdered Tablets taken, 25 mL of an aqueous extracting solution prepared just before use to contain, in each 100 mL, 1.29 g of dibasic sodium phosphate, 1.1 g of anhydrous citric acid, and 1.0 g of sodium metabisulfite. Autoclave the mixture at 121° for 10 min. Allow any undissolved particles of the extract to settle, and filter or centrifuge if necessary. Dilute an aliquot of the clear solution with water to obtain a final solution containing vitamin B_{12} activity equivalent to the nominal activity of the *Standard solution*.

Acid-hydrolyzed casein solution: Prepare as directed in *Biotin, Method 2*.

Asparagine solution: Dissolve 2.0 g of L-asparagine in water to make 200 mL. Store under toluene in a refrigerator.

Adenine–guanine–uracil solution: Prepare as directed in *Biotin, Method 2*.

Xanthine solution: Suspend 0.20 g of xanthine in 30–40 mL of water, heat to 70°, add 6.0 mL of 6 N ammonium hydroxide, and stir until the solid is dissolved. Cool, and dilute with water to make 200 mL. Store under toluene in a refrigerator.

Salt solution A: Dissolve 10 g of monobasic potassium phosphate and 10 g of dibasic potassium phosphate in water to make 200 mL. Add 2 drops of hydrochloric acid. Store this solution under toluene.

Salt solution B: Dissolve 4.0 g of magnesium sulfate, 0.20 g of sodium chloride, 0.20 g of ferrous sulfate, and 0.20 g of manganese sulfate in water to make 200 mL. Add 2 drops of hydrochloric acid. Store this solution under toluene.

Polysorbate 80 solution: Dissolve 20 g of polysorbate 80 in alcohol to make 200 mL. Store in a refrigerator.

Vitamin solution A: Dissolve 10 mg of riboflavin, 10 mg of thiamine hydrochloride, 100 µg of biotin, and 20 mg of niacin in 0.02 N acetic acid to make 400 mL. Store under toluene, protected from light, in a refrigerator.

Vitamin solution B: Dissolve 20 mg of *p*-aminobenzoic acid, 10 mg of calcium pantothenate, 40 mg of pyridoxine hydrochloride, 40 mg of pyridoxal hydrochloride, 8 mg of pyridoxamine dihydrochloride, and 2 mg of folic acid in a mixture of water and neutralized alcohol (3:1) to make 400 mL. Store, protected from light, in a refrigerator.

Basal medium stock solution: Prepare the medium according to the following formula and directions. A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium comparable to that obtained from the formula given herein.

Add the ingredients in the order listed in ▲*Table 3*▲ (USP 1-May-2020) carefully dissolving cystine and tryptophan in the 1 N hydrochloric acid before adding the next 8 solutions to the resulting solution. Add 100 mL of water, and dissolve thedextros, sodium acetate, and ascorbic acid. Filter, if necessary. Add the *Polysorbate 80 solution*, adjust with 1 N sodium hydroxide to a pH 5.5–6.0, and dilute with Purified Water to make 250 mL.

▲**Table 3**▲ (USP 1-May-2020)

L-Cystine	0.1 g
L-Tryptophan	0.05 g
1 N hydrochloric acid	10 mL
Adenine–guanine–uracil solution	5 mL
Xanthine solution	5 mL
Vitamin solution A	10 mL
Vitamin solution B	10 mL
Salt solution A	5 mL
Salt solution B	5 mL
Asparagine solution	5 mL
Acid-hydrolyzed casein solution	25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Ascorbic acid	1 g
Polysorbate 80 solution	5 mL

Tomato juice preparation: Centrifuge commercially canned tomato juice so that most of the pulp is removed. Suspend 5 g/L of analytical filter aid in the supernatant, and pass, with the aid of reduced pressure, through a layer of the filter aid. Repeat, if necessary, until a clear, straw-colored filtrate is obtained. Store under toluene in a refrigerator.

Culture medium: [NOTE—A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium equivalent to that obtained from the formula given herein.] Dissolve 0.75 g of yeast extract, 0.75 g of dried peptone, 1.0 g of anhydrous dextrose, and 0.20 g of monobasic potassium phosphate in 60–70 mL of water. Add 10 mL of *Tomato juice preparation* and 1 mL of *Polysorbate 80 solution*. Adjust with 1 N sodium hydroxide to a pH of 6.8, and dilute with water to make 100 mL. Place 10-mL portions of the solution in test tubes, and plug with cotton. Sterilize the tubes and contents in an autoclave at 121° for 15 min. Cool as rapidly as possible to avoid color formation resulting from overheating the medium.

Suspension medium: Dilute a measured volume of *Basal medium stock solution* with an equal volume of water. Place 10-mL portions of the diluted medium in test tubes. Sterilize, and cool as directed for *Culture medium*.

Stock culture of *Lactobacillus leichmannii*: To 100 mL of *Culture medium* add 1.0–1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Place 10-mL portions of the hot solution in test tubes, cover the tubes, sterilize at 121° for 15 min in an autoclave, and allow the tubes to cool in an upright position. Inoculate 3 or more of the tubes by stab transfer of a pure culture of *Lactobacillus leichmannii*.[3] [NOTE—Before first using a fresh culture in this assay, make NLT 10 successive transfers of the culture in a 2-week period.] Incubate for 16–24 h at a temperature of between 30° and 40° held constant to within ±0.5°. Store in a refrigerator.

Prepare fresh stab cultures at least 3 times each week, and do not use them for preparing the *Inoculum* if more than 4 days old. The activity of the microorganism can be increased by daily or twice-daily transfer of the stab culture, to the point where definite turbidity in the liquid *Inoculum* can be observed 2–4 h after inoculation. A slow-growing culture seldom gives a suitable response curve and may lead to erratic results.

[3] Pure cultures of *Lactobacillus leichmannii* (listed as *Lactobacillus delbrueckii*) may be obtained as No. 7830 from ATCC, 10801 University Blvd., Manassas, VA 20110-2209 (www.atcc.org).

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Inoculum: [NOTE—A frozen suspension of *Lactobacillus leichmannii* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Transfer cells from the *Stock culture of Lactobacillus leichmannii* to 2 sterile tubes containing 10 mL each of the *Culture medium*. Incubate these cultures for 16–24 h at a temperature of between 30° and 40° held constant to within ±0.5°. Under aseptic conditions centrifuge the cultures, and decant the supernatant. Suspend the cells from the culture in 5 mL of sterile *Suspension medium*, and combine. Using sterile *Suspension medium*, adjust the volume so that a 1-in-20 dilution in saline TS produces 70% transmittance when read on a suitable spectrophotometer that has been set at a wavelength of 530 nm, equipped with a 10-mm cell, and read against saline TS set at 100% transmittance. Prepare a 1-in-400 dilution of the adjusted suspension using sterile *Basal medium stock solution*. [NOTE—This dilution may be altered, when necessary, to obtain the desired test response.] The cell suspension is the *Inoculum*.

Calibration of spectrophotometer: Check the wavelength of the spectrophotometer periodically, using a standard wavelength cell or other suitable device. Before reading any tests, calibrate the spectrophotometer for 0% and 100% transmittance, using water and with the wavelength set at 530 nm.

Analysis

Samples: *Standard solution* and *Sample solution*

Because of the high sensitivity of the test organism to minute amounts of vitamin B_{12} activity and to traces of many cleansing agents, cleanse meticulously by suitable means, followed preferably by heating at 250° for 2 h, using hard-glass 20-mm × 150-mm test tubes and other necessary glassware.

To separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each of these tubes and to 4 similar but empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*. To each tube add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes to prevent bacterial contamination, and sterilize in an autoclave at 121° for 5 min, arranging to reach this temperature in NMT 10 min by preheating the autoclave if necessary. Cool as rapidly as possible to avoid color formation resulting from overheating the medium. Take precautions to maintain uniformity of sterilizing and cooling conditions throughout the assay, because packing the tubes too closely in the autoclave or overloading it may cause variation in the heating rate.

Aseptically add 0.5 mL of *Inoculum* to each tube so prepared, except 2 of the 4 containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature of between 30° and 40°, held constant to within ±0.5° for 16–24 h.

Terminate growth by heating to a temperature NLT 80° for 5 min. Cool to room temperature. After agitating its contents, read the transmittance at 530 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of the inoculated blank. If the difference is greater than 5%, or if there is evidence of contamination with a foreign microorganism, disregard the results of the assay.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of each of the remaining tubes. Disregard the results of the assay if the slope of the standard curve indicates a problem with sensitivity.

Calculation: Prepare a standard concentration-response curve by the following procedure. Test for and replace any aberrant individual transmittances. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittances (Σ_S) as the difference, $y = 2.00 - \Sigma_S$. Plot this response on the ordinate of cross-section paper against the logarithm of the milliliter of the *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_U$, adding together the two transmittances (Σ_U) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X, for each dosage level. Average the values of X for each of 3 or more dosage levels to obtain $\overline{X}$, which equals the log-relative potency, M', of the *Sample solution*. Determine the quantity, in µg, of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$) in the portion of Tablets taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = amount of cyanocobalamin assumed to be present in the portion of Tablets taken (µg)

Calculate the percentage of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$) in the portion of Tablets taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of cyanocobalamin in the portion of the Tablets taken (µg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, $\overline{M}$, is the assayed log-potency of the test material (see *Vitamin B_{12} Activity* in *Design and Analysis of Biological Assays* ⟨111⟩, *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$)

Change to read:

- **FOLIC ACID, *Method 1***

▲Proceed as directed in *Folic Acid Assay* ⟨411⟩, *Assay, Procedure 1*.▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–150.0% of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$)

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Change to read:

- **FOLIC ACID,** ***Method 2***

▲Proceed as directed in *Folic Acid Assay* ⟨411⟩, *Assay, Procedure 2*.▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–150.0% of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$)

- **CALCIUM PANTOTHENATE,** ***Method 1***

Mobile phase: Phosphoric acid and water (1:1000)

Internal standard solution: 80 mg of *p*-hydroxybenzoic acid in 3 mL of alcohol. Add 50 mL of water and 7.1 g of dibasic sodium phosphate, and dilute with water to 1000 mL. Adjust with phosphoric acid to a pH of 6.7.

Standard solution: 0.6 mg/mL of USP Calcium Pantothenate RS in *Internal standard solution*

Sample solution: Finely powder NLT 30 Tablets. Transfer a portion of the powder, equivalent to 15 mg of calcium pantothenate, to a centrifuge tube. Add 25.0 mL of the *Internal standard solution*, and shake vigorously for 10 min. Centrifuge, filter, and use the clear filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 210 nm

Column: 3.9-mm × 15-cm; packing L1

Flow rate: 1.5 mL/min

Injection volume: 10 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for calcium pantothenate and *p*-hydroxybenzoic acid are about 0.5 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of calcium pantothenate and the internal standard. Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Tablets taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak area ratio of calcium pantothenate to *p*-hydroxybenzoic acid from the *Sample solution*

R_S = peak area ratio of calcium pantothenate to *p*-hydroxybenzoic acid from the *Standard solution*

C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

Change to read:

- **CALCIUM PANTOTHENATE,** ***Method 2***

Standard stock solution: Dissolve 50 mg of USP Calcium Pantothenate RS ▲▲ (USP 1-May-2020) in 500 mL of water in a 1000-mL volumetric flask. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), and dilute with water to volume to obtain a concentration of 50 µg/mL of USP Calcium Pantothenate RS. Store under toluene in a refrigerator.

Standard solution: On the day of the assay, dilute a volume of the *Standard stock solution* with water to obtain a concentration of 0.01–0.04 µg/mL of calcium pantothenate, the exact concentration being such that the responses obtained as directed in the *Analysis*, 2.0 and 4.0 mL of the *Standard solution*, are within the linear portion of the log-concentration response curve.

Sample solution: Finely powder NLT 30 Tablets. Transfer a portion of the powder, equivalent to 50 mg of calcium pantothenate, to a 1000-mL volumetric flask containing 500 mL of water. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), dilute with water to volume, and filter. Dilute a volume of this solution to obtain a solution having approximately the same concentration as that of the *Standard solution*.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and dilute with water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and dilute with water to make 1000 mL. Store under toluene in a cool place at a temperature not below 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and dilute with water to make 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin–thiamine hydrochloride–biotin solution: 20 µg/mL of riboflavin, 10 µg/mL of thiamine hydrochloride, and 0.04 µg/mL of biotin in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

***p*-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution:** 10 µg/mL of *p*-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Basal medium stock solution: Dissolve the anhydrous dextrose and anhydrous sodium acetate in the solutions previously mixed according to ▲*Table 4*,▲ (USP 1-May-2020) and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

▲Table 4▲ (USP 1-May-2020)

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g

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Table 4 (USP 1-May-2020) *(continued)*

Adenine–guanine–uracil solution	5 mL
Riboflavin–thiamine hydrochloride–biotin solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water. Add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to the test tubes, close or cover the tubes, sterilize in an autoclave at 121° for 15 min, and allow the tubes to cool in an upright position. Prepare stab cultures in 3 or more of the tubes, using a pure culture of *Lactobacillus plantarum*,[1] incubating for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution* add 5.0 mL of water containing 0.2 µg of calcium pantothenate. Plug the tubes with cotton, sterilize in an autoclave at 121° for 15 min, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.]

Transfer cells from the *Stock culture of Lactobacillus plantarum* to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. The cell suspension is the *Inoculum*.

Analysis

Samples: *Standard solution* and *Sample solution*

To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to 4 similar but empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, volumes of the *Sample solution* corresponding to 3 or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool, and add 1 drop of *Inoculum* to each tube, except 2 of the 4 tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37°, held constant to within ±0.5° until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of Standard during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to an optical container if necessary. Read the transmittance between 540 and 660 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittance (Σ_S) as the difference, $y = 2.00 - \Sigma_S$. Plot this response on the ordinate of cross-section paper against the logarithm of the milliliter of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_U$, adding together the two transmittances (Σ_U) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X, for each dosage level. Average the values of X for each of 3 or more dosage levels to obtain $\overline{X}$, which equals the log-relative potency, M', of the *Sample solution*. Determine the quantity, in mg, of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Tablets taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = amount of calcium pantothenate assumed to be present in the portion of the Tablets taken (mg)

Calculate the percentage of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Tablets taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of calcium pantothenate in the portion of Tablets taken (mg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, $\overline{M}$, is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* ⟨111⟩, *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• **CALCIUM PANTOTHENATE, *Method 3***

Buffer solution: Dissolve 10.0 g of monobasic potassium phosphate in 2000 mL of water, and adjust with phosphoric acid to a pH of 3.5.

Mobile phase: Methanol and *Buffer solution* (1:9)

Standard stock solution: 0.25 mg/mL of USP Calcium Pantothenate RS in water. Prepare fresh every 4 weeks. Store in a refrigerator.

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Standard solution: 40 µg/mL of USP Calcium Pantothenate RS from the *Standard stock solution* diluted with water
Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 10 mg of calcium pantothenate, to a 250-mL volumetric flask. Add 10 mL of methanol, and swirl the flask to disperse. Dilute with water to volume, mix, and filter.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 205 nm
Column: 3.9-mm × 30-cm; 5-µm packing L1
Column temperature: 50°
Flow rate: 2 mL/min
Injection volume: 25 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 3.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas of calcium pantothenate. Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of calcium pantothenate from the *Sample solution*
r_S = peak area of calcium pantothenate from the *Standard solution*
C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

Change to read:

- **NIACIN OR NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, RIBOFLAVIN,** and **THIAMINE,** ***Method 1***

[NOTE—Use low-actinic glassware throughout this procedure.]
Diluent: Acetonitrile, glacial acetic acid, and water (5:1:94)
Mobile phase: A mixture of methanol, glacial acetic acid, and water (27:1:73) containing 140 mg of sodium 1-hexanesulfonate per 100 mL
Standard solution: [NOTE—Use USP Niacin RS in place of USP Niacinamide RS for formulations containing niacin.] Transfer 80 mg of USP Niacinamide RS, 20 mg of USP Pyridoxine Hydrochloride RS, 20 mg of USP Riboflavin RS, and 20 mg of USP Thiamine Hydrochloride RS to a 200-mL volumetric flask, and add 180 mL of *Diluent*. Immerse the flask in a hot water bath maintained at 65°–70° for 10 min with regular shaking or using a vortex mixer, until all the solid materials are dissolved. Chill rapidly in a cold water bath for 10 min to room temperature, and dilute with *Diluent* to volume.
Sample solution: Finely powder NLT 30 Tablets. Transfer a portion of the powder, equivalent to 10 mg of niacinamide and 2.5 mg each of pyridoxine hydrochloride, riboflavin, and thiamine hydrochloride, to a 50-mL centrifuge tube. Add 25.0 mL of *Diluent*, and mix using a vortex mixer for 30 s to completely suspend the powder. Immerse the centrifuge tube in a hot water bath maintained at 65°–70°, heat for 5 min, and mix on a vortex mixer for 30 s. Return the tube to the hot water bath, heat for another 5 min, and mix on a vortex mixer for 30 s. Filter a portion of the solution, cool to room temperature, and use the clear filtrate. [NOTE—Use the filtrate within 3 h of filtration.]
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 280 nm
Column: 3.9-mm × 30-cm; packing L1
Flow rate: 1 mL/min
Injection volume: 10 µL
System suitability
Sample: *Standard solution*
▲▲ (USP 1-May-2020)
Suitability requirements
Relative standard deviation: NMT 3.0%
Analysis
Samples: *Standard solution* and *Sample solution*
▲Calculate the percentage of the labeled amount of vitamin B_1 as thiamine ion ($C_{12}H_{17}N_4OS+$), vitamin B_2 as riboflavin ($C_{17}H_{20}N_4O_6$), vitamin B_3 as niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), vitamin B_6 as pyridoxine ($C_8H_{11}NO_3$), calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), and folic acid ($C_{19}H_{19}N_7O_6$), in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin from the corresponding *Sample solution*
r_S = peak area of the relevant vitamin from the corresponding *Standard solution*
C_S = concentration of the relevant vitamin Reference Standard in the corresponding *Standard solution* (µg/mL)
C_U = nominal concentration of the relevant vitamin in the corresponding *Sample solution* (µg/mL)▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–150.0% of the labeled amount of ▲each individual vitamin▲ (USP 1-May-2020)

Change to read:

- **NIACIN,** ***Method 2***

▲Proceed as directed in *Niacin or Niacinamide Assay* ⟨441⟩, *Assay, Chromatographic Methods, Procedure 2*.▲ (USP 1-May-2020)
Acceptance criteria: 90.0%–150.0% of the labeled amount of niacin ($C_6H_5NO_2$)

Change to read:

- **NIACINAMIDE,** ***Method 2***

▲Proceed as directed in *Niacin or Niacinamide Assay* ⟨441⟩, *Assay, Chromatographic Methods, Procedure 2*.▲ (USP 1-May-2020)
Acceptance criteria: 90.0%–150.0% of the labeled amount of niacinamide ($C_6H_6N_2O$)

- **PYRIDOXINE HYDROCHLORIDE,** ***Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]
Extraction solvent, Mobile phase, and **Sample solution:** Prepare as directed in *Niacin, Method 2*.
Standard stock solution: 0.1 mg/mL of USP Pyridoxine Hydrochloride RS in *Extraction solvent*

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Standard solution: Transfer 5.0 mL of the *Standard stock solution* to a 25-mL volumetric flask, and dilute with *Extraction solvent* to volume.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; packing L1
Flow rate: 1 mL/min
Injection volume: 20 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 3.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas of pyridoxine. Calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of pyridoxine from the *Sample solution*
r_S = peak area of pyridoxine from the *Standard solution*
C_S = concentration of USP Pyridoxine Hydrochloride RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of pyridoxine hydrochloride in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$)

Change to read:

- **RIBOFLAVIN,** ***Method 2***

▲Proceed as directed in *Riboflavin Assay* ⟨481⟩, *Assay, Chromatographic Methods, Procedure 2*.▲ (USP 1-May-2020)
Acceptance criteria: 90.0%–150.0% of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$)

Change to read:

- **THIAMINE,** ***Method 2***

▲Proceed as directed in *Thiamine Assay* ⟨531⟩, *Assay, Chromatographic Methods, Procedure 2*.▲ (USP 1-May-2020)
Acceptance criteria: 90.0%–150.0% of the labeled amount of thiamine ▲as thiamine ion ($C_{12}H_{17}N_4OS+$)▲ (USP 1-May-2020)

Change to read:

- **NIACIN OR NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, RIBOFLAVIN,** and **THIAMINE,** ***Method 3***

[NOTE—Use low-actinic glassware throughout this procedure.]
Reagent: 25 mg/mL of edetate disodium in water
Mobile phase: Transfer 0.4 mL of triethylamine, 15.0 mL of glacial acetic acid, and 350 mL of methanol to a 2000-mL volumetric flask. Dilute with 0.008 M sodium 1-hexanesulfonate to volume.
Standard stock solution: 1.5 mg/mL of USP Niacin RS or USP Niacinamide RS, 0.24 mg/mL of USP Pyridoxine Hydrochloride RS, 0.08 mg/mL of USP Riboflavin RS, and 0.24 mg/mL of USP Thiamine Hydrochloride RS in the *Reagent*, with heating if necessary
Standard solution: Transfer 5.0 mL of the *Standard stock solution* to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few milliliters of the filtrate.
Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 7.5 mg of niacin or niacinamide, 1.2 mg of pyridoxine hydrochloride, 0.4 mg of riboflavin, and 1.2 mg of thiamine hydrochloride to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1), and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few milliliters of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 270 nm
Column: 4.6-mm × 25-cm; packing L7
Column temperature: 50°
Flow rate: 2.0 mL/min
Injection volume: 5 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 2.0%
Analysis
Samples: *Standard solution* and *Sample solution*
▲Calculate the percentage of the labeled amount of vitamin B_1 as thiamine ion ($C_{12}H_{17}N_4OS+$), vitamin B_2 as riboflavin ($C_{17}H_{20}N_4O_6$), vitamin B_3 as niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), vitamin B_6 as pyridoxine ($C_8H_{11}NO_3$), calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), and folic acid ($C_{19}H_{19}N_7O_6$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin from the relevant *Sample solution*
r_S = peak area of the relevant vitamin from the relevant *Standard solution*
C_S = concentration of the relevant vitamin Reference Standard in the relevant *Standard solution* (µg/mL)
C_U = nominal concentration of the relevant vitamin in the relevant *Sample solution* (µg/mL)▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–150.0% of the labeled amount of ▲each individual vitamin▲ (USP 1-May-2020)

Add the following:

▲• **FOLIC ACID,** ***Method 3*****; ASCORBIC ACID, NIACIN OR NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, CALCIUM PANTOTHENATE, RIBOFLAVIN,** and **THIAMINE,** ***Method 4***

[NOTE—Use low-actinic glassware.]
Solution A: 0.56 g of edetate disodium and 2.04 g of potassium phosphate monobasic in 1000 mL of water. Adjust with phosphoric acid to a pH of 3.0.
Solution B: Acetonitrile, acetic acid, glacial, and water (5:1:94)
Solution C: 2 mL of ammonium hydroxide and 1 g of sodium perchlorate in 100 mL of water
Solution D: 2 mL of ammonium hydroxide, 1 g of sodium perchlorate, and 2 g of sodium ascorbate in 100 mL of water
Mobile phase A: 0.02% trifluoroacetic acid in water

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Mobile phase B: Acetonitrile
Mobile phase: See *Table 5*.

Table 5

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
0	100	0
1.0	100	0
6.0	95	5
15.0	83	17
17.0	80	20
19.0	80	20
19.1	100	0
25.0	100	0

Ascorbic acid standard stock solution: 1.0 mg/mL of ascorbic acid from USP Ascorbic Acid RS in *Solution A*
Thiamine standard stock solution: 0.2 mg/mL of thiamine from USP Thiamine Hydrochloride RS in *Solution B*. The solution can be placed on a steam bath for about 5–10 min with frequent swirling to assist in dissolution.
Riboflavin standard stock solution: 0.2 mg/mL of USP Riboflavin RS in *Solution B*. Heat the solution to 65°–70° for 10 min to assist in dissolution.
Niacin or niacinamide standard stock solution: 1.0 mg/mL of USP Niacin RS or USP Niacinamide RS in *Solution A*
Pyridoxine standard stock solution: 0.2 mg/mL of pyridoxine from USP Pyridoxine Hydrochloride RS in *Solution C*
Folic acid standard stock solution: 0.2 mg/mL of folic acid from USP Folic Acid RS in *Solution C*
Calcium pantothenate standard stock solution: 1.0 mg/mL of calcium pantothenate from USP Calcium Pantothenate RS in *Mobile phase A*
System suitability solution: Transfer 0.5 mL of *Ascorbic acid standard stock solution*, 2.5 mL of *Thiamine standard stock solution*, and 0.5 mL of *Niacin or niacinamide standard stock solution* into a 10-mL volumetric flask, and dilute with *Mobile phase A* to volume.
Sample solution 1: Weigh and finely powder NLT 20 Tablets. Transfer a calculated amount of accurately weighed powder into a 100-mL volumetric flask to obtain a solution containing known nominal concentrations of thiamine, riboflavin, and calcium pantothenate in the ranges of 5–30 µg/mL for thiamine, 10–60 µg/mL for riboflavin, and 25–150 µg/mL for calcium pantothenate. Add *Solution B* to about half the volume, and mix on a vortex mixer for 5 min. Heat the flask at 65°–70° for 5 min, swirling frequently. Sonicate additionally for 5 min, cool to room temperature, dilute with *Mobile phase A* to volume, and mix well. Pass a portion of the solution through a suitable filter of 0.45-µm pore size, and discard the first milliliter of the filtrate.
Sample solution 2: Transfer a portion of finely powdered Tablets into a 100-mL volumetric flask to obtain a solution containing known nominal concentrations of ascorbic acid and niacin or niacinamide in the ranges of 50–300 µg/mL for ascorbic acid and 12–65 µg/mL for niacin or niacinamide. Add *Solution A* to about half the volume, mix on a vortex mixer for 5 min, and sonicate for 10 min with occasional shaking. Cool to room temperature, dilute with *Solution A* to volume, and mix well. Pass a portion of the solution through a suitable filter of 0.45-µm pore size, and discard the first milliliter of the filtrate.
Sample solution 3: Transfer a portion of finely powdered Tablets into a 100-mL volumetric flask to obtain a solution containing known nominal concentrations of pyridoxine hydrochloride and folic acid in the ranges of 5–30 µg/mL for pyridoxine hydrochloride and 2–12 µg/mL for folic acid, add *Solution D* to about half the volume, heat on a water bath at 40° for 5 min, and sonicate for 10 min with occasional shaking. Cool to room temperature, dilute with *Solution D* to volume, and mix well. Pass a portion of the solution through a suitable filter of 0.45-µm pore size, and discard the first milliliter of the filtrate.
Standard solution 1: Accurately transfer calculated volumes of *Thiamine standard stock solution*, *Riboflavin standard stock solution*, and *Calcium pantothenate standard stock solution* into a suitable volumetric flask to obtain a solution with final vitamin concentrations similar to the nominal concentrations in *Sample solution 1* for the corresponding vitamins, dilute with *Mobile phase A* to volume, and mix well.
Standard solution 2: Accurately transfer calculated volumes of *Ascorbic acid standard stock solution* and *Niacin or niacinamide standard stock solution*, into a suitable volumetric flask to obtain a solution with final vitamin concentrations similar to the nominal concentrations in *Sample solution 2* for the corresponding vitamins, dilute with *Mobile phase A* to volume, and mix well.
Standard solution 3: Accurately transfer calculated volumes of *Pyridoxine standard stock solution* and *Folic acid standard stock solution* into a suitable volumetric flask to obtain a solution with final vitamin concentrations similar to the nominal concentrations in *Sample solution 3* for the corresponding vitamins, dilute with *Solution C* to volume, and mix well.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detectors
For calcium pantothenate: UV 210 nm
For thiamine, ascorbic acid, niacin or niacinamide, and riboflavin: UV 254 nm
For pyridoxine hydrochloride and folic acid: UV 280 nm
Column: 4.6-mm × 15-cm; 5-µm packing L1
Column temperature: 25°
Flow rate: 1.0 mL/min
Injection volume: 20 µL
System suitability
Samples: *System suitability solution*, *Standard solution 1*, *Standard solution 2*, and *Standard solution 3*
Suitability requirements
Resolution: NLT 3.5 between the thiamine and ascorbic acid peaks and NLT 4.0 between the ascorbic acid and niacin or niacinamide peaks, *System suitability solution*
Relative standard deviation: NMT 2.0% for each individual peak, *Standard solution 1*, *Standard solution 2*, and *Standard solution 3*
Analysis
Samples: *Sample solutions 1–3* and *Standard solutions 1–3*
Calculate the percentage of the labeled amount of vitamin B_1 as thiamine ion ($C_{12}H_{17}N_4OS^+$), vitamin B_2 as riboflavin ($C_{17}H_{20}N_4O_6$), vitamin B_3 as niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), vitamin B_6 as pyridoxine ($C_8H_{11}NO_3$), calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), and folic acid ($C_{19}H_{19}N_7O_6$), in the portion of Tablets taken:

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$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin from the corresponding *Sample solution*

r_S = peak area of the relevant vitamin from the corresponding *Standard solution*

C_S = concentration of the relevant vitamin Reference Standard in the corresponding *Standard solution* (µg/mL)

C_U = nominal concentration of the relevant vitamin in the corresponding *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of each individual vitamin▲ (USP 1-May-2020)

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩, *Dissolution*: Meet the requirements
- **WEIGHT VARIATION** ⟨2091⟩: Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic microbial count does not exceed 3×10^3 cfu/g, and the combined molds and yeasts count does not exceed 3×10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures*, *Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meet the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

Change to read:

- **LABELING:**[4] The label states that the product is Oil- and Water-Soluble Vitamins Tablets. The label also states the quantity of each vitamin ▲in mg/Tablet or µg/Tablet▲ (USP 1-May-2020) and, where necessary, the chemical form in which it is present. Where the product contains vitamin E, the label indicates whether it is the ▲*RRR*▲ (USP 1-May-2020) or ▲*all-rac*▲ (USP 1-May-2020) form. Where more than one assay method is given for a particular vitamin, the labeling states with which assay method the product complies only if *Method 1* is not used.

Change to read:

- **USP REFERENCE STANDARDS** ⟨11⟩

USP Alpha Tocopherol RS
USP Alpha Tocopheryl Acetate RS
USP Alpha Tocopheryl Acid Succinate RS
▲USP Ascorbic Acid RS▲ (USP 1-May-2020)
USP Biotin RS
1*H*-Thieno[3,4-*d*]imidazole-4-pentanoic acid, hexahydro-2-oxo-, 3a*S*-[(3aα,4β,6aα)]-;
(3a*S*,4*S*,6a*R*)-Hexahydro-2-oxo-1*H*-thieno[3,4-*d*] imidazole-4-valeric acid.
$C_{10}H_{16}N_2O_3S$ 244.31
USP Calcium Pantothenate RS
β-Alanine, *N*-(2,4-dihydroxy-3,3-dimethyl-1-oxobutyl)-, calcium salt (2:1), (*R*)-;
Calcium D-pantothenate (1:2).
$C_{18}H_{32}CaN_2O_{10}$ 476.53
USP Cholecalciferol RS
9,10-Secocholesta-5,7,10(19)-trien-3-ol, (3β,5*Z*,7*E*)-;
Cholecalciferol.
$C_{27}H_{44}O$ 384.64
USP Cyanocobalamin (Crystalline) RS
Vitamin B_{12}.
$C_{63}H_{88}CoN_{14}O_{14}P$ 1355.37
USP Ergocalciferol RS
9,10-Secoergosta-5,7,10(19),22-tetraen-3-ol, (3β,5*Z*,7*E*,22*E*)-;
Ergocalciferol.
$C_{28}H_{44}O$ 396.65
USP Folic Acid RS
L-Glutamic acid, *N*-[4-[[(2-amino-1,4-dihydro-4-oxo-6-pteridinyl)methyl]amino]benzoyl]-;
Folic acid;
N-[*p*-[[(2-Amino-4-hydroxy-6-pteridinyl)methyl]amino]-benzoyl]-L-glutamic acid.
$C_{19}H_{19}N_7O_6$ 441.40
USP Niacin RS
3-Pyridinecarboxylic acid;
Nicotinic acid.
$C_6H_5NO_2$ 123.11
USP Niacinamide RS
3-Pyridinecarboxamide;
Nicotinamide.
$C_6H_6N_2O$ 122.12
USP Phytonadione RS
1,4-Naphthalenedione, 2-methyl-3-(3,7,11,15-tetramethyl-2-hexadecenyl)-, [*R*-[*R**,*R**-(*E*)]]-;
Phylloquinone.
$C_{31}H_{46}O_2$ 450.70
USP Pyridoxine Hydrochloride RS
3,4-Pyridinedimethanol, 5-hydroxy-6-methyl-, hydrochloride;
Pyridoxol hydrochloride.
$C_8H_{11}NO_3 \cdot HCl$ 205.64
▲USP Retinyl Acetate RS
3,7-Dimethyl-9-(2,6,6-trimethyl-1- cyclohexen-1-yl) 2,4,6,8-nonatetraen-1-ol acetate.
USP Retinyl Palmitate RS
[(2*E*,4*E*,6*E*,8*E*)-3,7-Dimethyl-9-(2,6,6-trimethyl-1-cyclohex-1-en-1-yl)nona-2,4,6,8-tetraen-1-yl] hexadecanoate.▲ (USP 1-May-2020)
USP Riboflavin RS
Riboflavine.
$C_{17}H_{20}N_4O_6$ 376.36

[4] ▲▲ (USP 1-May-2020)Where articles are labeled in terms of Units in addition to the required labeling, the relationship of the USP Units or IU to mass is as follows. One USP Vitamin A Unit = 0.3 µg of all-*trans*-retinol (vitamin A alcohol) or 0.344 µg of all-*trans*-retinyl acetate (vitamin A acetate) or 0.55 µg of all-*trans*-retinyl palmitate (vitamin A palmitate), and 1 µg of retinol (3.3 USP Vitamin A Units) = 1 retinol equivalent (RE); 1 IU of beta carotene = 0.6 µg of all-*trans*-beta carotene; 1 USP Vitamin D Unit = 0.025 µg of ergocalciferol or cholecalciferol; and 1 mg of ▲*all-rac*▲ (USP 1-May-2020)-alpha-tocopherol = 1.1 former USP Vitamin E Units; 1 mg of ▲*all-rac*▲ (USP 1-May-2020)-alpha-tocopheryl acetate = 1 former USP Vitamin E unit; 1 mg of ▲*all-rac*▲ (USP 1-May-2020)-alpha-tocopheryl acid succinate = 0.89 former USP Vitamin E Units; 1 mg of ▲*RRR*▲ (USP 1-May-2020)-alpha-tocopherol = 1.49 former USP Vitamin E Units; 1 mg of ▲*RRR*▲ (USP 1-May-2020)-alpha-tocopheryl acetate = 1.36 former USP Vitamin E Units; and 1 mg of ▲*RRR*▲ (USP 1-May-2020)-alpha-tocopheryl acid succinate = 1.21 former USP Vitamin E Units. In terms of ▲*RRR*▲ (USP 1-May-2020)-alpha-tocopherol equivalents, 1 mg of ▲*RRR*▲ (USP 1-May-2020)-alpha-tocopheryl acetate = 0.91; 1 mg of ▲*RRR*▲ (USP 1-May-2020)-alpha-tocopheryl acid succinate = 0.81; 1 mg of ▲*all-rac*▲ (USP 1-May-2020)-alpha-tocopherol = 0.74; 1 mg of ▲*all-rac*▲ (USP 1-May-2020)-alpha-tocopheryl acetate = 0.67; and 1 mg of ▲*all-rac*▲ (USP 1-May-2020)-alpha-tocopheryl acid succinate = 0.60. ▲Note that 1 mg of Institute of Medicine (IOM) alpha-tocopherol equivalent = 1 mg of 2*R*-alpha-tocopherol = 1 mg of *RRR*-alpha-tocopherol = 2 mg of *all-rac*-alpha-tocopherol.▲ (USP 1-May-2020)

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USP Thiamine Hydrochloride RS

Thiazolium, 3-[(4-amino-2-methyl-5-pyrimidinyl) methyl]-5-(2-hydroxyethyl)-4-methyl-, chloride, monohydrochloride;
Thiamine monohydrochloride.
$C_{12}H_{17}ClN_4OS \cdot HCl$ 337.27

▲▲ (USP 1-May-2020)

Oil- and Water-Soluble Vitamins with Minerals Capsules

DEFINITION

Oil- and Water-Soluble Vitamins with Minerals Capsules contain one or more of the following oil-soluble vitamins: Vitamin A, vitamin D as Ergocalciferol (vitamin D_2) or Cholecalciferol (vitamin D_3), Vitamin E, Phytonadione (vitamin K_1), and Beta Carotene; one or more of the following water-soluble vitamins: Ascorbic Acid or its equivalent as Calcium Ascorbate or Sodium Ascorbate, Biotin, Cyanocobalamin, Folic Acid, Niacin or Niacinamide, Dexpanthenol or Panthenol, vantothenic vcid (as Calcium Pantothenate or Racemic Calcium Pantothenate), Pyridoxine Hydrochloride, Riboflavin, and Thiamine Hydrochloride or Thiamine Mononitrate; and one or more minerals derived from substances generally recognized as safe, furnishing one or more of the following elements in ionizable form: boron, calcium, chromium, copper, fluorine, iodine, iron, magnesium, manganese, molybdenum, nickel, phosphorus, potassium, selenium, tin, vanadium, and zinc. Capsules contain NLT 90.0% and NMT 165.0% of the labeled amounts of vitamin A ($C_{20}H_{30}O$) as retinol or esters of retinol in the form of retinyl acetate ($C_{22}H_{32}O_2$) or retinyl palmitate ($C_{36}H_{60}O_2$); vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$); vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$) or alpha tocopheryl acetate ($C_{31}H_{52}O_3$) or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$); phytonadione ($C_{31}H_{46}O_2$); and beta carotene ($C_{40}H_{56}$); NLT 90.0% and NMT 150.0% of the labeled amounts of ascorbic acid ($C_6H_8O_6$) or its salts as calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$) or sodium ascorbate ($C_6H_7NaO_6$); biotin ($C_{10}H_{16}N_2O_3S$); cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$); folic acid ($C_{19}H_{19}N_7O_6$); niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$); dexpanthenol ($C_9H_{19}NO_4$) or panthenol ($C_9H_{19}NO_4$); calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$); pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$); riboflavin ($C_{17}H_{20}N_4O_6$); and thiamine ($C_{12}H_{17}ClN_4OS$) as thiamine hydrochloride or thiamine mononitrate; and NLT 90.0% and NMT 125.0% of the labeled amount of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), potassium (K), and zinc (Zn); and NLT 90.0% and NMT 160.0% of the labeled amounts of boron (B), chromium (Cr), fluorine (F), iodine (I), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), and vanadium (V).

They may contain other labeled added substances that are generally recognized as safe, in amounts that are unobjectionable.

STRENGTH

[NOTE—In the following assays, where more than one assay method is given for an individual ingredient, the requirements may be met by following any one of the specified methods, the method used being stated in the labeling only if *Method 1* is not used.]

• **VITAMIN A,** ***Method 1***

[NOTE—Where the use of a vitamin A ester (retinyl acetate or retinyl palmitate) is specified in the following procedure, use the chemical form present in the formulation. USP Vitamin A RS is retinyl acetate. It is to be used where USP Vitamin A RS is specified. Use low-actinic glassware throughout this procedure.]

Mobile phase: *n*-Hexane

Standard solution: 15 µg/mL of retinyl acetate from USP Vitamin A RS in *n*-hexane

System suitability stock solution: 15 µg/mL of retinyl palmitate in *n*-hexane

System suitability solution: Mix equal volumes of *System suitability stock solution* and the *Standard solution* to obtain concentrations of 7.5 µg/mL each of retinyl acetate and retinyl palmitate.

Sample stock solution: Transfer the contents of NLT 20 Capsules to a suitable container, mix, and weigh. Transfer a portion of the mixture, equivalent to 5 Capsules, to a container with a polytef-lined screw cap. [NOTE—For hard gelatin Capsules, remove, as completely as possible, the contents of NLT 20 Capsules by cutting open the Capsule shells, transferring the shells and their contents to a suitable container, and triturating to a homogeneous mass. Transfer a portion of the mass, equivalent to 5 Capsules, to a container with a polytef-lined screw cap.] Add 10 mL of dimethyl sulfoxide and 15 mL of *n*-hexane, and shake for 45 min on a wrist-action shaker in a water bath maintained at 60°. [NOTE—Set up the wrist-action shaker to ensure that the contents of the container are mixed vigorously and thoroughly.] Centrifuge at 3000 rpm for 10 min, and transfer the hexane layer by means of a pipet to a 100-mL volumetric flask. Add 15 mL of *n*-hexane to the dimethyl sulfoxide layer, shake thoroughly for 5 min, and transfer the hexane layer by means of a pipet to the 100-mL volumetric flask. Repeat this extraction with three additional 15-mL portions of *n*-hexane. Dilute the extracts in the volumetric flask with *n*-hexane to volume.

Sample solution: Dilute a volume of the *Sample stock solution* with *n*-hexane to obtain a solution with a concentration of 15 µg/mL of vitamin A as retinol ($C_{20}H_{30}O$).

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 325 nm

Column: 4.6-mm × 15-cm; 3-µm packing L8

Flow rate: 1 mL/min

Injection volume: 40 µL

System suitability

Sample: *System suitability solution*

Suitability requirements

Resolution: NLT 10 between all-*trans*-retinyl acetate and all-*trans*-retinyl palmitate

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak area for all-*trans*-retinyl acetate from the *Standard solution* and the peak area for all-*trans*-retinyl acetate or all-*trans*-retinyl palmitate in the chromatogram of the *Sample solution*. For products containing vitamin A acetate or vitamin A palmitate, calculate the percentage of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$), in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

DS Monographs

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r_U = peak area of the all-*trans*-retinyl ester from the *Sample solution*
r_S = peak area of the all-*trans*-retinyl ester from the *Standard solution*
C_S = concentration of retinyl acetate ($C_{22}H_{32}O_2$) from USP Vitamin A RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of vitamin A, as retinol ($C_{20}H_{30}O$), in the *Sample solution* (µg/mL)
F = factor used to convert retinyl acetate, the ester form present in USP Vitamin A RS, to retinol, 0.872

[NOTE—The molar responses of retinyl acetate and retinyl palmitate are equivalent.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$)

• **VITAMIN A, *Method 2***

[NOTE—Where a vitamin A ester (retinyl acetate or retinyl palmitate) is indicated in the following procedure, use the chemical form present in the formulation. USP Vitamin A RS is retinyl acetate. It is to be used where USP Vitamin A RS is specified. Use low-actinic glassware throughout this procedure.]

3 N methanolic sulfuric acid solution: Cautiously add 9 mL of sulfuric acid to 80 mL of methanol in a 100-mL volumetric flask. Cool, and dilute with methanol to volume.

Sodium ascorbate–pyrogallol solution: Transfer 10 g of sodium ascorbate and 5 g of pyrogallol to a 100-mL volumetric flask, and add sufficient water to dissolve. Add 1.7 mL of sulfuric acid, and dilute with water to volume.

Lecithin solution: 5 mg/mL of lecithin in 2,2,4-trimethylpentane

Mobile phase: *n*-Hexane and ethyl acetate (99.7:0.3)

Standard solution: 15 µg/mL of retinyl acetate from USP Vitamin A RS in 2,2,4-trimethylpentane

System suitability stock solution: 15 µg/mL of retinyl palmitate in 2,2,4-trimethylpentane

System suitability solution: Mix equal volumes of the *System suitability stock solution* and the *Standard solution* to obtain concentrations of 7.5 µg/mL each of retinyl acetate and retinyl palmitate.

Sample solution: [NOTE—This preparation is suitable for the determination of vitamin A, vitamin D, and vitamin E, when present in the formulation.] Weigh NLT 20 Capsules in a tared weighing bottle. Using a sharp blade if necessary, carefully open the Capsules, without loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to 30 µg of the labeled amount of cholecalciferol or ergocalciferol (vitamin D), to a container with a polytef-lined screw cap. If vitamin D is not present in the formulation, use a portion, equivalent to 90 mg of the labeled amount of vitamin E. If vitamin E is not present in the formulation, use a portion, equivalent to 2.5 mg of the labeled amount of vitamin A, as retinol. Add 0.5 g of sodium bicarbonate, 1.5 mL of *Lecithin solution*, and 12.5 mL of 2,2,4-trimethylpentane, and disperse on a vortex mixer. Add 6 mL of *Sodium ascorbate–pyrogallol solution*, shake slowly, and allow the solution to degas. Continue shaking until the evolution of gas has ceased, and then shake for an additional 12 min. Add 6 mL of dimethyl sulfoxide, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 6 mL of *3 N methanolic sulfuric acid solution*, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 12.5 mL of 2,2,4-trimethylpentane, mix on a vortex mixer to form a suspension, and shake for 10 min. Centrifuge for 10 min to break up the emulsion and to clarify the supernatant. [NOTE—The supernatant is used for the determination of vitamin A, and also vitamin D and vitamin E, if present in the formulation.] If necessary, quantitatively dilute a volume of the supernatant with 2,2,4-trimethylpentane to obtain a concentration close to that of the *Standard solution*.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 325 nm
Column: 4.6-mm × 25-cm; 5-µm packing L24
Flow rate: 1.5 mL/min
Injection volume: 40 µL

System suitability
Sample: *System suitability solution*
Suitability requirements
Resolution: NLT 8.0 between all-*trans*-retinyl acetate and all-*trans*-retinyl palmitate
Relative standard deviation: NMT 3.0%

Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak area for all-*trans*-retinyl acetate from the *Standard solution* and the peak area of all-*trans*-retinyl acetate or all-*trans*-retinyl palmitate from the *Sample solution*.
Calculate the percentage of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$), in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of the all-*trans*-retinyl ester from the *Sample solution*
r_S = peak area of the all-*trans*-retinyl ester from the *Standard solution*
C_S = concentration of retinyl acetate ($C_{22}H_{32}O_2$) from USP Vitamin A RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of vitamin A, as retinol ($C_{20}H_{30}O$), in the *Sample solution* (µg/mL)
F = factor used to convert retinyl acetate, the ester form present in USP Vitamin A RS, to retinol, 0.872

[NOTE—Account for the initial extraction volume of 26.5 mL of 2,2,4-trimethylpentane to calculate the nominal concentration. The molar responses of retinyl acetate and retinyl palmitate are equivalent.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$)

• **VITAMIN A, *Method 3***

[NOTE—Where a vitamin A ester (retinyl acetate or retinyl palmitate) is indicated in the following procedure, use the chemical form present in the formulation. USP Vitamin A RS is retinyl acetate. It is to be used where USP Vitamin A RS is specified. Use low-actinic glassware throughout this procedure.]

Extraction solvent: *n*-Hexane and methylene chloride (3:1)

Potassium hydroxide solution: 800 mg/mL of potassium hydroxide in water. [NOTE—Cautiously add potassium hydroxide in water. Mix, and cool.]

Diluent: 10 mg/mL of pyrogallol in alcohol

Mobile phase: *n*-Hexane and isopropyl alcohol (92:8)

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Standard stock solution: 30 µg/mL of retinyl acetate from USP Vitamin A RS in *Diluent*. [NOTE—This solution may be stored in a refrigerator for 1 week.]

Standard solution: Dilute a volume of *Standard stock solution* with *Diluent* to obtain a concentration of 1 µg/mL of retinyl acetate from USP Vitamin A RS. Transfer 10.0 mL of this solution to a stoppered 125-mL flask, and add 5 mL of water, 5 mL of *Diluent*, and 3 mL of *Potassium hydroxide solution*. Insert the stopper tightly, shake for 15 min over a water bath maintained at 60 ± 5°, and cool to room temperature. Add 7 mL of water and 25.0 mL of *Extraction solvent*. Insert the stopper tightly, and shake vigorously for 60 s. Rinse the sides of the flask with 60 mL of water, and allow to stand for 10 min until the layers separate. Withdraw a portion of the organic layer for injection into the chromatograph. This *Standard solution* contains 0.34 µg/mL of retinol.

Sample solution: Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to 1.5 mg of retinyl acetate, to a stoppered 125-mL flask. Add 5 mL of water, 15 mL of *Diluent*, and 3 mL of *Potassium hydroxide solution*. Insert the stopper tightly, shake for 15 min over a water bath maintained at 60 ± 5°, and cool to room temperature. Add 7 mL of water and 25.0 mL of *Extraction solvent*. Insert the stopper tightly, and shake vigorously for 60 s or longer, if necessary, for complete extraction. Rinse the sides of the flask with 60 mL of water, and allow to stand for 10 min until the layers separate. [NOTE—Do not shake, because an emulsion may form.] Withdraw a portion of the organic layer, and dilute quantitatively, and stepwise if necessary, with *Extraction solvent* to obtain a concentration of 0.34 µg/mL of retinol.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 335 nm
Column: 6.2-mm × 8-cm; packing L3
Column temperature: 40°
Flow rate: 4 mL/min
Injection volume: 50 µL

System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for 13-*cis*-retinol and all-*trans*-retinol are about 0.92 and 1.0, respectively.]
Suitability requirements
Relative standard deviation: NMT 5.0%

Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas for all-*trans*-retinol and 13-*cis*-retinol.
Calculate the percentage of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$), in the portion of Capsules taken:

$$\text{Result} = (r_{T1}/r_{T2}) \times (C_S/C_U) \times F \times 100$$

r_{T1} = sum of the areas of the all-*trans*-retinol and 13-*cis*-retinol peaks from the *Sample solution*
r_{T2} = sum of the areas of all-*trans*-retinol and 13-*cis*-retinol peaks from the *Standard solution*
C_S = concentration of retinyl acetate ($C_{23}H_{32}O_2$) from USP Vitamin A RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of vitamin A, as retinol ($C_{20}H_{30}O$), in the *Sample solution* (µg/mL)
F = factor used to convert retinyl acetate, the ester form present in USP Vitamin A RS, to retinol, 0.872

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$)

• **CHOLECALCIFEROL** or **ERGOCALCIFEROL (VITAMIN D),** ***Method 1***

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Mobile phase: *n*-Hexane and isopropyl alcohol (99:1)

Standard solution: 2 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in *n*-hexane

System suitability solution: Heat a volume of the *Standard solution* at 60° for 1 h to partially isomerize vitamin D (cholecalciferol or ergocalciferol) to its corresponding precursor.

Sample solution: Transfer NLT 20 mL of the solution prepared as directed for the *Sample stock solution* in *Vitamin A, Method 1* to a suitable container, and evaporate, if necessary, under vacuum at room temperature to obtain a solution with an expected concentration of 2 µg/mL of cholecalciferol or ergocalciferol.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 265 nm
Column: 4.6-mm × 15-cm; 3-µm packing L8
Flow rate: 1 mL/min
Injection volume: 100 µL

System suitability
Samples: *Standard solution* and *System suitability solution*
Suitability requirements
Resolution: NLT 10 between the vitamin D form present and its corresponding precursor, *System suitability solution*
Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas for vitamin D. Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*
r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*
C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)
F = correction factor to account for the average amount of previtamin D present in the *Sample solution*, 1.09

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Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

• **CHOLECALCIFEROL** or **ERGOCALCIFEROL (VITAMIN D),** ***Method 2***

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

3 N methanolic sulfuric acid solution, Sodium ascorbate–pyrogallol solution, Lecithin solution, and **Sample solution:** Proceed as directed in *Vitamin A, Method 2.*

Mobile phase: *n*-Hexane and tertiary butyl alcohol (98.75: 1.25)

Standard solution: 1 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in 2,2,4-trimethylpentane

System suitability solution: Heat a volume of the *Standard solution* at 60° for 1 h to partially isomerize vitamin D (cholecalciferol or ergocalciferol) to its corresponding precursor.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability.*)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 25-cm; 5-µm packing L24

Flow rate: 1 mL/min

Injection volume: 40 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 4.0 between the vitamin D form present and its corresponding precursor, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for vitamin D. Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*

r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*

C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

• **CHOLECALCIFEROL** or **ERGOCALCIFEROL (VITAMIN D),** ***Method 3***

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Diluted acetic acid: Glacial acetic acid solution (1 in 10) in water

Phenolphthalein solution: 10 mg/mL of phenolphthalein in alcohol

Potassium hydroxide solution: Slowly dissolve 14 g of potassium hydroxide in a mixture of 31 mL of dehydrated alcohol and 5 mL of water. Prepare fresh daily.

Extraction solvent: Methylene chloride and isopropyl alcohol (99.8: 0.2)

Mobile phase: Acetonitrile and methanol (91:9)

Standard stock solution: 0.2 mg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in dehydrated alcohol. [NOTE—Prepare fresh every 4 weeks. Store in a freezer.]

Standard solution: [NOTE—Condition the solid-phase extraction column specified for use in the *Standard solution* and the *Sample solution* by initially washing the column with 4.0 mL of a mixture of methylene chloride and isopropyl alcohol (4:1), followed by 5.0 mL of *Extraction solvent.* Do not allow the column to dry.] Dilute a volume of *Standard stock solution* with dehydrated alcohol to obtain a concentration of 5 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS. Prepare this solution fresh daily. Transfer 2.0 mL of this solution to a stoppered 125-mL flask. Add 15.0 mL of water and 15.0 mL of *Potassium hydroxide solution,* insert the stopper, and shake for 30 min in a water bath maintained at 60°. Allow to cool to room temperature, and transfer the contents of the flask to a 250-mL separatory funnel. Add 15.0 mL of water to the flask, insert the stopper, shake vigorously, and transfer this solution to the separatory funnel. Rinse the flask with 60 mL of *n*-hexane, and transfer the rinsing to the separatory funnel. Insert the stopper, shake vigorously for 90 s, and allow to stand for 15 min until the layers separate. Drain, and discard the aqueous layer. Add 15.0 mL of water to the hexane layer in the separatory funnel, insert the stopper, and shake vigorously. Allow to stand for 10 min until the layers separate, and discard the aqueous layer. Add 1 drop of *Phenolphthalein solution* and 15.0 mL of water to the separatory funnel. Add *Diluted acetic acid* dropwise, with shaking, until the washing is neutral. Allow to stand for 10 min until the layers separate. Drain, and discard the aqueous layer. Filter the hexane layer through anhydrous sodium sulfate supported by a small pledget of cotton into a 100-mL round-bottom flask. Rinse the funnel and sodium sulfate with a few mL of *n*-hexane, and collect the rinsings in the same flask. Evaporate the hexane in the flask on a rotary evaporator at 50° to dryness. Immediately add 2.0 mL of *Extraction solvent* to dissolve the residue. Transfer this solution to a freshly conditioned solid-phase extraction column containing silica packing with a sorbent mass-to-column volume ratio of 500 mg to 2.8 mL or equivalent, rinse the round-bottom flask with 1.0 mL of *Extraction solvent,* and transfer to the column. Elute the column with 2.0 mL of *Extraction solvent,* and discard this fraction. Elute the column with 7.0 mL of *Extraction solvent,* and collect the eluate in a suitable flask. Place the flask in a warm water bath maintained at 42°, and evaporate the solvent with the aid of a stream of nitrogen. Immediately add 2.0 mL of acetonitrile to the residue, and use the solution for injection into the chromatograph.

Sample solution: Proceed as directed in *Vitamin A, Method 3,* through "calculate the net weight of the Capsule contents." Transfer a portion of the Capsule contents, equivalent to 10 µg of cholecalciferol or ergocalciferol, to a stoppered 125-mL flask, and proceed as directed for the *Standard solution,* beginning with "Add 15.0 mL of water and 15.0 mL of *Potassium hydroxide solution*".

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability.*)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

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Column temperature: 27°
Flow rate: 0.7 mL/min
Injection volume: 15 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 4.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas for vitamin D. Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*
r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*
C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

• **VITAMIN E, *Method 1***

[NOTE—Where vitamin E (alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Solution A: Phosphoric acid solution (1 in 100) in water
Mobile phase: Methanol and *Solution A* (19:1)
System suitability solution: Prepare a 0.65-mg/mL solution of USP Ergocalciferol RS in methanol. Transfer 1.0 mL of this solution to a 100-mL volumetric flask containing 100 mg of USP Alpha Tocopheryl Acetate RS. Dissolve in 30 mL of methanol, with the aid of sonication if necessary, and dilute with methanol to volume. Store this solution in a refrigerator.
Standard solution: 2 mg/mL of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, or USP Alpha Tocopheryl Acid Succinate RS in methanol
Sample solution: Transfer NLT 20 mL of the solution prepared as directed for the *Sample stock solution* in *Vitamin A, Method 1* to a suitable container, and evaporate, under vacuum at room temperature to dryness. Transfer the contents of the flask to a suitable volumetric flask with the aid of methanol, and dilute with methanol to volume, to obtain a solution with an expected concentration of 2 mg/mL of alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 8-mm × 10-cm; 5-µm packing L1
Flow rate: 2 mL/min
Injection volume: 100 µL
System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The relative retention times for ergocalciferol and alpha tocopheryl acetate are about 0.5 and 1.0, respectively.]
Suitability requirements
Resolution: NLT 12 between ergocalciferol and alpha tocopheryl acetate, *System suitability solution*
Tailing factor: Between 0.8 and 1.2, *System suitability solution*
Relative standard deviation: NMT 3.0%, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas. Calculate the percentage of the labeled amount of alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*
r_S = peak area of the relevant vitamin E form from the *Standard solution*
C_S = concentration of the corresponding USP Reference Standard in the *Standard solution* (mg/mL)
C_U = nominal concentration of the corresponding form of vitamin E in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$)

• **VITAMIN E, *Method 2***

[NOTE—Where vitamin E (alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Mobile phase: Mix 240 mL of methanol with 10 mL of water, followed by 0.5 mL of 50% phosphoric acid, and dilute with acetonitrile to 1000 mL.
System suitability solution: 2 mg/mL each of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, and USP Alpha Tocopheryl Acid Succinate RS in methanol
Standard solution: 2 mg/mL of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, or USP Alpha Tocopheryl Acid Succinate RS in methanol
3 N methanolic sulfuric acid solution: Cautiously mix sulfuric acid and methanol (9 in 100) in a 100-mL volumetric flask. [NOTE—Dissolve in a portion of methanol, cool, and then dilute to final volume.]
Sodium ascorbate–pyrogallol solution: Transfer 10 g of sodium ascorbate and 5 g of pyrogallol to a 100-mL volumetric flask. Add sufficient water to dissolve. Add 1.7 mL of sulfuric acid, and dilute with water to volume.
Lecithin solution: 5 mg/mL of lecithin in 2,2,4-trimethylpentane
Sample solution: Proceed as directed in *Vitamin A, Method 2*, through "calculate the net weight of the Capsule contents." Transfer a portion of the Capsule contents, equivalent to 55 mg of vitamin E, to a container with a polytef-lined screw cap. Add 0.5 g of sodium bicarbonate, 1.5 mL of *Lecithin solution*, and 12.5 mL of 2,2,4-trimethylpentane, and disperse on a vortex mixer. Add 6 mL of *Sodium ascorbate–pyrogallol solution*, shake slowly, and allow the solution to degas. Continue shaking until the evolution of gas has ceased, and then shake for an additional 12 min. Add 6 mL of dimethyl sulfoxide, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 6 mL of *3 N methanolic sulfuric acid solution*, mix on a

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vortex mixer to form a suspension, and shake for 12 min. Add 12.5 mL of 2,2,4-trimethylpentane, mix on a vortex mixer to form a suspension, and shake for 10 min. Centrifuge for 10 min to break up the emulsion and to clarify the supernatant layer. Transfer a volume of the supernatant 2,2,4-trimethylpentane layer to a suitable volumetric flask, the volume of the specimen withdrawn from the 2,2,4-trimethylpentane layer and the size of the volumetric flask being such that the final concentration of the *Sample solution* is equivalent to that of the *Standard solution*. Evaporate nearly to dryness, add several mL of methanol, and evaporate the remaining 2,2,4-trimethylpentane. Dilute with methanol to volume.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 280 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Flow rate: 1.5 mL/min
Injection volume: 25 µL

System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The relative retention times for alpha tocopheryl acid succinate, alpha tocopherol, and alpha tocopheryl acetate are about 0.6, 0.8, and 1.0, respectively.]
Suitability requirements
Resolution: NLT 4.0 between alpha tocopheryl acid succinate and alpha tocopherol and NLT 3.0 between alpha tocopherol and alpha tocopheryl acetate, *System suitability solution*
Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas. Calculate the percentage of the labeled amount of alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*
r_S = peak area of the relevant vitamin E form from the *Standard solution*
C_S = concentration of the corresponding USP Reference Standard in the *Standard solution* (mg/mL)
C_U = nominal concentration of the corresponding form of vitamin E in the *Sample solution* (mg/mL)

[NOTE—Account for the initial extraction volume of 26.5 mL of 2,2,4-trimethylpentane and the dilution factor to exchange the solvent from 2,2,4-trimethylpentane to methanol to calculate the nominal concentration.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$)

• **VITAMIN E, *Method 3***
Diluent: Acetonitrile and ethyl acetate (1:1)
Mobile phase: Methanol, acetonitrile, and *n*-hexane (46.5:46.5:7.0)
Standard solution: 0.3 mg/mL of USP Alpha Tocopherol RS in methanol
Sample solution: Proceed as directed in *Vitamin A, Method 3*, through "calculate the net weight of the Capsule contents." Transfer a portion of the Capsule contents, equivalent to 8.0 mg of alpha tocopherol, to a glass-stoppered conical flask. Add 25.0 mL of water, 25.0 mL of dehydrated alcohol, and 3.5 g of potassium hydroxide pellets. Shake for 1 h in a water bath maintained at 55°. Cool, and transfer with the aid of a minimum volume of water to a 125-mL separatory funnel. Rinse the flask with 50 mL of *n*-hexane, and add the rinsing to the separatory funnel. Insert the stopper, shake vigorously for 60 s, and allow the layers to separate. Drain the aqueous layer into a second 250-mL separatory funnel, and repeat the extraction with 50 mL of *n*-hexane. Discard the aqueous layer, and combine the hexane extracts. Wash the combined extracts with 25 mL of water, allow the layers to separate, and discard the aqueous layer. Add 3 drops of glacial acetic acid, and repeat the washing procedure two more times. Filter the washed hexane layer through anhydrous sodium sulfate into a 250-mL round-bottom flask. Rinse the funnel and sodium sulfate with a few mL of *n*-hexane, and add the rinsing to the hexane solution in the flask. Place the flask in a water bath maintained at 50°, and evaporate the hexane solution with the aid of a rotary evaporator to dryness. Immediately add 25.0 mL of *Diluent*, and swirl to dissolve the residue.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 291 nm
Column: 4.6-mm × 25-cm; packing L1
Column temperature: 40°
Flow rate: 3 mL/min
Injection volume: 20 µL

System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 5.0%

Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas. Calculate the percentage of the labeled amount of vitamin E, as alpha tocopherol ($C_{29}H_{50}O_2$), in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of alpha tocopherol from the *Sample solution*
r_S = peak area of alpha tocopherol from the *Standard solution*
C_S = concentration of alpha tocopherol in the *Standard solution* (mg/mL)
C_U = nominal concentration of vitamin E, as alpha tocopherol, in the *Sample solution* (mg/mL)

[NOTE—Calculate the alpha tocopherol equivalent ($C_{29}H_{50}O_2$) of alpha tocopheryl acetate ($C_{31}H_{52}O_3$) or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$) by multiplying their contents by the factors 0.91 or 0.81, respectively.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin E

• **PHYTONADIONE**
[NOTE—Use low-actinic glassware throughout this procedure.]
Mobile phase: Methanol and water (19:1)
Standard stock solution: 200 µg/mL of USP Phytonadione RS in methanol. Dissolve with the aid of sonication if necessary.
System suitability solution: 0.65 mg/mL of USP Alpha Tocopheryl Acetate RS and 20 µg/mL of USP Phytonadione RS from *Standard stock solution* diluted with

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methanol. [NOTE—Dissolve USP Alpha Tocopheryl Acetate RS in a portion of methanol, add the *Standard stock solution*, and then dilute with methanol to volume.]

Standard solution: 20 µg/mL of USP Phytonadione RS from *Standard stock solution* diluted with methanol

Sample solution: Transfer NLT 20 mL of the solution prepared as directed for the *Sample stock solution* in *Vitamin A, Method 1* to a suitable container, and evaporate, if necessary, under vacuum at room temperature to dryness. Transfer the contents of the flask to a suitable volumetric flask with the aid of methanol, and dilute with methanol to volume to obtain a solution with an expected concentration of 20 µg/mL of phytonadione.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 8-mm × 10-cm; 5-µm packing L1

Flow rate: 2.0 mL/min

Injection volume: 100 µL

System suitability

Samples: *System suitability solution* and *Standard solution*

[NOTE—The relative retention times for alpha tocopheryl acetate and phytonadione are about 0.68 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 5 between alpha tocopheryl acetate and phytonadione, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas. Calculate the percentage of the labeled amount of phytonadione ($C_{31}H_{46}O_2$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Phytonadione RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of phytonadione in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of phytonadione ($C_{31}H_{46}O_2$)

• **BETA CAROTENE**

[NOTE—Use low-actinic glassware throughout this procedure.]

Potassium hydroxide solution: Dissolve 58.8 g of potassium hydroxide in 50 mL of water.

Iodine solution: Transfer 10 mg of iodine to a 100-mL volumetric flask. Dissolve in cyclohexane, and dilute with cyclohexane to volume. Dilute 10 mL of this solution with cyclohexane to 100 mL. [NOTE—Prepare this solution fresh daily.]

Sample solution A (for preparations containing beta carotene in oil solutions): Proceed as directed in *Vitamin A, Method 1*, except use cyclohexane instead of *n*-hexane as the extraction solvent, and dilute the filtered extracts with cyclohexane, to obtain a concentration of 2 µg/mL of beta carotene.

Sample solution B (for preparations containing beta carotene in dry powder): Remove the contents of NLT 20 Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the Capsule contents, equivalent to 2 mg of beta carotene, to a 500-mL saponification flask. Add 100 mL of alcohol, 6 mL of *Potassium hydroxide solution*, and a magnetic stirring bar. Attach an air condenser to the flask, and heat under reflux for 45 min with constant stirring. Cool to room temperature. Add 170 mL of solvent hexane, and stir for 30 min. Quantitatively transfer the contents of the flask to a 500-mL separatory funnel with portions of solvent hexane. Allow the layers to separate for 5–10 min, and transfer the upper organic layer to a 500-mL volumetric flask. Transfer the lower aqueous layer into the saponification flask. Add 170 mL of solvent hexane, and stir for an additional 20 min. Quantitatively transfer the contents of the saponification flask to the separatory funnel with the aid of portions of solvent hexane. Allow the layers to separate for 10 min. Drain the lower aqueous layer, and discard. Transfer the organic layer to the volumetric flask containing the previously collected organic layer. Rinse the separatory funnel with small portions of solvent hexane, and transfer the washings to the volumetric flask. Dilute the hexane extracts with solvent hexane to volume. Add 3 g of anhydrous sodium sulfate, shake, and allow to settle. Quantitatively transfer a volume of this solution, equivalent to 100 µg of beta carotene, to a 50-mL volumetric flask. Evaporate under a stream of nitrogen to dryness, and immediately add cyclohexane. Add 2 mL of *Iodine solution*, and heat for 15 min in a water bath maintained at 65°. Cool rapidly, and dilute with cyclohexane to volume.

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Mode: Vis

Analytical wavelength: 452 nm

Blank: Cyclohexane

Analysis

Sample: *Sample solution*

Determine the absorbance against the *Blank*. Calculate the percentage of the labeled amount of beta carotene ($C_{40}H_{56}$) in the portion of Capsules taken:

$$\text{Result} = (A_U/F) \times (100/C_U)$$

A_U = absorbance of the *Sample solution*

F = absorptivity of beta carotene at 452 nm, 223

C_U = nominal concentration of beta carotene in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of beta carotene ($C_{40}H_{56}$)

• **ASCORBIC ACID, CALCIUM ASCORBATE,** and **SODIUM ASCORBATE**

(See *Vitamin C Assay* ⟨580⟩.)

[NOTE—For labeling purposes, consider *Method I—Titrimetric Method* as *Method 1*.]

Acceptance criteria: 90.0%–150.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$) or its salts as calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$) or sodium ascorbate ($C_6H_7NaO_6$)

• **BIOTIN, *Method 1***

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Mix 85 mL of acetonitrile, 1 g of sodium perchlorate, and 1 mL of phosphoric acid, and dilute with water to 1000 mL.

Standard stock solution: 0.333 mg/mL of USP Biotin RS in dimethyl sulfoxide

Standard solution: 5 µg/mL of USP Biotin RS prepared by diluting the *Standard stock solution* in water

Sample solution: Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without the loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing, if necessary, with several portions of ether.

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Discard the washings, and dry the Capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the average net weight per Capsule. Transfer a portion of the Capsule contents, equivalent to 1 mg of biotin, to a 200-mL volumetric flask. Add 3 mL of dimethyl sulfoxide, and swirl to wet the contents. Place the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with water to volume, and filter.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 200 nm
Column: 4.6-mm × 15-cm; 3-µm packing L7
Flow rate: 1.2 mL/min
Injection volume: 100 µL

System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 3.0%

Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas of biotin. Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of biotin from the *Sample solution*
r_S = peak area of biotin from the *Standard solution*
C_S = concentration of USP Biotin RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of biotin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

• **BIOTIN, *Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Dehydrated mixtures yielding formulations similar to the media described herein may be used provided that, when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described herein.

Standard stock solution: 50 µg/mL of USP Biotin RS in 50% alcohol. Store this solution in a refrigerator.

Standard solution: 0.1 ng/mL of USP Biotin RS in water, prepared by dilution of the *Standard stock solution* with water on the day of the assay

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." Transfer a portion of the Capsule contents, equivalent to 100 µg of biotin, to a 200-mL volumetric flask. Add 3 mL of 50% alcohol, and swirl to wet the contents. Heat the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with 50% alcohol to volume, and filter. Dilute a volume of the filtrate quantitatively, and stepwise if necessary, with water to obtain a solution with a concentration of 0.1 ng/mL.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and dilute with water to 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature NLT 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water. Heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and dilute with water to 1000 mL. Store under toluene in a cool place at a temperature NLT 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and dilute with water to 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Calcium pantothenate solution: 10 µg/mL of calcium pantothenate in 50% alcohol. Store in a refrigerator.

Riboflavin–thiamine hydrochloride solution: 20 µg/mL of riboflavin and 10 µg/mL of thiamine hydrochloride in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

***p*-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution:** 10 µg/mL of *p*-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid, and mix. Store under toluene.

Basal medium stock solution: Dissolve the *anhydrous Dextrose* and *anhydrous Sodium acetate* in the solutions previously mixed according to *Table 1*, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Table 1

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Calcium pantothenate solution	5 mL
Riboflavin–thiamine hydrochloride solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water. Add 500 mg of *anhydrous Dextrose*, 500 mg of *anhydrous Sodium acetate*, and 1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to test tubes, close or cover the tubes, sterilize in an autoclave at 121° for 15 min, and allow the tubes to cool in an upright position. Prepare stab cultures in three or

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more of the tubes, using a pure culture of *Lactobacillus plantarum*,[1] incubating for 16–24 h at a temperature of between 30° and 37° held constant to within ±0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution* add 5.0 mL of water containing 0.5 ng of biotin. Plug the tubes with cotton, sterilize in an autoclave at 121° for 15 min, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Transfer cells from the *Stock culture of Lactobacillus plantarum* to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature of between 30° and 37° held constant to within ±0.5°. The cell suspension so obtained is the *Inoculum*.

Analysis

Samples: *Standard solution* and *Sample solution*

To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool. Add 1 drop of *Inoculum* to each tube, except two of the four tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature of between 30° and 37° held constant to within ±0.5° until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of Standard during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to a spectrophotometer cell. Place the cell in a spectrophotometer that has been set at a specific wavelength of 540–660 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittance (Σ_S) as the difference, $y = 2.00 - \Sigma_S$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_U$, adding together the two transmittances (Σ_U) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X, for each dosage level. Average the values of X for each of three or more dosage levels to obtain $\overline{X}$, which equals the log-relative potency, M', of the *Sample solution*.

Determine the quantity, in µg, of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Capsules taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of µg of biotin assumed to be present in the portion of the Capsules taken

Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of the Capsules taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of biotin in the portion of Capsules taken (µg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, $\overline{M}$, is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* ⟨111⟩, *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

• **CYANOCOBALAMIN, *Method 1***

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Methanol and water (7:13)

Standard stock solution: 10 µg/mL of USP Cyanocobalamin (Crystalline) RS in water. [NOTE—Store this stock solution in a dark place, and discard after 1 week.]

Standard solution: 1 µg/mL of USP Cyanocobalamin (Crystalline) RS from *Standard stock solution* diluted with water

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." Transfer a portion of the Capsule contents, equivalent to 100 µg of cyanocobalamin, to a 250-mL flask. Quantitatively, add 100.0 mL of water, and carefully extract for 2 min. Filter 10 mL of the extract, and use the clear filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: 550 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Flow rate: 0.5 mL/min

Injection volume: 200 µL

System suitability

Sample: *Standard solution*

[1] ATCC No. 8014 is suitable. This strain was formerly known as *Lactobacillus arabinosus* 17-5.

DS Monographs

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Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak area of cyanocobalamin. Calculate the percentage of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the *Sample solution*

r_S = peak area of the *Standard solution*

C_S = concentration of USP Cyanocobalamin (Crystalline) RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cyanocobalamin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$)

• **CYANOCOBALAMIN, *Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Standard stock solution: 1.0 µg/mL of USP Cyanocobalamin (Crystalline) RS in 25% alcohol. Store in a refrigerator.

Standard solution: Dilute a suitable volume of *Standard stock solution* with water to a measured volume such that after the incubation period as described in the *Analysis*, the difference in transmittance between the inoculated blank and the 5.0-mL level of the *Standard solution* is NLT that which corresponds to a difference of 1.25 mg in dried cell weight. This concentration usually falls between 0.01 and 0.04 ng/mL of the *Standard solution*. Prepare this solution fresh for each assay.

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." Transfer a portion of the Capsule contents, equivalent to 1.0 µg of cyanocobalamin, to an appropriate vessel containing, for each g of Capsule contents taken, 25 mL of an aqueous extracting solution prepared just before use to contain 12.9 mg/mL of dibasic sodium phosphate, 11.0 mg/mL of anhydrous citric acid, and 10 mg/mL of sodium metabisulfite. Autoclave the mixture at 121° for 10 min. Allow any undissolved particles of the extract to settle, and filter or centrifuge, if necessary. Dilute an aliquot of the clear solution with water to obtain a final solution containing vitamin B_{12} activity approximately equivalent to that of the *Standard solution*.

Acid-hydrolyzed casein solution: Prepare as directed in *Calcium Pantothenate, Method 2*.

Asparagine solution: Dissolve 2.0 g of L-asparagine in water to make 200 mL. Store under toluene in a refrigerator.

Adenine–guanine–uracil solution: Prepare as directed in *Calcium Pantothenate, Method 2*.

Xanthine solution: Suspend 0.20 g of xanthine in 30–40 mL of water, heat to 70°, add 6.0 mL of 6 N ammonium hydroxide, and stir until the solid is dissolved. Cool, and dilute with water to 200 mL. Store under toluene in a refrigerator.

Salt solution A: Dissolve 10 g of monobasic potassium phosphate and 10 g of dibasic potassium phosphate in water to make 200 mL, and add 2 drops of hydrochloric acid. Store this solution under toluene.

Salt solution B: Dissolve 4.0 g of magnesium sulfate, 0.20 g of sodium chloride, 0.20 g of ferrous sulfate, and 0.20 g of manganese sulfate in water to make 200 mL. Add 2 drops of hydrochloric acid. Store this solution under toluene.

Polysorbate 80 solution: Dissolve 20 g of polysorbate 80 in alcohol to make 200 mL. Store in a refrigerator.

Vitamin solution A: Dissolve 10 mg of riboflavin, 10 mg of thiamine hydrochloride, 100 µg of biotin, and 20 mg of niacin in 0.02 N acetic acid to make 400 mL. Store under toluene, protected from light, in a refrigerator.

Vitamin solution B: Dissolve 20 mg of *p*-aminobenzoic acid, 10 mg of calcium pantothenate, 40 mg of pyridoxine hydrochloride, 40 mg of pyridoxal hydrochloride, 8 mg of pyridoxamine dihydrochloride, and 2 mg of folic acid in a mixture of water and neutralized alcohol (3:1) to make 400 mL. Store, protected from light, in a refrigerator.

Basal medium stock solution: Prepare the medium according to the following formula and directions. A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium comparable to that obtained from the formula given herein.

Add the ingredients in the order listed in *Table 2*, carefully dissolving *Cystine* and *Tryptophan* in the *hydrochloric acid* before adding the next eight solutions to the resulting solution. Add 100 mL of water, and dissolve the *Dextrose*, *Sodium acetate*, and *Ascorbic acid*. Filter, if necessary. Add the *Polysorbate 80 solution*, adjust with 1 N sodium hydroxide to a pH of 5.5–6.0, and dilute with Purified Water to 250 mL.

Table 2

L-Cystine	0.1 g
L-Tryptophan	0.05 g
1 N hydrochloric acid	10 mL
Adenine–guanine–uracil solution	5 mL
Xanthine solution	5 mL
Vitamin solution A	10 mL
Vitamin solution B	10 mL
Salt solution A	5 mL
Salt solution B	5 mL
Asparagine solution	5 mL
Acid-hydrolyzed casein solution	25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Ascorbic acid	1 g
Polysorbate 80 solution	5 mL

Tomato juice preparation: Centrifuge commercially canned tomato juice so that most of the pulp is removed. Suspend 5 g/L of analytical filter aid in the supernatant, and pass, with the aid of reduced pressure, through a layer of the filter aid. Repeat, if necessary, until a clear, straw-colored filtrate is obtained. Store under toluene in a refrigerator.

Culture medium: [NOTE—A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium equivalent to that obtained from the formula given herein.] Dissolve 0.75 g of yeast extract, 0.75 g of dried peptone, 1.0 g of anhydrous dextrose, and 0.20 g of monobasic potassium phosphate in 60–70 mL of water. Add 10 mL of *Tomato juice preparation* and 1 mL of *Polysorbate 80 solution*. Adjust with 1 N sodium hydroxide to a pH of 6.8, and add water to make 100 mL. Place 10-mL portions of the solution in test tubes, and plug with cotton. Sterilize the tubes and contents in an autoclave at 121° for

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15 min. Cool as rapidly as possible to avoid color formation resulting from overheating the medium.

Suspension medium: Dilute a measured volume of *Basal medium stock solution* with an equal volume of water. Place 10-mL portions of the diluted medium in test tubes. Sterilize, and cool as directed for *Culture medium*.

Stock culture of *Lactobacillus leichmannii*: To 100 mL of *Culture medium* add 1.0–1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Place 10-mL portions of the hot solution in test tubes, cover the tubes, sterilize at 121° for 15 min in an autoclave, and allow the tubes to cool in an upright position. Inoculate three or more of the tubes by stab transfer of a pure culture of *Lactobacillus leichmannii*[2] [NOTE—Before first using a fresh culture in this assay, make NLT 10 successive transfers of the culture in a 2-week period.] Incubate for 16–24 h at a temperature of between 30° and 40° held constant to within ±0.5°. Store in a refrigerator.

Prepare fresh stab cultures at least three times each week, and do not use them for preparing the *Inoculum* if more than 4 days old. The activity of the microorganism can be increased by daily or twice-daily transfer of the stab culture, to the point where definite turbidity in the liquid *Inoculum* can be observed 2–4 h after inoculation. A slow-growing culture seldom gives a suitable response curve and may lead to erratic results.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus leichmannii* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Transfer cells from the *Stock culture of Lactobacillus leichmannii* to two sterile tubes containing 10 mL each of the *Culture medium*. Incubate these cultures for 16–24 h at a temperature of between 30° and 40° held constant to within ±0.5°. Under aseptic conditions centrifuge the cultures, and decant the supernatant. Suspend the cells from the culture in 5 mL of sterile *Suspension medium*, and combine. Using sterile *Suspension medium*, adjust the volume so that a 1-in-20 dilution in saline TS produces 70% transmittance when read on a suitable spectrophotometer that has been set at a wavelength of 530 nm, equipped with a 10-mm cell, and read against saline TS set at 100% transmittance. Prepare a 1-in-400 dilution of the adjusted suspension using sterile *Basal medium stock solution*. [NOTE—This dilution may be altered, when necessary, to obtain the desired test response.] The cell suspension so obtained is the *Inoculum*.

Calibration of spectrophotometer: Check the wavelength of the spectrophotometer periodically, using a standard wavelength cell or other suitable device. Before reading any tests, calibrate the spectrophotometer for 0% and 100% transmittance, using water, and with the wavelength set at 530 nm.

Analysis

Samples: *Standard solution* and *Sample solution*

Because of the high sensitivity of the test organism to minute amounts of vitamin B_{12} activity and to traces of many cleansing agents, cleanse meticulously by suitable means, followed preferably by heating at 250° for 2 h, using hard-glass 20-mm × 150-mm test tubes and other necessary glassware.

To separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each of these tubes and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*. To each tube add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes to prevent bacterial contamination, and sterilize in an autoclave at 121° for 5 min, arranging to reach this temperature in NMT 10 min by preheating the autoclave if necessary. Cool as rapidly as possible to avoid color formation resulting from overheating the medium. Take precautions to maintain uniformity of sterilizing and cooling conditions throughout the assay, because packing the tubes too closely in the autoclave or overloading it may cause variation in the heating rate.

Aseptically add 0.5 mL of *Inoculum* to each tube so prepared, except two of the four containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature of between 30° and 40°, held constant to within ±0.5°, for 16–24 h.

Terminate growth by heating to a temperature NLT 80° for 5 min. Cool to room temperature. After agitating its contents, read the transmittance at 530 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of the inoculated blank. If the difference is greater than 5% or if there is evidence of contamination with a foreign microorganism, disregard the results of the assay.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of each of the remaining tubes. Disregard the results of the assay if the slope of the standard curve indicates a problem with sensitivity.

Calculation: Prepare a standard concentration-response curve by the following procedure. Test for and replace any aberrant individual transmittances. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittances (Σ_S) as the difference, $y = 2.00 - \Sigma_S$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_U$, adding together the two transmittances (Σ_U) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X, for each dosage level. Average the values of X for each of three or more dosage levels to obtain $\overline{X}$, which equals the log-relative potency, M', of the *Sample solution*.

Determine the quantity, in µg, of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$) in the portion of Capsules taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of µg of cyanocobalamin assumed to be present in each mg in the portion of Capsules taken

Calculate the percentage of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$) in the portion of the Capsules taken:

[2] Pure cultures of *Lactobacillus leichmannii* (listed as *Lactobacillus delbruekii*) may be obtained as No. 7830 from ATCC, 10801 University Blvd., Manassas, VA 20110-2209 (www.atcc.org).

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Result = [(antilog M)/N] × 100

N = nominal amount of cyanocobalamin in the portion of Capsules taken (µg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, $\overline{M}$, is the assayed log-potency of the test material (see *Vitamin B_{12} Activity* in *Design and Analysis of Biological Assays* ⟨111⟩, *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$)

• **Folic Acid, *Method 1***

[Note—Use low-actinic glassware throughout this procedure.]

Reagent A: 25% solution of tetrabutylammonium hydroxide in methanol

Reagent B: Transfer 5.0 g of pentetic acid to a 50-mL volumetric flask. Using sonication if necessary, dissolve in and dilute with 1 N sodium hydroxide to volume.

Mobile phase: 2 g of monobasic potassium phosphate in 650 mL of water. Add 12.0 mL of *Reagent A*, 7.0 mL of 3 N phosphoric acid, and 240 mL of methanol. Cool to room temperature, adjust with phosphoric acid or ammonia TS to a pH of 7.0, dilute with water to 1000 mL, and filter. Recheck the pH before use by adding water or methanol to the prepared *Mobile phase* to obtain baseline separation of folic acid and the internal standard. The pH may be increased up to 7.15 to obtain better separation. [Note—The methanol and water content may be varied (1%–3%).]

Internal standard solution: Transfer 40 mg of methylparaben to a 1000-mL volumetric flask, and add 220 mL of methanol to dissolve. Dissolve 2.0 g of monobasic potassium phosphate in 300 mL of water in a separate beaker, quantitatively transfer this solution to the flask containing the methylparaben solution, and add an additional 300 mL of water. Add 19 mL of *Reagent A*, 7 mL of 3 N phosphoric acid, and 30 mL of *Reagent B*. Adjust with ammonia TS to a pH of 9.8, bubble nitrogen through the solution for 30 min, dilute with water to volume, and mix.

Standard solution: 0.016 mg/mL of USP Folic Acid RS in *Internal standard solution*

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." Transfer an amount of Capsule contents to a suitable centrifuge tube, and add a volume of *Internal standard solution* to obtain a nominal concentration of 0.016 mg/mL of folic acid. Shake by mechanical means for 10 min, and centrifuge. Filter a portion of the clear supernatant, and use the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 3.9-mm × 30-cm; packing L1

Flow rate: 1 mL/min

Injection volume: 15 µL

System suitability

Sample: *Standard solution*

[Note—The relative retention times for folic acid and methylparaben are about 0.8 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for folic acid and methylparaben. Calculate the percentage of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$) in the portion of Capsules taken:

Result = (R_U/R_S) × (C_S/C_U) × 100

R_U = peak area ratio of folic acid to methylparaben from the *Sample solution*

R_S = peak area ratio of folic acid to methylparaben from the *Standard solution*

C_S = concentration of USP Folic Acid RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of folic acid in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$)

• **Folic Acid, *Method 2***

[Note—Use low-actinic glassware throughout this procedure.]

Reagent: Dissolve 7.5 g of edetate disodium, with stirring, in 500 mL of water containing 10 mL of ammonium hydroxide.

Diluent: 60 µg/mL of ammonium hydroxide

Mobile phase: Transfer 0.4 mL of triethylamine, 15 mL of glacial acetic acid, and 350 mL of methanol to a 2000-mL volumetric flask, and dilute with 0.008 M sodium 1-hexanesulfonate to volume.

Standard stock solution: 60 µg/mL of USP Folic Acid RS in *Diluent*. Prepare this solution fresh daily.

Standard solution: Mix 5.0 mL of *Standard stock solution* with 10.0 mL of methanol and 35.0 mL of *Reagent*. Shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the net weight of the Capsule contents." Transfer a portion of the Capsule contents, equivalent to 0.3 mg of folic acid, to a 125-mL stoppered flask. Add 10.0 mL of methanol and 35.0 mL of *Reagent*. Shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 270 nm

Column: 4.6-mm × 25-cm; packing L7

Column temperature: 50°

Flow rate: 2 mL/min

Injection volume: 5 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the areas of the major peaks. Calculate the percentage of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$) in the portion of Capsules taken:

Result = (r_U/r_S) × (C_S/C_U) × 100

r_U = peak area of folic acid from the *Sample solution*

r_S = peak area of folic acid from the *Standard solution*

C_S = concentration of USP Folic Acid RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of folic acid in the *Sample solution* (µg/mL)

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Acceptance criteria: 90.0%–150.0% of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$)

• **DEXPANTHENOL or PANTHENOL**

[NOTE—The following procedure is applicable also to the determination of the dextrorotatory component of racemic panthenol in preparations containing panthenol.]

Dehydrated mixtures yielding formulations similar to the media described herein may be used provided that, when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described herein.

Standard stock solution: 800 µg/mL of USP Dexpanthenol RS or 1600 µg/mL of USP Racemic Panthenol RS in water. Store in a refrigerator, protected from light, and use within 30 days.

Standard solution: On the day of the assay, prepare a dilution of 1.2 µg/mL of dexpanthenol or 2.4 µg/mL of panthenol from *Standard stock solution* diluted with water.

Sample solution: Weigh NLT 30 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents as completely as possible to a beaker. Remove any contents adhering to the empty Capsule shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the average net weight per Capsule. Dissolve a portion of the Capsule contents, nominally equivalent to 1.2 mg of dexpanthenol or 2.4 mg of panthenol, in 100.0 mL of water. Quantitatively dilute a portion of this solution with water to obtain a concentration of 1.2 µg/mL of dexpanthenol or 2.4 µg/mL of panthenol.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in about 500 mL of water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature NLT 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to 75 ± 5°, and add hydrochloric acid solution (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and dilute with water to make 1000 mL. Store under toluene in a cool place at a temperature NLT 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin–thiamine hydrochloride–biotin solution: 20 µg/mL of riboflavin, 10 µg/mL of thiamine hydrochloride, and 0.04 µg/mL of biotin in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

***p*-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution:** 10 µg/mL of *p*-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in neutral 25% alcohol. Store in a refrigerator.

Salt solution A: 50 mg/mL of monobasic potassium phosphate and 50 mg/mL of dibasic potassium phosphate in water. Add 10 drops of hydrochloric acid per L of solution. Store under toluene.

Salt solution B: 20 mg/mL of magnesium sulfate, 1 mg/mL of sodium chloride, 1 mg/mL of ferrous sulfate, and 1 mg/mL of manganese sulfate in water. Add 10 drops of hydrochloric acid per L of the solution. Store under toluene.

Pyridoxal–calcium pantothenate solution: 200 µg/mL of pyridoxal hydrochloride and 1.875 µg/mL of calcium pantothenate in 10% alcohol. Store in a refrigerator, and use within 30 days.

Polysorbate 40–oleic acid solution: 50 mg/mL of polysorbate 40 and 0.5 mg/mL of oleic acid in 20% alcohol. Store in a refrigerator, and use within 30 days.

Modified pantothenate medium: Dissolve *anhydrous Dextrose* and *Sodium acetate* in the solutions previously mixed according to *Table 3*, and adjust with 1 N sodium hydroxide to a pH of 6.8. Finally, dilute with water to 250 mL.

Table 3

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Riboflavin–thiamine hydrochloride–biotin solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL
Pyridoxal–calcium pantothenate solution	5 mL
Polysorbate 40–oleic acid solution	5 mL

Double-strength modified pantothenate medium: Prepare as directed in *Modified pantothenate medium*, but make the final dilution to 125 mL instead of 250 mL. Prepare fresh.

Stock culture of *Pediococcus acidilactici*: Dissolve in 800 mL of water, with the aid of heat, 6.0 g of peptone, 4.0 g of pancreatic digest of casein, 3.0 g of yeast extract, 1.5 g of beef extract, 1.0 g of dextrose, and 15.0 g of agar. Adjust with 0.1 N sodium hydroxide or 0.1 N hydrochloric acid to a pH of 6.5–6.6, and dilute with water to 1000 mL. Add 10-mL portions of the solution to culture tubes, place caps on the tubes, and sterilize in an autoclave at 121° for 15 min. Cool on a slant, and store in a refrigerator. Prepare a stock culture of *Pediococcus acidilactici*[3] on a slant of this medium. Incubate at 35° for 20–24 h, and store in a refrigerator. Maintain the stock culture by monthly transfer onto fresh slants.

Inoculum: Inoculate three 250-mL portions of *Modified pantothenate medium* from a stock culture slant, and incubate at 35° for 20–24 h. Centrifuge the suspension from the combined portions, and wash the cells with sterile *Modified pantothenate medium*. Resuspend the cells in sufficient *Modified pantothenate medium* so that a 1-in-50 dilution, when tested in a 13-mm diameter test tube, gives 80% light transmission at 530 nm. Transfer 1.2-mL portions of this stock suspension to glass ampuls, seal, freeze in liquid nitrogen, and store in a freezer. On the day of the assay, allow the ampuls to reach room temperature, mix the

[3] ATCC No. 8042 is suitable.

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contents, and dilute 1 mL of thawed culture with sterile saline TS to 150 mL. [NOTE—This dilution may be altered when necessary to obtain the desired test response.]

Analysis: Prepare in triplicate a series of eight culture tubes by adding the following quantities of water to the tubes within a set: 5.0, 4.5, 4.0, 3.5, 3.0, 2.0, 1.0, and 0.0 mL. To these same tubes and in the same order add 0.0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. Prepare in duplicate a series of five culture tubes by adding the following quantities of water to the tubes within a set: 4.0, 3.5, 3.0, 2.0, and 1.0 mL. To these same tubes and in the same order add 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*.

Add 5.0 mL of *Double-strength modified pantothenate medium* to each tube. Cover the tubes with metal caps, and sterilize in an autoclave at 121° for 5 min. Cool to room temperature in a chilled water bath, and inoculate each tube with 0.5 mL of the *Inoculum*. Allow to incubate at 37° for 16 h. Terminate growth by heating to a temperature NLT 80°, such as by steaming at atmospheric pressure in a sterilizer for 5–10 min. Cool, and determine the percentage transmittance of the suspensions, in cells of equal pathlength, on a suitable spectrophotometer, at a wavelength of 530 nm.

Calculation: Draw a dose-response curve on arithmetic graph paper by plotting the average response, in percentage of transmittance, for each set of tubes of the standard curve against the standard level concentrations. The curve is drawn by connecting each adjacent pair of points with a straight line. From this standard curve, determine by interpolation the potency of each tube containing portions of the *Sample solution*. To obtain the individual responses, divide the potency of each tube by the amount of the *Sample solution* added to it. Calculate the mean response by averaging the individual responses that vary from their mean by NMT 15%, using NLT half the total number of tubes. Calculate the potency of the portion of the material taken for the assay by multiplying the mean response by the appropriate dilution factor.

Calculate the percentage of the labeled amount of dexpanthenol or panthenol ($C_9H_{19}NO_4$) in the portion of Capsules taken:

$$\text{Result} = (P/N) \times 100$$

P = potency of dexpanthenol or panthenol in the portion of Capsules taken (mg)

N = nominal amount of dexpanthenol or panthenol in the portion of Capsules taken (mg)

Acceptance criteria: 90.0%–150.0% of the labeled amount of dexpanthenol or panthenol ($C_9H_{19}NO_4$)

• **CALCIUM PANTOTHENATE, *Method 1***

Mobile phase: Phosphoric acid and water (1:1000)

Internal standard solution: 80 mg of *p*-hydroxybenzoic acid in 3 mL of alcohol. Add 50 mL of water and 7.1 g of dibasic sodium phosphate, and dilute with water to 1000 mL. Adjust with phosphoric acid to a pH of 6.7.

Standard solution: 0.6 mg/mL of USP Calcium Pantothenate RS in *Internal standard solution*

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." To a centrifuge tube transfer an amount of mixed Capsule contents and a volume of *Internal standard solution* to obtain a concentration of 0.6 mg/mL in the *Sample solution*.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 210 nm

Column: 3.9-mm × 15-cm; packing L1

Flow rate: 1.5 mL/min

Injection volume: 10 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for calcium pantothenate and *p*-hydroxybenzoic acid are about 0.5 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of calcium pantothenate and the internal standard. Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Capsules taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak area ratio of calcium pantothenate to *p*-hydroxybenzoic acid from the *Sample solution*

R_S = peak area ratio of calcium pantothenate to *p*-hydroxybenzoic acid from the *Standard solution*

C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• **CALCIUM PANTOTHENATE, *Method 2***

Standard stock solution: Dissolve 50 mg of USP Calcium Pantothenate RS, previously dried and stored in the dark over phosphorus pentoxide and protected from absorption of moisture while weighing, in 500 mL of water in a 1000-mL volumetric flask. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), and dilute with water to volume, to obtain a concentration of 50 µg/mL of USP Calcium Pantothenate RS. Store under toluene in a refrigerator.

Standard solution: On the day of the assay, dilute a volume of *Standard stock solution* with water to obtain a concentration of 0.01–0.04 µg/mL of calcium pantothenate, the exact concentration being such that the responses obtained as directed in the *Analysis*, 2.0 and 4.0 mL of the *Standard solution* being used, are within the linear portion of the log-concentration response curve.

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." Transfer a portion of the Capsule contents, equivalent to an amount of 50 mg of calcium pantothenate, to a 1000-mL volumetric flask containing 500 mL of water. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), dilute with water to volume, and filter. Dilute a volume of this solution to obtain a solution with approximately the same concentration as that of the *Standard solution*.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and dilute with water to 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature NLT 10°. Filter the solution if a precipitate forms during storage.

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Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and dilute with water to 1000 mL. Store under toluene in a cool place at a temperature NLT 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and dilute with water to 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin–thiamine hydrochloride–biotin solution: 20 µg/mL of riboflavin, 10 µg/mL of thiamine hydrochloride, and 0.04 µg/mL of biotin in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

***p*-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution:** 10 µg/mL of *p*-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Basal medium stock solution: Dissolve the *anhydrous Dextrose* and *anhydrous Sodium acetate* in the solutions previously mixed according to *Table 4*, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Table 4

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Riboflavin–thiamine hydrochloride–biotin solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water. Add 500 mg of *anhydrous Dextrose*, 500 mg of *anhydrous Sodium acetate*, and 1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to the test tubes, close or cover the tubes, sterilize in an autoclave at 121° for 15 min, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*, [1] incubating for 16–24 h at a temperature of 30°–37° held constant to within ±0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution* add 5.0 mL of water containing 0.2 µg of calcium pantothenate. Plug the tubes with cotton, sterilize in an autoclave at 121° for 15 min, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Transfer cells from the *Stock culture of Lactobacillus plantarum* to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature of between 30° and 37° held constant to within ±0.5°. The cell suspension so obtained is the *Inoculum*.

Analysis

Samples: *Standard solution* and *Sample solution*

To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool, and add 1 drop of *Inoculum* to each tube, except two of the four tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature of between 30° and 37°, held constant to within ±0.5° until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of Standard during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to an optical container if necessary. Read the transmittance between 540 and 660 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittance (Σ_S) as the difference, $y = 2.00 - \Sigma_S$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_U$, adding together the two transmittances (Σ_U) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume,

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in mL, of the *Sample solution* to obtain the difference, X, for each dosage level. Average the values of X for each of three or more dosage levels to obtain $\overline{X}$, which equals the log-relative potency, M', of the *Sample solution*. Determine the quantity, in mg, of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Capsules taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of mg of calcium pantothenate assumed to be present in the portion of the Capsules taken

Calculate the percentage of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Capsules taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of calcium pantothenate in the portion of the Capsules taken (mg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, $\overline{M}$, is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* ⟨111⟩, *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• **CALCIUM PANTOTHENATE, *Method 3***

Buffer solution: Dissolve 10.0 g of monobasic potassium phosphate in 2000 mL of water, and adjust with phosphoric acid to a pH of 3.5.

Mobile phase: Methanol and *Buffer solution* (1:9)

Standard stock solution: 0.25 mg/mL of USP Calcium Pantothenate RS in water. Prepare fresh every 4 weeks. Store in a refrigerator.

Standard solution: 40 µg/mL of USP Calcium Pantothenate RS from *Standard stock solution* diluted with water

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the net weight of the Capsule contents." Transfer a portion of the Capsule contents, equivalent to 10 mg of calcium pantothenate, to a 250-mL volumetric flask. Add 10 mL of methanol, and swirl the flask to disperse the Capsules contents. Dilute with water to volume, mix, and filter.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 205 nm

Column: 3.9-mm × 30-cm; 5-µm packing L1

Column temperature: 50°

Flow rate: 2 mL/min

Injection volume: 25 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for calcium pantothenate. Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• **NIACIN OR NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, RIBOFLAVIN,** and **THIAMINE, *Method 1***

[NOTE—Use low-actinic glassware throughout this procedure.]

Diluent: Acetonitrile, glacial acetic acid, and water (5:1:94)

Mobile phase: A mixture of methanol, glacial acetic acid, and water (27:1:73) containing 140 mg of sodium 1-hexanesulfonate per 100 mL

Standard solution: [NOTE—Use USP Niacin RS in place of USP Niacinamide RS for formulations containing niacin.] Transfer 80 mg of USP Niacinamide RS, 20 mg of USP Pyridoxine Hydrochloride RS, 20 mg of USP Riboflavin RS, and 20 mg of USP Thiamine Hydrochloride RS to a 200-mL volumetric flask, and add 180 mL of *Diluent*. Immerse the flask in a hot water bath maintained at 65°–70° for 10 min with regular shaking or using a vortex mixer, until all the solid materials are dissolved. Chill rapidly in a cold water bath for 10 min to room temperature, and dilute with *Diluent* to volume.

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." Transfer a portion of the Capsule contents, equivalent to 10 mg of niacinamide and 2.5 mg each of pyridoxine hydrochloride, riboflavin, and thiamine hydrochloride to a 50-mL centrifuge tube. Add 25.0 mL of *Diluent*, and mix using a vortex mixer for 30 s to completely suspend the powder. Immerse the centrifuge tube in a hot water bath maintained at 65°–70°, heat for 5 min, and mix on a vortex mixer for 30 s. Return the tube to the hot water bath, heat for another 5 min, and mix on a vortex mixer for 30 s. Filter a portion of the solution, cool to room temperature, and use the clear filtrate. [NOTE—Use the filtrate within 3 h of filtration.]

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 3.9-mm × 30-cm; packing L1

Flow rate: 1 mL/min

Injection volume: 10 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for niacinamide, pyridoxine, riboflavin, and thiamine are about 0.3, 0.5, 0.8, and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for niacin or niacinamide, pyridoxine, riboflavin, and thiamine. Calculate the percentage of the labeled amount of niacinamide ($C_6H_6N_2O$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacinamide from the *Sample solution*

r_S = peak area of niacinamide from the *Standard solution*

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C_S = concentration of USP Niacinamide RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of niacinamide in the *Sample solution* (mg/mL)

For formulations containing niacin:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacin from the *Sample solution*
r_S = peak area of niacin from the *Standard solution*
C_S = concentration of USP Niacin RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of niacin in the *Sample solution* (mg/mL)

Separately calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the corresponding vitamin from the *Sample solution*
r_S = peak area of the corresponding vitamin from the *Standard solution*
C_S = concentration of the relevant USP Reference Standard in the *Standard solution* (mg/mL)
C_U = nominal concentration of the corresponding vitamin in the *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of thiamine from the *Sample solution*
r_S = peak area of thiamine from the *Standard solution*
C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL)
M_{r1} = molecular weight of thiamine mononitrate, 327.36
M_{r2} = molecular weight of thiamine hydrochloride, 337.27

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine as thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$)

• **NIACIN, *Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A: Transfer 1 mL of glacial acetic acid and 2.5 g of edetate disodium to a 100-mL volumetric flask. Dissolve in and dilute with water to volume.

Extraction solvent: *Solution A* and methanol (3:1)

Mobile phase: 0.1 M sodium acetate solution (13.6 mg/mL of sodium acetate in water). Adjust with acetic acid to a pH of 5.4. [NOTE—A small amount of methanol (up to 1%) may be added to the *Mobile phase* to improve resolution.]

Standard stock solution: 1 mg/mL of USP Niacin RS in *Extraction solvent*

Standard solution: Transfer 5.0 mL of *Standard stock solution* to a 25-mL volumetric flask and dilute with *Extraction solvent* to volume.

Sample solution: [NOTE—This preparation is suitable for the determination of niacin or niacinamide, pyridoxine, and riboflavin, when present in the formulation.] Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents to a beaker. Remove any contents adhering to the shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to 2 mg of riboflavin, to a 200-mL volumetric flask. If riboflavin is not present in the formulation, use a portion equivalent to a nominal amount of 2 mg of pyridoxine. If pyridoxine is not present in the formulation, use a portion equivalent to 20 mg of niacin or niacinamide. Add 100.0 mL of *Extraction solvent*, and mix for 20 min, using a wrist-action shaker. Immerse the flask in a water bath maintained at 70°–75°, and heat for 20 min. Mix on a vortex mixer for 30 s, cool to room temperature, and filter. Use the clear filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection volume: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

[NOTE—If necessary, flush the column with methanol between injections.]

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of niacin. Calculate the percentage of the labeled amount of niacin ($C_6H_5NO_2$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacin from the *Sample solution*
r_S = peak area of niacin from the *Standard solution*
C_S = concentration of USP Niacin RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of niacin in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacin ($C_6H_5NO_2$)

• **NIACINAMIDE, *Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A, Extraction solvent, Mobile phase, Standard stock solution, Standard solution, Sample solution, Chromatographic system, and **System suitability solution:** Using USP Niacinamide RS in place of USP Niacin RS, proceed as directed in *Niacin, Method 2*.

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of niacinamide. Calculate the percentage of the labeled amount of niacinamide ($C_6H_6N_2O$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

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r_U = peak area of niacinamide from the *Sample solution*
r_S = peak area of niacinamide from the *Standard solution*
C_S = concentration of USP Niacinamide RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of niacinamide in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacinamide ($C_6H_6N_2O$)

• **PYRIDOXINE HYDROCHLORIDE, *Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Extraction solvent, Mobile phase, and **Sample solution:** Prepare as directed in *Niacin, Method 2*.

Standard stock solution: 0.1 mg/mL of USP Pyridoxine Hydrochloride RS in *Extraction solvent*

Standard solution: Transfer 20 µg/mL of USP Pyridoxine Hydrochloride RS from *Standard stock solution*, and dilute with *Extraction solvent* to volume.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; packing L1
Flow rate: 1 mL/min
Injection volume: 20 µL

System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 3.0%

Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas of pyridoxine. Calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of pyridoxine from the *Sample solution*
r_S = peak area of pyridoxine from the *Standard solution*
C_S = concentration of USP Pyridoxine Hydrochloride RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of pyridoxine hydrochloride in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$)

• **RIBOFLAVIN, *Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Extraction solvent and **Sample solution:** Prepare as directed in *Niacin, Method 2*.

Solution A: 6.8 g/L of sodium acetate in water

Mobile phase: Prepare a mixture of *Solution A* and methanol (13:7). Add 2 mL of triethylamine per L of the mixture, and adjust with glacial acetic acid to a pH of 5.2.

Standard stock solution: Transfer 20 mg of USP Riboflavin RS to a 200-mL volumetric flask, and add 180 mL of *Extraction solvent*. Immerse the flask for 5 min in a water bath maintained at 65°–75°. Mix well, and repeat if necessary until dissolved. Chill rapidly in a cold water bath to room temperature, and dilute with *Extraction solvent* to volume.

Standard solution: Dilute 5.0 mL of *Standard stock solution* with *Extraction solvent* to 25.0 mL.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; packing L1
Flow rate: 1 mL/min
Injection volume: 20 µL

System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 3.0%

Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas of riboflavin. Calculate the percentage of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of riboflavin from the *Sample solution*
r_S = peak area of riboflavin from the *Standard solution*
C_S = concentration of USP Riboflavin RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of riboflavin in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$)

• **THIAMINE, *Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A: 1.88 g/L of sodium 1-hexanesulfonate in 0.1% phosphoric acid

Mobile phase: *Solution A* and acetonitrile (46:9)

Standard stock solution: 0.1 mg/mL of USP Thiamine Hydrochloride RS in 0.2 N hydrochloric acid

Standard solution: 0.02 mg/mL of USP Thiamine Hydrochloride RS from *Standard stock solution* diluted with 0.2 N hydrochloric acid

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the net weight of the Capsule contents." Mix a portion of the Capsule contents with a volume of 0.2 N hydrochloric acid to obtain a concentration of 0.02 mg/mL of thiamine. Shake for 15 min with a wrist-action shaker, and heat to boiling for 30 min. Cool to room temperature, and filter. Use the clear filtrate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; packing L1
Flow rate: 2 mL/min
Injection volume: 20 µL

System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 3.0%

Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas for the major peaks. For products containing thiamine hydrochloride, calculate the percentage of the labeled amount of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of thiamine from the *Sample solution*
r_S = peak area of thiamine from the *Standard solution*

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C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of thiamine hydrochloride in the *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of thiamine from the *Sample solution*
r_S = peak area of thiamine from the *Standard solution*
C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL)
M_{r1} = molecular weight of thiamine mononitrate, 327.36
M_{r2} = molecular weight of thiamine hydrochloride, 337.27

Acceptance criteria: 90.0%–150.0% of the labeled amount of thiamine as thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$)

• **NIACIN OR NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, RIBOFLAVIN,** and **THIAMINE,** ***Method 3***

[NOTE—Use low-actinic glassware throughout this procedure.]

Reagent: 25 mg/mL of edetate disodium in water

Mobile phase: Transfer 0.4 mL triethylamine, 15.0 mL of glacial acetic acid, and 350 mL of methanol to a 2000-mL volumetric flask. Dilute with 0.008 M sodium 1-hexanesulfonate to volume.

Standard stock solution: 1.5 mg/mL of USP Niacin RS or USP Niacinamide RS, 0.24 mg/mL of USP Pyridoxine Hydrochloride RS, 0.08 mg/mL of USP Riboflavin RS, and 0.24 mg/mL of USP Thiamine Hydrochloride RS in the *Reagent*, with heating if necessary

Standard solution: Transfer 5.0 mL of *Standard stock solution* to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the net weight of the Capsule contents." Transfer a portion of the Capsule contents, equivalent to 7.5 mg of niacin or niacinamide, 1.2 mg of pyridoxine hydrochloride, 0.4 mg of riboflavin, and 1.2 mg of thiamine hydrochloride, to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 270 nm
Column: 4.6-mm × 25-cm; packing L7
Column temperature: 50°
Flow rate: 2.0 mL/min
Injection volume: 5 µL

System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 2.0%

Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas of niacin or niacinamide. Calculate the percentage of the labeled amount of niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacin or niacinamide from the *Sample solution*
r_S = peak area of niacin or niacinamide from the *Standard solution*
C_S = concentration of USP Niacin RS or USP Niacinamide RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of niacin or niacinamide in the *Sample solution* (mg/mL)

Separately calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine ($C_{12}H_{17}ClN_4OS$) (for products containing thiamine hydrochloride) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the corresponding vitamin from the *Sample solution*
r_S = peak area of the corresponding vitamin from the *Standard solution*
C_S = concentration of the relevant USP Reference Standard in the *Standard solution* (mg/mL)
C_U = nominal concentration of the corresponding vitamin in the *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of thiamine from the *Sample solution*
r_S = peak area of thiamine from the *Standard solution*
C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL)
M_{r1} = molecular weight of thiamine mononitrate, 327.36
M_{r2} = molecular weight of thiamine hydrochloride, 337.27

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacinamide ($C_6H_6N_2O$) or niacin ($C_6H_5NO_2$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine as thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$)

[NOTE—Commercially available atomic absorption standard solutions for the minerals, where applicable, may be used where preparation of a *Standard stock solution* is described in the following assays. Use deionized water where water is specified. Where atomic absorption spectrophotometry is specified in the assay, the *Standard solutions* and the *Sample solution* may be diluted quantitatively with the solvent specified, if

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necessary, to yield solutions of suitable concentrations adaptable to the linear or working range of the instrument.]

• **CALCIUM, *Method 1***

Lanthanum chloride solution: 267 mg/mL of lanthanum chloride heptahydrate in 0.125 N hydrochloric acid

Calcium standard solution: 400 µg/mL of calcium. Dissolve 1.001 g of calcium carbonate, previously dried at 300° for 3 h and cooled in a desiccator for 2 h, in 25 mL of 1 N hydrochloric acid. Boil to expel carbon dioxide, and dilute with water to 1000 mL.

Standard stock solution: 100 µg/mL of calcium from *Calcium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Into separate 100-mL volumetric flasks pipet 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, and dilute with water to volume to obtain concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 µg/mL of calcium.

Polysorbate 80 solution: Polysorbate 80 and alcohol (1:10)

Sample solution: Transfer 5 Capsules to a 100-mL volumetric flask. [NOTE—For hard gelatin Capsules, weigh NLT 20 Capsules. Open the Capsules, without loss of shell material, and transfer the contents to a suitable container. Remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and allow the Capsule shells to dry. Weigh the empty Capsule shells, calculate the net weight of the Capsule contents, and transfer a portion of the Capsule contents, equivalent to 5 Capsules, to a 100-mL volumetric flask.] Add 15 mL of water, 10 mL of 6 N hydrochloric acid, and 1 mL of *Polysorbate 80 solution* to the flask. Heat on a hot plate or steam bath, with intermittent swirling, until the Capsules are completely disintegrated or the contents are dissolved. Boil gently for an additional 15 min. Cool, dilute with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute this solution with 0.125 N hydrochloric acid, to obtain a concentration of 2 µg/mL of calcium, adding 1 mL of *Lanthanum chloride solution* per 100 mL of the final volume.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Calcium emission line at 422.7 nm

Lamp: Calcium hollow-cathode

Flame: Nitrous oxide–acetylene

Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution* per 100 mL

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of calcium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in mg/mL, of calcium in the *Sample solution*.

Calculate the percentage of the labeled amount of calcium (Ca) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of calcium in the *Sample solution* (µg/mL)

C_U = nominal concentration of calcium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of calcium (Ca)

• **CHROMIUM, *Method 1***

Chromium standard solution: 1000 µg/mL of chromium from potassium dichromate, previously dried at 120° for 4 h in water. Store in a polyethylene bottle.

Standard stock solution: 10 µg/mL of chromium from *Chromium standard solution* diluted with 6 N hydrochloric acid and water (1 in 20)

Standard solutions: Transfer 10.0 and 20.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks, and transfer 15.0 and 20.0 mL of the *Standard stock solution* to separate 50-mL volumetric flasks. Dilute the contents of each of the four flasks with 0.125 N hydrochloric acid to volume to obtain concentrations of 1.0, 2.0, 3.0, and 4.0 µg/mL of chromium.

Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain 1 µg/mL of chromium and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Chromium emission line at 357.9 nm

Lamp: Chromium hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of chromium, and draw the straight line best fitting the four plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of chromium in the *Sample solution*.

Calculate the percentage of the labeled amount of chromium (Cr) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of chromium in the *Sample solution* (µg/mL)

C_U = nominal concentration of chromium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of chromium (Cr)

• **COPPER, *Method 1***

Copper standard solution: Dissolve 1.00 g of copper foil in a minimum volume of a 50% solution of nitric acid, and dilute with a 1% solution of nitric acid to 1000 mL. This solution contains 1000 µg/mL of copper.

Standard stock solution: 100 µg/mL of copper from *Copper standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 200-mL volumetric flasks transfer 1.0, 2.0, 4.0, 6.0, and 8.0 mL of the *Standard stock solution*. Dilute with water to volume to obtain concentrations of 0.5, 1.0, 2.0, 3.0, and 4.0 µg/mL of copper.

Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain 2 µg/mL of copper and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Copper emission line at 324.7 nm

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Lamp: Copper hollow-cathode
Flame: Air–acetylene
Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of copper, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of copper in the *Sample solution*.

Calculate the percentage of the labeled amount of copper (Cu) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of copper in the *Sample solution* (µg/mL)
C_U = nominal concentration of copper in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of copper (Cu)

• **FLUORIDE, *Method 1***

[NOTE—Store all solutions in plastic containers.]

3 M sodium acetate solution: Dissolve 408 g of sodium acetate in 600 mL of water contained in a 1000-mL volumetric flask. Allow the solution to equilibrate to room temperature, and dilute with water to volume. Adjust with a few drops of acetic acid to a pH of 7.0.

Sodium citrate solution: Dissolve 222 g of sodium citrate in 250 mL of water in a 1000-mL volumetric flask. Add 28 mL of perchloric acid, and dilute with water to volume.

Fluoride standard stock solution: 500 µg/mL of fluoride from a quantity of sodium fluoride, previously dried at 100° for 4 h and cooled in a desiccator, in water

Intermediate stock solution A: 100 µg/mL of fluoride from *Fluoride standard stock solution* diluted with water

Intermediate stock solution B: 10 µg/mL of fluoride from *Fluoride standard stock solution* diluted with water

Standard solutions: To five separate 100-mL volumetric flasks transfer 3.0, 5.0, and 10.0 mL of *Intermediate stock solution B* and 5.0 and 10.0 mL of *Intermediate stock solution A*. To each flask add 10.0 mL of 1 N hydrochloric acid, 25 mL of *3 M sodium acetate solution*, and 25.0 mL of *Sodium citrate solution*. Dilute the contents of each flask with water to volume to obtain concentrations of 0.3, 0.5, 1.0, 5.0, and 10.0 µg/mL of fluoride.

Sample solution: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the mixed Capsule contents, equivalent to 200 µg of fluoride, to a 100-mL volumetric flask. Add 10.0 mL of 1 N hydrochloric acid, 25.0 mL of *3 M sodium acetate solution*, and 25.0 mL of *Sodium citrate solution*. Dilute with water to volume.

Analysis

Samples: *Standard solutions* and *Sample solution*

To separate plastic beakers, each containing a plastic-coated stirring bar, transfer 50.0 mL each of the *Standard solutions* and the *Sample solution*. Measure the potentials (see *pH* ⟨791⟩), in mV, of the *Standard solutions* and the *Sample solution*, with a pH meter capable of a minimum reproducibility of ±0.2 mV and equipped with a fluoride-specific ion-indicating electrode and a calomel reference electrode. [NOTE—When taking measurements, immerse the electrodes in the solution, stir on a magnetic stirrer with an insulated top until equilibrium is attained (1–2 min), and record the potential. Rinse and dry the electrodes between measurements, taking care to avoid damaging the crystal of the specific-ion electrode.]

Plot the logarithms of fluoride concentrations, in µg/mL, of the *Standard solutions* versus potential, in mV. From the standard response curve so obtained and the measured potential of the *Sample solution*, determine the concentration, *C*, in mg/mL, of fluoride in the *Sample solution*.

Calculate the percentage of the labeled amount of fluorine (F) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of fluoride in the *Sample solution* (µg/mL)
C_U = nominal concentration of fluorine in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (F)

• **FLUORIDE, *Method 2***

[NOTE—Use plastic containers and deionized water throughout this procedure.]

pH 10.0 buffer: Add 214 mL of 0.1 N sodium hydroxide to 1000 mL of 0.05 M sodium bicarbonate.

Mobile phase: Alcohol, 0.1 N sulfuric acid, and water (20:5:175)

Standard stock solution: 220 µg/mL of USP Sodium Fluoride RS in water. This solution contains 100 µg/mL of fluoride.

Standard solution: [NOTE—Condition the solid-phase extraction column specified for use in the *Standard solution* and the *Sample solution* in the following manner. Using a vacuum at a pressure not exceeding 5 mm of mercury, wash the column with one column volume of methanol followed by one column volume of *pH 10.0 buffer*. Do not allow the column top to dry. If the top of the column becomes dry, recondition the column.] Transfer 10.0 mL of *Standard stock solution* to a 100-mL volumetric flask. Add 75 mL of water, and adjust with 0.1 N sodium hydroxide to a pH of 10.4 ± 0.1. Dilute with water to volume. Filter, discarding the first 15 mL of the filtrate. Transfer 25.0 mL of the filtrate to a 50-mL volumetric flask, add 15.0 mL of water, and adjust with 0.1 N sodium hydroxide to a pH of 10.0. Dilute with *pH 10.0 buffer* to volume. Elute a portion of this solution through a 3-mL solid-phase extraction column containing L1 packing that is connected through an adaptor to a second solid-phase extraction column containing sulfonylpropyl strong cation-exchange packing. Discard the first 3 mL of the eluate, and collect the rest of the eluate in a suitable flask for injection into the chromatograph.

Sample solution: Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents to a 100-mL container. If necessary, remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to 1 mg of fluorine, to a 100-mL volumetric flask. Add 15 mL of water, and shake vigorously. Rinse the sides of the flask with 15 mL of water, and allow to stand for 10 min. Dilute with water to 85 mL, adjust with 1 N sodium hydroxide to a pH of 10.4 ± 0.1, and dilute with water to 100 mL. Proceed as directed for the *Standard solution*, beginning with "Filter, discarding the first 15 mL of the filtrate."

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Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Conductivity
Columns
Guard: 4.6-mm × 3-cm; packing L17
Analytical: 7.8-mm × 30-cm; packing L17
Flow rate: 0.5 mL/min
Injection volume: 100 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 2.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas of fluoride. Calculate the percentage of the labeled amount of fluorine (F) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*
r_S = peak area from the *Standard solution*
C_S = concentration of fluoride in the *Standard solution* (µg/mL)
C_U = nominal concentration of fluorine in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (F)

• **IODIDE**
Bromine water: To 20 mL of bromine in a glass-stoppered bottle add 100 mL of water. Insert the stopper into the bottle, and shake. Allow to stand for 30 min, and use the supernatant.
Analysis
Sample: Capsules
Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the contents, equivalent to 3 mg of iodine, to a nickel crucible. Add 5 g of sodium carbonate, 5 mL of 50% (w/v) sodium hydroxide solution, and 10 mL of alcohol, taking care that the entire specimen is moistened. Heat the crucible on a steam bath to evaporate the alcohol, then dry the crucible at 100° for 30 min to prevent spattering upon subsequent heating. Transfer the crucible with its contents to a furnace heated to 500°, and heat the crucible for 15 min. [NOTE—Heating at 500° is necessary to carbonize any organic matter present; a higher temperature may be used, if necessary, to ensure complete carbonization of all organic matter.]
Cool the crucible, add 25 mL of water, cover the crucible with a watchglass, and boil gently for 10 min. Filter the solution, and wash the crucible with boiling water, collecting the filtrate and washings in a beaker. Add phosphoric acid until the solution is neutral to methyl orange, then add 1 mL excess of phosphoric acid. Add excess of *Bromine water*, and boil the solution gently until colorless and then for 5 min longer. Add a few crystals of salicylic acid, and cool the solution to 20°. Add 1 mL of phosphoric acid and 0.5 g of potassium iodide, and titrate the liberated iodine with 0.005 N sodium thiosulfate VS, adding starch TS when the liberated iodine color has nearly disappeared.
Calculate the percentage of the labeled amount of iodine (I) in the portion of Capsules taken:

$$\text{Result} = V \times N_A \times F \times Ime \times (Aw/W) \times (100/L)$$

V = volume of sodium thiosulfate consumed (mL)
N_A = actual normality of the sodium thiosulfate solution used
F = correction factor to convert mg to µg, 1000 µg/mL
Ime = milliequivalent of I, 21.16 mg/meq
Aw = average weight of the Capsules content
W = weight of the sample of Capsules content taken
L = labeled amount of iodine (µg/Capsule)

Acceptance criteria: 90.0%–160.0% of the labeled amount of iodine (I)

• **IRON, *Method 1***
Iron standard stock solution: Transfer 100 mg of iron powder to a 1000-mL volumetric flask. Dissolve in 25 mL of 6 N hydrochloric acid, dilute with water to volume, and mix.
Standard solutions: To separate 100-mL volumetric flasks transfer 2.0, 4.0, 5.0, 6.0, and 8.0 mL of *Iron standard stock solution*. Dilute the contents of each flask with water to volume to obtain concentrations of 2.0, 4.0, 5.0, 6.0, and 8.0 µg/mL of iron.
Polysorbate 80 solution: Prepare as directed in *Calcium, Method 1*.
Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain a concentration of 5 µg/mL of iron and omit the use of the *Lanthanum chloride solution*.
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Analytical wavelength: Iron emission line at 248.3 nm
Lamp: Iron hollow-cathode
Flame: Air–acetylene
Blank: 0.125 N hydrochloric acid
Analysis
Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of iron, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C, in µg/mL, of iron in the *Sample solution*.
Calculate the percentage of the labeled amount of iron (Fe) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of iron in the *Sample solution* (µg/mL)
C_U = nominal concentration of iron in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of iron (Fe)

• **MAGNESIUM, *Method 1***
Lanthanum chloride solution: Prepare as directed in *Calcium, Method 1*.
Magnesium standard solution: Transfer 1.0 g of magnesium ribbon to a 1000-mL volumetric flask, dissolve in 50 mL of 6 N hydrochloric acid, dilute with water to volume, and mix to obtain a solution with a known concentration of 1000 µg/mL of magnesium.
Standard stock solution: 20 µg/mL of magnesium from *Magnesium standard solution* diluted with 0.125 N hydrochloric acid
Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, and dilute with 0.125 N hydrochloric acid to

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volume to obtain concentrations of 0.2, 0.3, 0.4, 0.5, and 0.6 µg/mL of magnesium.
Polysorbate 80 solution: Prepare as directed in *Calcium, Method 1*.
Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain 0.4 µg/mL of magnesium.
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Analytical wavelength: Magnesium emission line at 285.2 nm
Lamp: Magnesium hollow-cathode
Flame: Air–acetylene
Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution* per 100 mL
Analysis
Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of magnesium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of magnesium in the *Sample solution*.
Calculate the percentage of the labeled amount of magnesium (Mg) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of magnesium in the *Sample solution* (µg/mL)
C_U = nominal concentration of magnesium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of magnesium (Mg)

• **MANGANESE, *Method 1***
Manganese standard stock solution: Transfer 1.00 g of manganese to a 1000-mL volumetric flask. Dissolve in 20 mL of nitric acid, dilute with 6 N hydrochloric acid to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of manganese.
Standard stock solution: 50 µg/mL of manganese from *Manganese standard stock solution* diluted with 0.125 N hydrochloric acid
Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 3.0, and 4.0 mL of *Standard stock solution*. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions with known concentrations of 0.5, 0.75, 1.0, 1.5, and 2.0 µg/mL of manganese.
Polysorbate 80 solution: Prepare as directed in *Calcium, Method 1*.
Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain to 1 µg/mL of manganese and omit the use of the *Lanthanum chloride solution*.
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Analytical wavelength: Manganese emission line at 279.5 nm
Lamp: Manganese hollow-cathode
Flame: Air–acetylene
Blank: 0.125 N hydrochloric acid
Analysis
Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of manganese, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of manganese in the *Sample solution*.
Calculate the percentage of the labeled amount of manganese (Mn) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of manganese in the *Sample solution* (µg/mL)
C_U = nominal concentration of manganese in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of manganese (Mn)

• **MOLYBDENUM, *Method 1***
Diluent: 20 mg/mL of ammonium chloride in water
Molybdenum standard solution: Transfer 1.0 g of molybdenum wire to a 1000-mL volumetric flask, and dissolve in 50 mL of nitric acid, warming if necessary. Dilute with water to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of molybdenum.
Standard stock solution: 100 µg/mL of molybdenum from *Molybdenum standard solution* diluted with water
Standard solutions: To separate 100-mL volumetric flasks transfer 5.0, 10.0, and 25.0 mL of the *Standard stock solution*, and add 5.0 mL of perchloric acid to each flask. Gently boil the solution in each flask for 15 min. Cool to room temperature, and dilute each with *Diluent* to volume to obtain concentrations of 5.0, 10.0, and 25.0 µg/mL of molybdenum.
Polysorbate 80 solution: Prepare as directed in *Calcium, Method 1*.
Sample solution: Proceed as directed in *Calcium, Method 1*, except take a number of Capsules or a portion of Capsule contents equivalent to 1000 µg of molybdenum and make appropriate dilutions to obtain a final concentration of 10 µg/mL of molybdenum, omitting the addition of the *Lanthanum chloride solution*.
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Analytical wavelength: Molybdenum emission line at 313.3 nm
Lamp: Molybdenum hollow-cathode
Flame: Nitrous oxide–acetylene
Blank: *Diluent* and perchloric acid (20:1)
Analysis
Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of molybdenum, and draw the straight line best fitting the three plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of molybdenum in the *Sample solution*.
Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of molybdenum in the *Sample solution* (µg/mL)
C_U = nominal concentration of molybdenum in the *Sample solution* (µg/mL)

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Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

• **MOLYBDENUM, *Method 2***

Sodium fluoride solution: Add 200 mL of water to 10 g of sodium fluoride, stir until the solution is saturated, and filter. Store in a polyethylene bottle.

Ferrous sulfate solution: 4.98 mg/mL of ferrous sulfate in water

Potassium thiocyanate solution: 200 mg/mL of potassium thiocyanate in water

20% Stannous chloride solution: Transfer 40 mg of stannous chloride to a beaker, add 20 mL of 6.5 N hydrochloric acid solution, and heat the solution until the stannous chloride is dissolved. Cool, and dilute with water to 100 mL.

Diluted stannous chloride solution: *20% Stannous chloride solution* diluted with water (1 in 25). Prepare this solution fresh at the time of use.

Standard solution: 20 µg/mL of molybdenum in water

Sample: Remove the contents of a counted number of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Use a quantity of the Capsule contents, equivalent to 40 µg of molybdenum.

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Mode: UV-Vis

Analytical wavelength: 465 nm

Cell: 1-cm

Blank: Amyl alcohol

Analysis

Samples: *Standard solution* and *Sample*

Transfer the *Sample* and 2.0 mL of the *Standard solution* to separate 200-mL beakers. Add 20 mL of nitric acid to each beaker. Cover each beaker with a watchglass, and boil slowly on a hot plate for 45 min. Cool to room temperature. Add 6 mL of perchloric acid, cover the beakers with a watchglass, and continue the heating until digestion is complete, as indicated when the liquid becomes colorless or pale yellow. Evaporate the solutions in the beakers to dryness. Rinse the sides of the beakers and the watchglasses with water, and dilute with water to 50 mL in each beaker. Gently boil the water solution for a few min. Cool to room temperature. Add 2 drops of methyl orange TS, and neutralize with ammonium hydroxide. Add 8.2 mL of hydrochloric acid. Quantitatively transfer the contents of the beakers to separate 100-mL volumetric flasks, rinse the beakers with water, transfer the rinsings to the corresponding volumetric flasks, and dilute with water to volume. Transfer 50.0 mL of each solution to separatory funnels. To each separatory funnel add 1.0 mL of *Sodium fluoride solution*, 0.5 mL of *Ferrous sulfate solution*, 4.0 mL of *Potassium thiocyanate solution*, 1.5 mL of *20% Stannous chloride solution*, and 15.0 mL of amyl alcohol, and shake the separatory funnel for 1 min. Allow the layers to separate, and discard the aqueous layers. Add 25 mL of *Diluted stannous chloride solution* to each separatory funnel, and shake gently for 15 s. Allow the layers to separate, and discard the aqueous layers. Transfer the organic layer from each separatory funnel to a centrifuge tube, and centrifuge at 2000 rpm for 10 min. Determine the absorbances of the organic phases obtained from the *Standard solution* and the *Sample*, and correct with the *Blank*.

Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Capsules taken:

$$\text{Result} = (A_U/A_S) \times [(V \times C_S)/M_U] \times 100$$

A_U = absorbance of the *Sample*

A_S = absorbance of the *Standard solution*

V = volume of the *Standard solution* analyzed, 2.0 mL

C_S = concentration of molybdenum in the *Standard solution* (µg/mL)

M_U = nominal amount of molybdenum in the *Sample* (µg)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

• **PHOSPHORUS, *Method 1***

Sulfuric acid solution: Cautiously add sulfuric acid to water (37.5: 100), and mix.

Ammonium molybdate solution: 50 mg/mL of ammonium molybdate in *Sulfuric acid solution* and water (2:3). [NOTE—Dissolve in water first, and then dilute with *Sulfuric acid solution* to volume.]

Hydroquinone solution: 5 mg/mL of hydroquinone in water. Add 1 drop of sulfuric acid per 100 mL of solution.

Sodium bisulfite solution: 200 mg/mL of sodium bisulfite in water

Phosphorus standard stock solution: Weigh 4.395 g of monobasic potassium phosphate, previously dried at 105° for 2 h and stored in a desiccator, and transfer to a 1000-mL volumetric flask. Dissolve in water, add 6 mL of sulfuric acid as a preservative, dilute with water to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of phosphorus.

Standard solution: 20 µg/mL of phosphorus from *Phosphorus standard stock solution* diluted with water

Sample solution: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the Capsule contents, equivalent to 100 mg of phosphorus, to 25 mL of nitric acid, and digest on a hot plate for 30 min. Add 15 mL of hydrochloric acid, and continue the digestion to the cessation of brown fumes. Cool, and transfer the contents of the flask to a 500-mL volumetric flask with the aid of small portions of water. Dilute with water to volume. Transfer 10.0 mL of this solution to a 100-mL volumetric flask, and dilute with water to volume.

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Mode: Vis

Analytical wavelength: 650 nm

Cell: 1-cm

Analysis

Samples: *Standard solution* and *Sample solution*

To three separate 25-mL volumetric flasks transfer 5.0 mL each of the *Standard solution*, the *Sample solution*, and water to provide the blank. To each of the three flasks add 1.0 mL each of *Ammonium molybdate solution*, *Hydroquinone solution*, and *Sodium bisulfite solution*, and swirl to mix. Dilute the contents of each flask with water to volume, and allow the flasks to stand for 30 min. Determine the absorbances of the solutions against the blank.

Calculate the percentage of the labeled amount of phosphorus (P) in the portion of Capsules taken:

$$\text{Result} = (A_U/A_S) \times (C_S/C_U) \times 100$$

A_U = absorbance of the *Sample solution*

A_S = absorbance of the *Standard solution*

C_S = concentration of phosphorus in the *Standard solution* (µg/mL)

C_U = nominal concentration of phosphorus in the *Sample solution* (µg/mL)

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Acceptance criteria: 90.0%–125.0% of the labeled amount of phosphorus (P)

• **POTASSIUM**

Potassium standard solution: 100 µg/mL of potassium from potassium chloride, previously dried at 105° for 2 h, in water

Standard stock solution: 10 µg/mL of potassium from *Potassium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Transfer 5.0, 10.0, 15.0, 20.0, and 25.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions containing 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of potassium.

Polysorbate 80 solution: Prepare as directed in *Calcium, Method 1*.

Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain 1 µg/mL of potassium and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Potassium emission line at 766.5 nm

Lamp: Potassium hollow-cathode

Flame: Air–acetylene

Blank: Water

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of potassium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of potassium in the *Sample solution*.

Calculate the percentage of the labeled amount of potassium (K) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of potassium in the *Sample solution* (µg/mL)

C_U = nominal concentration of potassium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of potassium (K)

• **SELENIUM, *Method 1***

Diluent: Prepare as directed in *Molybdenum, Method 1*.

Selenium standard solution: [**CAUTION**—Selenium is toxic; handle it with care.] Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness, add 2 mL of water, and evaporate to dryness. Repeat the addition of water and the evaporation to dryness three times. Dissolve the residue in 3 N hydrochloric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume to obtain a concentration of 1000 µg/mL of selenium.

Standard stock solution: 100 µg/mL of selenium from *Selenium standard solution* diluted with water

Standard solutions: To separate 100-mL volumetric flasks transfer 5.0, 10.0, and 25.0 mL of the *Standard stock solution*, and add 5.0 mL of perchloric acid to each flask. Gently boil the solutions for 15 min, cool to room temperature, and dilute each with *Diluent* to volume to obtain solutions with concentrations of 5.0, 10.0, and 25.0 µg/mL of selenium.

Sample solution: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the Capsule contents, equivalent to 1000 µg of selenium, to a suitable flask, and add 12 mL of nitric acid. [NOTE—The volume of nitric acid may be varied to ensure that the powder is uniformly dispersed.] Carefully swirl the flask to disperse the sample specimen. Sonicate for 10 min or until the sample specimen is completely dissolved. Gently boil the solution for 15 min, and cool to room temperature. Carefully add 8 mL of perchloric acid to the flask, heat the flask until perchloric acid fumes appear, and swirl the flask to dissipate the fumes. Repeat the heating and swirling until the fumes appear again. Cool to room temperature. Transfer the contents of the flask to a 50-mL volumetric flask with the aid of the *Diluent*, and dilute with *Diluent* to volume.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Selenium emission line at 196.0 nm

Lamp: Selenium hollow-cathode

Flame: Air–acetylene

Blank: *Diluent* and perchloric acid (20:1)

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of selenium, and draw the straight line best fitting the three plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of selenium in the *Sample solution*.

Calculate the percentage of the labeled amount of selenium (Se) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of selenium in the *Sample solution* (µg/mL)

C_U = nominal concentration of selenium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of selenium (Se)

• **SELENIUM, *Method 2***

Hydrochloric acid solution: Hydrochloric acid diluted with water (1 in 10)

50% Ammonium hydroxide solution: Ammonium hydroxide diluted with water (1 in 2)

Reagent A: 9 mg/mL of edetate disodium and 25 mg/mL of hydroxylamine hydrochloride in water. [NOTE—Dissolve edetate disodium in a portion of water first, then add hydroxylamine hydrochloride, and dilute with water to volume.]

Reagent B: Transfer 200 mg of 2,3-diaminonaphthalene to a 250-mL separatory funnel, and add 200 mL of 0.1 N hydrochloric acid. Wash the solution with three 40-mL portions of cyclohexane, and discard the cyclohexane layer. Filter the solution into a brown bottle, and cover the solution with a 1-cm layer of cyclohexane. This solution is stable for 1 week if stored in a refrigerator.

Standard stock solution: [**CAUTION**—Selenium is toxic; handle it with care.] Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness, add 2 mL of water, and evaporate to dryness. Repeat the addition of water and evaporation to dryness three times. Dissolve the residue in 3 N hydrochloric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume to obtain a solution with a concentration of

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1000 µg/mL of selenium. Dilute a volume of the solution with 0.125 N hydrochloric acid to obtain a concentration of 2.0 µg/mL of selenium.

Standard solution: Transfer 10 mL of the *Standard stock solution* to a glass-stoppered flask. Add 1 mL of perchloric acid and 1 mL of *Hydrochloric acid solution*, and dilute with water to 20 mL.

Sample solution: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the Capsule contents, equivalent to 20 µg of selenium, to a suitable flask. Add 10 mL of nitric acid, and warm gently on a hot plate. Continue heating until the initial nitric acid reaction has subsided, then add 3 mL of perchloric acid.

[**CAUTION**—Exercise care at this stage, because perchloric acid reaction becomes vigorous.]

Continue heating on the hot plate until the appearance of white fumes of perchloric acid or until the digest begins to darken. Add 0.5 mL of nitric acid, and resume heating, adding additional amounts of nitric acid if further darkening occurs. Digest for 10 min after the first appearance of perchloric acid fumes or until the digest becomes colorless. Cool the flask. Add 2.5 mL of *Hydrochloric acid solution*, and return the flask to the hot plate to expel residual nitric acid. Heat the mixture for 3 min after it begins to boil. Cool the flask to room temperature, and dilute with water to 20 mL.

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Mode: UV

Analytical wavelength: 380 nm

Cell: 1-cm

Blank: 1 mL of perchloric acid and 1 mL of *Hydrochloric acid solution* diluted with water to 20 mL

Analysis

Samples: *Standard solution* and *Sample solution*

Treat the *Sample solution*, the *Standard solution*, and the *Blank* as follows. Add 5 mL of *Reagent A* to each flask, and swirl gently to mix. Adjust the solution in each flask with *50% Ammonium hydroxide solution* to a pH of 1.1 ± 0.1. Add 5 mL of *Reagent B* to each flask, and swirl gently to mix. Place the flasks in a water bath maintained at 50°, and equilibrate for 30 min, taking care that the flasks are covered to protect them from light. Cool to room temperature, and transfer the contents of each flask to separate separatory funnels. Transfer 10.0 mL of cyclohexane to each separatory funnel, and extract vigorously for 1 min. Discard the aqueous layer. Transfer the cyclohexane layer to a centrifuge tube, and centrifuge at 1000 rpm for 1 min to remove any remaining water. Determine the absorbances of the solutions obtained from the *Samples* against the solution obtained from the *Blank*.

Calculate the percentage of the labeled amount of selenium (Se) in the portion of Capsules taken:

$$\text{Result} = (A_U/A_S) \times [(V \times C_S)/M_U] \times 100$$

A_U = absorbances of the cyclohexane layer from the *Sample solution*

A_S = absorbances of the cyclohexane layer from the *Standard solution*

V = volume of the *Standard stock solution* used to prepare the *Standard solution*, 10 mL

C_S = concentration of selenium in the *Standard stock solution* (µg/mL)

M_U = nominal amount of selenium in the *Sample solution* (µg)

Acceptance criteria: 90.0%–160.0% of the labeled amount of selenium (Se)

• **ZINC, *Method 1***

Zinc standard solution: 1000 µg/mL of zinc from zinc oxide in 5 M hydrochloric acid (3.89 mg/mL) and diluted with water to final volume. [NOTE—Dissolve in 5 M hydrochloric acid by warming, if necessary, cool, and then dilute to final volume.]

Standard stock solutions: 50 µg/mL of zinc from *Zinc standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Transfer 1.0, 2.0, 3.0, 4.0, and 5.0 mL of *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of zinc.

Polysorbate 80 solution: Prepare as directed in *Calcium, Method 1*.

Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain 2 µg/mL of zinc and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Zinc emission line at 213.8 nm

Lamp: Zinc hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of zinc, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C, in µg/mL, of zinc in the *Sample solution*.

Calculate the percentage of the labeled amount of zinc (Zn) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of zinc in the *Sample solution* (µg/mL)

C_U = nominal concentration of zinc in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of zinc (Zn)

• **BORON, NICKEL, TIN, AND VANADIUM, *Method 1*; CALCIUM, CHROMIUM, COPPER, IRON, MAGNESIUM, MANGANESE, PHOSPHORUS,** and **ZINC, *Method 2*; MOLYBDENUM AND SELENIUM, *Method 3***

Stock aqua regia solution: Prepare a mixture of hydrochloric acid and nitric acid (3:1) by adding the nitric acid to the hydrochloric acid. [NOTE—Periodically vent the solution in an appropriate fume hood.]

Diluent: Prepare a mixture of *Stock aqua regia solution* and water (1:9) by adding 1 volume of *Stock aqua regia solution* to 2 volumes of water. Dilute with additional water to volume, and mix well.

System suitability solution: Prepare a mixture of 1000 mg/L of yttrium in 5% nitric acid solution, 1000 mg/L of scandium in 5% nitric acid solution, and *Diluent* (1:1:198), and mix.

Standard stock solution 1 (Ca, Cu, Fe, Mg, Mn, P, and Zn) : [NOTE—It is only necessary to include the minerals of interest in the solution.] Using commercially available element standard (single- or multi-element) solutions in 5% nitric acid solution, pipet the appropriate amount of element standard solution into a volumetric flask, and dilute

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with 5% nitric acid solution to obtain a solution having final concentrations of about 1000 mg/L of calcium, 100 mg/L of copper, 250 mg/L of iron, 500 mg/L of magnesium, 100 mg/L of manganese, 800 mg/L of phosphorus, and 250 mg/L of zinc.

Standard stock solution 2 (B, Cr, Mo, Ni, Se, Sn, and V): [NOTE—It is only necessary to include the minerals of interest in the solution.] Using commercially available element standard (single- or multi-element) solutions in 20% hydrochloric acid solution, pipet the appropriate amount of element standard solution into a volumetric flask, and dilute with 20% hydrochloric acid solution to obtain a solution having final concentrations of about 200 mg/L of boron, and 100 mg/L each of chromium, molybdenum, nickel, selenium, tin, and vanadium.

Standard solutions: Prepare a mixture of *Standard stock solution 1* and *Standard stock solution 2*, as required, in *Diluent*, to prepare a six-point calibration curve to bracket the concentration range of each mineral of interest.

Sample solution: Weigh, then transfer 5 Capsules to a 250-mL volumetric flask, and heat gently on a hot plate until the contents begin to release. Cautiously add 25 mL of *Stock aqua regia solution* in 5-mL increments, and swirl. Heat, continue to swirl until the Capsules dissolve into the acid, immediately remove from the heat source, and add 150 mL of water. Cool, and dilute with water to volume. Filter about 30 mL into a centrifuge tube, using a 5-µm pore size nylon syringe filter. If necessary, make any further adjustments using the *Diluent*.

Instrumental conditions

(See *Plasma Spectrochemistry* ⟨730⟩.)

Mode: Inductively coupled plasma spectrometry, using a spectrometer set to measure the emission of each mineral of interest at about the corresponding wavelength. [NOTE—The operating conditions may be developed and optimized based on the manufacturer's recommendation. The wavelengths selected should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision.]

System suitability

[NOTE—Analyze the *System suitability solution*, and obtain the response as directed in the *Analysis*.]

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the emission of each mineral of interest in the *Standard solutions* and *Sample solution* with an inductively coupled plasma system using the *Diluent* as the blank. Plot the emission of the *Standard solutions* versus the concentration, in mg/L, of the minerals of interest, and draw the straight line best fitting the plotted points. From the graph so obtained, determine the concentration, *C*, in mg/L, for each mineral of interest in the *Sample solution*. Calculate the percentage of the labeled amount for each mineral taken:

$$\text{Result} = C \times (V/W) \times F \times (C_W/L) \times 100$$

C = measured concentration of the *Sample solution* (mg/L)
V = volume of the *Sample solution* (L)
W = sample weight (mg)
F = dilution factor of the *Sample solution*
C_W = average Capsule weight (mg)
L = labeled amount per Capsule (mg)

Acceptance criteria: 90.0%–125.0% of the labeled amount of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), and zinc (Zn); and 90.0%–160.0% of the labeled amounts of boron (B), chromium (Cr), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), and vanadium (V)

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** ⟨2040⟩**:** Meet the requirements for *Dissolution*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩**:** Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 3×10^3 cfu/g, and the combined molds and yeasts count does not exceed 3×10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meet the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:**[4] The label states that the product is Oil- and Water-Soluble Vitamins with Minerals Capsules. The label also states the quantity of each vitamin and mineral per dosage unit and, where necessary, the chemical form in which a vitamin is present and also states the salt form of the mineral used as the source of each element. Where the product contains vitamin E, the label indicates whether it is the *d*- or *dl*- form. Where more than one assay method is given for a particular vitamin or mineral, the labeling states with which assay method the product complies only if *Method 1* is not used.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Biotin RS
  USP Calcium Pantothenate RS
  USP Cholecalciferol RS
  USP Cyanocobalamin (Crystalline) RS
  USP Dexpanthenol RS
  USP Ergocalciferol RS
  USP Folic Acid RS
  USP Niacin RS
  USP Niacinamide RS
  USP Phytonadione RS
  USP Pyridoxine Hydrochloride RS
  USP Racemic Panthenol RS
  USP Riboflavin RS
  USP Sodium Fluoride RS
  USP Thiamine Hydrochloride RS

[4] USP Units of activity for vitamins, where such exist or formerly existed, are equivalent to the corresponding international units (IU), where such formerly existed. The USP Unit for Vitamin E has been discontinued. IU for vitamins also have been discontinued; however, the use of IU on the labels of vitamin products continues. Where articles are labeled in terms of Units in addition to the required labeling, the relationship of the USP Units or IU to mass is as follows. One USP Vitamin A Unit = 0.3 µg of all-*trans*-retinol (vitamin A alcohol) or 0.344 µg of all-*trans*-retinyl acetate (vitamin A acetate) or 0.55 µg of all-*trans*-retinyl palmitate (vitamin A palmitate), and 1 µg of retinol (3.3 USP Vitamin A Units) = 1 retinol equivalent (RE); 1 IU of beta carotene = 0.6 µg of all-*trans*-beta carotene; 1 USP Vitamin D Unit = 0.025 µg of cholecalciferol or ergocalciferol; and 1 mg of *dl*-alpha tocopherol = 1.1 former USP Vitamin E Units, 1 mg of *dl*-alpha tocopheryl acetate = 1 former USP Vitamin E Unit, 1 mg of *dl*-alpha tocopheryl acid succinate = 0.89 former USP Vitamin E Unit, 1 mg of *d*-alpha tocopherol = 1.49 former USP Vitamin E Units, and 1 mg of *d*-alpha tocopheryl acetate = 1.36 former USP Vitamin E Units; and 1 mg of *d*-alpha tocopheryl acid succinate = 1.21 former USP Vitamin E Units. In terms of *d*-alpha tocopherol equivalents, 1 mg of *d*-alpha tocopheryl acetate = 0.91, 1 mg of *d*-alpha tocopheryl acid succinate = 0.81, 1 mg of *dl*-alpha tocopherol = 0.74, 1 mg of *dl*-alpha tocopheryl acetate = 0.67, and 1 mg of *dl*-alpha tocopheryl acid succinate = 0.60.

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USP Alpha Tocopherol RS
USP Alpha Tocopheryl Acetate RS
USP Alpha Tocopheryl Acid Succinate RS
USP Vitamin A RS

Oil- and Water-Soluble Vitamins with Minerals Oral Solution

DEFINITION

Oil- and Water-Soluble Vitamins with Minerals Oral Solution contains one or more of the following oil-soluble vitamins: Vitamin A, vitamin D as Ergocalciferol (vitamin D_2) or Cholecalciferol (vitamin D_3), and Vitamin E; one or more of the following water-soluble vitamins: Ascorbic Acid or its equivalent as Calcium Ascorbate or Sodium Ascorbate, Biotin, Cyanocobalamin, Niacin or Niacinamide, Dexpanthenol or Panthenol, pantothenic acid (as Calcium Pantothenate or Racemic Calcium Pantothenate), Pyridoxine Hydrochloride, Riboflavin or Riboflavin-5′-Phosphate Sodium, and Thiamine Hydrochloride or Thiamine Mononitrate; and one or more minerals derived from substances generally recognized as safe, furnishing one or more of the following elements in ionizable form: chromium, fluorine, iodine, iron, magnesium, manganese, molybdenum, and zinc. Oral Solution contains NLT 90.0% and NMT 200.0% of the labeled amounts of vitamin A ($C_{20}H_{30}O$) as retinol or esters of retinol in the form of retinyl acetate ($C_{22}H_{32}O_2$) or retinyl palmitate ($C_{36}H_{60}O_2$), vitamin D as ergocalciferol ($C_{28}H_{44}O$) or cholecalciferol ($C_{27}H_{44}O$), vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$) or alpha tocopheryl acetate ($C_{31}H_{52}O_3$) or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$), ascorbic acid ($C_6H_8O_6$) or its salts as calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$) or sodium ascorbate ($C_6H_7NaO_6$), and thiamine ($C_{12}H_{17}ClN_4OS$) as thiamine hydrochloride or thiamine mononitrate; NLT 90.0% and NMT 150.0% of the labeled amounts of biotin ($C_{10}H_{16}N_2O_3S$), calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), dexpanthenol ($C_9H_{19}NO_4$) or panthenol ($C_9H_{19}NO_4$), niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$) or riboflavin-5′-phosphate sodium ($C_{17}H_{20}N_4NaO_9P$); NLT 90.0% and NMT 450.0% of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$); and NLT 90.0% and NMT 160.0% of the labeled amounts of chromium (Cr), fluorine (F), iodine (I), and molybdenum (Mo); and NLT 90.0% and NMT 125.0% of the labeled amounts of iron (Fe), magnesium (Mg), manganese (Mn), and zinc (Zn).

STRENGTH

[NOTE—In the following assays, where more than one assay method is given for an individual ingredient, the requirements may be met by following any one of the specified methods, the method used being stated in the labeling only if *Method 1* is not used.]

• **VITAMIN A**

[NOTE—Use low-actinic glassware throughout this procedure.]

Diluent: Tetrahydrofuran and acetonitrile (1:1)

Mobile phase: Methanol, acetonitrile, and *n*-hexane (46.5: 46.5: 7.0)

Standard solution: 0.33 mg/mL of retinol ($C_{20}H_{30}O$) from USP Vitamin A RS in *Diluent*. [NOTE—USP Vitamin A RS is retinyl acetate. Use it to analyze Oral Solution that contains vitamin A as retinol, retinyl acetate, or retinyl palmitate.]

Sample solution: Transfer an accurately measured volume of Oral Solution, equivalent to 3.3 mg of retinol, to a 500-mL separatory funnel containing 10 mL of water and 20 mL of dehydrated alcohol. Add 150 mL of solvent hexane, insert the stopper, and shake for 1 min. Add another 150 mL of solvent hexane, insert the stopper, shake, and allow the layers to separate. Discard the aqueous layer, and filter the solvent hexane extract through anhydrous sodium sulfate into a 500-mL round-bottom flask. Evaporate the solution to dryness with the aid of a rotary evaporator over a water bath maintained at about 65°. Immediately add 10.0 mL of *Diluent*, swirl to dissolve the residue, and filter.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 50-cm (prepared from two concatenated 4.6-mm × 25-cm columns); packing L1

Column temperature: 40°

Flow rate: 1.5 mL/min

Injection volume: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of vitamin A as retinol ($C_{20}H_{30}O$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of retinol or retinyl ester from the *Sample solution*

r_S = peak area of retinyl acetate from the *Standard solution*

C_S = concentration of retinyl acetate ($C_{22}H_{32}O_2$) in the *Standard solution* (µg/mL)

C_U = nominal concentration of vitamin A as retinol ($C_{20}H_{30}O$) in the *Sample solution* (µg/mL)

F = factor used to convert retinyl acetate, the ester form present in USP Vitamin A RS, to retinol, 0.872

Acceptance criteria: 90.0%–200.0% of the labeled amount of vitamin A as retinol ($C_{20}H_{30}O$)

• **CHOLECALCIFEROL OR ERGOCALCIFEROL (VITAMIN D)**

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Diluent and **Mobile phase:** Prepare as directed for *Vitamin A*.

Standard solution: 5 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in *Diluent*

Sample solution: Transfer an accurately measured volume of Oral Solution, equivalent to 50 µg of cholecalciferol or ergocalciferol, to a 500-mL separatory funnel containing 10 mL of water and 20 mL of dehydrated alcohol. Add 150.0 mL of solvent hexane, insert the stopper, and shake for 1 min. Add another 150 mL of solvent hexane, insert the stopper, shake, and allow the layers to separate. Discard the aqueous layer. Drain the solvent hexane extract through anhydrous sodium sulfate into a 500-mL round-bottom flask. Evaporate the solution to dryness with the aid of a rotary evaporator over a water bath maintained at about

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65°. Immediately add 10.0 mL of *Diluent*, swirl to dissolve the residue, and filter.

Chromatographic system: Prepare as directed for *Vitamin A*.

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of vitamin D. Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak areas of cholecalciferol or ergocalciferol from the *Sample solution*

r_S = peak areas of cholecalciferol or ergocalciferol from the *Standard solution*

C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

F = correction factor to account for the average amount of previtamin D present in the formulation, 1.09

Acceptance criteria: 90.0%–200.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

• **VITAMIN E**

[NOTE—Use low-actinic glassware throughout this procedure.]

Diluent: Acetonitrile and ethyl acetate (1:1)

Potassium hydroxide solution: Transfer 90 g of potassium hydroxide pellets to a 100-mL volumetric flask containing 60 mL of water. Mix to dissolve, cool, and dilute with water to volume.

Mobile phase: Methanol, acetonitrile, and *n*-hexane (46.5: 46.5: 7.0)

Standard solution: 0.3 mg/mL of USP Alpha Tocopherol RS in *Diluent*

Sample solution: Transfer an amount of Oral Solution, equivalent to 1.5 mg of alpha tocopherol, to a 125-mL conical flask fitted with a ground-glass joint, and add 25.0 mL of dehydrated alcohol. Attach a reflux condenser, and reflux in a boiling water bath for 1 min. Cautiously add 3 mL of *Potassium hydroxide solution* through the condenser, and continue to reflux for 30 min. Remove the flask from the bath, and rinse the condenser with about 15 mL of water. Cool, and transfer with a minimum volume of water to a 250-mL separatory funnel. Rinse the flask with 50 mL of *n*-hexane, and add the rinsings to the separatory funnel. Insert the stopper, shake vigorously for 1 min, and allow the layers to separate. Drain the aqueous layer into a second 250-mL separatory funnel, and repeat the extraction with 50 mL of *n*-hexane. Discard the aqueous layer, and combine the hexane extracts. Wash the combined extracts with 25 mL of water, allow the layers to separate, and discard the aqueous layer. Add 3 drops of glacial acetic acid, and repeat the washing procedure two more times. Filter the washed hexane layer through anhydrous sodium sulfate into a 250-mL round-bottom flask. Rinse the funnel and sodium sulfate with *n*-hexane, and add the rinsing to the hexane solution in the flask. Evaporate the hexane solution to dryness with the aid of a rotary evaporator over a water bath maintained at about 50°. Immediately add 5.0 mL of *Diluent*, and swirl to dissolve the residue.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 291 nm

Column: 4.6-mm × 25-cm; packing L1

Column temperature: 40°

Flow rate: 3.0 mL/min

Injection volume: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas. Calculate the percentage of the labeled amount of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of alpha tocopherol from the *Sample solution*

r_S = peak area of alpha tocopherol from the *Standard solution*

C_S = concentration of alpha tocopherol in the *Standard solution* (mg/mL)

C_U = nominal concentration of vitamin E as alpha tocopherol in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–200.0% of the labeled amount of vitamin E

• **ASCORBIC ACID, CALCIUM ASCORBATE,** and **SODIUM ASCORBATE**

(See *Vitamin C Assay* ⟨580⟩.)

[NOTE—For labeling purposes, consider *Method I—Titrimetric Method* as *Method 1*.]

Acceptance criteria: 90.0%–200.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$) or its salts as calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$) or sodium ascorbate ($C_6H_7NaO_6$)

• **BIOTIN, *Method 1***

[NOTE—Use low-actinic glassware throughout this procedure.]

Dehydrated mixtures, yielding formulations similar to the media described herein, may be used provided that, when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described herein.

Standard stock solution: 50 µg/mL of USP Biotin RS in 50% alcohol. Store this solution in a refrigerator.

Standard solution: On the day of the assay, prepare 0.1 ng/mL of biotin from the *Standard stock solution* in water.

Sample solution: Dilute an accurately measured portion of the Oral Solution with water to an assumed concentration of 0.1 ng/mL.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and dilute with water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

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Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water. Heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and dilute with water to make 1000 mL. Store under toluene in a cool place at a temperature NLT 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and dilute with water to make 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Calcium pantothenate solution: 10 µg/mL of calcium pantothenate in 50% alcohol. Store in a refrigerator.

Riboflavin–thiamine hydrochloride solution: 20 µg/mL of riboflavin and 10 µg/mL of thiamine hydrochloride in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

***p*-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution:** 10 µg/mL of *p*-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: Dissolve 50 mg/mL of monobasic potassium phosphate and 50 mg/mL of dibasic potassium phosphate in water. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: Dissolve 20 g of magnesium sulfate, 1 mg/mL of sodium chloride, 1 mg/mL of ferrous sulfate, and 1 mg/mL of manganese sulfate in water. Add 5 drops of hydrochloric acid, and mix. Store under toluene.

Basal medium stock solution: Dissolve the *anhydrous Dextrose* and *anhydrous Sodium acetate* in the solutions previously mixed according to *Table 1*, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Table 1

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Calcium pantothenate solution	5 mL
Riboflavin–thiamine hydrochloride solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water. Add 500 mg of *anhydrous Dextrose*, 500 mg of *anhydrous Sodium acetate*, and 1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to test tubes, close or cover the tubes, sterilize in an autoclave at 121° for 15 min, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*,[1] incubating for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution* add 5.0 mL of water containing 0.5 ng of biotin. Plug the tubes with cotton, sterilize in an autoclave at 121° for 15 min, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an inoculum comparable to a fresh culture.]

Transfer cells from the *Stock culture of Lactobacillus plantarum* to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. The cell suspension so obtained is the *Inoculum*.

Analysis

Samples: *Standard solution* and *Sample solution*

To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool. Add 1 drop of *Inoculum* to each tube, except two of the four tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37° held constant to within ±0.5° until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of Standard during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to a spectrophotometer cell. Place the cell in a spectrophotometer that has been set at a specific wavelength of 540–660 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittance (Σ_S) as the difference, $y = 2.00 - \Sigma_S$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better

[1] ATCC No. 8014 is suitable. This strain was formerly known as *Lactobacillus arabinosus* 17-5.

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approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_U$, adding together the two transmittances (Σ_U) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X, for each dosage level. Average the values of X for each of three or more dosage levels to obtain $\overline{X}$, which equals the log-relative potency, M', of the *Sample solution*.

Calculate the quantity, in ng, of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Oral Solution taken:

$$\text{antilog M} = \text{antilog}\,(M' + \log R)$$

R = number of ng of biotin assumed to be present in the portion of Oral Solution taken

Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Oral Solution taken:

$$\text{Result} = [(\text{antilog}\,M)/N] \times 100$$

N = nominal amount of biotin, in ng, in the portion of Oral Solution taken

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, $\overline{M}$, is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* ⟨111⟩, *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

• **BIOTIN, *Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A: Transfer 800 mL of water and 100 mL of triethylamine to a 1000-mL volumetric flask. Add 80 mL of 85% phosphoric acid, and dilute with water to volume.

Mobile phase: Transfer 80 mL of acetonitrile and 10 mL of *Solution A* to a 1000-mL volumetric flask. Dilute with water to volume.

Standard solution: 0.6 µg/mL of USP Biotin RS in water. [NOTE—A portion of the *Standard solution* will be used to determine the percent recovery of biotin from the *Solid-phase extraction* procedure.]

Sample solution: 0.6 µg/mL of biotin, from the Oral Solution in water. Adjust the solution with either dilute acetic acid or 0.1 N sodium hydroxide to a pH of 6.0–7.0.

Solid-phase extraction: [NOTE—Condition the extraction column specified in this procedure in the following manner. Wash the column with a 2-mL portion of methanol. Equilibrate with a 2-mL portion of water.] Separately pipet 5.0 mL each of the *Sample solution* and *Standard solution* into freshly conditioned solid-phase extraction columns consisting of a mixed-mode packing with a sorbent-mass of 60 mg. [NOTE—The mixed-mode packing consists of anion-exchange and reversed-phase sorbents. The reverse-phase component is a copolymer of *N*-vinylpyrrolidone and divinylbenzene. The anion exchange moiety is a trialkylamino group.[2]]

Wash the column with 10 mL of 30% (v/v) methanol in water. Apply an appropriate volume (4.9 mL) of 30% (v/v) methanol in 0.1 N hydrochloric acid to the column. Collect the eluate in a 5-mL volumetric flask, containing 100 µL of 40% (w/v) sodium acetate in water, and dilute with 30% (v/v) methanol in 0.1 N hydrochloric acid to volume.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 200 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 2 mL/min

Injection volume: 100 µL

System suitability

Samples: *Standard solution* and a portion of *Standard solution* that has undergone *Solid-phase extraction*

Suitability requirements

Tailing factor: NMT 1.5, *Standard solution*

Relative standard deviation: NMT 2.0%, *Standard solution* and *Standard solution* that has undergone *Solid-phase extraction*

Recovery: 95%–100%, *Standard solution* that has undergone *Solid-phase extraction*

Analysis

Samples: *Standard solution* and *Sample solution* that have both undergone *Solid-phase extraction*

Measure the peak areas of biotin. Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of biotin from the *Sample solution*

r_S = peak area of biotin from the *Standard solution*

C_S = concentration of USP Biotin RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of biotin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

• **CYANOCOBALAMIN**

[NOTE—Use low-actinic glassware throughout this procedure.]

Standard stock solution: 1.0 µg/mL of cyanocobalamin from USP Cyanocobalamin (Crystalline) RS in 25% alcohol. Store in a refrigerator.

Standard solution: Dilute a suitable volume of *Standard stock solution* with water to a measured volume such that after the incubation period as described in *Analysis*, the difference in transmittance between the inoculated blank and the 5.0-mL level of the *Standard solution* is NLT that which corresponds to a difference of 1.25 mg in dried cell weight. This concentration usually falls between 0.01 and 0.04 ng/mL of *Standard solution*. Prepare this solution fresh for each assay.

Sample solution: Transfer an accurately measured volume of Oral Solution, assumed to contain 1.0 µg of cyanocobalamin, to an appropriate vessel containing, for each mL of the Oral Solution taken, 25 mL of an aqueous extracting solution prepared just before use to contain, in each 100 mL, 1.29 g of dibasic sodium phosphate, 1.1 g of anhydrous citric acid, and 1.0 g of sodium metabisulfite.

[2] A suitable cartridge is the Waters Oasis MAX Vac RC cartridge, particle size 30 µm, part 186000371.

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Autoclave the mixture at 121° for 10 min. Allow any undissolved particles of the extract to settle, and filter or centrifuge if necessary. Dilute an aliquot of the clear solution with water to obtain a final solution containing vitamin B_{12} activity approximately equivalent to that of the *Standard solution*.

Acid-hydrolyzed casein solution: Prepare as directed in *Biotin, Method 1*.

Asparagine solution: Dissolve 2.0 g of L-asparagine in water to make 200 mL. Store under toluene in a refrigerator.

Adenine–guanine–uracil solution: Prepare as directed for *Biotin, Method 1*.

Xanthine solution: Suspend 0.20 g of xanthine in 30–40 mL of water, heat to 70°, add 6.0 mL of 6 N ammonium hydroxide, and stir until the solid is dissolved. Cool, and dilute with water to make 200 mL. Store under toluene in a refrigerator.

Salt solution A: Dissolve 10 g of monobasic potassium phosphate and 10 g of dibasic potassium phosphate in water to make 200 mL, and add 2 drops of hydrochloric acid. Store this solution under toluene.

Salt solution B: Dissolve 4.0 g of magnesium sulfate, 0.20 g of sodium chloride, 0.20 g of ferrous sulfate, and 0.20 g of manganese sulfate in water to make 200 mL, and add 2 drops of hydrochloric acid. Store this solution under toluene.

Polysorbate 80 solution: 20 g of polysorbate 80 in alcohol to make 200 mL. Store in a refrigerator.

Vitamin solution A: 10 mg of riboflavin, 10 mg of thiamine hydrochloride, 100 µg of biotin, and 20 mg of niacin in 0.02 N acetic acid to make 400 mL. Store under toluene protected from light in a refrigerator.

Vitamin solution B: 20 mg of *p*-aminobenzoic acid, 10 mg of calcium pantothenate, 40 mg of pyridoxine hydrochloride, 40 mg of pyridoxal hydrochloride, 8 mg of pyridoxamine dihydrochloride, and 2 mg of folic acid in a mixture of water and neutralized alcohol (3:1) to make 400 mL. Store, protected from light, in a refrigerator.

Basal medium stock solution: Prepare the medium according to the following formula and directions. A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium comparable to that obtained from the formula given herein.

Add the ingredients in the order listed in *Table 2*, carefully dissolving *Cystine* and *Tryptophan* in the *hydrochloric acid* before adding the next eight solutions. In the resulting solution add 100 mL of water, and dissolve *Dextrose*, *Sodium acetate*, and *Ascorbic acid*. Filter if necessary. Add the *Polysorbate 80 solution*, adjust with 1 N sodium hydroxide to a pH of 5.5–6.0, and dilute with Purified Water to make 250 mL.

Table 2

L-Cystine	0.1 g
L-Tryptophan	0.05 g
1 N hydrochloric acid	10 mL
Adenine–guanine–uracil solution	5 mL
Xanthine solution	5 mL
Vitamin solution A	10 mL
Vitamin solution B	10 mL
Salt solution A	5 mL
Salt solution B	5 mL
Asparagine solution	5 mL
Acid-hydrolyzed casein solution	25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Ascorbic acid	1 g
Polysorbate 80 solution	5 mL

Tomato juice preparation: Centrifuge commercially canned tomato juice so that most of the pulp is removed. Suspend 5 g/L of analytical filter aid in the supernatant, and filter with the aid of reduced pressure through a layer of the filter aid. Repeat, if necessary, until a clear, straw-colored filtrate is obtained. Store under toluene in a refrigerator.

Culture medium: [NOTE—A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium equivalent to that obtained from the formula given herein.] Dissolve 0.75 g of yeast extract, 0.75 g of dried peptone, 1.0 g of *anhydrous Dextrose*, and 0.20 g of monobasic potassium phosphate in 60–70 mL of water. Add 10 mL of *Tomato juice preparation* and 1 mL of *Polysorbate 80 solution*. Adjust with 1 N sodium hydroxide to a pH of 6.8, and dilute with water to make 100 mL. Place 10-mL portions of the solution in test tubes, and plug with cotton. Sterilize the tubes and contents in an autoclave at 121° for 15 min. Cool as rapidly as possible to avoid color formation resulting from overheating the medium.

Suspension medium: Dilute a measured volume of *Basal medium stock solution* with an equal volume of water. Place 10-mL portions of the diluted medium in test tubes. Sterilize, and cool as directed for *Culture medium*.

Stock culture of *Lactobacillus leichmannii*: To 100 mL of *Culture medium* add 1.0–1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Place 10-mL portions of the hot solution in test tubes, cover the tubes, sterilize at 121° for 15 min in an autoclave, and allow the tubes to cool in an upright position. Inoculate three or more of the tubes by stab transfer of a pure culture of *Lactobacillus leichmannii*.[3] [NOTE—Before first using a fresh culture in this assay, make NLT 10 successive transfers of the culture in a 2-week period.]

Incubate for 16–24 h at a temperature between 30° and 40° held constant to within ±0.5°. Store in a refrigerator.

Prepare fresh stab cultures at least three times each week, and do not use them for preparing the *Inoculum* if more than 4 days old. The activity of the microorganism can be increased by daily or twice-daily transfer of the stab culture to the point where definite turbidity in the liquid *Inoculum* can be observed 2–4 h after inoculation. A slow-growing culture seldom gives a suitable response curve and may lead to erratic results.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus leichmannii* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.]

Transfer cells from the *Stock culture of Lactobacillus leichmannii* to two sterile tubes each containing 10 mL of the *Culture medium*. Incubate these cultures for 16–24 h at a temperature between 30° and 40° held constant to within ±0.5°. Under aseptic conditions centrifuge the cultures, and decant the supernatant. Suspend the cells from the culture in 5 mL of sterile *Suspension medium*, and combine. Using sterile *Suspension medium*, adjust the volume so that a 1-in-20 dilution in saline TS produces

[3] Pure cultures of *Lactobacillus leichmannii* (listed as *Lactobacillus delbrueckii*) may be obtained as No. 7030 from ATCC, 10801 University Blvd., Manassas, VA 20110-2209 (www.atcc.org).

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70% transmittance when read on a suitable spectrophotometer that has been set at a wavelength of 530 nm, equipped with a 10-mm cell, and read against saline TS set at 100% transmittance. Prepare a 1-in-400 dilution of the adjusted suspension using sterile *Basal medium stock solution*. [NOTE—This dilution may be altered, when necessary, to obtain the desired test response.] The cell suspension so obtained is the *Inoculum*.

Calibration of spectrophotometer: Check the wavelength of the spectrophotometer periodically using a standard wavelength cell or other suitable device. Before reading any tests, calibrate the spectrophotometer for 0% and 100% transmittance using water and with the wavelength set at 530 nm.

Analysis

Samples: *Standard solution* and *Sample solution*

Because of the high sensitivity of the test organism to minimum amounts of vitamin B_{12} activity and to traces of many cleansing agents, cleanse meticulously by suitable means, followed preferably by heating at 250° for 2 h, using hard-glass 20-mm × 150-mm test tubes and other necessary glassware.

To separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each of these tubes and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*. To each tube add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes to prevent bacterial contamination, and sterilize in an autoclave at 121° for 5 min, arranging to reach this temperature in NMT 10 min by preheating the autoclave if necessary. Cool as rapidly as possible to avoid color formation resulting from overheating the medium. Take precautions to maintain uniformity of sterilizing and cooling conditions throughout the assay, because packing the tubes too closely in the autoclave or overloading it may cause variation in the heating rate.

Aseptically add 0.5 mL of *Inoculum* to each tube so prepared, except two of the four containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 40°, held constant to within ±0.5°, for 16–24 h.

Terminate growth by heating to NLT 80° for 5 min. Cool to room temperature. After agitating the contents, read the transmittance at 530 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of the inoculated blank. If the difference is greater than 5% or if there is evidence of contamination with a foreign microorganism, disregard the results of the assay.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of each of the remaining tubes. Disregard the results of the assay if the slope of the standard curve indicates a problem with sensitivity.

Calculation: Prepare a standard concentration-response curve by the following procedure. Test for and replace any aberrant individual transmittances. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittances (Σ_S) as the difference, $y = 2.00 - \Sigma_S$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_U$, adding together the two transmittances (Σ_U) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X, for each dosage level. Average the values of X for each of three or more dosage levels to obtain $\overline{X}$, which equals the log-relative potency, M', of the *Sample solution*.

Calculate the quantity, in µg, of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$) in the portion of Oral Solution taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = µg of cyanocobalamin assumed to be present in the portion of Oral Solution taken

Calculate the percentage of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$) in the portion of the Oral Solution taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of cyanocobalamin in the portion of Oral Solution taken (µg)

Replication: Repeat the entire determination at least once using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, $\overline{M}$, is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* ⟨111⟩, *The Confidence Interval and Limits of Potency, Vitamin B_{12} Activity*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–450.0% of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$)

• **CALCIUM PANTOTHENATE, *Method 1***

Mobile phase: 0.2 M monobasic sodium phosphate and methanol (97:3). Adjust with 1.7 M phosphoric acid to a pH of 3.2 ± 0.1.

Standard solution: 80 µg/mL of USP Calcium Pantothenate RS in *Mobile phase*

System suitability solution: 80 µg/mL of USP Racemic Panthenol RS in *Mobile phase*. Mix the resulting solution and *Standard solution* (1:1).

Sample solution: Equivalent to 80 µg/mL of calcium pantothenate from the Oral Solution in *Mobile phase*

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 210 nm

Column: 4.0-mm × 10-cm; packing L1

Flow rate: 1 mL/min

Injection volume: 20 µL

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System suitability
Samples: *Standard solution* and *System suitability solution*
Suitability requirements
Resolution: NLT 1.5 between panthenol and calcium pantothenate, *System suitability solution*
Tailing factor: NMT 2.0 for both the calcium pantothenate and the panthenol peaks, *Standard solution*
Relative standard deviation: NMT 2.0%, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas of calcium pantothenate.
Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of calcium pantothenate from the *Sample solution*
r_S = peak area of calcium pantothenate from the *Standard solution*
C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• **CALCIUM PANTOTHENATE, *Method 2***

Standard stock solution: Dissolve 50 mg of USP Calcium Pantothenate RS, previously dried and stored in the dark over phosphorus pentoxide and protected from absorption of moisture while weighing, in 500 mL of water in a 1000-mL volumetric flask. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), and dilute with water to volume to obtain a concentration of 50 µg/mL of USP Calcium Pantothenate RS. Store under toluene in a refrigerator.

Standard solution: On the day of the assay, dilute a volume of *Standard stock solution* with water to obtain a concentration of 0.01–0.04 µg/mL of calcium pantothenate, the exact concentration being such that the responses obtained as directed in the *Analysis*, 2.0 and 4.0 mL of the *Standard solution* being used, are within the linear portion of the log-concentration response curve.

Sample solution: Transfer an accurately measured volume of Oral Solution, equivalent to 50 mg of calcium pantothenate, to a 1000-mL volumetric flask containing 500 mL of water. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), dilute with water to volume, and filter. Dilute a measured volume of this solution quantitatively, and stepwise if necessary, with water to obtain a solution having about the same concentration as that of the *Standard solution*.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and dilute with water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature NLT 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and dilute with water to make 1000 mL. Store under toluene in a cool place at a temperature NLT 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and dilute with water to make 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin–thiamine hydrochloride–biotin solution: 20 µg/mL of riboflavin, 10 µg/mL of thiamine hydrochloride, and 0.04 µg/mL of biotin in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

***p*-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution:** 10 µg/mL of *p*-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Basal medium stock solution: Dissolve the *anhydrous Dextrose* and *anhydrous Sodium acetate* in the solutions previously mixed according to *Table 3*, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Table 3

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Riboflavin–thiamine hydrochloride–biotin solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water. Add 500 mg of *anhydrous Dextrose*, 500 mg of *anhydrous Sodium acetate*, and 1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to the test tubes, close or cover the tubes, sterilize in an autoclave at 121° for 15 min, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*,[1] incubating for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution* add 5.0 mL of water containing 0.2 µg of calcium pantothenate. Plug the tubes

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with cotton, sterilize in an autoclave at 121° for 15 min, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Transfer cells from the *Stock culture of Lactobacillus plantarum* to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. The cell suspension so obtained is the *Inoculum*.

Analysis

Samples: *Standard solution* and *Sample solution*

To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool. Add 1 drop of *Inoculum* to each tube, except two of the four tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37°, held constant to within ±0.5°, following 16–24 h of incubation, until there has been no substantial increase in turbidity in the tubes containing the highest level of Standard during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to an optical container if necessary. Read the transmittance between 540 and 660 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittance (Σ_S) as the difference, $y = 2.00 - \Sigma_S$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_U$, adding together the two transmittances (Σ_U) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X, for each dosage level. Average the values of X for each of three or more dosage levels to obtain $\overline{X}$, which equals the log-relative potency, M', of the *Sample solution*.

Determine the quantity, in mg, of USP Calcium Pantothenate RS corresponding to the calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Oral Solution taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of mg of calcium pantothenate assumed to be present in the portion of Oral Solution taken

Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Oral Solution taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of calcium pantothenate in the portion of Oral Solution (mg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, $\overline{M}$, is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* ⟨111⟩, *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• **DEXPANTHENOL OR PANTHENOL, *Method 1***

Mobile phase and **Chromatographic system:** Proceed as directed in the assay for *Calcium Pantothenate, Method 1*.

Standard solution: 80 µg/mL of USP Dexpanthenol RS or USP Racemic Panthenol RS in *Mobile phase*. [NOTE—Use USP Dexpanthenol RS to analyze Oral Solution that contains dexpanthenol and USP Racemic Panthenol RS to analyze Oral Solution that contains panthenol.]

System suitability solution: 80 µg/mL of USP Calcium Pantothenate RS in *Mobile phase*. Mix the resulting solution and *Standard solution* (1:1).

Sample solution: Equivalent to 80 µg/mL of dexpanthenol or panthenol from the Oral Solution in *Mobile phase*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of panthenol.

Calculate the percentage of the labeled amount of dexpanthenol or panthenol ($C_9H_{19}NO_4$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of dexpanthenol or panthenol from the *Sample solution*

r_S = peak area of dexpanthenol or panthenol from the *Standard solution*

C_S = concentration of USP Dexpanthenol RS or USP Racemic Panthenol RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of panthenol or dexpanthenol in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of dexpanthenol or panthenol ($C_9H_{19}NO_4$)

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• **DEXPANTHENOL OR PANTHENOL, *Method 2***

[NOTE—The following procedure is applicable also to the determination of the dextrorotatory component of racemic panthenol in preparations containing panthenol.]

Dehydrated mixtures yielding formulations similar to the media described herein may be used provided that, when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described herein.

Standard stock solution: 800 µg/mL of USP Dexpanthenol RS or 1600 µg/mL of USP Racemic Panthenol RS in water. Store in a refrigerator, protected from light, and use within 30 days. [NOTE—Use USP Dexpanthenol RS to analyze Oral Solution that contains dexpanthenol and USP Racemic Panthenol RS to analyze Oral Solution that contains panthenol.]

Standard solution: On the day of the assay, prepare 1.2 µg/mL of dexpanthenol or 2.4 µg/mL of racemic panthenol from the *Standard stock solution* with water.

Sample solution: 1.2 µg/mL of dexpanthenol or 2.4 µg/mL of panthenol from Oral Solution in water

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of dilute hydrochloric acid (1 in 2), and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in about 500 mL of water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and dilute with water to 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to 75 ± 5°, and add 6 M hydrochloric acid solution dropwise, with stirring, until the solids are dissolved. Cool, dilute with water to 1000 mL, and mix. Store under toluene in a cool place at a temperature not below 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, dilute with water to 200 mL, and mix. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin–thiamine hydrochloride–biotin solution: Prepare a solution of riboflavin, thiamine hydrochloride, and biotin in 0.02 N acetic acid containing 20 µg/mL of riboflavin, 10 µg/mL of thiamine hydrochloride, and 0.04 µg/mL of biotin. Store under toluene, protected from light, in a refrigerator.

***p*-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution:** Prepare a solution in neutral 25% alcohol containing 10 µg/mL of *p*-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride. Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid, and mix. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid, and mix. Store under toluene.

Pyridoxal–calcium pantothenate solution: Dissolve 40 mg of pyridoxal hydrochloride and 375 µg of calcium pantothenate in 10% alcohol to make 200 mL, and mix. Store in a refrigerator, and use within 30 days.

Polysorbate 40–oleic acid solution: Dissolve 25 g of polysorbate 40 and 0.25 g of oleic acid in 20% alcohol to make 500 mL, and mix. Store in a refrigerator, and use within 30 days.

Modified pantothenate medium: Dissolve *anhydrous Dextrose* and *Sodium acetate* in the solutions previously mixed according to *Table 4*, adjust with 1 N sodium hydroxide to a pH of 6.8, dilute with water to 250 mL, and mix.

Table 4

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Riboflavin–thiamine hydrochloride–biotin solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL
Pyridoxal–calcium pantothenate solution	5 mL
Polysorbate 40–oleic acid solution	5 mL

Double-strength modified pantothenate medium: Prepare as directed in *Modified pantothenate medium*, but make the final dilution to 125 mL instead of 250 mL. Prepare fresh.

Stock culture of *Pediococcus acidilactici*: Dissolve in 800 mL of water, with the aid of heat, 6.0 g of peptone, 4.0 g of pancreatic digest of casein, 3.0 g of yeast extract, 1.5 g of beef extract, 1.0 g of dextrose, and 15.0 g of agar. Adjust with 0.1 N sodium hydroxide or 0.1 N hydrochloric acid to a pH between 6.5 and 6.6, dilute with water to 1000 mL, and mix. Add 10-mL portions of the solution to culture tubes, place caps on the tubes, and sterilize in an autoclave at 121° for 15 min. Cool on a slant, and store in a refrigerator. Prepare a stock culture of *Pediococcus acidilactici*[4] on a slant of this medium. Incubate at 35° for 20–24 h, and store in a refrigerator. Maintain the stock culture by monthly transfer onto fresh slants.

Inoculum: Inoculate three 250-mL portions of sterile *Modified pantothenate medium* from a stock culture slant, and incubate at 35° for 20–24 h. Centrifuge the suspension from the combined portions, and wash the cells with sterile *Modified pantothenate medium*. Resuspend the cells in sufficient sterile *Modified pantothenate medium* so that a 1-in-50 dilution, when tested in a 13-mm diameter test tube, gives 80% light transmission at 530 nm. Transfer 1.2-mL portions of this stock suspension to sterile glass ampuls, seal, freeze in liquid nitrogen, and store in a freezer. On the day of the assay, allow the ampuls to reach room temperature, mix the contents, and dilute 1 mL of thawed culture with sterile saline TS to 150 mL. [NOTE—This dilution may be altered when necessary to obtain the desired test response.]

Analysis

Samples: *Standard solution* and *Sample solution*

[4] ATCC No. 8042 is suitable. This strain was formerly known as *Lactobacillus arabinosus* 17-5.

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Prepare in triplicate a series of eight culture tubes by adding the following quantities of water to the tubes within a set: 5.0, 4.5, 4.0, 3.5, 3.0, 2.0, 1.0, and 0.0 mL. To these same tubes, and in the same order, add 0.0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*.

Prepare in duplicate a series of five culture tubes by adding the following quantities of water to the tubes within a set: 4.0, 3.5, 3.0, 2.0, and 1.0 mL. To these same tubes, and in the same order, add 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*.

Add 5.0 mL of *Double-strength modified pantothenate medium* to each tube, and mix. Cover the tubes with metal caps, and sterilize in an autoclave at 121° for 5 min. Cool to room temperature in a chilled water bath, and inoculate each tube with 0.5 mL of the *Inoculum*. Allow to incubate at 37° for 16 h. Terminate growth by heating to a temperature NLT 80°, such as by steaming at atmospheric pressure in a sterilizer for 5–10 min. Cool, and concomitantly determine the percentage transmittance of the suspensions, in cells of equal path length, on a suitable spectrophotometer at a wavelength of 530 nm.

Calculation: Draw a dose-response curve on arithmetic graph paper by plotting the average response, in percent transmittance, for each set of tubes of the standard curve against the standard level concentrations. The curve is drawn by connecting each adjacent pair of points with a straight line. From this standard curve, determine by interpolation the potency, in terms of dexpanthenol, of each tube containing portions of the *Sample solution*. Divide the potency of each tube by the amount of the *Sample solution* added to it to obtain the individual responses. Calculate the mean response by averaging the individual responses that vary from their mean by NMT 15%, using NLT half the total number of tubes. Calculate the potency of the portion of the Oral Solution taken for assay by multiplying the mean response by the appropriate dilution factor.

Calculate the percentage of the labeled amount of dexpanthenol or panthenol in the portion of Oral Solution taken:

$$\text{Result} = (P/N) \times 100$$

P = calculated potency of dexpanthenol or panthenol in the portion of Oral Solution taken (mg)

N = nominal amount of dexpanthenol or panthenol in the portion of Oral Solution taken (mg)

Acceptance criteria: 90.0%–150.0% of the labeled amount of dexpanthenol or panthenol ($C_9H_{19}NO_4$)

• **NIACIN OR NIACINAMIDE**

[NOTE—Use low-actinic glassware throughout this procedure.]

Diluent: 25 mg/mL of edetate disodium in water

Mobile phase: Mix 350 mL of methanol, 15.0 mL of glacial acetic acid, 0.4 mL of triethylamine, and dilute with 0.008 M sodium 1-hexanesulfonate to 2000 mL.

Standard solution: 0.10 mg/mL of USP Niacin RS or USP Niacinamide RS in *Diluent*. [NOTE—Use USP Niacin RS for Oral Solution that contains niacin, and USP Niacinamide RS for Oral Solution that contains niacinamide.]

Sample solution: Dilute an accurately measured volume of Oral Solution with *Diluent* to obtain a solution with a concentration of 0.1 mg/mL of niacin or niacinamide.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 270 nm

Column: 4.6-mm × 25-cm; packing L7

Flow rate: 2 mL/min

Injection volume: 5 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacin or niacinamide from the *Sample solution*

r_S = peak area of niacin or niacinamide from the *Standard solution*

C_S = concentration of USP Niacin RS or USP Niacinamide RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacin or niacinamide in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% the labeled amount of niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$)

• **PYRIDOXINE HYDROCHLORIDE**

Diluent, Mobile phase, and **Chromatographic system:** Proceed as directed in *Niacin* or *Niacinamide*.

Standard solution: Equivalent to 24 µg/mL of USP Pyridoxine Hydrochloride RS in *Diluent*

Sample solution: Equivalent to 24 µg/mL of Pyridoxine Hydrochloride from Oral Solution in *Diluent*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of pyridoxine hydrochloride from the *Sample solution*

r_S = peak area of pyridoxine hydrochloride from the *Standard solution*

C_S = concentration of USP Pyridoxine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of pyridoxine hydrochloride in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$)

• **RIBOFLAVIN-5′-PHOSPHATE SODIUM, *Method 1***

[NOTE—Riboflavin-5′-phosphate sodium is quantitated against USP Riboflavin RS in this procedure. In the chromatogram of the *Sample solution*, the riboflavin-5′-phosphate peak is the only peak measured for calculation.]

Diluent, Mobile phase, and **Chromatographic system:** Proceed as directed in *Niacin* or *Niacinamide*.

Standard solution: Equivalent to 8 µg/mL of USP Riboflavin RS in *Diluent*, by heating if necessary

Sample solution: Equivalent to 8 µg/mL of riboflavin from the Oral Solution in *Diluent*

Analysis

Samples: *Standard solution* and *Sample solution*

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[NOTE—The relative retention times for riboflavin-5′-phosphate and riboflavin are about 0.18 and 1.0, respectively.]

Calculate the percentage of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of riboflavin-5′-phosphate from the *Sample solution*
r_S = peak area of riboflavin from the *Standard solution*
C_S = concentration of USP Riboflavin RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of riboflavin in the *Sample solution* (mg/mL)
F = factor for converting the response obtained for riboflavin-5′-phosphate to riboflavin, 1.493. [NOTE—Riboflavin phosphate sodium is a mixture of isomeric monophosphates and diphosphates containing an average amount of 67% of riboflavin-5′-monophosphate, which separates in this chromatographic system. The factor 1.493 assumes 67% of riboflavin-5′-monophosphate.]

Acceptance criteria: 90.0%–150.0% of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$)

• **RIBOFLAVIN OR RIBOFLAVIN-5′-PHOSPHATE SODIUM, *Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Solvent blank: 1 N hydrochloric acid, 2.5 M sodium acetate, and water (1:2:97)

Standard stock solution: Transfer 0.16 mg/mL of USP Riboflavin RS in 1 N hydrochloric acid, 2.5 M sodium acetate, and water (1:2:97). Mix the resulting solution and water (1:9).

Standard solution: Transfer 5.0 mL of the *Standard stock solution* to a 500-mL volumetric flask, add 5.0 mL of 1 N hydrochloric acid, 10.0 mL of 2.5 M sodium acetate, and dilute with water to volume.

Sample solution: Transfer an accurately measured volume of Oral Solution, equivalent to 0.8 mg of riboflavin to a 100-mL volumetric flask, and dilute with water to volume. Transfer 10.0 mL of the resulting solution to a 500-mL volumetric flask, add 5.0 mL of 1 N hydrochloric acid and 10.0 mL of 2.5 M sodium acetate, and dilute with water to volume.

Instrumental conditions

(See *Fluorescence Spectroscopy* ⟨853⟩.)

Mode: Fluorescence

Analytical wavelengths

Excitation: 440 nm

Emission: 530 nm

Blank: *Solvent blank*

Analysis

Samples: *Standard solution* and *Sample solution*

Determine the maximum fluorescence intensities, I_S and I_U, of the *Standard solution* and the *Sample solution*, respectively.

Calculate the percentage of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$) in the portion of Oral Solution taken:

$$\text{Result} = (I_U/I_S) \times (C_S/C_U) \times 100$$

I_U = fluorescence intensity from the *Sample solution*
I_S = fluorescence intensity from the *Standard solution*
C_S = concentration of USP Riboflavin RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of riboflavin in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$)

• **THIAMINE**

Diluent, Mobile phase, and **Chromatographic system:** Proceed as directed in *Niacin* or *Niacinamide*.

Standard solution: Equivalent to 24 µg/mL of USP Thiamine Hydrochloride RS in *Diluent*

Sample solution: Equivalent to 24 µg/mL of thiamine hydrochloride or thiamine mononitrate from Oral Solution in *Diluent*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the areas of the major peaks. Calculate the percentage of the labeled amount of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of thiamine from the *Sample solution*
r_S = peak area of thiamine from the *Standard solution*
C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of thiamine hydrochloride or thiamine mononitrate in the *Sample solution* (mg/mL)
F = 1 (for products containing thiamine hydrochloride) and 0.97 (for products containing thiamine mononitrate)

Acceptance criteria: 90.0%–200.0% of the labeled amount of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$)

[NOTE—Commercially available atomic absorption standard solutions for the minerals, where applicable, may be used where preparation of a standard stock solution is described in the following assays. Use deionized water where water is specified. Where atomic absorption spectrophotometry is specified in the assay, the *Standard solutions* and the *Sample solution* may be diluted quantitatively with the solvent specified, if necessary, to yield solutions of suitable concentrations adaptable to the linear or working range of the instrument.]

• **CHROMIUM**

Chromium standard solution: 1000 µg/mL of chromium from potassium dichromate, previously dried at 120° for 4 h in water. Store in a polyethylene bottle.

Standard stock solution: 10 µg/mL of chromium from *Chromium standard solution* diluted with 6 N hydrochloric acid and water (1 in 20)

Standard solutions: Transfer 10.0 and 20.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks, and transfer 15.0 and 20.0 mL of the *Standard stock solution* to separate 50-mL volumetric flasks. Dilute the contents of each of the four flasks with 0.125 N hydrochloric acid to volume to obtain concentrations of 1.0, 2.0, 3.0, and 4.0 µg/mL of chromium.

Sample solution: Dilute an accurately measured volume of Oral Solution to obtain a solution equivalent to 1 µg/mL of chromium in 0.125 N hydrochloric acid.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

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Mode: Atomic absorption spectrophotometry
Analytical wavelength: 357.9 nm
Lamp: Chromium hollow-cathode
Flame: Air–acetylene
Blank: 0.125 N hydrochloric acid

Analysis
Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the blank. Plot the absorbances of the *Standard solutions* versus concentration, in µg/mL, of chromium, and draw the straight line best fitting the four plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of chromium in the *Sample solution*.
Calculate the percentage of the labeled amount of chromium (Cr) in the portion of Oral Solution taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of chromium in the *Sample solution* (µg/mL)
C_U = nominal concentration of chromium in the *Sample solution*, (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of chromium (Cr)

• **FLUORIDE**
[NOTE—Store all solutions in plastic containers.]
Ascorbic acid solution: 70 mg/mL of ascorbic acid in water
Mobile phase: Alcohol, water, and 1 N sulfuric acid (50:449:1)
Standard stock solution: 220 µg/mL of USP Sodium Fluoride RS in water. This solution contains 100 µg/mL of fluoride.
Standard solution: Transfer 5.0 mL of *Standard stock solution* to a 100-mL volumetric flask. Add 2 mL of *Ascorbic acid solution*, 10 mL of alcohol, and about 70 mL of water, and mix. Adjust with 1 N sodium hydroxide to a pH of 4.25 ± 0.05. Dilute with water to volume, and mix to obtain 5 µg/mL of fluoride solution.
Sample solution: Transfer an accurately measured volume of the Oral Solution, equivalent to 0.5 mg of fluoride, to a 100-mL volumetric flask. Add 1 drop of hydrochloric acid, 10 mL of alcohol, and about 75 mL of water, and mix. Adjust with 1 N sodium hydroxide to a pH of 4.25 ± 0.05. Dilute with water to volume.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Conductivity
Columns
Guard: 4.6-mm × 3-cm; packing L17
Analytical: 7.8-mm × 30-cm; packing L17
Flow rate: 0.6 mL/min
Injection volume: 100 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 2.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas of fluoride. Calculate the percentage of the labeled amount of fluorine (F) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of fluoride from the *Sample solution*
r_S = peak area of fluoride from the *Standard solution*
C_S = concentration of fluoride in the *Standard solution* (µg/mL)
C_U = nominal concentration of fluorine in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (F)

• **IODIDE**
Mobile phase: Dissolve 5.15 g of tetrabutylammonium bromide in 320 mL of acetonitrile. Dilute with water to 2000 mL.
Standard stock solution: 1.3 mg/mL of potassium iodide in *Mobile phase*. This solution has a concentration of 1 mg/mL of iodide.
Standard solution: 2.5 µg/mL of iodide from the *Standard stock solution* in *Mobile phase*
System suitability solution: Transfer 0.13 g of potassium iodide and 0.5 g of potassium iodate to a 100-mL volumetric flask. Dissolve in *Mobile phase*, using sonication if necessary, dilute with *Mobile phase* to volume, and mix. Transfer 1.0 mL of this solution to a 100-mL volumetric flask, dilute with *Mobile phase* to volume, and mix. Transfer 25.0 mL of this solution to a 100-mL volumetric flask, and dilute with *Mobile phase* to volume.
Sample solution: Dilute an accurately measured volume of the Oral Solution to obtain a solution with a concentration of 2.5 µg/mL of iodine in *Mobile phase*.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 225 nm
Column: 4.6-mm × 15-cm; packing L1
Flow rate: 1.5 mL/min
Injection volume: 30 µL
System suitability
Samples: *Standard solution* and *System suitability solution*
[NOTE—The relative retention times for iodate and iodide are about 0.32 and 1.0, respectively.]
Suitability requirements
Resolution: NLT 2.5 between iodate and iodide, *System suitability solution*
Relative standard deviation: NMT 2.0% for the iodide peak, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of iodine (I) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of iodide from the *Sample solution*
r_S = peak area of iodide from the *Standard solution*
C_S = concentration of iodide in the *Standard solution* (µg/mL)
C_U = nominal concentration of iodine in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of iodine (I)

• **IRON**
Iron standard stock solution: Transfer 100 mg of iron powder to a 1000-mL volumetric flask, dissolve in 6 N hydrochloric acid, and dilute with water to volume.
Standard solutions: To separate 100-mL volumetric flasks, transfer 2.0, 4.0, 5.0, 6.0, and 8.0 mL of the *Iron standard stock solution*. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 2.0, 4.0, 5.0, 6.0, and 8.0 µg/mL of iron.

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Sample solution: 6 µg/mL of iron from Oral Solution in 0.125 N hydrochloric acid

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: 248.3 nm

Lamp: Iron hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus concentration, in µg/mL, of iron, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of iron in the *Sample solution*.

Calculate the percentage of the labeled amount of iron (Fe) in the portion of Oral Solution taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of iron in the *Sample solution* from the graph (µg/mL)

C_U = nominal concentration of iron in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of iron (Fe)

• **MAGNESIUM**

Magnesium standard solution: Transfer 1.00 g of magnesium ribbon to a 1000-mL volumetric flask. Dissolve in 50 mL of 6 N hydrochloric acid, and dilute with water to volume.

Standard stock solution: 20 µg/mL of magnesium from *Magnesium standard solution* in 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks, transfer 5.0, 7.5, 10.0, 12.5, and 15.0 mL of *Standard stock solution*. Dilute with 0.125 N hydrochloric acid to volume to obtain concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 µg/mL of magnesium.

Sample solution: 2.5 µg/mL of magnesium from Oral Solution in 0.125 N hydrochloric acid

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: 285.2 nm

Lamp: Magnesium hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus concentration, in µg/mL, of magnesium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, µg/mL, of magnesium in the *Sample solution*.

Calculate the percentage of the labeled amount of magnesium (Mg) in the portion of Oral Solution taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of magnesium in the *Sample solution* from the graph (µg/mL)

C_U = nominal concentration of magnesium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of magnesium (Mg)

• **MANGANESE**

Manganese standard solution: Transfer 1.0 g of manganese to a 1000-mL volumetric flask. Dissolve in 20 mL of nitric acid, and dilute with 6 N hydrochloric acid to volume.

Standard stock solution: 50 µg/mL of manganese from the *Manganese standard stock solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks, transfer 1.0, 1.5, 2.0, 3.0, and 4.0 mL of *Standard stock solution*. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions having known concentrations of 0.5, 0.75, 1.0, 1.5, and 2.0 µg/mL of manganese.

Sample solution: Dilute an accurately measured volume of the Oral Solution to obtain 1.5 µg/mL of manganese in 0.125 N hydrochloric acid.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: 279.5 nm

Lamp: Manganese hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus concentration, in µg/mL, of manganese, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of manganese in the *Sample solution*.

Calculate the percentage of the labeled amount of manganese (Mn) in the portion of Oral Solution taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of manganese in the *Sample solution* from the graph (µg/mL)

C_U = nominal concentration of manganese in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of manganese (Mn)

• **MOLYBDENUM**

Molybdenum standard solution: Transfer 1.0 g of molybdenum wire to a 1000-mL volumetric flask, and dissolve in 50 mL of nitric acid, warming if necessary. Dilute with water to volume.

Standard stock solution: 100 µg/mL of molybdenum from the *Molybdenum standard solution* in water

Standard solutions: To separate 100-mL volumetric flasks transfer 0.5, 1.0, 1.5, 2.0, and 3.0 mL of the *Standard stock solution*. Add 0.125 N hydrochloric acid to volume, and mix to obtain the solutions having known concentrations of about 0.5, 1.0, 1.5, 2.0, and 3.0 µg/mL of molybdenum.

Sample solution: Dilute an accurately measured volume of the Oral Solution to obtain 1 µg/mL of molybdenum from the Oral Solution in 0.125 N hydrochloric acid.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: 313.3 nm

Lamp: Molybdenum hollow-cathode

Flame: Nitrous oxide–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

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Determine the absorbances of the solutions, against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of molybdenum, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of molybdenum in the *Sample solution*.

Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Oral Solution taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of molybdenum in the *Sample solution* from the graph (µg/mL)

C_U = nominal concentration of molybdenum in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

- **ZINC**

Zinc standard solution: Transfer 311 mg of zinc oxide to a 250-mL volumetric flask, and add 80 mL of 6 N hydrochloric acid, warming if necessary to dissolve. Cool, dilute with water to volume, and mix to obtain a solution having a known concentration of 1000 µg/mL of zinc.

Standard stock solution: 50 µg/mL of zinc from the *Zinc standard solution* in 0.125 N hydrochloric acid

Standard solutions: Transfer 1.0, 2.0, 3.0, 4.0, and 5.0 mL of *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of zinc.

Sample solution: Dilute an accurately measured volume of the Oral Solution to obtain 1 µg/mL of zinc in 0.125 N hydrochloric acid.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: 213.8 nm

Lamp: Zinc hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus concentration, in µg/mL, of zinc, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of zinc in the *Sample solution*.

Calculate the percentage of the labeled amount of zinc (Zn) in the portion of Oral Solution taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of zinc in the *Sample solution* from the graph (µg/mL)

C_U = nominal concentration of zinc in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of zinc (Zn)

OTHER COMPONENTS

- **ALCOHOL DETERMINATION,** *Method I* ⟨611⟩ (if present): 90.0%–120.0% of the labeled amount of alcohol (C_2H_5OH)

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 3×10^3 cfu/mL, and the combined molds and yeasts count does not exceed 3×10^2 cfu/mL.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meet the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, under an inert gas or with a minimum of headspace.
- **LABELING:**[5] The label states that the product is Oil- and Water-Soluble Vitamins with Minerals Oral Solution. The label also states the quantity of each vitamin and mineral in a given volume of the Oral Solution and, where necessary, the chemical form in which a vitamin is present, and also states the salt form of the mineral used as the source of each element. Where the product contains vitamin E, the label indicates whether it is the *d*- or *dl*- form. Where the product is labeled to contain panthenol the label states the equivalent content of dexpanthenol. Where more than one assay method is given for a particular vitamin or mineral, the labeling states with which assay method the product complies only if *Method 1* is not used.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Alpha Tocopherol RS
2*H*-1-Benzopyran-6-ol, 3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-.
$C_{29}H_{50}O_2$ 430.70

USP Biotin RS
1*H*-Thieno[3,4-*d*]imidazole-4-pentanoic acid, hexahydro-2-oxo-, 3a*S*-[(3aα,4β,6aα)]-.
$C_{10}H_{16}N_2O_3S$ 244.31

USP Calcium Pantothenate RS
β-Alanine, *N*-(2,4-dihydroxy-3,3-dimethyl-1-oxobutyl)-, calcium salt (2:1), (*R*)-.
$C_{18}H_{32}CaN_2O_{10}$ 476.53

USP Cholecalciferol RS
9,10-Secocholesta-5,7,10(19)-trien-3-ol, (3β,5*Z*,7*E*)-.
$C_{27}H_{44}O$ 384.64

USP Cyanocobalamin (Crystalline) RS
Vitamin B_{12}.
$C_{63}H_{88}CoN_{14}O_{14}P$ 1355.37

USP Dexpanthenol RS
Butanamide, 2,4-dihydroxy-*N*-(3-hydroxypropyl)-3,3-dimethyl-, (*R*)-.
$C_9H_{19}NO_4$ 205.25

[5] USP Units of activity for vitamins, where such exist or formerly existed, are equivalent to the corresponding international units, where such formerly existed. The USP Unit for Vitamin E has been discontinued. International units (IU) for vitamins also have been discontinued; however the use of IU on the labels of vitamin products continues. Where articles are labeled in terms of Units in addition to the required labeling, the relationship of the USP Units or IU to mass is as follows. One USP Vitamin A Unit = 0.3 µg of all-*trans*-retinol (vitamin A alcohol) or 0.344 µg of all-*trans*-retinyl acetate (vitamin A acetate) or 0.55 µg of all-*trans*-retinyl palmitate (vitamin A palmitate), and 1 µg of retinol (3.3 USP Vitamin A Units) = 1 retinol equivalent (RE); 1 IU of beta carotene = 0.6 µg of all-*trans*-beta carotene; 1 USP Vitamin D Unit = 0.025 µg of ergocalciferol or cholecalciferol; and 1 mg of *dl*-alpha tocopherol = 1.1 former USP Vitamin E Units, 1 mg of *dl*-alpha tocopheryl acetate = 1 former USP Vitamin E Unit, 1 mg of *dl*-alpha tocopheryl acid succinate = 0.89 former USP Vitamin E Unit, 1 mg of *d*-alpha tocopherol = 1.49 former USP Vitamin E Units, and 1 mg of *d*-alpha tocopheryl acetate = 1.36 former USP Vitamin E Units, 1 mg of *d*-alpha tocopheryl acid succinate = 1.21 former USP Vitamin E Units. In terms of *d*-alpha tocopherol equivalents, 1 mg of *d*-alpha tocopheryl acetate = 0.91, 1 mg of *d*-alpha tocopheryl acid succinate = 0.81, 1 mg of *dl*-alpha tocopherol = 0.74, 1 mg of *dl*-alpha tocopheryl acetate = 0.67, and 1 mg of *dl*-alpha tocopheryl acid succinate = 0.60.

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USP Ergocalciferol RS
9,10-Secoergosta-5,7,10(19),22-tetraen-3-ol, (3β,5*Z*,7*E*,22*E*)-.
$C_{28}H_{44}O$ 396.65
USP Niacin RS
3-Pyridinecarboxylic acid.
$C_6H_5NO_2$ 123.11
USP Niacinamide RS
3-Pyridinecarboxamide.
$C_6H_6N_2O$ 122.12
USP Pyridoxine Hydrochloride RS
3,4-Pyridinedimethanol, 5-hydroxy-6-methyl-, hydrochloride.
$C_8H_{11}NO_3 \cdot HCl$ 205.64
USP Racemic Panthenol RS
Butanamide, 2,4-dihydroxy-*N*-(3-hydroxypropyl)-3,3-dimethyl-,(±)-.
$C_9H_{19}NO_4$ 205.25
USP Riboflavin RS
Riboflavine.
$C_{17}H_{20}N_4O_6$ 376.36
USP Sodium Fluoride RS
Sodium fluoride.
NaF 41.99
USP Thiamine Hydrochloride RS
Thiazolium, 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-5-(2-hydroxyethyl)-4-methyl-, chloride, monohydrochloride.
$C_{12}H_{17}ClN_4OS \cdot HCl$ 337.27
USP Vitamin A RS
3,7-Dimethyl-9-(2,6,6-trimethyl-1- cyclohexen-1-yl) 2,4,6,8-nonatetraen-1-ol acetate (vitamin A acetate).

Oil- and Water-Soluble Vitamins with Minerals Tablets

DEFINITION

Change to read:

Oil- and Water-Soluble Vitamins with Minerals Tablets contain one or more of the following oil-soluble vitamins: Vitamin A ▲as retinyl acetate or retinyl palmitate,▲ (USP 1-May-2020) vitamin D as Ergocalciferol (vitamin D_2) or Cholecalciferol (vitamin D_3), Vitamin E ▲as *RRR*- or *all-rac*-alpha-tocopherol, *RRR*- or *all-rac*-alpha-tocopheryl acetate, or *RRR*- or *all-rac*-alpha-tocopheryl acid succinate,▲ (USP 1-May-2020) Phytonadione (vitamin K_1), and Beta Carotene; and one or more of the following water-soluble vitamins: Ascorbic Acid or its equivalent as Calcium Ascorbate or Sodium Ascorbate, Biotin, Cyanocobalamin, Folic Acid, Niacin or Niacinamide, pantothenic acid (as Calcium Pantothenate or Racemic Calcium Pantothenate), Pyridoxine Hydrochloride, Riboflavin, and Thiamine Hydrochloride or Thiamine Mononitrate; and one or more minerals derived from substances generally recognized as safe, furnishing one or more of the following elements in ionizable form: boron, calcium, chromium, copper, fluorine, iodine, iron, magnesium, manganese, molybdenum, nickel, phosphorus, potassium, selenium, tin, vanadium, and zinc. Tablets contain NLT 90.0% and NMT 165.0% of the labeled amount of vitamin A ▲as retinol▲ (USP 1-May-2020) ($C_{20}H_{30}O$); vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$); vitamin E ▲as 2*R*-alpha-tocopherol▲ (USP 1-May-2020) ($C_{29}H_{50}O_2$); phytonadione ($C_{31}H_{46}O_2$); and beta carotene ($C_{40}H_{56}$); and NLT 90.0% and NMT 150.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$); ▲▲ (USP 1-May-2020) biotin ($C_{10}H_{16}N_2O_3S$), cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$), folic acid ($C_{19}H_{19}N_7O_6$), niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), pyridoxine ▲▲ (USP 1-May-2020) ($C_8H_{11}NO_3$▲▲ (USP 1-May-2020)), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine ▲as thiamine ion ($C_{12}H_{17}N_4OS$)▲ (USP 1-May-2020) and NLT 90.0% and NMT 125.0% of the labeled amount of calcium (Ca), copper (Cu), iron (Fe), manganese (Mn), magnesium (Mg), phosphorus (P), potassium (K), and zinc (Zn); and NLT 90.0% and NMT 160.0% of the labeled amount of boron (B), chromium (Cr), fluorine (F), iodine (I), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), and vanadium (V). They may contain other labeled added substances that are generally recognized as safe, in amounts that are unobjectionable.

IDENTIFICATION

Add the following:

▲• **A.** Characteristic absorption of each mineral as obtained in the tests in *Strength*▲ (USP 1-May-2020)

Add the following:

▲• **B.** The retention times of the vitamin peaks of the *Sample solutions* correspond to those of the corresponding vitamin peaks of the *Standard solutions* as obtained in the tests for *Strength*.▲ (USP 1-May-2020)

STRENGTH

[NOTE—Where more than one assay method is given for an individual ingredient, the requirements may be met by following any one of the specified methods, with the method used being stated in the labeling only if *Method 1* is not used.]

Change to read:

• **VITAMIN A, *Method 1***

▲Proceed as directed in *Vitamin A Assay* ⟨571⟩, *Assay, Chromatographic Methods, Procedure 1*, except for the *Sample stock solution* and *Sample solution*.▲ (USP 1-May-2020)

Sample stock solution: Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 5 Tablets, to a container having a polytef-lined screw-cap. Add 10 mL of dimethyl sulfoxide and 15 mL of *n*-hexane, and shake for 45 min on a wrist-action shaker in a water bath maintained at 60°. [NOTE—Set up the wrist-action shaker to ensure that the contents of the container are mixed vigorously and thoroughly.] Centrifuge at 3000 rpm for 10 min, and transfer the hexane layer by means of a pipet to a 100-mL volumetric flask. Add 15 mL of *n*-hexane to the dimethyl sulfoxide layer, shake thoroughly for 5 min, and transfer the hexane layer by means of a pipet to the 100-mL volumetric flask. Repeat this extraction with 3 additional 15-mL portions of *n*-hexane. Dilute the extracts in the volumetric flask with *n*-hexane to volume.

Sample solution: Dilute a 10-mL volume of the *Sample stock solution* with *n*-hexane to obtain a solution with a concentration of 15 µg/mL of vitamin A as retinol ($C_{20}H_{30}O$).

▲▲ (USP 1-May-2020)

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Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A as retinol ($C_{20}H_{30}O$)

Change to read:

- **VITAMIN A, *Method 2***

▲Proceed as directed in *Vitamin A Assay* ⟨571⟩, *Assay, Chromatographic Methods, Procedure 2*.▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A as retinol ($C_{20}H_{30}O$)

Change to read:

- **VITAMIN A, *Method 3***

▲Proceed as directed in *Vitamin A Assay* ⟨571⟩, *Assay, Chromatographic Methods, Procedure 3*.▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A as retinol ($C_{20}H_{30}O$)

Change to read:

- **VITAMIN D (CHOLECALCIFEROL OR ERGOCALCIFEROL), *Method 1***

▲Proceed as directed in *Vitamin D Assay* ⟨581⟩, *Assay, Chromatographic Methods, Procedure 1*, except for the *Sample solution*.▲ (USP 1-May-2020)

Sample solution: Prepare as directed for the *Sample stock solution* in *Vitamin A, Method 1*. Transfer NLT 20 mL of this solution to a suitable container, and if necessary, evaporate under vacuum at room temperature to obtain a concentration of 2 µg/mL of cholecalciferol or ergocalciferol.

▲▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

Change to read:

- **VITAMIN D (CHOLECALCIFEROL or ERGOCALCIFEROL), *Method 2***

▲Proceed as directed in *Vitamin D Assay* ⟨581⟩, *Assay, Chromatographic Methods, Procedure 2*.▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

Change to read:

- **VITAMIN D (CHOLECALCIFEROL or ERGOCALCIFEROL), *Method 3***

▲Proceed as directed in *Vitamin D Assay* ⟨581⟩, *Assay, Chromatographic Methods, Procedure 3*, except for the *Analysis*.▲ (USP 1-May-2020)

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for vitamin D.

Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*

r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*

C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

Change to read:

- **VITAMIN E, *Method 1***

▲Proceed as directed in *Vitamin E Assay* ⟨551⟩, *Assay, Procedure 1*, except for the *Sample solution* and *Analysis*.▲ (USP 1-May-2020)

Sample solution: Prepare as directed for the *Sample stock solution* in *Vitamin A, Method 1*. Transfer NLT 20 mL of this solution to a suitable container, and evaporate under vacuum at room temperature to dryness. Transfer the residue with the aid of methanol to a suitable volumetric flask, and dilute with methanol to volume to obtain a concentration of 2 mg/mL of alpha-tocopherol, alpha-tocopheryl acetate, or alpha-tocopheryl acid succinate.

▲▲ (USP 1-May-2020)

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas.

Calculate the percentage of the labeled amount of alpha-tocopherol ($C_{29}H_{50}O_2$), alpha-tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha-tocopheryl acid succinate ($C_{33}H_{54}O_5$) ▲as *2R*-alpha-tocopherol ($C_{29}H_{50}O_2$)▲ (USP 1-May-2020) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*

r_S = peak area of the relevant vitamin E form from the *Standard solution*

C_S = concentration of ▲*all-rac*-alpha-tocopherol from either USP Alpha Tocopherol RS or USP Alpha Tocopheryl Acetate RS or *RRR*-alpha-tocopherol from USP Alpha Tocopheryl Acid Succinate RS▲ (USP 1-May-2020) in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding form of vitamin E ▲as *2R*-alpha-tocopherol▲ (USP 1-May-2020) in the *Sample solution* (mg/mL)

▲F = conversion factor for the content of *all-rac*-alpha-tocopherol to *2R*-alpha-tocopherol, 1/2 (for products labeled to contain *all-rac* vitamin E sources) and 1 (for products labeled to contain *RRR* vitamin E sources)▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–165.0% of the labeled amount of alpha-tocopherol ($C_{29}H_{50}O_2$), alpha-tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha-tocopheryl acid succinate ($C_{33}H_{54}O_5$) as ▲*2R*-alpha-tocopherol▲ (USP 1-May-2020)

Change to read:

- **VITAMIN E, *Method 2***

▲Proceed as directed in *Vitamin E Assay* ⟨551⟩, *Assay, Procedure 2*, except for the *Analysis*.▲ (USP 1-May-2020)

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Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of alpha-tocopherol ($C_{29}H_{50}O_2$), alpha-tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha-tocopheryl acid succinate ($C_{33}H_{54}O_5$) as 2R-alpha-tocopherol ($C_{29}H_{50}O_2$) in the portion of Tablets taken as follows:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*
r_S = peak area of the relevant vitamin E form from the *Standard solution*
C_S = concentration of *all-rac*-alpha-tocopherol from either USP Alpha Tocopherol RS or USP Alpha Tocopheryl Acetate RS or *RRR*-alpha-tocopherol from USP Alpha Tocopheryl Acid Succinate RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of the corresponding form of vitamin E as *2R*-alpha-tocopherol in the *Sample solution* (mg/mL)
F = conversion factor for the content of *all-rac*-alpha-tocopherol to *2R*-alpha-tocopherol, 1/2 (for products labeled to contain *all-rac* vitamin E sources) and 1 (for products labeled to contain *RRR* vitamin E sources)

Acceptance criteria: 90.0%–165.0% of the labeled amount of alpha-tocopherol ($C_{29}H_{50}O_2$), alpha-tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha-tocopheryl acid succinate ($C_{33}H_{54}O_5$) as ▲2*R*-alpha-tocopherol▲ (USP 1-May-2020)

Change to read:

• **VITAMIN E,** ***Method 3***
▲Proceed as directed in *Vitamin E Assay* ⟨551⟩, *Assay, Procedure 3*, except for the *Analysis*.
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of alpha-tocopherol ($C_{29}H_{50}O_2$), alpha-tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha-tocopheryl acid succinate ($C_{33}H_{54}O_5$) as 2*R*-alpha-tocopherol ($C_{29}H_{50}O_2$) in the portion of Tablets taken as follows:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*
r_S = peak area of the relevant vitamin E form from the *Standard solution*
C_S = concentration of *all-rac*-alpha-tocopherol from either USP Alpha Tocopherol RS and USP Alpha Tocopheryl Acetate RS or *RRR*-alpha-tocopherol from USP Alpha Tocopheryl Acid Succinate RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of the corresponding form of vitamin E as *2R*-alpha-tocopherol in the *Sample solution* (mg/mL)
F = conversion factor for the content of *all-rac*-alpha-tocopherol to *2R*-alpha-tocopherol, 1/2 (for products labeled to contain *all-rac* vitamin E sources) and 1 (for products labeled to contain *RRR* vitamin E sources)▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–165.0% of the labeled amount of alpha-tocopherol ($C_{29}H_{50}O_2$), alpha-tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha-tocopheryl acid succinate ($C_{33}H_{54}O_5$) as ▲2*R*-alpha-tocopherol▲ (USP 1-May-2020)

• **PHYTONADIONE,** ***Method 1***
[NOTE—Use low-actinic glassware throughout this procedure.]
Mobile phase: Methanol and water (19:1)
Standard stock solution: 200 µg/mL of USP Phytonadione RS in methanol. Dissolve with the aid of sonication if necessary.
System suitability solution: 0.65 mg/mL of USP Alpha Tocopheryl Acetate RS and 20 µg/mL of USP Phytonadione RS from the *Standard stock solution* diluted with methanol. [NOTE—Dissolve USP Alpha Tocopheryl Acetate RS in a portion of methanol, add the *Standard stock solution*, and then dilute with methanol to volume.]
Standard solution: 20 µg/mL of USP Phytonadione RS from the *Standard stock solution* diluted with methanol
Sample solution: Prepared as directed for the *Sample stock solution* in *Vitamin A, Method 1*. Transfer NLT 20 mL of the solution to a suitable container, and, if necessary, evaporate under vacuum at room temperature to dryness. Transfer the residue to a suitable volumetric flask with the aid of methanol, and dilute with methanol to volume to obtain a concentration of 20 µg/mL of phytonadione.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 8-mm × 10-cm; 5-µm packing L1
Flow rate: 2 mL/min
Injection volume: 100 µL
System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The relative retention times for alpha-tocopheryl acetate and phytonadione are 0.68 and 1.0, respectively.]
Suitability requirements
Resolution: NLT 5 between alpha-tocopheryl acetate and phytonadione, *System suitability solution*
Relative standard deviation: NMT 3.0%, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas. Calculate the percentage of the labeled amount of phytonadione ($C_{31}H_{46}O_2$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of phytonadione from the *Sample solution*
r_S = peak area of phytonadione from the *Standard solution*
C_S = concentration of USP Phytonadione RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of phytonadione in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of phytonadione ($C_{31}H_{46}O_2$)

• **PHYTONADIONE,** ***Method 2***
[NOTE—Use low-actinic glassware throughout this procedure.]
Solvent: Methanol and isopropyl alcohol (19:1)
Mobile phase: Mix 800 mL of methanol, 200 mL of methylene chloride, 0.1 mL of glacial acetic acid, 1.36 g of zinc chloride, and 0.41 g of sodium acetate.
Internal standard solution: 5 µg/mL of menaquinone 4 (vitamin K_2) in the *Solvent*. [NOTE—A concentrated stock

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solution of menaquinone 4 (100 µg/mL) can be stored for 2 months in a refrigerator.]

Standard stock solution: 5 µg/mL of USP Phytonadione RS, prepared by dissolving in methylene chloride with the aid of sonication and diluting with *Solvent* to final volume

Standard solution: Transfer 1.0 mL of the *Standard stock solution* and 1.0 mL of the *Internal standard solution* to a suitable flask, and dilute with *Solvent* to 5 mL. Pass through a membrane filter of 0.45-µm or finer pore size.

Sample solution: Finely powder NLT 20 Tablets. To a centrifuge tube fitted with a cap transfer an amount of powder not exceeding 800 mg and equivalent to an amount of phytonadione not exceeding 50 µg. Add 4 mL of water. Insert the stopper, and mix using a vortex mixer until the sample is dispersed. Place the tube in a water bath at 60° for 5 min. Remove from the bath, and again shake or mix using a vortex mixer for 1 min while the preparation is still hot. Add 8 mL of alcohol, and swirl the contents to mix. Place the tube in a water bath at 60° for 5 min. Remove from the bath, and again shake or mix using a vortex mixer for 2 min while the preparation is still hot. Cool to room temperature. Add a volume of the *Internal standard solution*, equivalent to 1.0 mL per each 5 µg of the expected amount of phytonadione in the aliquot taken. Add 20.0 mL of petroleum ether, and cap the tube tightly. Shake or mix using a vortex mixer for 15 min to thoroughly mix the contents. Centrifuge to separate the 2 layers. Transfer a volume of the top layer of petroleum ether, equivalent to 5–50 µg of the nominal amount of phytonadione, to an appropriate flask. Place the flask in a water bath at 35°–45°, and evaporate the solvent under a stream of nitrogen until an oily residue is left. Dissolve the residue in a volume of the *Solvent* to obtain a concentration of 1 µg/mL of phytonadione.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Fluorometric detector

Excitation: 320 nm

Emission: 420 nm

Column: 4.6-mm × 25-cm; 5-µm, end-capped packing L1, and a postcolumn reactor constituted with a 4.6-mm × 3-cm PEEK column tightly packed with zinc powder. [NOTE—Prepare the postcolumn reactor daily, or as necessary, to meet the *System suitability* requirements.]

Flow rate: 1 mL/min

Injection volume: 25 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for the internal standard and phytonadione are 1.0 and 1.4, respectively.]

Suitability requirements

Column efficiency: NLT 2500 theoretical plates for the phytonadione peak

Tailing factor: NMT 1.5 for the phytonadione peak

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of phytonadione ($C_{31}H_{46}O_2$) in the portion of Tablets taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of phytonadione to the internal standard from the *Sample solution*

R_S = peak response ratio of phytonadione to the internal standard from the *Standard solution*

C_S = concentration of USP Phytonadione RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of phytonadione in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of labeled amount of phytonadione ($C_{31}H_{46}O_2$)

Add the following:

▲• **VITAMIN A, VITAMIN D (CHOLECALCIFEROL OR ERGOCALCIFEROL), and VITAMIN E, *Method 4*; PHYTONADIONE, *Method 3***

[NOTE—Where vitamin A (retinyl acetate or retinyl palmitate), vitamin D (cholecalciferol or ergocalciferol), or vitamin E (alpha-tocopherol, alpha-tocopheryl acetate, or alpha-tocopheryl acid succinate) are specified in the following procedure, use the chemical form present in the formulation and the relevant Reference Standard. Use low-actinic glassware.]

Diluent: Methanol and water (90:10, v/v)

Solution A: 0.1% (v/v) trifluoroacetic acid in *Diluent*

Solution B: 0.1% (v/v) trifluoroacetic acid in methanol

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
19	0	100
27	0	100
28	100	0
30	100	0

Vitamin A standard stock solution: A solution containing the equivalent of 0.2 mg/mL of retinol from USP Retinyl Acetate RS or USP Retinyl Palmitate RS in isopropyl alcohol. Dissolve with the aid of sonication if necessary.

Vitamin D standard stock solution: 0.05 mg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in isopropyl alcohol. Dissolve with the aid of sonication if necessary.

Vitamin E standard stock solution: 3.0 mg/mL of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, or USP Alpha Tocopheryl Acid Succinate RS in isopropyl alcohol. Dissolve with the aid of sonication if necessary.

Phytonadione standard stock solution: 0.04 mg/mL of USP Phytonadione RS in isopropyl alcohol. Dissolve with the aid of sonication if necessary.

Sample solution 1: Finely powder NLT 20 Tablets. Transfer an accurately weighed portion of the powder into a 50-mL volumetric flask to obtain a solution containing known nominal concentrations in the ranges of 0.05–0.1 mg/mL of vitamin A and 0.5–0.15 mg/mL of vitamin E. Add 10 mL of dimethyl sulfoxide and sonicate for 30 min with vigorous intermittent shaking. Cool to room temperature, dilute with isopropyl alcohol to volume, and mix well. Pass a portion of the solution through a nylon filter of 0.45-µm pore size, and discard the first milliliter of the filtrate.

Sample solution 2: Transfer an accurately weighed portion of the powder from NLT 20 finely powdered Tablets into a 50-mL volumetric flask to obtain a solution containing known nominal concentrations in the ranges of 1.0–3.5 µg/mL of vitamin D and 1.0–3.5 µg/mL of phytonadione. Add 5 mL of water and sonicate for 10 min with intermittent

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shaking. Add 10 mL of dimethyl sulfoxide and sonicate for 30 min with vigorous intermittent shaking. Cool to room temperature, dilute with isopropyl alcohol to volume, and mix well. Pass a portion of the solution through a nylon filter of 0.45-µm pore size, and discard the first milliliter of the filtrate.

Standard solution: Transfer calculated volumes of *Vitamin A standard stock solution*, *Vitamin D standard stock solution*, *Vitamin E standard stock solution*, and *Phytonadione standard stock solution* into a suitable volumetric flask, and dilute with isopropyl alcohol to volume to obtain a solution with final vitamin concentrations similar to those obtained for the corresponding vitamins in *Sample solution 1* and *Sample solution 2*.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detectors

For *Sample solution 1* containing retinyl acetate: UV 325 nm for 7 min, then switch to 265 nm

For *Sample solution 1* containing retinyl palmitate: UV 265 nm for 18 min, then switch to 325 nm

For *Sample solution 2*: UV 265 nm

Column: 4.6-mm × 10-cm; 3-µm packing L1

Temperatures

Sample: 4°

Column: 35°

Flow rate: 1.5 mL/min

Injection volume: 20 µL

System suitability

Sample: *Sample solution 1*, *Sample solution 2*, and *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0% for each individual peak, *Standard solution*

Peak purity of vitamin D and vitamin A (as retinyl palmitate): Monitor the chromatograms of *Sample solution 1* and *Sample solution 2* at 450 nm. The area of any peak at the retention time of retinyl palmitate from *Sample solution 1* and any peak at the retention time of vitamin D (as cholecalciferol or ergocalciferol) from *Sample solution 2* should be NMT 5% of the corresponding peak areas detected at 325 nm and 265 nm, respectively. [NOTE—This test is required because of possible interference with carotenoids (cryptoxanthin and lycopene) that may be present in the formulation. Use another procedure in the monograph for analysis of vitamin D and vitamin A (as retinyl palmitate) if the system is not suitable]

Analysis

Samples: *Sample solution 1*, *Sample solution 2*, and *Standard solution*

Calculate the percentage of the labeled amount of vitamin A activity, as retinol ($C_{20}H_{30}O$), in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the *all-trans*-retinyl ester from the *Sample solution*

r_S = peak area of the *all-trans*-retinyl ester from the *Standard solution*

C_S = concentration of retinol in the *Standard solution* (mg/mL)

C_U = nominal concentration of vitamin A, as retinol, in the *Sample solution* (mg/mL)

Calculate the percentage of the labeled amount of vitamin E, as 2*R*-alpha-tocopherol ($C_{29}H_{50}O_2$), in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*

r_S = peak area of the relevant vitamin E form from the *Standard solution*

C_S = concentration of *all-rac*-alpha-tocopherol from either USP Alpha Tocopherol RS or USP Alpha Tocopheryl Acetate RS or *RRR*-alpha-tocopherol from USP Alpha Tocopheryl Acid Succinate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of vitamin E, as 2*R*-alpha-tocopherol, in the *Sample solution* (mg/mL)

F = conversion factor for the content of *all-rac*-alpha-tocopherol to *RRR*-alpha-tocopherol equivalent, 1/2 (for products labeled to contain *all-rac* vitamin E sources) and 1 (for products labeled to contain *RRR* vitamin E sources)

Calculate the percentage of the labeled amount of vitamin D, as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$), and phytonadione ($C_{31}H_{46}O_2$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the related vitamin from the *Sample solution*

r_S = peak area of the related vitamin from the *Standard solution*

C_S = concentration of related vitamin in the *Standard solution* (µg/mL)

C_U = nominal concentration of related vitamin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of each individual vitamin ▲(USP 1-May-2020)

• **BETA CAROTENE**

[NOTE—Use low-actinic glassware throughout this procedure.]

Potassium hydroxide solution: Dissolve 58.8 g potassium hydroxide in 50 mL of water.

Iodine solution: 0.01 mg/mL of iodine in cyclohexane. [NOTE—Prepare this solution fresh daily.]

Sample solution: Weigh NLT 20 Tablets. Grind the Tablets to a fine powder, and transfer a quantity of the powder, equivalent to 2 mg of beta carotene, to a 500-mL saponification flask. Add 100 mL of alcohol, 6 mL of *Potassium hydroxide solution*, and a magnetic stirring bar. Attach an air condenser to the flask, and heat under reflux for 45 min with constant stirring. Cool to room temperature. Add 170 mL of solvent hexane, and stir for 30 min. Quantitatively transfer the contents of the flask to a 500-mL separatory funnel with portions of solvent hexane. Allow the layers to separate for 5–10 min, and transfer the upper organic layer to a 500-mL volumetric flask. Transfer the lower aqueous layer into the saponification flask. Add 170 mL of solvent hexane, and stir for an additional 20 min. Quantitatively transfer the contents of the saponification flask to the separatory funnel with the aid of portions of solvent hexane. Allow the layers to separate for 10 min. Drain the lower aqueous layer, and discard. Transfer the

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organic layer to the volumetric flask containing the previously collected organic layer. Rinse the separatory funnel with small portions of solvent hexane, and transfer the washings to the volumetric flask. Dilute the hexane extracts with solvent hexane to volume. Add 3 g of anhydrous sodium sulfate, shake, and allow to settle. Quantitatively transfer a volume of this solution, equivalent to 100 µg of beta carotene, to a 50-mL volumetric flask. Evaporate under a stream of nitrogen to dryness, and immediately add cyclohexane. Add 2 mL of *Iodine solution*, and heat for 15 min in a water bath maintained at 65°. Cool rapidly, and dilute with cyclohexane to volume.

Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: Vis
Analytical wavelength: 452 nm
Blank: Cyclohexane

Analysis
Sample: *Sample solution*
Determine the absorbance against the *Blank*.
Calculate the percentage of the labeled amount of beta carotene ($C_{40}H_{56}$) in the portion of Tablets taken:

$$\text{Result} = (A_U/F) \times (100/C_U)$$

A_U = absorbance of the *Sample solution*
F = absorptivity of beta carotene at 452 nm, 223
C_U = nominal concentration of beta carotene in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of beta carotene ($C_{40}H_{56}$)

Change to read:

- **ASCORBIC ACID, CALCIUM ASCORBATE,** and **SODIUM ASCORBATE**

(See *Vitamin C Assay* ⟨580⟩.)
[NOTE—For labeling purposes, consider ⟨580⟩, *Method I—Titrimetric Method* as *Method 1*; ▲⟨580⟩, *Method II—Chromatographic Method, Procedure 1* as *Method 2*; and ⟨580⟩, *Method II—Chromatographic Method, Procedure 2* as *Method 3*.▲ (USP 1-May-2020)]
Acceptance criteria: 90.0%–150.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$), or its salts as calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$) or sodium ascorbate ($C_6H_7NaO_6$)

- **BIOTIN,** ***Method 1***

[NOTE—Use low-actinic glassware throughout this procedure.]
Mobile phase: Mix 85 mL of acetonitrile, 1 g of sodium perchlorate, 1 mL of phosphoric acid, and dilute with water to 1000 mL.
Standard stock solution: 0.333 mg/mL of USP Biotin RS in dimethyl sulfoxide
Standard solution: 5 µg/mL of USP Biotin RS prepared by diluting the *Standard stock solution* in water
Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 1 mg of biotin, to a 200-mL volumetric flask. Add 3 mL of dimethyl sulfoxide, and swirl to wet the contents. Place the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with water to volume, and filter.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 200 nm
Column: 4.6-mm × 15-cm; 3-µm packing L7
Flow rate: 1.2 mL/min
Injection volume: 100 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 3.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas of biotin. Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of biotin from the *Sample solution*
r_S = peak area of biotin from the *Standard solution*
C_S = concentration of USP Biotin RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of biotin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

Change to read:

- **BIOTIN,** ***Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]
Dehydrated mixtures yielding formulations similar to the media described herein may be used provided that, when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described herein.
Standard stock solution: 50 µg/mL of USP Biotin RS in 50% alcohol. Store in a refrigerator.
Standard solution: 0.1 ng/mL of USP Biotin RS in water, prepared by dilution of the *Standard stock solution* with water on the day of the assay
Sample solution: Finely powder NLT 30 Tablets. Transfer a portion of the powder, equivalent to 100 µg of biotin, to a 200-mL volumetric flask. Add 3 mL of 50% alcohol, and swirl to wet the contents. Heat the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with 50% alcohol to volume, and filter. Dilute a volume of the filtrate quantitatively, and stepwise if necessary, with water to obtain a solution with a concentration of 0.1 ng/mL.
Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and dilute with water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature not below 10°. Filter the solution if a precipitate forms during storage.
Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water. Heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and add water to make 1000 mL. Store under toluene in a cool place at a temperature NLT 10°.
Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

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Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Calcium pantothenate solution: 10 µg/mL of calcium pantothenate in 50% alcohol. Store in a refrigerator.

Riboflavin–thiamine hydrochloride solution: 20 µg/mL of riboflavin and 10 µg/mL of thiamine hydrochloride in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

***p*-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution:** 10 µg/mL of *p*-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid, and mix. Store under toluene.

Basal medium stock solution: Dissolve the anhydrous dextrose and anhydrous sodium acetate in the solutions previously mixed according to ▲*Table 2*,▲ (USP 1-May-2020) and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

▲**Table 2**▲ (USP 1-May-2020)

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Calcium pantothenate solution	5 mL
Riboflavin–thiamine hydrochloride solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water. Add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to test tubes, close or cover the tubes, sterilize in an autoclave at 121° for 15 min, and allow the tubes to cool in an upright position. Prepare stab cultures in 3 or more of the tubes, using a pure culture of *Lactobacillus plantarum*,[1] incubating for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution* add 5.0 mL of water containing 0.5 ng of biotin. Plug the tubes with cotton, sterilize in an autoclave at 121° for 15 min, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an inoculum comparable to a fresh culture.] Transfer cells from the *Stock culture of Lactobacillus plantarum* to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. The cell suspension is the *Inoculum*.

Analysis

Samples: *Standard solution* and *Sample solution*

To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to 4 similar but empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar test tubes add, in duplicate, volumes of the *Sample solution* corresponding to 3 or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool. Add 1 drop of *Inoculum* to each tube, except 2 of the 4 tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37° held constant to within ±0.5° until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of Standard during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to a spectrophotometer cell. Place the cell in a spectrophotometer that has been set at a specific wavelength of 540–660 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittance (Σ_S) as the difference, $y = 2.00 - \Sigma_S$. Plot this response on the ordinate of cross-section paper against the logarithm of the milliliter of the *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_U$, adding together the two transmittances (Σ_U) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X, for each dosage level. Average the values of X for each of 3 or more dosage levels to obtain $\bar{X}$, which equals the log-relative potency, M', of the *Sample solution*.

Determine the quantity, in µg, of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Tablets taken:

[1] ATCC No. 8014 is suitable. This strain was formerly known as *Lactobacillus arabinosus* 17-5.

Supplement 3

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$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = amount of biotin assumed to be present in the portion of the Tablets taken (µg)

Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of the Tablets taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of biotin in the portion of the Tablets taken (µg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, $\overline{M}$, is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* ⟨111⟩, *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

• **BIOTIN, *Method 3***

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A: Transfer 800 mL of water and 100 mL of triethylamine to a 1000-mL volumetric flask. Add 80 mL of 85% phosphoric acid, and dilute with water to volume.

Mobile phase: Transfer 80 mL of acetonitrile and 10 mL of *Solution A* to a 1000-mL volumetric flask. Dilute with water to volume.

Standard solution: 0.6 µg/mL of USP Biotin RS in water. [NOTE—A portion of the *Standard solution* will be used to determine the percent recovery of biotin from the *Solid-phase extraction* procedure.]

Sample solution: Finely powder NLT 20 Tablets. Transfer an amount of powdered Tablets to a volumetric flask to obtain a nominal concentration of 0.6 µg/mL of biotin. Add water up to 80% of the flask capacity, and sonicate for 30–40 min, with occasional mixing, to dissolve. Dilute with water to volume, and filter. Adjust the pH of the solution with either dilute acetic acid or 0.1 N sodium hydroxide to a pH of 6.0–7.0.

Solid-phase extraction: [NOTE—Condition the extraction column specified in this procedure in the following manner. Wash the column with a 2-mL portion of methanol. Equilibrate with a 2-mL portion of water.] Separately pipet 5.0 mL each of the *Sample solution* and *Standard solution* into freshly conditioned solid-phase extraction columns consisting of a mixed-mode packing with a sorbent-mass of 60 mg. [NOTE—The mixed-mode packing consists of anion-exchange and reversed-phase sorbents. The reverse-phase component is a polymer of copolymer *N*-vinylpyrrolidone and divinylbenzene. The anion exchange moiety is a trialkylamino group.[2]]

Wash the column with 10 mL of 30% (v/v) methanol in water. Apply an appropriate volume (4.9 mL) of 30% (v/v) methanol in 0.1 N hydrochloric acid to the column. Collect the eluate in a 5-mL volumetric flask, containing 100 µL of 40% (w/v) sodium acetate in water, and dilute with 30% (v/v) methanol in 0.1 N hydrochloric acid to volume.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC
Detector: UV 200 nm
Column: 4.6-mm × 25-cm; packing L1
Flow rate: 2 mL/min
Injection volume: 100 µL

System suitability

Samples: *Standard solution* and portion of the *Standard solution* that has undergone *Solid-phase extraction*

Suitability requirements

Tailing factor: NMT 1.5, *Standard solution*
Relative standard deviation: NMT 2.0%, *Standard solution* and *Standard solution* that has undergone *Solid-phase extraction*
Recovery: 95%–100%, *Standard solution* that has undergone *Solid-phase extraction*

Analysis

Samples: *Standard solution* and the *Sample solution* that have both undergone *Solid-phase extraction*

Measure the peak areas of biotin. Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of biotin from the *Sample solution*
r_S = peak area of biotin from the *Standard solution*
C_S = concentration of USP Biotin RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of biotin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

• **CYANOCOBALAMIN, *Method 1***

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Methanol and water (7:13)

Standard stock solution: 10 µg/mL of USP Cyanocobalamin (Crystalline) RS in water. [NOTE—Store in a dark place, and discard after 1 week.]

Standard solution: 1 µg/mL of USP Cyanocobalamin (Crystalline) RS from *Standard stock solution* diluted with water

Sample solution: Finely powder NLT 30 Tablets. Transfer a portion of the powder, equivalent to 100 µg of cyanocobalamin, to a 250-mL flask. Quantitatively add 100.0 mL of water, and carefully extract for 2 min. Filter 10 mL of the extract, and use the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC
Detector: 550 nm
Column: 4.6-mm × 15-cm; 5-µm packing L1
Flow rate: 0.5 mL/min
Injection volume: 200 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of cyanocobalamin.

Calculate the percentage of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

[2] A suitable cartridge is the Waters Oasis MAX Vac RC, particle size 30 µm, part #186000371.

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r_S = peak area from the *Standard solution*
C_S = concentration of USP Cyanocobalamin (Crystalline) RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of cyanocobalamin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$)

Change to read:

- **CYANOCOBALAMIN, *Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Standard stock solution: 1.0 µg/mL of USP Cyanocobalamin (Crystalline) RS in 25% alcohol. Store in a refrigerator.

Standard solution: Dilute a suitable volume of the *Standard stock solution* with water to a measured volume such that after the incubation period as described in the *Analysis*, the difference in transmittance between the inoculated blank and the 5.0-mL level of the *Standard solution* is NLT that which corresponds to a difference of 1.25 mg in dried cell weight. This concentration usually falls between 0.01 and 0.04 ng/mL of the *Standard solution*. Prepare this solution fresh for each assay.

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powdered Tablets, equivalent to 1.0 µg of cyanocobalamin, to an appropriate vessel containing, for each gram of powdered Tablets taken, 25 mL of an aqueous extracting solution prepared just before use to contain, in each 100 mL, 1.29 g of dibasic sodium phosphate, 1.1 g of anhydrous citric acid, and 1.0 g of sodium metabisulfite. Autoclave the mixture at 121° for 10 min. Allow any undissolved particles of the extract to settle, and filter or centrifuge if necessary. Dilute an aliquot of the clear solution with water to obtain a final solution containing vitamin B_{12} activity equivalent to the nominal activity of the *Standard solution*.

Acid-hydrolyzed casein solution: Prepare as directed in *Biotin, Method 2*.

Asparagine solution: Dissolve 2.0 g of L-asparagine in water to make 200 mL. Store under toluene in a refrigerator.

Adenine–guanine–uracil solution: Prepare as directed in *Biotin, Method 2*.

Xanthine solution: Suspend 0.20 g of xanthine in 30–40 mL of water, heat to 70°, add 6.0 mL of 6 N ammonium hydroxide, and stir until the solid is dissolved. Cool, and dilute with water to make 200 mL. Store under toluene in a refrigerator.

Salt solution A: Dissolve 10 g of monobasic potassium phosphate and 10 g of dibasic potassium phosphate in water to make 200 mL, and add 2 drops of hydrochloric acid. Store this solution under toluene.

Salt solution B: Dissolve 4.0 g of magnesium sulfate, 0.20 g of sodium chloride, 0.20 g of ferrous sulfate, and 0.20 g of manganese sulfate in water to make 200 mL. Add 2 drops of hydrochloric acid. Store this solution under toluene.

Polysorbate 80 solution: Dissolve 20 g of polysorbate 80 in alcohol to make 200 mL. Store in a refrigerator.

Vitamin solution A: Dissolve 10 mg of riboflavin, 10 mg of thiamine hydrochloride, 100 µg of biotin, and 20 mg of niacin in 0.02 N acetic acid to make 400 mL. Store under toluene, protected from light, in a refrigerator.

Vitamin solution B: Dissolve 20 mg of *p*-aminobenzoic acid, 10 mg of calcium pantothenate, 40 mg of pyridoxine hydrochloride, 40 mg of pyridoxal hydrochloride, 8 mg of pyridoxamine dihydrochloride, and 2 mg of folic acid in a mixture of water and neutralized alcohol (3:1) to make 400 mL. Store, protected from light, in a refrigerator.

Basal medium stock solution: Prepare the medium according to the following formula and directions. A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium comparable to that obtained from the formula given herein.

Add the ingredients in the order listed in ▲*Table 3*▲ (USP 1-May-2020) carefully dissolving cystine and tryptophan in the 1N hydrochloric acid before adding the next 8 solutions to the resulting solution. Add 100 mL of water, and dissolve the dextrose, sodium acetate, and ascorbic acid. Filter, if necessary. Add the *Polysorbate 80 solution*, adjust with 1 N sodium hydroxide to a pH of 5.5–6.0, and dilute with Purified Water to make 250 mL.

▲**Table 3**▲ (USP 1-May-2020)

L-Cystine	0.1 g
L-Tryptophan	0.05 g
1 N hydrochloric acid	10 mL
Adenine–guanine–uracil solution	5 mL
Xanthine solution	5 mL
Vitamin solution A	10 mL
Vitamin solution B	10 mL
Salt solution A	5 mL
Salt solution B	5 mL
Asparagine solution	5 mL
Acid-hydrolyzed casein solution	25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Ascorbic acid	1 g
Polysorbate 80 solution	5 mL

Tomato juice preparation: Centrifuge commercially canned tomato juice so that most of the pulp is removed. Suspend 5 g/L of analytical filter aid in the supernatant, and pass with the aid of reduced pressure through a layer of the filter aid. Repeat if necessary until a clear, straw-colored filtrate is obtained. Store under toluene in a refrigerator.

Culture medium: [NOTE—A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium equivalent to that obtained from the formula given herein.] Dissolve 0.75 g of yeast extract, 0.75 g of dried peptone, 1.0 g of anhydrous dextrose, and 0.20 g of monobasic potassium phosphate in 60–70 mL of water. Add 10 mL of *Tomato juice preparation* and 1 mL of *Polysorbate 80 solution*. Adjust with 1 N sodium hydroxide to a pH of 6.8, and add water to make 100 mL. Place 10-mL portions of the solution in test tubes, and plug with cotton. Sterilize the tubes and contents in an autoclave at 121° for 15 min. Cool as rapidly as possible to avoid color formation resulting from overheating the medium.

Suspension medium: Dilute a measured volume of the *Basal medium stock solution* with an equal volume of water. Place 10-mL portions of the diluted medium in test tubes. Sterilize, and cool as directed for *Culture medium*.

Stock culture of *Lactobacillus leichmannii*: To 100 mL of *Culture medium* add 1.0–1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Place 10-mL portions of the hot solution in test tubes, cover the

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tubes, sterilize at 121° for 15 min in an autoclave, and allow the tubes to cool in an upright position. Inoculate 3 or more of the tubes by stab transfer of a pure culture of *Lactobacillus leichmannii*.[3] [NOTE—Before first using a fresh culture in this assay, make NLT 10 successive transfers of the culture in a 2-week period.] Incubate for 16–24 h at a temperature between 30° and 40° held constant to within ±0.5°. Store in a refrigerator.

Prepare fresh stab cultures at least 3 times each week, and do not use them for preparing the *Inoculum* if more than 4 days old. The activity of the microorganism can be increased by daily or twice-daily transfer of the stab culture, to the point where definite turbidity in the liquid *Inoculum* can be observed 2–4 h after inoculation. A slow-growing culture seldom gives a suitable response curve and may lead to erratic results.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus leichmannii* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Transfer cells from the *Stock culture of Lactobacillus leichmannii* to 2 sterile tubes containing 10 mL each of the *Culture medium*. Incubate these cultures for 16–24 h at a temperature between 30° and 40° held constant to within ±0.5°. Under aseptic conditions centrifuge the cultures, and decant the supernatant. Suspend the cells from the culture in 5 mL of sterile *Suspension medium*, and combine. Using sterile *Suspension medium*, adjust the volume so that a 1-in-20 dilution in saline TS produces 70% transmittance when read on a suitable spectrophotometer that has been set at a wavelength of 530 nm, equipped with a 10-mm cell, and read against saline TS set at 100% transmittance. Prepare a 1-in-400 dilution of the adjusted suspension using sterile *Basal medium stock solution*. [NOTE—This dilution may be altered, when necessary, to obtain the desired test response.] The cell suspension is the *Inoculum*.

Calibration of spectrophotometer: Check the wavelength of the spectrophotometer periodically using a standard wavelength cell or other suitable device. Before reading any tests, calibrate the spectrophotometer for 0% and 100% transmittance, using water, and with the wavelength set at 530 nm.

Analysis

Samples: *Standard solution* and *Sample solution*

Because of the high sensitivity of the test organism to minute amounts of vitamin B_{12} activity and to traces of many cleansing agents, cleanse meticulously by suitable means, followed preferably by heating at 250° for 2 h using hard-glass 20-mm × 150-mm test tubes and other necessary glassware.

To separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each of these tubes and to 4 similar but empty tubes add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes to prevent bacterial contamination, and sterilize in an autoclave at 121° for 5 min, arranging to reach this temperature in NMT 10 min by preheating the autoclave if necessary. Cool as rapidly as possible to avoid color formation resulting from overheating the medium. Take precautions to maintain uniformity of sterilizing and cooling conditions throughout the assay, because packing the tubes too closely in the autoclave or overloading it may cause variation in the heating rate.

Aseptically add 0.5 mL of *Inoculum* to each tube so prepared, except 2 of the 4 containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 40°, held constant to within ±0.5°, for 16–24 h.

Terminate growth by heating to a temperature NLT 80° for 5 min. Cool to room temperature. After agitating its contents, read the transmittance at 530 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of the inoculated blank. If the difference is greater than 5% or if there is evidence of contamination with a foreign microorganism, disregard the results of the assay.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of each of the remaining tubes. Disregard the results of the assay if the slope of the standard curve indicates a problem with sensitivity.

Calculation: Prepare a standard concentration-response curve by the following procedure. Test for and replace any aberrant individual transmittances. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittances (Σ_S) as the difference, $y = 2.00 - \Sigma_S$. Plot this response on the ordinate of cross-section paper against the logarithm of the milliliter of the *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_U$, adding together the two transmittances (Σ_U) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X, for each dosage level. Average the values of X for each of 3 or more dosage levels to obtain $\bar{X}$, which equals the log-relative potency, M', of the *Sample solution*.

Determine the quantity, in µg, of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$) in the portion of Tablets taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = amount of cyanocobalamin assumed to be present in the portion of Tablets taken (µg)

Calculate the percentage of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$) in the portion of the Tablets taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of cyanocobalamin in the portion of the Tablets taken (µg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08,

[3] Pure cultures of *Lactobacillus leichmannii* (listed as *Lactobacillus delbruekii*) may be obtained as #7830 from ATCC, 10801 University Blvd., Manassas, VA 20110-2209 (www.atcc.org).

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their mean, $\overline{M}$, is the assayed log-potency of the test material (see ▲ (USP 1-May-2020) *Design and Analysis of Biological Assays* ⟨111⟩, *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$)

Change to read:

- **FOLIC ACID, *Method 1***

▲Proceed as directed in *Folic Acid Assay* ⟨411⟩, *Assay, Procedure 1*.▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–150.0% of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$)

Change to read:

- **FOLIC ACID, *Method 2***

▲Proceed as directed in *Folic Acid Assay* ⟨411⟩, *Assay, Procedure 2*.▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–150.0% of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$)

- **CALCIUM PANTOTHENATE, *Method 1***

Mobile phase: Phosphoric acid and water (1:1000)

Internal standard solution: 80 mg of *p*-hydroxybenzoic acid in 3 mL of alcohol. Add 50 mL of water and 7.1 g of dibasic sodium phosphate, and dilute with water to 1000 mL. Adjust with phosphoric acid to a pH of 6.7.

Standard solution: 0.6 mg/mL of USP Calcium Pantothenate RS in the *Internal standard solution*

Sample solution: Finely powder NLT 30 Tablets. Transfer a portion of the powder, equivalent to 15 mg of calcium pantothenate, to a centrifuge tube. Add 25.0 mL of the *Internal standard solution*, and shake vigorously for 10 min. Centrifuge, filter, and use the clear filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 210 nm

Column: 3.9-mm × 15-cm; packing L1

Flow rate: 1.5 mL/min

Injection volume: 10 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for calcium pantothenate and *p*-hydroxybenzoic acid are about 0.5 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of calcium pantothenate and the internal standard.

Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Tablets taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak area ratio of calcium pantothenate to *p*-hydroxybenzoic acid from the *Sample solution*

R_S = peak area ratio of calcium pantothenate to *p*-hydroxybenzoic acid from the *Standard solution*

C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

Change to read:

- **CALCIUM PANTOTHENATE, *Method 2***

Standard stock solution: Dissolve 50 mg of USP Calcium Pantothenate RS ▲ (USP 1-May-2020) in 500 mL of water in a 1000-mL volumetric flask. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), and dilute with water to volume to obtain a concentration of 50 µg/mL of USP Calcium Pantothenate RS. Store under toluene in a refrigerator.

Standard solution: On the day of the assay, dilute a volume of the *Standard stock solution* with water to obtain a concentration of 0.01–0.04 µg/mL of calcium pantothenate, the exact concentration being such that the responses obtained as directed in the *Analysis*, using 2.0 and 4.0 mL of the *Standard solution*, are within the linear portion of the log-concentration-response curve.

Sample solution: Finely powder NLT 30 Tablets. Transfer a portion of the powder, equivalent to 50 mg of calcium pantothenate, to a 1000-mL volumetric flask containing 500 mL of water. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), dilute with water to volume, and filter. Dilute a volume of this solution to obtain a solution having approximately the same concentration as that of the *Standard solution*.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and dilute with water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and dilute with water to make 1000 mL. Store under toluene in a cool place at a temperature not below 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and dilute with water to make 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin–thiamine hydrochloride–biotin solution: 20 µg/mL of riboflavin, 10 µg/mL of thiamine hydrochloride, and 0.04 µg/mL of biotin in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

***p*-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution:** 10 µg/mL of *p*-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in

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water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Basal medium stock solution: Dissolve the anhydrous dextrose and anhydrous sodium acetate in the solutions previously mixed according to ▲*Table 4*▲ (USP 1-May-2020) and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

▲Table 4▲ (USP 1-May-2020)

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Riboflavin–thiamine hydrochloride–biotin solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water. Add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to the test tubes, close or cover the tubes, sterilize in an autoclave at 121° for 15 min, and allow the tubes to cool in an upright position. Prepare stab cultures in 3 or more of the tubes using a pure culture of *Lactobacillus plantarum*,[1] incubating for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for the *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution* add 5.0 mL of water containing 0.2 µg of calcium pantothenate. Plug the tubes with cotton, sterilize in an autoclave at 121° for 15 min, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.]

Transfer cells from the *Stock culture of Lactobacillus plantarum* to a sterile tube containing 10 mL of the *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. The cell suspension is the *Inoculum*.

Analysis

Samples: *Standard solution* and *Sample solution*

To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to 4 similar but empty tubes, add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, volumes of the *Sample solution* corresponding to 3 or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool, and add 1 drop of *Inoculum* to each tube, except 2 of the 4 tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37°, held constant to within ±0.5° until, following 16–24 h of incubation, until there has been no substantial increase in turbidity in the tubes containing the highest level of Standard during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to an optical container if necessary. Read the transmittance between 540 and 660 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the Standard calculate the response from the sum of the duplicate values of the transmittance (Σ_S) as the difference, $y = 2.00 - \Sigma_S$. Plot this response on the ordinate of cross-section paper against the logarithm of the milliliter of the *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_U$, adding together the two transmittances (Σ_U) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X, for each dosage level. Average the values of X for each of 3 or more dosage levels to obtain $\overline{X}$, which equals the log-relative potency, M', of the *Sample solution*. Determine the quantity, in mg, of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Tablets taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = amount of calcium pantothenate assumed to be present in the portion of the Tablets taken (mg)

Calculate the percentage of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of the Tablets taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of calcium pantothenate in the portion of the Tablets taken (mg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, $\overline{M}$, is the assayed log-potency of the test

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material (see *Design and Analysis of Biological Assays* ⟨111⟩, *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of *M* that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

- **CALCIUM PANTOTHENATE, *Method 3***

Buffer solution: Dissolve 10.0 g of monobasic potassium phosphate in 2000 mL of water, and adjust with phosphoric acid to a pH of 3.5.

Mobile phase: Methanol and *Buffer solution* (1:9)

Standard stock solution: 0.25 mg/mL of USP Calcium Pantothenate RS in water. Prepare fresh every 4 weeks. Store in a refrigerator.

Standard solution: 40 µg/mL of USP Calcium Pantothenate RS from the *Standard stock solution* diluted with water

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 10 mg of calcium pantothenate, to a 250-mL volumetric flask. Add 10 mL of methanol, and swirl the flask to disperse. Dilute with water to volume, mix, and filter.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 205 nm

Column: 3.9-mm × 30-cm; 5-µm packing L1

Column temperature: 50°

Flow rate: 2 mL/min

Injection volume: 25 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of calcium pantothenate.

Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of calcium pantothenate from the *Sample solution*

r_S = peak area of calcium pantothenate from the *Standard solution*

C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

Change to read:

- **NIACIN OR NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, RIBOFLAVIN, and THIAMINE, *Method 1***

[NOTE—Use low-actinic glassware throughout this procedure.]

Diluent: Acetonitrile, glacial acetic acid, and water (5:1:94)

Mobile phase: A mixture of methanol, glacial acetic acid, and water (27:1:73) containing 140 mg of sodium 1-hexanesulfonate per 100 mL

Standard solution: [NOTE—Use USP Niacin RS in place of USP Niacinamide RS for formulations containing niacin.] Transfer 80 mg of USP Niacinamide RS, 20 mg of USP Pyridoxine Hydrochloride RS, 20 mg of USP Riboflavin RS, and 20 mg of USP Thiamine Hydrochloride RS, to a 200-mL volumetric flask, and add 180 mL of *Diluent*. Immerse the flask in a hot water bath maintained at 65°–70° for 10 min with regular shaking or using a vortex mixer, until all the solid materials are dissolved. Chill rapidly in a cold water bath for 10 min to room temperature, and dilute with *Diluent* to volume.

Sample solution: Finely powder NLT 30 Tablets. Transfer a portion of the powder, equivalent to 10 mg of niacinamide and 2.5 mg each of pyridoxine hydrochloride, riboflavin, and thiamine hydrochloride, to a 50-mL centrifuge tube. Add 25.0 mL of *Diluent*, and mix using a vortex mixer for 30 s to completely suspend the powder. Immerse the centrifuge tube in a hot water bath maintained at 65°–70°, heat for 5 min, and mix on a vortex mixer for 30 s. Return the tube to the hot water bath, heat for another 5 min, and mix on a vortex mixer for 30 s. Filter a portion of the solution, cool to room temperature, and use the clear filtrate. [NOTE—Use the filtrate within 3 h of filtration.]

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 3.9-mm × 30-cm; packing L1

Flow rate: 1 mL/min

Injection volume: 10 µL

System suitability

Sample: *Standard solution* ▲(USP 1-May-2020)

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

▲Calculate the percentage of the labeled amount of vitamin B_1 as thiamine ion ($C_{12}H_{17}N_4OS+$), vitamin B_2 as riboflavin ($C_{17}H_{20}N_4O_6$), vitamin B_3 as niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), vitamin B_6 as pyridoxine ($C_8H_{11}NO_3$), calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), and folic acid ($C_{19}H_{19}N_7O_6$), in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin from the corresponding *Sample solution*

r_S = peak area of the relevant vitamin from the corresponding *Standard solution*

C_S = concentration of the relevant vitamin Reference Standard in the corresponding *Standard solution* (µg/mL)

C_U = nominal concentration of the relevant vitamin in the corresponding *Sample solution* (µg/mL)▲(USP 1-May-2020)

Acceptance criteria: 90.0%–150.0% of the labeled amount of ▲each individual vitamin▲(USP 1-May-2020)

Change to read:

- **NIACIN, *Method 2***

▲Proceed as directed in *Niacin or Niacinamide Assay* ⟨441⟩, *Assay, Chromatographic Methods, Procedure 2*.▲(USP 1-May-2020)

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacin ($C_6H_5NO_2$)

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Change to read:

- **NIACINAMIDE,** ***Method 2***

▲Proceed as directed in *Niacin or Niacinamide Assay* ⟨441⟩, *Assay, Chromatographic Methods, Procedure 2.*▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacinamide ($C_6H_6N_2O$)

- **PYRIDOXINE HYDROCHLORIDE,** ***Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Extraction solvent, Mobile phase, and **Sample solution:** Prepare as directed for *Niacin, Method 2.*

Standard stock solution: 0.1 mg/mL of USP Pyridoxine Hydrochloride RS in the *Extraction solvent*

Standard solution: Transfer 5.0 mL of *Standard stock solution* to a 25-mL volumetric flask, and dilute with the *Extraction solvent* to volume.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability.*)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection volume: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of pyridoxine. Calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of pyridoxine from the *Sample solution*

r_S = peak area of pyridoxine from the *Standard solution*

C_S = concentration of USP Pyridoxine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of pyridoxine hydrochloride in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$)

Change to read:

- **RIBOFLAVIN,** ***Method 2***

▲Proceed as directed in *Riboflavin Assay* ⟨481⟩, *Assay, Chromatographic Methods, Procedure 2.*▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–150.0% of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$)

Change to read:

- **THIAMINE,** ***Method 2***

▲Proceed as directed in *Thiamine Assay* ⟨531⟩, *Assay, Chromatographic Methods, Procedure 2.*▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–150.0% of the labeled amount of thiamine ▲as thiamine ion ($C_{12}H_{17}N_4OS+$)▲ (USP 1-May-2020)

Change to read:

- **NIACIN OR NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, RIBOFLAVIN,** and **THIAMINE,** ***Method 3***

[NOTE—Use low-actinic glassware throughout this procedure.]

Reagent: 25 mg/mL of edetate disodium in water

Mobile phase: Transfer 0.4 mL of triethylamine, 15.0 mL of glacial acetic acid, and 350 mL of methanol to a 2000-mL volumetric flask. Dilute with 0.008 M sodium 1-hexanesulfonate to volume.

Standard stock solution: 1.5 mg/mL of USP Niacin RS or USP Niacinamide RS, 0.24 mg/mL of USP Pyridoxine Hydrochloride RS, 0.08 mg/mL of USP Riboflavin RS, and 0.24 mg/mL of USP Thiamine Hydrochloride RS in the *Reagent,* with heating if necessary

Standard solution: Transfer 5.0 mL of the *Standard stock solution* to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few milliliters of the filtrate.

Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 7.5 mg of niacin or niacinamide, 1.2 mg of pyridoxine hydrochloride, 0.4 mg of riboflavin, and 1.2 mg of thiamine hydrochloride to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1), and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few milliliters of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability.*)

Mode: LC

Detector: UV 270 nm

Column: 4.6-mm × 25-cm; packing L7

Column temperature: 50°

Flow rate: 2.0 mL/min

Injection volume: 5 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

▲Calculate the percentage of the labeled amount of vitamin B_1 as thiamine ion ($C_{12}H_{17}N_4OS+$), vitamin B_2 as riboflavin ($C_{17}H_{20}N_4O_6$), vitamin B_3 as niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), vitamin B_6 as pyridoxine ($C_8H_{11}NO_3$), calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), and folic acid ($C_{19}H_{19}N_7O_6$), in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin from the corresponding *Sample solution*

r_S = peak area of the relevant vitamin from the corresponding *Standard solution*

C_S = concentration of the relevant vitamin Reference Standard in the corresponding *Standard solution* (µg/mL)

C_U = nominal concentration of the relevant vitamin in the corresponding *Sample solution* (µg/mL)▲ (USP 1-May-2020)

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Acceptance criteria: 90.0%–150.0% of the labeled amount of ▲each individual vitamin▲ (USP 1-May-2020)

Add the following:

▲• **FOLIC ACID *Method 3*; ASCORBIC ACID, NIACIN OR NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, CALCIUM PANTOTHENATE, RIBOFLAVIN, and THIAMINE, *Method 4***

[NOTE—Use low-actinic glassware.]

Solution A: 0.56 g of edetate disodium and 2.04 g of potassium phosphate monobasic per 1000 mL of water. Adjust with phosphoric acid to a pH of 3.0.

Solution B: Acetonitrile, acetic acid, glacial, and water (5:1:94)

Solution C: Aqueous solution containing 2 mL of ammonium hydroxide, 1 g of sodium perchlorate per 100 mL

Solution D: Aqueous solution containing 2 mL of ammonium hydroxide, 1 g of sodium perchlorate and 2 g of sodium ascorbate per 100 mL

Mobile phase A: 0.02% trifluoroacetic acid in water

Mobile phase B: Acetonitrile

Mobile phase: See *Table 5*.

Table 5

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
0	100	0
1.0	100	0
6.0	95	5
15.0	83	17
17.0	80	20
19.0	80	20
19.1	100	0
25.0	100	0

Ascorbic acid standard stock solution: 1.0 mg/mL of ascorbic acid from USP Ascorbic Acid RS in *Solution A*

Thiamine standard stock solution: 0.2 mg/mL of thiamine from USP Thiamine Hydrochloride RS in *Solution B*. The solution can be placed on a steam bath for about 5–10 min with frequent swirling to assist in dissolution.

Riboflavin standard stock solution: 0.2 mg/mL of USP Riboflavin RS in *Solution B*. Heat the solution to 65°–70° for 10 min to assist in dissolution.

Niacin or niacinamide standard stock solution: 1.0 mg/mL of USP Niacin RS or USP Niacinamide RS in *Solution A*

Pyridoxine standard stock solution: 0.2 mg/mL of pyridoxine from USP Pyridoxine Hydrochloride RS in *Solution C*

Folic acid standard stock solution: 0.2 mg/mL of folic acid from USP Folic Acid RS in *Solution C*

Calcium pantothenate standard stock solution: 1.0 mg/mL of calcium pantothenate from USP Calcium Pantothenate RS in *Mobile phase A*

System suitability solution: Transfer 0.5 mL of *Ascorbic Acid standard stock solution*, 2.5 mL of *Thiamine standard stock solution*, and 0.5 mL of *Niacin or niacinamide standard stock solution* into 10-mL volumetric flask and dilute to volume with *Mobile phase A*.

Sample solution 1: Weigh and finely powder NLT 20 Tablets. Transfer a calculated amount of accurately weighed powder into a 100-mL volumetric flask to obtain a solution containing known nominal concentrations of thiamine, riboflavin, and calcium pantothenate in the ranges of 5–30 µg/mL for thiamine, 10–60 µg/mL for riboflavin, and 25–150 µg/mL for calcium pantothenate. Add *Solution B* to about half the volume and mix on a vortex mixer for 5 min. Heat the flask at 65°–70° for 5 min, swirling frequently and sonicate additionally for 5 min. Cool to room temperature, dilute with *Mobile phase A* to volume, and mix well. Pass a portion of the solution through a suitable filter of 0.45-µm pore size and discard the first milliliter of the filtrate.

Sample solution 2: Transfer a portion of finely powdered Tablets into a 100-mL volumetric flask to obtain a solution containing known nominal concentrations of ascorbic acid and niacin or niacinamide in the ranges of 50–300 µg/mL for ascorbic acid and 12–65 µg/mL for niacin or niacinamide, add *Solution A* to about half the volume and mix on a vortex mixer for 5 min, sonicate for 10 min with occasional shaking. Cool to room temperature, dilute with *Solution A* to volume, mix well. Pass a portion of the solution through a suitable filter of 0.45-µm pore size and discard the first milliliter of the filtrate.

Sample solution 3: Transfer a portion of finely powdered Tablets into a 100-mL volumetric flask to obtain a solution containing known nominal concentrations of pyridoxine hydrochloride and folic acid in the ranges of 5–30 µg/mL for pyridoxine hydrochloride and 2–12 µg/mL for folic acid, add *Solution D* to about half the volume, heat on a water bath at 40° for 5 min and sonicate for 10 min with occasional shaking. Cool to room temperature, dilute with *Solution D* to volume, and mix well. Pass a portion of the solution through a suitable filter of 0.45-µm pore size and discard the first milliliter of the filtrate.

Standard solution 1: Accurately transfer calculated volumes of *Thiamine standard stock solution*, *Riboflavin standard stock solution*, and *Calcium pantothenate standard stock solution* into a suitable volumetric flask to obtain a solution with final vitamin concentrations similar to the nominal concentrations in the *Sample solution 1* for the corresponding vitamins, dilute with *Mobile phase A* to volume, mix well.

Standard solution 2: Accurately transfer calculated volumes of *Ascorbic acid standard stock solution* and *Niacin or niacinamide standard stock solution*, into a suitable volumetric flask to obtain a solution with final vitamin concentrations similar to the nominal concentrations in the *Sample solution 2* for the corresponding vitamins, dilute with *Mobile phase A* to volume, mix well.

Standard solution 3: Accurately transfer calculated volumes of *Pyridoxine standard stock solution* and *Folic acid standard stock solution* into a suitable volumetric flask to obtain a solution with final vitamin concentrations similar to the nominal concentrations in *Sample solution 3* for the corresponding vitamins, dilute with *Solution C* to volume, and mix well.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detectors

For calcium pantothenate: UV 210 nm

For thiamine, ascorbic acid, niacin or niacinamide, and riboflavin: UV 254 nm

For pyridoxine hydrochloride and folic acid: UV 280 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Column temperature: 25°

DS Monographs

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Flow rate: 1.0 mL/min
Injection volume: 20 µL
System suitability
Samples: *System suitability solution, Standard solution 1, Standard solution 2* and *Standard solution 3*
Suitability requirements
Resolution: NLT 3.5 between the thiamine and ascorbic acid peaks and NLT 4.0 between the ascorbic acid and niacin or niacinamide peaks, *System suitability solution*
Relative standard deviation: NMT 2.0% for each individual peak, *Standard solution 1, Standard solution 2,* and *Standard solution 3*
Analysis
Samples: *Sample solutions 1–3* and *Standard solutions 1–3*
Calculate the percentage of the labeled amount of vitamin B1 as thiamine ion ($C_{12}H_{17}N_4OS+$), vitamin B2 as riboflavin ($C_{17}H_{20}N_4O_6$), vitamin B3 as niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), vitamin B6 as pyridoxine ($C_8H_{11}NO_3$), calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), and folic acid ($C_{19}H_{19}N_7O_6$), in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin from the corresponding *Sample solution*
r_S = peak area of the relevant vitamin from the corresponding *Standard solution*
C_S = concentration of the relevant vitamin Reference Standard in the corresponding *Standard solution* (µg/mL)
C_U = nominal concentration of the relevant vitamin in the corresponding *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of each individual vitamin ▲(USP 1-May-2020)

[NOTE—In the following assays commercially available atomic absorption standard solutions for the minerals, where applicable, may be used where preparation of a *Standard stock solution* is described. Use deionized water where water is specified. Where atomic absorption spectrophotometry is specified in the assay, the *Standard solutions* and the *Sample solution* may be diluted quantitatively with the solvent specified, if necessary, to yield solutions of suitable concentrations adaptable to the linear or working range of the instrument.]

• **CALCIUM, *Method 1***
Lanthanum chloride solution: 267 mg/mL of lanthanum chloride heptahydrate in 0.125 N hydrochloric acid
Calcium standard solution: 400 µg/mL of calcium. Dissolve 1.001 g of calcium carbonate, previously dried at 300° for 3 h and cooled in a desiccator for 2 h, in 25 mL of 1 N hydrochloric acid. Boil to expel carbon dioxide, and dilute with water to 1000 mL.
Standard stock solution: 100 µg/mL of calcium from the *Calcium standard solution* diluted with 0.125 N hydrochloric acid
Standard solutions: Into separate 100-mL volumetric flasks pipet 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the *Standard stock solution*. To each flask add 1.0 mL of the *Lanthanum chloride solution*, and dilute with water to volume to obtain concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 µg/mL of calcium.
Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 5 Tablets, to a porcelain crucible. Heat the crucible in a muffle furnace maintained at 550° for 6–12 h, and cool. Add 60 mL of hydrochloric acid, and boil gently on a hot plate or steam bath for 30 min, intermittently rinsing the inner surface of the crucible with 6 N hydrochloric acid. Cool, and quantitatively transfer the contents of the crucible to a 100-mL volumetric flask. Rinse the crucible with small portions of 6 N hydrochloric acid, and add the rinsings to the flask. Dilute with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute this solution quantitatively, with 0.125 N hydrochloric acid, to obtain a concentration of 2 µg/mL of calcium, adding 1 mL of the *Lanthanum chloride solution* per 100 mL of the final volume.
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectroscopy
Analytical wavelength: Calcium emission line at 422.7 nm
Lamp: Calcium hollow-cathode
Flame: Nitrous oxide–acetylene
Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution* per 100 mL
Analysis
Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of calcium, and draw the straight line best fitting the 5 plotted points. From the graph, determine the concentration (C), in µg/mL, of calcium in the *Sample solution*.
Calculate the percentage of the labeled amount of calcium (Ca) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of calcium in the *Sample solution* (µg/mL)
C_U = nominal concentration of calcium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of calcium (Ca)

• **CHROMIUM, *Method 1***
Chromium standard solution: 1000 µg/mL of chromium from potassium dichromate, previously dried at 120° for 4 h in water. Store in a polyethylene bottle.
Standard stock solution: 10 µg/mL of chromium from the *Chromium standard solution* diluted with 6 N hydrochloric acid and water (1 in 20)
Standard solutions: Transfer 10.0 and 20.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks, and transfer 15.0 and 20.0 mL of the *Standard stock solution* to separate 50-mL volumetric flasks. Dilute the contents of each of the 4 flasks with 0.125 N hydrochloric acid to volume to obtain concentrations of 1.0, 2.0, 3.0, and 4.0 µg/mL of chromium.
Sample solution: Prepare as directed for *Calcium, Method 1*, except obtain a concentration of 1 µg/mL of chromium and omit the use of the *Lanthanum chloride solution*.
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Analytical wavelength: Chromium emission line at 357.9 nm
Lamp: Chromium hollow-cathode
Flame: Air–acetylene
Blank: 0.125 N hydrochloric acid
Analysis
Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of chromium, and

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draw the straight line best fitting the 4 plotted points. From the graph, determine the concentration (C), in µg/mL, of chromium in the *Sample solution*.

Calculate the percentage of the labeled amount of chromium (Cr) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of chromium in the *Sample solution* (µg/mL)

C_U = nominal concentration of chromium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of chromium (Cr)

• **COPPER, *Method 1***

Copper standard solution: Dissolve 1.00 g of copper foil in a minimum volume of a 50% solution of nitric acid, and dilute with a 1% solution of nitric acid to 1000 mL. This solution contains 1000 µg/mL of copper.

Standard stock solution: 100 µg/mL of copper from the *Copper standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 200-mL volumetric flasks transfer 1.0, 2.0, 4.0, 6.0, and 8.0 mL of the *Standard stock solution*. Dilute with water to volume to obtain concentrations of 0.5, 1.0, 2.0, 3.0, and 4.0 µg/mL of copper.

Sample solution: Prepare as directed for *Calcium, Method 1*, except obtain a concentration of 2 µg/mL of copper and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Copper emission line at 324.7 nm

Lamp: Copper hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of copper, and draw the straight line best fitting the 5 plotted points. From the graph, determine the concentration (C), in µg/mL, of copper in the *Sample solution*.

Calculate the percentage of the labeled amount of copper (Cu) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of copper in the *Sample solution* from the graph (µg/mL)

C_U = nominal concentration of copper in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of copper (Cu)

• **FLUORIDE, *Method 1***

[NOTE—Store all solutions in plastic containers.]

3 M sodium acetate solution: Dissolve 408 g of sodium acetate in 600 mL of water contained in a 1000-mL volumetric flask. Allow the solution to equilibrate to room temperature, and dilute with water to volume. Adjust with a few drops of acetic acid to a pH of 7.0.

Sodium citrate solution: Dissolve 222 g of sodium citrate in 250 mL of water in a 1000-mL volumetric flask. Add 28 mL of perchloric acid, and dilute with water to volume.

Fluoride standard stock solution: 500 µg/mL of fluoride from a quantity of sodium fluoride, previously dried at 100° for 4 h and cooled in a desiccator in water

Intermediate stock solution A: 100 µg/mL of fluoride from the *Fluoride standard stock solution* diluted with water

Intermediate stock solution B: 10 µg/mL of fluoride from the *Fluoride standard stock solution* diluted with water

Standard solutions: To 5 separate 100-mL volumetric flasks transfer 3.0, 5.0, and 10.0 mL of *Intermediate stock solution B* and 5.0 and 10.0 mL of *Intermediate stock solution A*. To each flask add 10.0 mL of 1 N hydrochloric acid, 25 mL of *3 M sodium acetate solution*, and 25.0 mL of *Sodium citrate solution*. Dilute the contents of each flask with water to volume to obtain concentrations of 0.3, 0.5, 1.0, 5.0, and 10.0 µg/mL of fluoride.

Sample solution: Transfer a quantity of the finely powdered Tablets, equivalent to 200 µg of fluoride, to a 100-mL volumetric flask. Add 10.0 mL of 1 N hydrochloric acid, 25.0 mL of *3 M sodium acetate solution*, and 25.0 mL of *Sodium citrate solution*, and dilute with water to volume.

Analysis

Samples: *Standard solutions* and *Sample solution*

To separate plastic beakers, each containing a plastic-coated stirring bar, transfer 50.0 mL each of the *Standard solutions* and the *Sample solution*. Measure the potentials (see *pH* ⟨791⟩), in mV, of the *Standard solutions* and the *Sample solution*, with a pH meter capable of a minimum reproducibility of ±0.2 mV and equipped with a fluoride-specific ion-indicating electrode and a calomel reference electrode. [NOTE—When taking measurements, immerse the electrodes in the solution, stir on a magnetic stirrer having an insulated top until equilibrium is attained (1–2 min), and record the potential. Rinse and dry the electrodes between measurements, taking care to avoid damaging the crystal of the specific-ion electrode.]

Plot the logarithms of fluoride concentrations, in µg/mL, of the *Standard solutions* versus potential, in mV. From the standard response curve and the measured potential of the *Sample solution*, determine the concentration (C), in µg/mL, of fluoride in the *Sample solution*.

Calculate the percentage of the labeled amount of fluorine (F) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of fluoride in the *Sample solution* (µg/mL)

C_U = nominal concentration of fluorine in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (F)

• **FLUORIDE, *Method 2***

[NOTE—Use plastic containers and deionized water throughout this procedure.]

pH 10.0 buffer: Add 214 mL of 0.1 N sodium hydroxide to 1000 mL of 0.05 M sodium bicarbonate.

Mobile phase: Alcohol, 0.1 N sulfuric acid, and water (20:5:175)

Standard stock solution: 220 µg/mL of USP Sodium Fluoride RS in water. This solution contains 100 µg/mL of fluoride.

Standard solution: [NOTE—Condition the solid-phase extraction column specified for use in the *Standard solution* and the *Sample solution* in the following manner. Using a vacuum at a pressure not exceeding 5 mm of mercury, wash the column with 1 column volume of methanol followed by 1 column volume of *pH 10.0 buffer*. Do not allow the column top to dry. If the top of the column becomes dry, recondition the column.] Transfer 10.0 mL of

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the *Standard stock solution* to a 100-mL volumetric flask. Add 75 mL of water, and adjust with 0.1 N sodium hydroxide to a pH of 10.4 ± 0.1. Dilute with water to volume. Filter, discarding the first 15 mL of the filtrate. Transfer 25.0 mL of the filtrate to a 50-mL volumetric flask, add 15.0 mL of water, and adjust with 0.1 N sodium hydroxide to a pH of 10.0. Dilute with *pH 10.0 buffer* to volume. Elute a portion of this solution through a 3-mL solid-phase extraction column containing L1 packing that is connected through an adapter to a second solid-phase extraction column containing sulfonylpropyl strong cation-exchange packing. Discard the first 3 mL of the eluate, and collect the rest of the eluate in a suitable flask for injection into the chromatograph.

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of powdered Tablets, equivalent to 1 mg of fluorine, in 15 mL of water, and shake vigorously. Rinse the sides of the flask with 15 mL of water, and allow to stand for 10 min. Dilute with water to 85 mL, adjust with 1 N sodium hydroxide to a pH of 10.4 ± 0.1, and dilute with water to 100 mL. Prepare as directed for the *Standard solution*, beginning with "Filter, discarding the first 15 mL of the filtrate".

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Conductivity
Columns
Guard: 4.6-mm × 3-cm; packing L17
Analytical: 7.8-mm × 30-cm; packing L17
Flow rate: 0.5 mL/min
Injection volume: 100 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 2.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas of fluoride. Calculate the percentage of the labeled amount of fluorine (F) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*
r_S = peak area from the *Standard solution*
C_S = concentration of fluoride in the *Standard solution* (µg/mL)
C_U = nominal concentration of fluorine in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (F)

• **IODIDE**

Bromine water: To 20 mL of bromine in a glass-stoppered bottle add 100 mL of water. Insert the stopper into the bottle, and shake. Allow to stand for 30 min, and use the supernatant.

Analysis: Transfer an amount of finely powdered Tablets, equivalent to 3 mg of iodide, to a nickel crucible. Add 5 g of sodium carbonate, 5 mL of 50% (w/v) sodium hydroxide solution, and 10 mL of alcohol, taking care that the entire specimen is moistened. Heat the crucible on a steam bath to evaporate the alcohol, then dry the crucible at 100° for 30 min to prevent spattering upon subsequent heating. Transfer the crucible with its contents to a furnace heated to 500°, and heat the crucible for 15 min. [NOTE—Heating at 500° is necessary to carbonize any organic matter present; a higher temperature may be used, if necessary, to ensure complete carbonization of all organic matter.] Cool the crucible, add 25 mL of water, cover the crucible with a watchglass, and boil gently for 10 min. Filter the solution, and wash the crucible with boiling water, collecting the filtrate and washings in a beaker. Add phosphoric acid until the solution is neutral to methyl orange, then add 1 mL excess of phosphoric acid. Add excess of *Bromine water*, and boil the solution gently until colorless and then for 5 min longer. Add a few crystals of salicylic acid, and cool the solution to 20°. Add 1 mL of phosphoric acid and 0.5 g of potassium iodide, and titrate the liberated iodine with 0.005 N sodium thiosulfate VS, adding starch TS when the liberated iodine color has nearly disappeared.

Calculate the percentage of the labeled amount of iodine (I) in the portion of Tablets taken:

$$\text{Result} = V \times N_A \times F \times I_{me} \times (Aw/W) \times (100/L)$$

V = volume of sodium thiosulfate consumed (mL)
N_A = actual normality of the sodium thiosulfate solution used
F = correction factor to convert mg to µg, 1000 µg/mL
I_{me} = milliequivalent weight of iodine, 21.16 mg/meq
Aw = average weight of the Tablets
W = weight of the portion of the Tablets taken
L = labeled amount of iodine (µg/Tablet)

Acceptance criteria: 90.0%–160.0% of the labeled amount of iodine (I)

• **IRON, *Method 1***

Iron standard stock solution: Transfer 100 mg of iron powder to a 1000-mL volumetric flask. Dissolve in 25 mL of 6 N hydrochloric acid, and dilute with water to volume.

Standard solutions: To separate 100-mL volumetric flasks, transfer 2.0, 4.0, 5.0, 6.0, and 8.0 mL of the *Iron standard stock solution*. Dilute the contents of each flask with water to volume to obtain concentrations of 2.0, 4.0, 5.0, 6.0, and 8.0 µg/mL of iron.

Sample solution: Prepare as directed for *Calcium, Method 1*, except obtain a concentration of 5 µg/mL of iron and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Analytical wavelength: Iron emission line at 248.3 nm
Lamp: Iron hollow-cathode
Flame: Air–acetylene
Blank: 0.125 N hydrochloric acid
Analysis
Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of iron, and draw the straight line best fitting the 5 plotted points. From the graph, determine the concentration (C), in µg/mL, of iron in the *Sample solution*.
Calculate the percentage of the labeled amount of iron (Fe) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of iron in the *Sample solution* (µg/mL)
C_U = nominal concentration of iron in the *Sample solution* (µg/mL)

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Acceptance criteria: 90.0%–125.0% of the labeled amount of iron (Fe)

• **MAGNESIUM, *Method 1***

Lanthanum chloride solution: Prepare as directed in *Calcium, Method 1*.

Magnesium standard solution: Transfer 1.0 g of magnesium ribbon to a 1000-mL volumetric flask, dissolve in 50 mL of 6 N hydrochloric acid, dilute with water to volume, and mix to obtain a solution with a known concentration of 1000 µg/mL of magnesium.

Standard stock solution: 20 µg/mL of magnesium from the *Magnesium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the *Standard stock solution*. To each flask add 1.0 mL of the *Lanthanum chloride solution*, and dilute with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.2, 0.3, 0.4, 0.5, and 0.6 µg/mL of magnesium.

Sample solution: Prepare as directed for *Calcium, Method 1*, except obtain a concentration of 0.4 µg/mL of magnesium.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Magnesium emission line at 285.2 nm

Lamp: Magnesium hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution* per 100 mL

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of magnesium, and draw the straight line best fitting the 5 plotted points. From the graph, determine the concentration (*C*), in µg/mL, of magnesium in the *Sample solution*.

Calculate the percentage of the labeled amount of magnesium (Mg) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of magnesium in the *Sample solution* (µg/mL)

C_U = nominal concentration of magnesium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of magnesium (Mg)

• **MANGANESE, *Method 1***

Manganese standard stock solution: Transfer 1.00 g of manganese to a 1000-mL volumetric flask. Dissolve in 20 mL of nitric acid, dilute with 6 N hydrochloric acid to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of manganese.

Standard stock solution: 50 µg/mL of manganese from the *Manganese standard stock solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Standard stock solution*. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions with known concentrations of 0.5, 0.75, 1.0, 1.5, and 2.0 µg/mL of manganese.

Sample solution: Prepare as directed for *Calcium, Method 1*, except obtain a concentration of 1 µg/mL of manganese and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Manganese emission line at 279.5 nm

Lamp: Manganese hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of manganese, and draw the straight line best fitting the 5 plotted points. From the graph, determine the concentration (*C*), in µg/mL, of manganese in the *Sample solution*.

Calculate the percentage of the labeled amount of manganese (Mn) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of manganese in the *Sample solution* (µg/mL)

C_U = nominal concentration of manganese in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of manganese (Mn)

• **MOLYBDENUM, *Method 1***

Diluent: 20 mg/mL of ammonium chloride in water

Molybdenum standard solution: Transfer 1.0 g of molybdenum wire to a 1000-mL volumetric flask, and dissolve in 50 mL of nitric acid, warming if necessary. Dilute with water to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of molybdenum.

Standard stock solution: 100 µg/mL of molybdenum from the *Molybdenum standard solution* diluted with water

Standard solutions: To separate 100-mL volumetric flasks transfer 2.0, 10.0, and 25.0 mL of the *Standard stock solution*, and add 5.0 mL of perchloric acid to each flask. Gently boil the solution in each flask for 15 min, cool to room temperature, and dilute each with *Diluent* to volume to obtain concentrations of 5.0, 10.0, and 25.0 µg/mL of molybdenum.

Sample solution: Transfer a portion of the powder, equivalent to 1000 µg of molybdenum, to a suitable flask, and add 12 mL of nitric acid. [NOTE—The volume of nitric acid may be varied to ensure that the powder is uniformly dispersed.] Carefully swirl the flask to disperse the test specimen. Sonicate for 10 min or until the test specimen is completely dissolved. Gently boil the solution for 15 min, and cool to room temperature. Carefully add 8 mL of perchloric acid, heat until perchloric acid fumes appear, and swirl the flask to dissipate the fumes. Repeat the heating and swirling until the fumes appear again. Cool to room temperature. Quantitatively transfer the contents of the flask to a 100-mL volumetric flask with the aid of the *Diluent*, and dilute with *Diluent* to volume.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Molybdenum emission line at 313.3 nm

Lamp: Molybdenum hollow-cathode

Flame: Nitrous oxide–acetylene

Blank: *Diluent* and perchloric acid (20:1)

Analysis

Samples: *Standard solutions* and *Sample solution*

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Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of molybdenum, and draw the straight line best fitting the 3 plotted points. From the graph, determine the concentration (C), in µg/mL, of molybdenum in the *Sample solution*.

Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of molybdenum in the *Sample solution* (µg/mL)

C_U = nominal concentration of molybdenum in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

• **MOLYBDENUM, *Method 2***

Sodium fluoride solution: Add 200 mL of water to 10 g of sodium fluoride, stir until the solution is saturated, and filter. Store in a polyethylene bottle.

Ferrous sulfate solution: 4.98 mg/mL of ferrous sulfate in water

Potassium thiocyanate solution: 200 mg/mL of potassium thiocyanate in water

20% stannous chloride solution: Transfer 40 mg of stannous chloride to a beaker, add 20 mL of 6.5 N hydrochloric acid solution, and heat the solution until the stannous chloride is dissolved. Cool, and dilute with water to 100 mL.

Diluted stannous chloride solution: *20% stannous chloride solution* diluted with water (1 in 25). Prepare this solution fresh at the time of use.

Standard solution: 20 µg/mL of molybdenum in water

Sample: A portion of finely powdered Tablets equivalent to 40 µg of molybdenum

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Vis

Analytical wavelength: 465 nm

Cell: 1 cm

Blank: Amyl alcohol

Analysis

Samples: *Standard solution* and *Sample*

Transfer the *Sample* and 2.0 mL of the *Standard solution* to separate 200-mL beakers. Add 20 mL of nitric acid to each beaker. Cover each beaker with a watchglass, and boil slowly on a hot plate for 45 min. Cool to room temperature. Add 6 mL of perchloric acid, cover the beakers with a watchglass, and continue the heating until digestion is complete, as indicated when the liquid becomes colorless or pale yellow. Evaporate the solutions in the beakers to dryness. Rinse the sides of the beakers and the watchglasses with water, and add more water to complete 50 mL in each beaker. Gently boil the water solution for a few min. Cool to room temperature. Add 2 drops of methyl orange TS, and neutralize with ammonium hydroxide. Add 8.2 mL of hydrochloric acid. Quantitatively transfer the contents of the beakers to separate 100-mL volumetric flasks, rinse the beakers with water, transfer the rinsings to the corresponding volumetric flasks, and dilute with water to volume. Transfer 50.0 mL of each solution to separatory funnels. To each separatory funnel add 1.0 mL of *Sodium fluoride solution*, 0.5 mL of *Ferrous sulfate solution*, 4.0 mL of *Potassium thiocyanate solution*, 1.5 mL of *20% stannous chloride solution*, and 15.0 mL of amyl alcohol, and shake the separatory funnel for 1 min. Allow the layers to separate, and discard the aqueous layers. Add 25 mL of *Diluted stannous chloride solution* to each separatory funnel, and shake gently for 15 s. Allow the layers to separate, and discard the aqueous layers. Transfer the organic layer from each separatory funnel to a centrifuge tube, and centrifuge at 2000 rpm for 10 min. Determine the absorbances of the organic phases obtained from the *Standard solution* and the *Sample*, and correct with the *Blank*.

Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Tablets taken:

$$\text{Result} = (A_U/A_S) \times [(V \times C_S)/M_U] \times 100$$

A_U = absorbance of the *Sample*

A_S = absorbance of the *Standard solution*

V = volume of the *Standard solution* analyzed, 2.0 mL

C_S = concentration of molybdenum in the *Standard solution* (µg/mL)

M_U = nominal amount of molybdenum in the *Sample* (µg)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

• **PHOSPHORUS, *Method 1***

Sulfuric acid solution: Cautiously add sulfuric acid to water (37.5: 100), and mix.

Ammonium molybdate solution: 50 mg/mL of ammonium molybdate in the *Sulfuric acid solution* and water (2:3). [NOTE—Dissolve in water first, and then dilute with the *Sulfuric acid solution* to volume.]

Hydroquinone solution: 5 mg/mL of hydroquinone in water. Add 1 drop of sulfuric acid per 100 mL of solution.

Sodium bisulfite solution: 200 mg/mL of sodium bisulfite in water

Phosphorus standard stock solution: Weigh 4.395 g of monobasic potassium phosphate, previously dried at 105° for 2 h and stored in a desiccator, and transfer to a 1000-mL volumetric flask. Dissolve in water, add 6 mL of sulfuric acid as a preservative, dilute with water to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of phosphorus.

Standard solution: 20 µg/mL of phosphorus from *Phosphorus standard stock solution* diluted with water

Sample solution: [NOTE—Finely powder and weigh a counted number of Tablets.] Transfer a portion of the powder, equivalent to 100 mg of phosphorus, to 25 mL of nitric acid, and digest on a hot plate for 30 min. Add 15 mL of hydrochloric acid, and continue the digestion to the cessation of brown fumes. Cool, and transfer the contents of the flask to a 500-mL volumetric flask with the aid of small portions of water. Dilute with water to volume. Transfer 10.0 mL of this solution to a 100-mL volumetric flask, and dilute with water to volume.

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Mode: Vis

Analytical wavelength: 650 nm

Cell: 1 cm

Analysis

Samples: *Standard solution* and *Sample solution*

To 3 separate 25-mL volumetric flasks transfer 5.0 mL each of the *Standard solution*, the *Sample solution*, and water to provide the blank. To each of the 3 flasks add 1.0 mL each of *Ammonium molybdate solution*, *Hydroquinone solution*, and *Sodium bisulfite solution*, and swirl to mix. Dilute the contents of each flask with water to volume, and allow the flasks to stand for 30 min. Determine the absorbances of the solutions against the blank.

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Calculate the percentage of the labeled amount of phosphorus (P) in the portion of Tablets taken:

$$\text{Result} = (A_U/A_S) \times (C_S/C_U) \times 100$$

A_U = absorbance of the *Sample solution*
A_S = absorbance of the *Standard solution*
C_S = concentration of phosphorus in the *Standard solution* (µg/mL)
C_U = nominal concentration of phosphorus in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of phosphorus (P)

• **POTASSIUM**

Potassium standard solution: 100 µg/mL of potassium from potassium chloride, previously dried at 105° for 2 h, in water

Standard stock solution: 10 µg/mL of potassium from the *Potassium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Transfer 5.0, 10.0, 15.0, 20.0, and 25.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions containing 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of potassium.

Sample solution: Prepare as directed for *Calcium, Method 1*, except obtain a concentration of 1 µg/mL of potassium and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Potassium emission line at 766.5 nm

Lamp: Potassium hollow-cathode

Flame: Air–acetylene

Blank: Water

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of potassium, and draw the straight line best fitting the 5 plotted points. From the graph, determine the concentration (C), in µg/mL, of potassium in the *Sample solution*.

Calculate the percentage of the labeled amount of potassium (K) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of potassium in the *Sample solution* (µg/mL)
C_U = nominal concentration of potassium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of potassium (K)

• **SELENIUM, *Method 1***

Diluent: Prepare as directed in *Molybdenum, Method 1*.

Selenium standard solution: [**CAUTION**—Selenium is toxic; handle it with care.] Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness. Add 2 mL of water, and evaporate to dryness. Repeat the addition of water and the evaporation to dryness 3 times. Dissolve the residue in 3 N hydrochloric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume to obtain a concentration of 1000 µg/mL of selenium.

Standard stock solution: 100 µg/mL of selenium from the *Selenium standard solution* diluted with water

Standard solutions: To separate 100-mL volumetric flasks transfer 5.0, 10.0, and 25.0 mL of the *Standard stock solution*, and add 5.0 mL of perchloric acid to each flask. Gently boil the solutions for 15 min, cool to room temperature, and dilute each with *Diluent* to volume to obtain solutions with concentrations of 5.0, 10.0, and 25.0 µg/mL of selenium.

Sample solution: Transfer a portion of the powder, equivalent to 1000 µg of selenium, to a suitable flask, and add 12 mL of nitric acid. [NOTE—The volume of nitric acid may be varied to ensure that the powder is uniformly dispersed.] Carefully swirl the flask to disperse the test specimen. Sonicate for 10 min or until the test specimen is completely dissolved. Gently boil the solution for 15 min, and cool to room temperature. Carefully add 8 mL of perchloric acid to the flask, heat the flask until perchloric acid fumes appear, and swirl the flask to dissipate the fumes. Repeat the heating and swirling until the fumes appear again. Cool to room temperature. Transfer the contents of the flask to a 50-mL volumetric flask with the aid of the *Diluent*, and dilute with *Diluent* to volume.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Selenium emission line at 196.0 nm

Lamp: Selenium hollow-cathode

Flame: Air–acetylene

Blank: *Diluent* and perchloric acid (20:1)

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of selenium, and draw the straight line best fitting the 3 plotted points. From the graph, determine the concentration (C), in µg/mL, of selenium in the *Sample solution*.

Calculate the percentage of the labeled amount of selenium (Se) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of selenium in the *Sample solution* from the graph (µg/mL)
C_U = nominal concentration of selenium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of selenium (Se)

• **SELENIUM, *Method 2***

Hydrochloric acid solution: Hydrochloric acid diluted with water (1 in 10)

50% ammonium hydroxide solution: Ammonium hydroxide diluted with water (1 in 2)

Reagent A: 9 mg/mL of edetate disodium and 25 mg/mL of hydroxylamine hydrochloride in water. [NOTE—Dissolve edetate disodium in a portion of water first, then add hydroxylamine hydrochloride, and dilute with water to volume.]

Reagent B: Transfer 200 mg of 2,3-diaminonaphthalene to a 250-mL separatory funnel, and add 200 mL of 0.1 N hydrochloric acid. Wash the solution with 3 40-mL portions of cyclohexane, and discard the cyclohexane layer. Filter the solution into a brown bottle, and cover the solution with a 1-cm layer of cyclohexane. This solution is stable for 1 week if stored in a refrigerator.

Standard stock solution: [**CAUTION**—Selenium is toxic; handle it with care.] Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness. Add

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2 mL of water, and evaporate to dryness. Repeat the addition of water and evaporation to dryness 3 times. Dissolve the residue in 3 N hydrochloric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume to obtain a solution with a concentration of 1000 µg/mL of selenium. Dilute a volume of the solution with 0.125 N hydrochloric acid to obtain a concentration of 2.0 µg/mL of selenium.

Standard solution: Transfer 10 mL of the *Standard stock solution* to a glass-stoppered flask. Add 1 mL of perchloric acid and 1 mL of *Hydrochloric acid solution*, and dilute with water to 20 mL.

Sample solution: Transfer a portion of finely powdered Tablets, equivalent to 20 µg of selenium, to a suitable flask. Add 10 mL of nitric acid, and warm gently on a hot plate. Continue heating until the initial nitric acid reaction has subsided, then add 3 mL of perchloric acid. [**CAUTION**—Exercise care at this stage because the perchloric acid reaction becomes vigorous.] Continue heating on the hot plate until the appearance of white fumes of perchloric acid or until the digest begins to darken. Add 0.5 mL of nitric acid and resume heating, adding additional amounts of nitric acid if further darkening occurs. Digest for 10 min after the first appearance of perchloric acid fumes or until the digest becomes colorless. Cool the flask, add 2.5 mL of *Hydrochloric acid solution*, and return the flask to the hot plate to expel residual nitric acid. Heat the mixture for 3 min after it begins to boil. Cool the flask to room temperature, and dilute with water to 20 mL.

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Mode: UV

Analytical wavelength: 380 nm

Cell: 1 cm

Blank: 1 mL of perchloric acid and 1 mL of *Hydrochloric acid solution* diluted with water to 20 mL

Analysis

Samples: *Standard solution* and *Sample solution*

Treat the *Sample solution*, the *Standard solution*, and the *Blank* as follows. Add 5 mL of *Reagent A* to each flask, and swirl gently to mix. Adjust the solution in each flask with 50% *ammonium hydroxide solution* to a pH of 1.1 ± 0.1. Add 5 mL of *Reagent B* to each flask, and swirl gently to mix. Place the flasks in a water bath maintained at 50°, and equilibrate for 30 min, taking care that the flasks are covered to protect them from light. Cool to room temperature, and transfer the contents of each flask to separate separatory funnels. Transfer 10.0 mL of cyclohexane to each separatory funnel, and extract vigorously for 1 min. Discard the aqueous layer. Transfer the cyclohexane layer to a centrifuge tube, and centrifuge at 1000 rpm for 1 min to remove any remaining water. Determine the absorbances of the solutions obtained from the *Samples* against the solution obtained from the *Blank*.

Calculate the percentage of the labeled amount of selenium (Se) in the portion of Tablets taken:

$$\text{Result} = (A_U/A_S) \times [(V \times C_S)/M_U] \times 100$$

A_U = absorbances of the cyclohexane layer from the *Sample solution*

A_S = absorbances of the cyclohexane layer from the *Standard solution*

V = volume of the *Standard stock solution* used to prepare the *Standard solution*, 10 mL

C_S = concentration of selenium in the *Standard stock solution* (µg/mL)

M_U = nominal amount of selenium in the *Sample solution* (µg)

Acceptance criteria: 90.0%–160.0% of the labeled amount of selenium (Se)

• **ZINC, *Method 1***

Zinc standard stock solution: 1000 µg/mL of zinc from zinc oxide in 5 M hydrochloric acid (3.89 mg/mL) and diluted with water to final volume. [NOTE—Dissolve in 5 M hydrochloric acid by warming, if necessary, cool, and then dilute to final volume.]

Standard stock solution: 50 µg/mL of zinc from the *Zinc standard stock solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Transfer 1.0, 2.0, 3.0, 4.0, and 5.0 mL of *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of zinc.

Sample solution: Prepare as directed for the *Sample solution* in *Calcium, Method 1*, except obtain a concentration of 2 µg/mL of zinc and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Zinc emission line at 213.8 nm

Lamp: Zinc hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of zinc, and draw the straight line best fitting the 5 plotted points. From the graph, determine the concentration (C), in µg/mL, of zinc in the *Sample solution*.

Calculate the percentage of the labeled amount of zinc (Zn) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of zinc in the *Sample solution* (µg/mL)

C_U = nominal concentration of zinc in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of zinc (Zn)

• **BORON, NICKEL, TIN,** and **VANADIUM, *Method 1*; CALCIUM, CHROMIUM, COPPER, IRON, MAGNESIUM, MANGANESE, PHOSPHORUS,** and **ZINC, *Method 2*; MOLYBDENUM** and **SELENIUM, *Method 3***

Stock aqua regia solution: Prepare a mixture of hydrochloric acid and nitric acid (3:1) by adding the nitric acid to the hydrochloric acid. [NOTE—Periodically vent the solution in an appropriate fume hood.]

Diluent: Prepare a mixture of the *Stock aqua regia solution* and water (1:9) by adding 1 volume of *Stock aqua regia solution* to 2 volumes of water. Dilute with additional water to volume, and mix well.

System suitability solution: Prepare a mixture of 1000 mg/L of yttrium in 5% nitric acid solution, 1000 mg/L of scandium in 5% nitric acid solution, and *Diluent* (1:1:198), and mix.

Standard stock solution 1 (Ca, Cu, Fe, Mg, Mn, P, and Zn): [NOTE—It is only necessary to include the minerals of interest in the solution.] Using commercially available element standard (single- or multi-element) solutions in 5%

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nitric acid solution, pipet the appropriate amount of element standard solution into a volumetric flask, and dilute with 5% nitric acid solution to obtain a solution having final concentrations of about 1000 mg/L of calcium, 100 mg/L of copper, 250 mg/L of iron, 500 mg/L of magnesium, 100 mg/L of manganese, 800 mg/L of phosphorus, and 250 mg/L of zinc.

Standard stock solution 2 (B, Cr, Mo, Ni, Se, Sn, and V): [NOTE—It is only necessary to include the minerals of interest in the solution.] Using commercially available element standard (single- or multi-element) solutions in 20% hydrochloric acid solution, pipet the appropriate amount of element standard solution into a volumetric flask, and dilute with 20% hydrochloric acid solution to obtain a solution having final concentrations of about 200 mg/L of boron, and 100 mg/L each of chromium, molybdenum, nickel, selenium, tin, and vanadium.

Standard solutions: Prepare a mixture of *Standard stock solution 1* and *Standard stock solution 2*, as required, in the *Diluent* to prepare a 6-point calibration curve to bracket the concentration range of each mineral of interest.

Sample solution 1 (for Tablets containing minerals in ***Standard stock solution 1*** and ***Standard stock solution 2***): Weigh and finely powder NLT 20 Tablets. Transfer a portion, equal to 3.5 times the average Tablet weight, to a 250-mL volumetric flask. Slowly add 25 mL of the *Stock aqua regia solution* in 5-mL increments, followed by mixing. [NOTE—If the sample contains a carbonate, bubbling will occur. Wait until bubbling ends to proceed.] Bring the solution to a boil on a hot plate. Continue to heat gently until fumes cease (about 1 h). [NOTE—If the sample contains selenium, digest for NMT 15 min.] Remove from heat, cool, and dilute with water to volume. Pass about 30 mL into a centrifuge tube using a 5-µm pore size nylon syringe filter. If necessary, make any further dilutions using the *Diluent*.

Sample solution 2 (for Tablets containing minerals only in ***Standard stock solution 2***): Weigh and finely powder NLT 20 Tablets. Transfer a portion, equal to 3.5 times the average Tablet weight, to a 250-mL volumetric flask. Slowly add 25 mL of the *Stock aqua regia solution* in 5-mL increments followed by mixing. [NOTE—If the sample contains a carbonate, bubbling will occur. Wait until bubbling ends to proceed.] Bring the solution to a boil on a hot plate. Continue to heat gently until fumes cease (about 1 h). [NOTE—If the sample contains selenium, digest for NMT 15 min.] Remove from heat, cool, and dilute with water to volume. Pass about 30 mL into a centrifuge tube using a nylon syringe filter of 5-µm pore size. If necessary, make any further dilutions using the *Diluent*.

Sample solution 3 (for Tablets containing minerals only in ***Standard stock solution 1***): Weigh and finely powder NLT 20 Tablets. Transfer a portion, equal to the average Tablet weight, to a 250-mL volumetric flask. Slowly add 25 mL of the *Stock aqua regia solution* in 5-mL increments, followed by mixing. [NOTE—If the sample contains a carbonate, bubbling will occur. Wait until bubbling ends to proceed.] Bring the solution to a boil on a hot plate. Continue to heat gently (about 1 h) until fumes cease. Remove from heat, cool, and dilute with water to volume. Pass about 30 mL into a centrifuge tube using a nylon syringe filter of 5-µm pore size. If necessary, make any further dilutions using the *Diluent*.

Instrumental conditions

(See *Plasma Spectrochemistry* ⟨730⟩.)

Mode: Inductively coupled plasma spectrometry, using a spectrometer set to measure the emission of each mineral of interest at about the corresponding wavelength. [NOTE—The operating conditions may be developed and optimized based on the manufacturer's recommendation. The wavelengths selected should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision.]

System suitability

Sample: *System suitability solution*

[NOTE—Analyze the *System suitability solution*, and obtain the response as directed in the *Analysis*.]

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the emission of each mineral of interest in the *Standard solutions* and *Sample solution* with an inductively coupled plasma system using the *Diluent* as the blank. Plot the emission of the *Standard solutions* versus the concentration, in mg/L, of the minerals of interest, and draw the straight line best fitting the plotted points. From the graph, determine the concentration (*C*), in mg/L, for each mineral of interest in the *Sample solution*.

Calculate the percentage of the labeled amount for each mineral taken:

$$\text{Result} = C \times (V/W) \times F \times (C_W/L) \times 100$$

C = measured concentration of the relevant element in the *Sample solution* (mg/L)
V = volume of the *Sample solution* (L)
W = sample weight (mg)
F = dilution factor of the *Sample solution*
C_W = average Tablet weight (mg)
L = labeled amount (mg/Tablet)

Acceptance criteria: 90.0%–125.0% of the labeled amount of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), and zinc (Zn); and 90.0%–160.0% of the labeled amount of boron (B), chromium (Cr), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), and vanadium (V)

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩, *Dissolution*: Meet the requirements
- **WEIGHT VARIATION** ⟨2091⟩: Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic microbial count does not exceed 3×10^3 cfu/g, and the combined molds and yeasts count does not exceed 3×10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meet the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

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Change to read:

- **LABELING:**[4] The label states that the product is Oil- and Water-Soluble Vitamins with Minerals Tablets. The label also states the quantity of each vitamin and mineral ▲in mg/Tablet or µg/Tablet▲ (USP 1-May-2020) and where necessary the chemical form in which a vitamin is present and also states the salt form of the mineral used as the source of each element. Where the product contains vitamin E, the label indicates whether it is the ▲*RRR*▲ (USP 1-May-2020) or ▲*all-rac*▲ (USP 1-May-2020) form. Where more than one assay method is given for a particular vitamin, the labeling states with which assay method the product complies only if *Method 1* is not used.

Change to read:

- **USP REFERENCE STANDARDS** ⟨11⟩

USP Alpha Tocopherol RS
2*H*-1-Benzopyran-6-ol, 3,4-dihydro-2,5,7,8,-tetramethyl-2-(4,8,12-trimethyltridecyl)-.
$C_{29}H_{50}O_2$ 430.70

USP Alpha Tocopheryl Acetate RS

USP Alpha Tocopheryl Acid Succinate RS

▲USP Ascorbic Acid RS▲ (USP 1-May-2020)

USP Biotin RS
1*H*-Thieno[3,4-*d*]imidazole-4-pentanoic acid, hexahydro-2-oxo-, 3a*S*-[(3aα,4β,6aα)]-.
$C_{10}H_{16}N_2O_3S$ 244.31

USP Calcium Pantothenate RS
β-Alanine, *N*-(2,4-dihydroxy-3,3-dimethyl-1-oxobutyl)-, calcium salt (2:1), (*R*)-.
$C_{18}H_{32}CaN_2O_{10}$ 476.53

USP Cholecalciferol RS
9,10-Secocholesta-5,7,10(19)-trien-3-ol, (3β,5*Z*,7*E*)-.
$C_{27}H_{44}O$ 384.64

USP Cyanocobalamin (Crystalline) RS
Vitamin B_{12}.
$C_{63}H_{88}CoN_{14}O_{14}P$ 1355.37

USP Ergocalciferol RS
9,10-Secoergosta-5,7,10(19),22-tetraen-3-ol, (3β,5*Z*,7*E*,22*E*)-.
$C_{28}H_{44}O$ 396.65

USP Folic Acid RS

USP Niacin RS
3-Pyridinecarboxylic acid.
$C_6H_5NO_2$ 123.11

USP Niacinamide RS
3-Pyridinecarboxamide.
$C_6H_6N_2O$ 122.12

USP Phytonadione RS

USP Pyridoxine Hydrochloride RS
3,4-Pyridinedimethanol, 5-hydroxy-6-methyl-, hydrochloride.
$C_8H_{11}NO_3 \cdot HCl$ 205.64

▲USP Retinyl Acetate RS
3,7-Dimethyl-9-(2,6,6-trimethyl-1- cyclohexen-1-yl) 2,4,6,8-nonatetraen-1-ol acetate.

USP Retinyl Palmitate RS
[(2*E*,4*E*,6*E*,8*E*)-3,7-Dimethyl-9-(2,6,6-trimethyl-1-cyclohex-1-en-1-yl)nona-2,4,6,8-tetraen-1-yl] hexadecanoate.▲ (USP 1-May-2020)

USP Riboflavin RS
Riboflavine.
$C_{17}H_{20}N_4O_6$ 376.36

USP Sodium Fluoride RS
Sodium fluoride.
NaF 41.99

USP Thiamine Hydrochloride RS
Thiazolium, 3-[(4-amino-2-methyl-5-pyrimidinyl) methyl]-5-(2-hydroxyethyl)-4-methyl-, chloride, monohydrochloride.
$C_{12}H_{17}ClN_4OS \cdot HCl$ 337.27

▲▲ (USP 1-May-2020)

Water-Soluble Vitamins Capsules

DEFINITION

Water-Soluble Vitamins Capsules contain two or more of the following water-soluble vitamins: Ascorbic Acid or its equivalent as Calcium Ascorbate or Sodium Ascorbate, Biotin, Cyanocobalamin, Folic Acid, Dexpanthenol or Panthenol, Niacin or Niacinamide, pantothenic acid (as Calcium Pantothenate or Racemic Calcium Pantothenate), Pyridoxine Hydrochloride, Riboflavin, and Thiamine Hydrochloride or Thiamine Mononitrate. Capsules contain NLT 90.0% and NMT 150.0% of the labeled amounts of ascorbic acid ($C_6H_8O_6$), biotin ($C_{10}H_{16}N_2O_3S$), calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$), folic acid ($C_{19}H_{19}N_7O_6$), dexpanthenol or panthenol ($C_9H_{19}NO_4$), niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine ($C_{12}H_{17}ClN_4OS$) as thiamine hydrochloride or thiamine mononitrate.

They do not contain any form of Beta Carotene or Vitamin A, D, E, or K. They do not contain any minerals for which nutritional value is claimed. They may contain other labeled added substances in quantities that are unobjectionable.

STRENGTH

[NOTE—In the following assays, where more than one assay method is given for an individual ingredient, the requirements may be met by following any one of the specified methods, the method used being stated in the labeling only if *Method 1* is not used.]

- **ASCORBIC ACID, CALCIUM ASCORBATE,** and **SODIUM ASCORBATE**

(See *Vitamin C Assay* ⟨580⟩.)

[NOTE—For labeling purposes, consider *Method I—Titrimetric Method* as *Method 1*.]

[4] ▲▲ (USP 1-May-2020) Where articles are labeled in terms of Units in addition to the required labeling, the relationship of the USP Units or IU to mass is as follows. One USP Vitamin A Unit = 0.3 µg of all-*trans*-retinol (vitamin A alcohol) or 0.344 µg of all-*trans*-retinyl acetate (vitamin A acetate) or 0.55 µg of all-*trans*-retinyl palmitate (vitamin A palmitate), and 1 µg of retinol (3.3 USP Vitamin A Units) = 1 retinol equivalent (RE); 1 IU of beta carotene = 0.6 µg of all-*trans*-beta carotene; 1 USP Vitamin D Unit = 0.025 µg of ergocalciferol or cholecalciferol; and 1 mg of ▲*all-rac*▲ (USP 1-May-2020)-alpha-tocopherol = 1.1 former USP Vitamin E Units; 1 mg of ▲*all-rac*▲ (USP 1-May-2020)-alpha-tocopheryl acetate = 1 former USP Vitamin E unit; 1 mg of ▲*all-rac*▲ (USP 1-May-2020)-alpha-tocopheryl acid succinate = 0.89 former USP Vitamin E Units; 1 mg of ▲*RRR*▲ (USP 1-May-2020)-alpha-tocopherol = 1.49 former USP Vitamin E Units; 1 mg of ▲*RRR*▲ (USP 1-May-2020)-alpha-tocopheryl acetate = 1.36 former USP Vitamin E Units; and 1 mg of ▲*RRR*▲ (USP 1-May-2020)-alpha-tocopheryl acid succinate = 1.21 former USP Vitamin E Units. In terms of ▲*RRR*▲ (USP 1-May-2020)-alpha-tocopherol equivalents, 1 mg of ▲*RRR*▲ (USP 1-May-2020)-alpha-tocopheryl acetate = 0.91; 1 mg of ▲*RRR*▲ (USP 1-May-2020)-alpha-tocopheryl acid succinate = 0.81; 1 mg of ▲*all-rac*▲ (USP 1-May-2020)-alpha-tocopherol = 0.74; 1 mg of ▲*all-rac*▲ (USP 1-May-2020)-alpha-tocopheryl acetate = 0.67; and 1 mg of ▲*all-rac*▲ (USP 1-May-2020)-alpha-tocopheryl acid succinate = 0.60. ▲Note that 1 mg of Institute of Medicine (IOM) alpha-tocopherol equivalent = 1 mg of 2*R*-alpha-tocopherol = 1 mg of *RRR*-alpha-tocopherol = 2 mg of *all-rac*-alpha-tocopherol.▲ (USP 1-May-2020)

DS Monographs

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Acceptance criteria: 90.0%–150.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$)

• **BIOTIN, *Method 1***

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Mix 85 mL of acetonitrile, 1 g of sodium perchlorate, and 1 mL of phosphoric acid, and dilute with water to 1000 mL.

Standard stock solution: 0.333 mg/mL of USP Biotin RS in dimethyl sulfoxide

Standard solution: 5 µg/mL of USP Biotin RS prepared by diluting the *Standard stock solution* in water

Sample solution: Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without the loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing, if necessary, with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the average net weight per Capsule. Transfer a portion of the Capsule contents, equivalent to a nominal amount of 1 mg of biotin, to a 200-mL volumetric flask. Add 3 mL of dimethyl sulfoxide, and swirl to wet the contents. Place the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with water to volume, and filter.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 200 nm

Column: 4.6-mm × 15-cm; 3-µm packing L7

Flow rate: 1.2 mL/min

Injection volume: 100 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of biotin. Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of biotin from the *Sample solution*

r_S = peak area of biotin from the *Standard solution*

C_S = concentration of USP Biotin RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of biotin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

• **BIOTIN, *Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Dehydrated mixtures yielding formulations similar to the media described herein may be used provided that, when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described herein.

Standard stock solution: 50 µg/mL of USP Biotin RS in 50% alcohol. Store this solution in a refrigerator.

Standard solution: 0.1 ng/mL of USP Biotin RS in water, prepared by dilution of the *Standard stock solution* with water on the day of the assay.

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." Transfer a portion of the Capsule contents, equivalent to 100 µg of biotin, to a 200-mL volumetric flask. Add 3 mL of 50% alcohol, and swirl to wet the contents. Heat the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with 50% alcohol to volume, and filter. Dilute a volume of the filtrate quantitatively, and stepwise if necessary, with water to obtain a solution with a concentration of 0.1 ng/mL.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and dilute with water to 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature NLT 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water. Heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and dilute with water to 1000 mL. Store under toluene in a cool place at a temperature NLT 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and dilute with water to 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Calcium pantothenate solution: 10 µg/mL of calcium pantothenate in 50% alcohol. Store in a refrigerator.

Riboflavin–thiamine hydrochloride solution: 20 µg/mL of riboflavin and 10 µg/mL of thiamine hydrochloride in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

***p*-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution:** 10 µg/mL of *p*-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid, and mix. Store under toluene.

Basal medium stock solution: Dissolve *anhydrous Dextrose* and *anhydrous Sodium acetate* in the solutions previously mixed according to *Table 1*, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Table 1

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL

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Table 1 *(continued)*

Calcium pantothenate solution	5 mL
Riboflavin–thiamine hydrochloride solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water. Add 500 mg of *anhydrous Dextrose*, 500 mg of *anhydrous Sodium acetate*, and 1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to test tubes, close or cover the tubes, sterilize in an autoclave at 121° for 15 min, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*,[1] incubating for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution* add 5.0 mL of water containing 0.5 ng of biotin. Plug the tubes with cotton, sterilize in an autoclave at 121° for 15 min, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Transfer cells from the *Stock culture of Lactobacillus plantarum* to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. The cell suspension so obtained is the *Inoculum*.

Analysis

Samples: *Standard solution* and *Sample solution*

To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool. Add 1 drop of *Inoculum* to each tube, except two of the four tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37° held constant to within ±0.5° until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of Standard during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to a spectrophotometer cell. Place the cell in a spectrophotometer that has been set at a specific wavelength 540–660 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittance (Σ_S) as the difference, $y = 2.00 - \Sigma_S$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response $y = 2.00 - \Sigma_U$, adding together the two transmittances (Σ_U) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that fall within the range of lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X, for each dosage level. Average the values of X for each of three or more dosage levels to obtain $\overline{X}$, which equals the log-relative potency, M', of the *Sample solution*.

Determine the quantity, in µg, of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Capsules taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of µg of biotin assumed to be present in the portion of Capsules taken

Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Capsules taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of biotin in the portion of Capsules taken (µg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, $\overline{M}$, is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* ⟨111⟩, *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

• **CYANOCOBALAMIN, *Method 1***

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Methanol and water (7:13)

Standard stock solution: 10 µg/mL of USP Cyanocobalamin (Crystalline) RS in water. [NOTE—Store this stock solution in a dark place, and discard after 1 week.]

[1] ATCC No. 8014 is suitable. This strain was formerly known as *Lactobacillus arabinosus* 17-5.

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Standard solution: 1 µg/mL of USP Cyanocobalamin (Crystalline) RS from the *Standard stock solution* diluted with water

Sample solution: Weigh NLT 30 Capsules in a tared weighing bottle. Open the Capsules, without the loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing, if necessary, with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the average net weight per Capsule. Transfer a portion of the Capsule contents, equivalent to 100 µg of cyanocobalamin, to a 250-mL flask. Quantitatively add 100.0 mL of water, and carefully extract for 2 min. Filter 10 mL of the extract, and use the clear filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: 550 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Flow rate: 0.5 mL/min

Injection volume: 200 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of cyanocobalamin. Calculate the percentage of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of cyanocobalamin from the *Sample solution*

r_S = peak area of cyanocobalamin from the *Standard solution*

C_S = concentration of USP Cyanocobalamin (Crystalline) RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cyanocobalamin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$)

• **CYANOCOBALAMIN, *Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Standard stock solution: 1.0 µg/mL of USP Cyanocobalamin (Crystalline) RS in 25% alcohol. Store in a refrigerator.

Standard solution: Dilute a suitable volume of *Standard stock solution* with water to a measured volume such that after the incubation period as described in the *Analysis*, the difference in transmittance between the inoculated blank and the 5.0-mL level of the *Standard solution* is NLT that which corresponds to a difference of 1.25 mg in dried cell weight. This concentration usually falls between 0.01 and 0.04 ng/mL of the *Standard solution*. Prepare this solution fresh for each assay.

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." Transfer a portion of the Capsule contents, equivalent to 1.0 µg of cyanocobalamin, to an appropriate vessel containing, for each g of Capsule contents taken, 25 mL of an aqueous extracting solution prepared just before use to contain 12.9 mg/mL of dibasic sodium phosphate, 11.0 mg/mL of anhydrous citric acid, and 10 mg/mL of sodium metabisulfite. Autoclave the mixture at 121° for 10 min. Allow any undissolved particles of the extract to settle, and filter or centrifuge, if necessary. Dilute an aliquot of the clear solution with water to obtain a final solution containing vitamin B_{12} activity approximately equivalent to that of the *Standard solution*.

Acid-hydrolyzed casein solution: Prepare as directed in *Calcium Pantothenate, Method 2*.

Asparagine solution: Dissolve 2.0 g of L-asparagine in water to make 200 mL. Store under toluene in a refrigerator.

Adenine–guanine–uracil solution: Prepare as directed in *Calcium Pantothenate, Method 2*.

Xanthine solution: Suspend 0.20 g of xanthine in 30–40 mL of water, heat to 70°, add 6.0 mL of 6 N ammonium hydroxide, and stir until the solid is dissolved. Cool, and dilute with water to 200 mL. Store under toluene in a refrigerator.

Salt solution A: Dissolve 10 g of monobasic potassium phosphate and 10 g of dibasic potassium phosphate in water to make 200 mL, and add 2 drops of hydrochloric acid. Store this solution under toluene.

Salt solution B: Dissolve 4.0 g of magnesium sulfate, 0.20 g of sodium chloride, 0.20 g of ferrous sulfate, and 0.20 g of manganese sulfate in water to make 200 mL. Add 2 drops of hydrochloric acid. Store this solution under toluene.

Polysorbate 80 solution: Dissolve 20 g of polysorbate 80 in alcohol to make 200 mL. Store in a refrigerator.

Vitamin solution A: Dissolve 10 mg of riboflavin, 10 mg of thiamine hydrochloride, 100 µg of biotin, and 20 mg of niacin in 0.02 N acetic acid to make 400 mL. Store under toluene, protected from light, in a refrigerator.

Vitamin solution B: Dissolve 20 mg of *p*-aminobenzoic acid, 10 mg of calcium pantothenate, 40 mg of pyridoxine hydrochloride, 40 mg of pyridoxal hydrochloride, 8 mg of pyridoxamine dihydrochloride, and 2 mg of folic acid in a mixture of water and neutralized alcohol (3:1) to make 400 mL. Store, protected from light, in a refrigerator.

Basal medium stock solution: Prepare the medium according to the following formula and directions. A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium comparable to that obtained from the formula given herein.

Add the ingredients in the order listed in *Table 2*, carefully dissolving *Cystine* and *Tryptophan* in the *hydrochloric acid* before adding the next eight solutions to the resulting solution. Add 100 mL of water, and dissolve the *Dextrose*, *Sodium acetate*, and *Ascorbic acid*. Filter, if necessary. Add the *Polysorbate 80 solution*, adjust with 1 N sodium hydroxide to a pH of 5.5–6.0, and dilute with Purified Water to 250 mL.

Table 2

L-Cystine	0.1 g
L-Tryptophan	0.05 g
1 N hydrochloric acid	10 mL
Adenine–guanine–uracil solution	5 mL
Xanthine solution	5 mL
Vitamin solution A	10 mL
Vitamin solution B	10 mL
Salt solution A	5 mL
Salt solution B	5 mL
Asparagine solution	5 mL

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Table 2 *(continued)*

Acid-hydrolyzed casein solution	25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Ascorbic acid	1 g
Polysorbate 80 solution	5 mL

Tomato juice preparation: Centrifuge commercially canned tomato juice so that most of the pulp is removed. Suspend 5 g/L of analytical filter aid in the supernatant, and pass, with the aid of reduced pressure, through a layer of the filter aid. Repeat, if necessary, until a clear, straw-colored filtrate is obtained. Store under toluene in a refrigerator.

Culture medium: [NOTE—A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium equivalent to that obtained from the formula given herein.] Dissolve 0.75 g of yeast extract, 0.75 g of dried peptone, 1.0 g of anhydrous dextrose, and 0.20 g of monobasic potassium phosphate in 60–70 mL of water. Add 10 mL of *Tomato juice preparation* and 1 mL of *Polysorbate 80 solution*. Adjust with 1 N sodium hydroxide to a pH of 6.8, and dilute with water to 100 mL. Place 10-mL portions of the solution in test tubes, and plug with cotton. Sterilize the tubes and contents in an autoclave at 121° for 15 min. Cool as rapidly as possible to avoid color formation resulting from overheating the medium.

Suspension medium: Dilute a measured volume of *Basal medium stock solution* with an equal volume of water. Place 10-mL portions of the diluted medium in test tubes. Sterilize, and cool as directed for *Culture medium*.

Stock culture of *Lactobacillus leichmannii*: To 100 mL of *Culture medium* add 1.0–1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Place 10-mL portions of the hot solution in test tubes, cover the tubes, sterilize at 121° for 15 min in an autoclave, and allow the tubes to cool in an upright position. Inoculate three or more of the tubes by stab transfer of a pure culture of *Lactobacillus leichmannii*.[2] [NOTE—Before first using a fresh culture in this assay, make NLT 10 successive transfers of the culture in a 2-week period.] Incubate for 16–24 h at a temperature between 30° and 40° held constant to within ±0.5°. Store in a refrigerator.

Prepare fresh stab cultures at least three times each week, and do not use them for preparing the *Inoculum* if more than 4 days old. The activity of the microorganism can be increased by daily or twice-daily transfer of the stab culture, to the point where definite turbidity in the liquid *Inoculum* can be observed 2–4 h after inoculation. A slow-growing culture seldom gives a suitable response curve and may lead to erratic results.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus leichmannii* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Make a transfer of cells from the *Stock culture of Lactobacillus leichmannii* to two sterile tubes containing 10 mL each of the *Culture medium*. Incubate these cultures for 16–24 h at a temperature between 30° and 40° held constant to within ±0.5°. Under aseptic conditions, centrifuge the cultures, and decant the supernatant. Suspend the cells from the culture in 5 mL of sterile *Suspension medium*, and combine. Using sterile *Suspension medium*, adjust the volume so that a 1-in-20 dilution in saline TS produces 70% transmittance when read on a suitable spectrophotometer that has been set at a wavelength of 530 nm, equipped with a 10-mm cell, and read against saline TS set at 100% transmittance. Prepare a 1-in-400 dilution of the adjusted suspension using sterile *Basal medium stock solution*. [NOTE—This dilution may be altered, when necessary, to obtain the desired test response.] The cell suspension so obtained is the *Inoculum*.

Calibration of spectrophotometer: Check the wavelength of the spectrophotometer periodically, using a standard wavelength cell or other suitable device. Before reading any tests, calibrate the spectrophotometer for 0% and 100% transmittance, using water, with the wavelength set at 530 nm.

Analysis

Samples: *Standard solution* and *Sample solution*

Because of the high sensitivity of the test organism to minute amounts of vitamin B_{12} activity and to traces of many cleansing agents, cleanse meticulously by suitable means, followed preferably by heating at 250° for 2 h, using hard-glass 20-mm × 150-mm test tubes and other necessary glassware.

To separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each of these tubes and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*. To each tube add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes to prevent bacterial contamination, and sterilize in an autoclave at 121° for 5 min, arranging to reach this temperature in NMT 10 min by preheating the autoclave if necessary. Cool as rapidly as possible to avoid color formation resulting from overheating the medium. Take precautions to maintain uniformity of sterilizing and cooling conditions throughout the assay, because packing the tubes too closely in the autoclave or overloading it may cause variation in the heating rate.

Aseptically add 0.5 mL of *Inoculum* to each tube so prepared, except two of the four containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 40°, held constant to within ±0.5°, for 16–24 h.

Terminate growth by heating to a temperature NLT 80° for 5 min. Cool to room temperature. After agitating contents, read the transmittance at 530 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of the inoculated blank. If the difference is greater than 5% or if there is evidence of contamination with a foreign microorganism, disregard the results of the assay.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of each of the remaining tubes. Disregard the results of the assay if the slope of the standard curve indicates a problem with sensitivity.

Calculation: Prepare a standard concentration-response curve by the following procedure. Test for and replace any aberrant individual transmittances. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittances (Σ_S) as the difference,

[2] Pure cultures of *Lactobacillus leichmannii* (listed as *Lactobacillus delbruekii*) may be obtained as No. 7830 from ATCC, 10801 University Blvd., Manassas, VA 20110-2209 (www.atcc.org).

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$y = 2.00 - \Sigma_S$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_U$, adding together the two transmittances (Σ_U) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X, for each dosage level. Average the values of X for each of three or more dosage levels to obtain $\bar{X}$, which equals the log-relative potency, M', of the *Sample solution*.

Determine the quantity, in µg, of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$) in the portion of Capsules taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of µg of cyanocobalamin assumed to be present in the portion of Capsules taken

Calculate the percentage of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$) in the portion of Capsules taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of cyanocobalamin in the portion of Capsules taken (µg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, $\bar{M}$, is the assayed log-potency of the test material (see *Vitamin B_{12} Activity* in *Design and Analysis of Biological Assays* ⟨111⟩, *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$)

• **Folic Acid, *Method 1***

[Note—Use low-actinic glassware throughout this procedure.]

Reagent A: 25% solution of tetrabutylammonium hydroxide in methanol

Reagent B: Transfer 5.0 g of pentetic acid to a 50-mL volumetric flask. Using sonication if necessary, dissolve in and dilute with 1 N sodium hydroxide to volume.

Mobile phase: 2 g of monobasic potassium phosphate in 650 mL of water. Add 12.0 mL of *Reagent A*, 7.0 mL of 3 N phosphoric acid, and 240 mL of methanol. Cool to room temperature, adjust with phosphoric acid or ammonia TS to a pH of 7.0, dilute with water to 1000 mL, and filter. Recheck the pH before use by adding water or methanol to the prepared *Mobile phase* to obtain baseline separation of folic acid and the internal standard. The pH may be increased up to 7.15 to obtain better separation. [Note—The methanol and water content may be varied (between 1% and 3%).]

Internal standard solution: Transfer 40 mg of methylparaben to a 1000-mL volumetric flask, and add 220 mL of methanol to dissolve. Dissolve 2.0 g of monobasic potassium phosphate in 300 mL of water in a separate beaker, quantitatively transfer this solution to the flask containing the methylparaben solution, and add an additional 300 mL of water. Add 19 mL of *Reagent A*, 7 mL of 3 N phosphoric acid, and 30 mL of *Reagent B*. Adjust with ammonia TS to a pH of 9.8, bubble nitrogen through the solution for 30 min, dilute with water to volume, and mix.

Standard solution: 0.016 mg/mL of USP Folic Acid RS in *Internal standard solution*

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." Transfer an amount of Capsule contents to a suitable centrifuge tube, and add a volume of *Internal standard solution* to obtain a nominal concentration of 0.016 mg/mL of folic acid. Shake by mechanical means for 10 min, and centrifuge. Filter a portion of the clear supernatant, and use the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 3.9-mm × 30-cm; packing L1

Flow rate: 1 mL/min

Injection volume: 15 µL

System suitability

Sample: *Standard solution*

[Note—The relative retention times for folic acid and methylparaben are about 0.8 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for folic acid and methylparaben. Calculate the percentage of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$) in the portion of Capsules taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak area ratio of folic acid to methylparaben from the *Sample solution*

R_S = peak area ratio of folic acid to methylparaben from the *Standard solution*

C_S = concentration of USP Folic Acid RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of folic acid in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$)

• **Folic Acid, *Method 2***

[Note—Use low-actinic glassware throughout this procedure.]

Diluent: 60 µg/mL of ammonium hydroxide

Mobile phase: Transfer 0.4 mL of triethylamine, 15.0 mL of glacial acetic acid, and 350 mL of methanol to a 2000-mL volumetric flask, and dilute with 0.008 M sodium 1-hexanesulfonate to volume.

Standard stock solution: 60 µg/mL of USP Folic Acid RS in *Diluent*. Prepare this solution fresh daily.

Standard solution: Mix 5.0 mL of the *Standard stock solution* with 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the net average weight per Capsule." Transfer a portion of the Capsule contents, equivalent to 0.3 mg of folic acid, to a 125-mL stoppered flask. Add

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10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 270 nm
Column: 4.6-mm × 25-cm; packing L7
Column temperature: 50°
Flow rate: 2 mL/min
Injection volume: 5 µL

System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 2.0%

Analysis
Samples: *Standard solution* and *Sample solution*
Measure the areas of the major peaks. Calculate the percentage of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of folic acid from the *Sample solution*
r_S = peak area of folic acid from the *Standard solution*
C_S = concentration of USP Folic Acid RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of folic acid in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$)

• **DEXPANTHENOL OR PANTHENOL**

[NOTE—The following procedure is applicable also to the determination of the dextrorotatory component of racemic panthenol in preparations containing panthenol.]

Dehydrated mixtures yielding formulations similar to the media described herein may be used provided that, when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described herein.

Standard stock solution: 800 µg/mL of USP Dexpanthenol RS or 1600 µg/mL of USP Racemic Panthenol RS in water. Store in a refrigerator protected from light, and use within 30 days.

Standard solution: On the day of the assay, prepare a dilution of 1.2 µg/mL of dexpanthenol or 2.4 µg/mL of panthenol from the *Standard stock solution* diluted with water.

Sample solution: Weigh NLT 30 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents as completely as possible to a beaker. Remove any contents adhering to the empty Capsule shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the average net weight per Capsule. Dissolve a portion of the Capsule contents, nominally equivalent to 1.2 mg of dexpanthenol or 2.4 mg of panthenol, in 100.0 mL of water. Quantitatively dilute a portion of this solution with water to obtain a nominal concentration of 1.2 µg/mL of dexpanthenol or 2.4 µg/mL of panthenol.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in about 500 mL of water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and dilute with water to 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature NLT 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to 75 ± 5°, and add hydrochloric acid solution (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and dilute with water to 1000 mL. Store under toluene in a cool place at a temperature NLT 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and dilute with water to 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin–thiamine hydrochloride–biotin solution: 20 µg/mL of riboflavin, 10 µg/mL of thiamine hydrochloride, and 0.04 µg/mL of biotin in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

***p*-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution:** 10 µg/mL of *p*-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in neutral 25% alcohol. Store in a refrigerator.

Salt solution A: 50 mg/mL of monobasic potassium phosphate and 50 mg/mL of dibasic potassium phosphate in water. Add 10 drops of hydrochloric acid per L of solution. Store under toluene.

Salt solution B: 20 mg/mL of magnesium sulfate, 1 mg/mL of sodium chloride, 1 mg/mL of ferrous sulfate, and 1 mg/mL of manganese sulfate in water. Add 10 drops of hydrochloric acid per L of the solution. Store under toluene.

Pyridoxal–calcium pantothenate solution: 200 µg/mL of pyridoxal hydrochloride and 1.875 µg/mL of calcium pantothenate in 10% alcohol. Store in a refrigerator, and use within 30 days.

Polysorbate 40–oleic acid solution: 50 mg/mL of polysorbate 40 and 0.5 mg/mL of oleic acid in 20% alcohol. Store in a refrigerator, and use within 30 days.

Modified pantothenate medium: Dissolve anhydrous dextrose and sodium acetate in the solutions previously mixed according to *Table 3*, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Table 3

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Riboflavin–thiamine hydrochloride–biotin solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL
Pyridoxal–calcium pantothenate solution	5 mL

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Table 3 *(continued)*

Polysorbate 40–oleic acid solution	5 mL

Double-strength modified pantothenate medium: Prepare as directed in *Modified pantothenate medium*, but make the final dilution to 125 mL instead of 250 mL. Prepare fresh.

Stock culture of *Pediococcus acidilactici*: Dissolve in 800 mL of water, with the aid of heat, 6.0 g of peptone, 4.0 g of pancreatic digest of casein, 3.0 g of yeast extract, 1.5 g of beef extract, 1.0 g of dextrose, and 15.0 g of agar. Adjust with 0.1 N sodium hydroxide or 0.1 N hydrochloric acid to a pH of 6.5–6.6, and dilute with water to 1000 mL. Add 10-mL portions of the solution to culture tubes, place caps on the tubes, and sterilize in an autoclave at 121° for 15 min. Cool on a slant, and store in a refrigerator. Prepare a stock culture of *Pediococcus acidilactici*[3] on a slant of this medium. Incubate at 35° for 20–24 h, and store in a refrigerator. Maintain the stock culture by monthly transfer onto fresh slants.

Inoculum: Inoculate three 250-mL portions of sterile *Modified pantothenate medium* from a stock culture slant, and incubate at 35° for 20–24 h. Centrifuge the suspension from the combined portions, and wash the cells with sterile *Modified pantothenate medium*. Resuspend the cells in sufficient *Modified pantothenate medium* so that a 1-in-50 dilution, when tested in a 13-mm diameter test tube, gives 80% light transmission at 530 nm. Transfer 1.2-mL portions of this stock suspension to sterile glass ampuls, seal, freeze in liquid nitrogen, and store in a freezer. On the day of the assay, allow the ampuls to reach room temperature, mix the contents, and dilute 1 mL of thawed culture with sterile saline TS to 150 mL. [NOTE—This dilution may be altered when necessary to obtain the desired test response.]

Analysis: Prepare in triplicate a series of eight culture tubes by adding the following quantities of water to the tubes within a set: 5.0, 4.5, 4.0, 3.5, 3.0, 2.0, 1.0, and 0.0 mL. To these same tubes and in the same order add 0.0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. Prepare in duplicate a series of five culture tubes by adding the following quantities of water to the tubes within a set: 4.0, 3.5, 3.0, 2.0, and 1.0 mL. To these same tubes and in the same order add 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*.

Add 5.0 mL of *Double-strength modified pantothenate medium* to each tube. Cover the tubes with metal caps, and sterilize in an autoclave at 121° for 5 min. Cool to room temperature in a chilled water bath, and inoculate each tube with 0.5 mL of the *Inoculum*. Allow to incubate at 37° for 16 h. Terminate growth by heating to a temperature NLT 80°, such as by steaming at atmospheric pressure in a sterilizer for 5–10 min. Cool, and determine the percentage transmittance of the suspensions, in cells of equal path length, on a suitable spectrophotometer at a wavelength of 530 nm.

Calculation: Draw a dose-response curve on arithmetic graph paper by plotting the average response, in percentage of transmittance, for each set of tubes of the standard curve against the Standard level concentrations. The curve is drawn by connecting each adjacent pair of points with a straight line. From this standard curve, determine by interpolation the potency of each tube containing portions of the *Sample solution*. To obtain the individual responses, divide the potency of each tube by the amount of the *Sample solution* added to it. Calculate the mean response by averaging the individual responses that vary from their mean by NMT 15%, using NLT half the total number of tubes. Calculate the potency of the portion of the material taken for assay by multiplying the mean response by the appropriate dilution factor.

Calculate the percentage of the labeled amount of dexpanthenol or panthenol ($C_9H_{19}NO_4$) in the portion of Capsules taken:

$$\text{Result} = (P/N) \times 100$$

P = potency of dexpanthenol or panthenol in the portion of Capsules taken (mg)

N = nominal amount of dexpanthenol or panthenol in the portion of Capsules taken (mg)

Acceptance criteria: 90.0%–150.0% of the labeled amount of dexpanthenol or panthenol ($C_9H_{19}NO_4$)

• **CALCIUM PANTOTHENATE, *Method 1***

Mobile phase: Phosphoric acid and water (1:1000)

Internal standard solution: 80 mg of *p*-hydroxybenzoic acid in 3 mL of alcohol. Add 50 mL of water and 7.1 g of dibasic sodium phosphate, and dilute with water to 1000 mL. Adjust with phosphoric acid to a pH of 6.7.

Standard solution: 0.6 mg/mL of USP Calcium Pantothenate RS in *Internal standard solution*

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." To a centrifuge tube, transfer an amount of mixed Capsule contents and a volume of *Internal standard solution* to obtain a nominal concentration of 0.6 mg/mL in the *Sample solution*.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 210 nm

Column: 3.9-mm × 15-cm; packing L1

Flow rate: 1.5 mL/min

Injection volume: 10 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for calcium pantothenate and *p*-hydroxybenzoic acid are about 0.5 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of calcium pantothenate and the internal standard. Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Capsules taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak area ratio of calcium pantothenate to *p*-hydroxybenzoic acid from the *Sample solution*

R_S = peak area ratio of calcium pantothenate to *p*-hydroxybenzoic acid from the *Standard solution*

C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• **CALCIUM PANTOTHENATE, *Method 2***

Standard stock solution: Dissolve 50 mg of USP Calcium Pantothenate RS, previously dried and stored in the dark over phosphorus pentoxide and protected from absorption

[3] ATCC No. 8042 is suitable.

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of moisture while weighing, in 500 mL of water in a 1000-mL volumetric flask. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), and dilute with water to volume to obtain a concentration of 50 µg/mL of USP Calcium Pantothenate RS. Store under toluene in a refrigerator.

Standard solution: On the day of the assay, dilute a volume of *Standard stock solution* with water to obtain a concentration of 0.01–0.04 µg/mL of calcium pantothenate, the exact concentration being such that the responses obtained as directed in the *Analysis*, 2.0 and 4.0 mL of the *Standard solution* being used, are within the linear portion of the log-concentration response curve.

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." Transfer a portion of the Capsule contents, equivalent to a nominal amount of 50 mg of calcium pantothenate, to a 1000-mL volumetric flask containing 500 mL of water. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), dilute with water to volume, and filter. Dilute a volume of this solution to obtain a solution with approximately the same concentration as that of the *Standard solution*.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and dilute with water to 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature NLT 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and dilute with water to 1000 mL. Store under toluene in a cool place at a temperature NLT 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and dilute with water to 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin–thiamine hydrochloride–biotin solution: 20 µg/mL of riboflavin, 10 µg/mL of thiamine hydrochloride, and 0.04 µg/mL of biotin in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

***p*-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution:** 10 µg/mL of *p*-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Basal medium stock solution: Dissolve *anhydrous Dextrose* and *anhydrous Sodium acetate* in the solutions previously mixed according to *Table 4*, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Table 4

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Riboflavin–thiamine hydrochloride–biotin solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water. Add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to the test tubes, close or cover the tubes, sterilize in an autoclave at 121° for 15 min, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*[1] incubating for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution*, add 5.0 mL of water containing 0.2 µg of calcium pantothenate. Plug the tubes with cotton, sterilize in an autoclave at 121° for 15 min, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Transfer cells from the *Stock culture of Lactobacillus plantarum* to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. The cell suspension so obtained is the *Inoculum*.

Analysis

Samples: *Standard solution* and *Sample solution*

To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool, and add 1 drop of *Inoculum* to each tube, except two of the four tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37°, held constant to within ±0.5°, until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of Standard during a 2-h period.

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Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to an optical container if necessary. Read the transmittance between 540 and 660 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittance (Σ_S) as the difference, $y = 2.00 - \Sigma_S$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_U$, adding together the two transmittances (Σ_U) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that fall within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X, for each dosage level. Average the values of X for each of three or more dosage levels to obtain $\bar{X}$, which equals the log-relative potency, M', of the *Sample solution*.

Determine the quantity, in mg, of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Capsules taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of mg of calcium pantothenate assumed to be present in the portion of Capsules taken

Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Capsules taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of calcium pantothenate in the portion of Capsules taken (mg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, $\bar{M}$, is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* ⟨111⟩, *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• **CALCIUM PANTOTHENATE, *Method 3***

Buffer solution: Dissolve 10.0 g of monobasic potassium phosphate in 2000 mL of water, and adjust with phosphoric acid to a pH of 3.5.

Mobile phase: Methanol and *Buffer solution* (1:9)

Standard stock solution: 0.25 mg/mL of USP Calcium Pantothenate RS in water. Prepare fresh every 4 weeks. Store in a refrigerator.

Standard solution: 40 µg/mL of USP Calcium Pantothenate RS from the *Standard stock solution* diluted with water

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." Transfer a portion of the Capsule contents, equivalent to a nominal amount of 10 mg of calcium pantothenate, to a 250-mL volumetric flask. Add 10 mL of methanol, and swirl the flask to disperse the Capsule contents. Dilute with water to volume, mix, and filter.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 205 nm

Column: 3.9-mm × 30-cm; 5-µm packing L1

Column temperature: 50°

Flow rate: 2 mL/min

Injection volume: 25 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of calcium pantothenate. Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of calcium pantothenate from the *Sample solution*

r_S = peak area of calcium pantothenate from the *Standard solution*

C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• **NIACIN OR NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, RIBOFLAVIN,** and **THIAMINE, *Method 1***

[NOTE—Use low-actinic glassware throughout this procedure.]

Diluent: Acetonitrile, glacial acetic acid, and water (5:1:94)

Mobile phase: A mixture of methanol, glacial acetic acid, and water (27:1:73) containing 140 mg of sodium 1-hexanesulfonate per 100 mL

Standard solution: [NOTE—Use USP Niacin RS in place of USP Niacinamide RS for formulations containing niacin.] Transfer 80 mg of USP Niacinamide RS, 20 mg of USP Pyridoxine Hydrochloride RS, 20 mg of USP Riboflavin RS, and 20 mg of USP Thiamine Hydrochloride RS to a 200-mL volumetric flask, and add 180 mL of *Diluent*. Immerse the flask in a hot water bath maintained at 65°–70° for 10 min with regular shaking or using a vortex mixer, until all the solid materials are dissolved. Chill rapidly in a cold water bath for 10 min to room temperature, and dilute with *Diluent* to volume.

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." Transfer a portion of the Capsule contents, equivalent to 10 mg of niacinamide and 2.5 mg each of pyridoxine

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hydrochloride, riboflavin, and thiamine hydrochloride, to a 50-mL centrifuge tube. Add 25.0 mL of *Diluent*, and mix using a vortex mixer for 30 s to completely suspend the powder. Immerse the centrifuge tube in a hot water bath maintained at 65°–70°, heat for 5 min, and mix using a vortex mixer for 30 s. Return the tube to the hot water bath, heat for another 5 min, and mix using a vortex mixer for 30 s. Filter a portion of the solution, cool to room temperature, and use the clear filtrate. [NOTE—Use the filtrate within 3 h of filtration.]

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 280 nm
Column: 3.9-mm × 30-cm; packing L1
Flow rate: 1 mL/min
Injection volume: 10 µL

System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for niacinamide, pyridoxine, riboflavin, and thiamine are about 0.3, 0.5, 0.8, and 1.0, respectively.]
Suitability requirements
Relative standard deviation: NMT 3.0%

Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas for niacin or niacinamide, pyridoxine, riboflavin, and thiamine. Calculate the percentage of the labeled amount of niacinamide ($C_6H_6N_2O$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacinamide from the *Sample solution*
r_S = peak area of niacinamide from the *Standard solution*
C_S = concentration of USP Niacinamide RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of niacinamide in the *Sample solution* (mg/mL)

For formulations containing niacin:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacin from the *Sample solution*
r_S = peak area of niacin from the *Standard solution*
C_S = concentration of USP Niacin RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of niacin in the *Sample solution* (mg/mL)

Separately calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the corresponding vitamin from the *Sample solution*
r_S = peak area of the corresponding vitamin from the *Standard solution*
C_S = concentration of the relevant USP Reference Standard in the *Standard solution* (mg/mL)
C_U = nominal concentration of the corresponding vitamin in the *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of thiamine from the *Sample solution*
r_S = peak area of thiamine from the *Standard solution*
C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL)
M_{r1} = molecular weight of thiamine mononitrate, 327.36
M_{r2} = molecular weight of thiamine hydrochloride, 337.27

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacinamide ($C_6H_6N_2O$) or niacin ($C_6H_5NO_2$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine as thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$)

• **NIACIN, *Method 2***
[NOTE—Use low-actinic glassware throughout this procedure.]
Solution A: Transfer 1 mL of glacial acetic acid and 2.5 g of edetate disodium to a 100-mL volumetric flask. Dissolve in and dilute with water to volume.
Extraction solvent: *Solution A* and methanol (3:1)
Mobile phase: 0.1 M sodium acetate solution (13.6 mg/mL of sodium acetate in water). Adjust with acetic acid to a pH of 5.4. [NOTE—A small amount of methanol (up to 1%) may be added to the *Mobile phase* to improve resolution.]
Standard stock solution: 1 mg/mL of USP Niacin RS in *Extraction solvent*
Standard solution: Transfer 5.0 mL of *Standard stock solution* to a 25-mL volumetric flask, and dilute with *Extraction solvent* to volume.
Sample solution: [NOTE—This preparation is suitable for the determination of niacin or niacinamide, pyridoxine, and riboflavin, when present in the formulation.] Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents to a beaker. Remove any contents adhering to the shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to a nominal amount of 2 mg of riboflavin, to a 200-mL volumetric flask. If riboflavin is not present in the formulation, use a portion equivalent to a nominal amount of 2 mg of pyridoxine. If pyridoxine is not present in the formulation, use a portion equivalent to a nominal amount of 20 mg of niacin or niacinamide. Add 100.0 mL of *Extraction solvent*, and mix for 20 min, using a wrist-action shaker. Immerse the flask in a water bath maintained at 70°–75°, and heat for 20 min. Mix on a vortex mixer for 30 s, cool to room temperature, and filter. Use the clear filtrate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; packing L1
Flow rate: 1 mL/min
Injection volume: 20 µL

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System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 3.0%
[NOTE—If necessary, flush the column with methanol between injections.]
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas of niacin. Calculate the percentage of the labeled amount of niacin ($C_6H_5NO_2$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacin from the *Sample solution*
r_S = peak area of niacin from the *Standard solution*
C_S = concentration of USP Niacin RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of niacin in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacin ($C_6H_5NO_2$)

• **NIACINAMIDE, *Method 2***
[NOTE—Use low-actinic glassware throughout this procedure.]
Extraction solvent, Mobile phase, Standard stock solution, Standard solution, Sample solution, and **Chromatographic system:** Using USP Niacinamide RS in place of USP Niacin RS, proceed as directed in *Niacin, Method 2*.
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas of niacinamide. Calculate the percentage of the labeled amount of niacinamide ($C_6H_6N_2O$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacinamide from the *Sample solution*
r_S = peak area of niacinamide from the *Standard solution*
C_S = concentration of USP Niacinamide RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of niacinamide in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacinamide ($C_6H_6N_2O$)

• **PYRIDOXINE HYDROCHLORIDE, *Method 2***
[NOTE—Use low-actinic glassware throughout this procedure.]
Extraction solvent, Mobile phase, and **Sample solution:** Prepare as directed in *Niacin, Method 2*.
Standard stock solution: 0.1 mg/mL of USP Pyridoxine Hydrochloride RS in *Extraction solvent*
Standard solution: 20 µg/mL of USP Pyridoxine Hydrochloride RS from the *Standard stock solution* diluted with *Extraction solvent*
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; packing L1
Flow rate: 1 mL/min
Injection volume: 20 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 3.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas of pyridoxine. Calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of pyridoxine from the *Sample solution*
r_S = peak area of pyridoxine from the *Standard solution*
C_S = concentration of USP Pyridoxine Hydrochloride RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of pyridoxine hydrochloride in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$)

• **RIBOFLAVIN, *Method 2***
[NOTE—Use low-actinic glassware throughout this procedure.]
Extraction solvent and **Sample solution:** Prepare as directed in *Niacin, Method 2*.
Solution A: 6.8 mg/mL of sodium acetate in water
Mobile phase: Prepare a mixture of *Solution A* and methanol (13:7). Add 2 mL of triethylamine per L of the mixture, and adjust with glacial acetic acid to a pH of 5.2.
Standard stock solution: Transfer 20 mg of USP Riboflavin RS to a 200-mL volumetric flask, and add 180 mL of *Extraction solvent*. Immerse the flask for 5 min in a water bath maintained at 65°–75°. Mix well, and repeat if necessary until dissolved. Chill rapidly in a cold water bath to room temperature, and dilute with *Extraction solvent* to volume.
Standard solution: Dilute 5.0 mL of *Standard stock solution* with *Extraction solvent* to 25.0 mL.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; packing L1
Flow rate: 1 mL/min
Injection volume: 20 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 3.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas of riboflavin. Calculate the percentage of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of riboflavin from the *Sample solution*
r_S = peak area of riboflavin from the *Standard solution*
C_S = concentration of USP Riboflavin RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of riboflavin in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$)

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• **THIAMINE, *Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A: 1.88 mg/mL of sodium 1-hexanesulfonate in 0.1% phosphoric acid

Mobile phase: *Solution A* and acetonitrile (46:9)

Standard stock solution: 0.1 mg/mL of USP Thiamine Hydrochloride RS in 0.2 N hydrochloric acid

Standard solution: 0.02 mg/mL of USP Thiamine Hydrochloride RS from the *Standard stock solution* diluted with 0.2 N hydrochloric acid

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." Mix a portion of the Capsule contents with a volume of 0.2 N hydrochloric acid to obtain a nominal concentration of 0.02 mg/mL of thiamine. Shake for 15 min with a wrist-action shaker, and heat to boiling for 30 min. Cool to room temperature, and filter. Use the clear filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 2 mL/min

Injection volume: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of the major peaks. For products containing thiamine hydrochloride, calculate the percentage of the labeled amount of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of thiamine from the *Sample solution*

r_S = peak area of thiamine from the *Standard solution*

C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of thiamine hydrochloride in the *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of thiamine from the *Sample solution*

r_S = peak area of thiamine from the *Standard solution*

C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of thiamine mononitrate, 327.36

M_{r2} = molecular weight of thiamine hydrochloride, 337.27

Acceptance criteria: 90.0%–150.0% of the labeled amount of thiamine as thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$)

• **NIACIN OR NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, RIBOFLAVIN, and THIAMINE, *Method 3***

[NOTE—Use low-actinic glassware throughout this procedure.]

Reagent: 25 mg/mL of edetate disodium in water

Mobile phase: Transfer 0.4 mL of triethylamine, 15.0 mL of glacial acetic acid, and 350 mL of methanol to a 2000-mL volumetric flask. Dilute with 0.008 M sodium 1-hexanesulfonate to volume.

Standard stock solution: 1.5 mg/mL of USP Niacin RS or USP Niacinamide RS, 0.24 mg/mL of USP Pyridoxine Hydrochloride RS, 0.08 mg/mL of USP Riboflavin RS, and 0.24 mg/mL of USP Thiamine Hydrochloride RS in the *Reagent*, with heating if necessary

Standard solution: Transfer 5.0 mL of *Standard stock solution* to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." Transfer a portion of the Capsule contents, equivalent to 7.5 mg of niacin or niacinamide, 1.2 mg of pyridoxine hydrochloride, 0.4 mg of riboflavin, and 1.2 mg of thiamine hydrochloride, to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1), and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 270 nm

Column: 4.6-mm × 25-cm; packing L7

Column temperature: 50°

Flow rate: 2.0 mL/min

Injection volume: 5 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of niacin or niacinamide. Calculate the percentage of the labeled amount of niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacin or niacinamide from the *Sample solution*

r_S = peak area of niacin or niacinamide from the *Standard solution*

C_S = concentration of USP Niacin RS or USP Niacinamide RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacin or niacinamide in the *Sample solution* (mg/mL)

Separately calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) (for products containing thiamine hydrochloride) in the portion of Capsules taken:

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$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the corresponding vitamin from the *Sample solution*
r_S = peak area of the corresponding vitamin from the *Standard solution*
C_S = concentration of the relevant USP Reference Standard in the *Standard solution* (mg/mL)
C_U = nominal concentration of the corresponding vitamin in the *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of thiamine from the *Sample solution*
r_S = peak area of thiamine from the *Standard solution*
C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL)
M_{r1} = molecular weight of thiamine mononitrate, 327.36
M_{r2} = molecular weight of thiamine hydrochloride, 337.27

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine as thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$)

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** ⟨2040⟩**:** Meet the requirements for *Dissolution*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩**:** Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 3×10^3 cfu/g, and the combined molds and yeasts count does not exceed 3×10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meet the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** The label states that the product is Water-Soluble Vitamins Capsules. The label also states the quantity of each vitamin in terms of metric units per dosage unit, and where necessary, the salt form in which it is present. Where more than one assay method is given for a particular vitamin, the labeling states which assay method is used only if *Method 1* is not used. Where products are labeled to contain panthenol, the label states the equivalent content of dexpanthenol.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Biotin RS
(3a*S*,4*S*,6a*R*)-Hexahydro-2-oxo-1*H*-thieno[3,4-*d*] imidazole-4-valeric acid.
$C_{10}H_{16}N_2O_3S$ 244.31

USP Calcium Pantothenate RS
Calcium D-pantothenate (1:2).
$C_{18}H_{32}CaN_2O_{10}$ 476.53

USP Cyanocobalamin (Crystalline) RS
Vitamin B_{12}.
$C_{63}H_{88}CoN_{14}O_{14}P$ 1355.37

USP Dexpanthenol RS
D-(+)-2,4-Dihydroxy-*N*-(3-hydroxypropyl)-3,3-dimethylbutyramide.
$C_9H_{19}NO_4$ 205.25

USP Folic Acid RS
L-Glutamic acid, *N*-[4-[[(2-amino-1,4-dihydro-4-oxo-6-pteridinyl)methyl]amino]benzoyl]-.
$C_{19}H_{19}N_7O_6$ 441.40

USP Niacin RS
Nicotinic acid.
$C_6H_5NO_2$ 123.11

USP Niacinamide RS
Nicotinamide.
$C_6H_6N_2O$ 122.12

USP Racemic Panthenol RS
Butanamide, 2,4-dihydroxy-*N*-(3-hydroxypropyl)-3,3-dimethyl-, (±)-.
$C_9H_{19}NO_4$ 205.25

USP Pyridoxine Hydrochloride RS
Pyridoxol hydrochloride.
$C_8H_{11}NO_3 \cdot HCl$ 205.64

USP Riboflavin RS
Riboflavine.
$C_{17}H_{20}N_4O_6$ 376.36

USP Thiamine Hydrochloride RS
Thiamine monohydrochloride.
$C_{12}H_{17}ClN_4OS \cdot HCl$ 337.27

Water-Soluble Vitamins Tablets

DEFINITION

Change to read:

Water-Soluble Vitamins Tablets contain two or more of the following water-soluble vitamins: Ascorbic Acid or its equivalent as Calcium Ascorbate or Sodium Ascorbate, Biotin, Cyanocobalamin, Folic Acid, Niacin or Niacinamide, pantothenic acid (as Calcium Pantothenate or Racemic Calcium Pantothenate), Pyridoxine Hydrochloride, Riboflavin, and Thiamine Hydrochloride or Thiamine Mononitrate. Tablets contain NLT 90.0% and NMT 150.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$), ▲▲ (USP 1-May-2020) biotin ($C_{10}H_{16}N_2O_3S$), calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$), folic acid ($C_{19}H_{19}N_7O_6$), niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), pyridoxine ▲▲ (USP 1-May-2020) ($C_8H_{11}NO_3 \cdot$ ▲▲ (USP 1-May-2020)), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine ▲as thiamine ion ($C_{12}H_{17}N_4OS^+$)▲ (USP 1-May-2020)

They do not contain any form of Beta Carotene or Vitamin A, D, E, or K. They do not contain any minerals for which nutritional value is claimed. They may contain other labeled added substances in quantities that are unobjectionable.

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IDENTIFICATION

Add the following:

▲• **A.** The retention times of the vitamin peaks of the *Sample solutions* correspond to those of the corresponding vitamin peaks of the *Standard solutions* as obtained in the tests for *Strength*.▲ (USP 1-May-2020)

STRENGTH

[NOTE—In the following assays, where more than one assay method is given for an individual ingredient, the requirements may be met by following any one of the specified methods, with the method used being stated in the labeling only if *Method 1* is not used.]

Change to read:

• **ASCORBIC ACID, CALCIUM ASCORBATE,** and **SODIUM ASCORBATE**

(See *Vitamin C Assay* ⟨580⟩.)

[NOTE—For labeling purposes, consider ⟨580⟩, *Method I—Titrimetric Method* as *Method 1*; ▲⟨580⟩, *Method II—Chromatographic Method, Procedure 1* as *Method 2* and ⟨580⟩, *Method II—Chromatographic Method, Procedure 2* as *Method 3*.▲ (USP 1-May-2020)]

Acceptance criteria: 90.0%–150.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$), or its equivalent as calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$), or sodium ascorbate ($C_6H_7NaO_6$)

• **BIOTIN,** ***Method 1***

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Mix 85 mL of acetonitrile, 1 g of sodium perchlorate, and 1 mL of phosphoric acid, and dilute with water to 1000 mL.

Standard stock solution: 0.333 mg/mL of USP Biotin RS in dimethyl sulfoxide

Standard solution: 5 µg/mL of USP Biotin RS prepared by diluting the *Standard stock solution* in water

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to a nominal amount of 1 mg of biotin, to a 200-mL volumetric flask. Add 3 mL of dimethyl sulfoxide, and swirl to wet the contents. Place the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with water to volume, and filter.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 200 nm

Column: 4.6-mm × 15-cm; 3-µm packing L7

Flow rate: 1.2 mL/min

Injection volume: 100 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of biotin. Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of biotin from the *Sample solution*

r_S = peak area of biotin from the *Standard solution*

C_S = concentration of USP Biotin RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of biotin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

• **BIOTIN,** ***Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Dehydrated mixtures yielding formulations similar to the media described herein may be used provided that, when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described.

Standard stock solution: 50 µg/mL of USP Biotin RS in 50% alcohol. Store in a refrigerator.

Standard solution: 0.1 ng/mL of USP Biotin RS in water, prepared by diluting the *Standard stock solution* with water on the day of the assay

Sample solution: Finely powder NLT 30 Tablets. Transfer a portion of the powder, equivalent to 100 µg of biotin, to a 200-mL volumetric flask. Add 3 mL of 50% alcohol, and swirl to wet the contents. Heat the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with 50% alcohol to volume, and filter. Dilute a volume of the filtrate with water quantitatively, and stepwise if necessary, to obtain a solution with a concentration of 0.1 ng/mL.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and dilute with water to 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature NLT 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water. Heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and dilute with water to 1000 mL. Store under toluene in a cool place at a temperature NLT 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and dilute with water to 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Calcium pantothenate solution: 10 µg/mL of calcium pantothenate in 50% alcohol. Store in a refrigerator.

Riboflavin–thiamine hydrochloride solution: 20 µg/mL of riboflavin and 10 µg/mL of thiamine hydrochloride in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

***p*-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution:** 10 µg/mL of *p*-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of

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manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid, and mix. Store under toluene.

Basal medium stock solution: Dissolve anhydrous dextrose and anhydrous sodium acetate in the solutions previously mixed according to *Table 1*, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Table 1

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Calcium pantothenate solution	5 mL
Riboflavin–thiamine hydrochloride solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water. Add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to test tubes, close or cover the tubes, sterilize in an autoclave at 121° for 15 min, and allow the tubes to cool in an upright position. Prepare stab cultures in 3 or more of the tubes, using a pure culture of *Lactobacillus plantarum*,[1] incubating for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution* add 5.0 mL of water containing 0.5 ng of biotin. Plug the tubes with cotton, sterilize in an autoclave at 121° for 15 min, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Transfer cells from the *Stock culture of Lactobacillus plantarum* to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. The cell suspension so obtained is the *Inoculum*.

Analysis

Samples: *Standard solution* and *Sample solution*

To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to four similar but empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar test tubes add, in duplicate, volumes of the *Sample solution* corresponding to 3 or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool. Add 1 drop of *Inoculum* to each tube, except 2 of the 4 tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37° held constant to within ±0.5° until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of Standard during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to a spectrophotometer cell. Place the cell in a spectrophotometer that has been set at a specific wavelength of 540–660 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittance (Σ_S) as the difference, $y = 2.00 - \Sigma_S$. Plot this response on the ordinate of cross-section paper against the logarithm of the milliliter of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_U$, adding together the two transmittances (Σ_U) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X, for each dosage level. Average the values of X for each of three or more dosage levels to obtain $\bar{X}$, which equals the log-relative potency, M', of the *Sample solution*.

Determine the quantity, in µg, of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Tablets taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = amount of biotin assumed to be present in the portion of Tablets taken (µg)

Calculate the percentage of the labeled amount of biotin in the portion of tablets taken: ($C_{10}H_{16}N_2O_3S$) in the portion of Tablets taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of biotin in the portion of Tablets taken (µg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, $\bar{M}$, is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* ⟨111⟩, *The Confidence Interval and Limits of Potency*). If the two determinations

[1] ATCC #8014 is suitable. This strain was formerly known as *Lactobacillus arabinosus* 17-5.

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differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of *M* that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

• **BIOTIN, *Method 3***

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A: Transfer 800 mL of water and 100 mL of triethylamine to a 1000-mL volumetric flask. Add 80 mL of 85% phosphoric acid, and dilute with water to volume.

Mobile phase: Transfer 80 mL of acetonitrile and 10 mL of *Solution A* to a 1000-mL volumetric flask. Dilute with water to volume.

Standard solution: 0.6 µg/mL of USP Biotin RS in water. [NOTE—A portion of the *Standard solution* will be used to determine the percent recovery of biotin from the *Solid-phase extraction* procedure.]

Sample solution: Finely powder NLT 20 Tablets. Transfer an amount of powdered Tablets to a volumetric flask to obtain a nominal concentration of 0.6 µg/mL of biotin. Add water up to 80% of the flask capacity, and sonicate for 30–40 min, with occasional mixing, to dissolve. Dilute with water to volume, and filter. Adjust the solution with either diluted acetic acid or 0.1 N sodium hydroxide to a pH of 6.0–7.0.

Solid-phase extraction: [NOTE—Condition the extraction column specified in this procedure in the following manner. Wash the column with a 2-mL portion of methanol. Equilibrate with a 2-mL portion of water.]

Separately pipet 5.0 mL of the *Sample solution* and *Standard solution* into freshly conditioned solid-phase extraction columns consisting of a mixed-mode packing with a sorbent mass of 60 mg. [NOTE—The mixed-mode packing consists of anion-exchange and reversed-phase sorbents. The reverse-phase component is a copolymer of *N*-vinylpyrrolidone and divinylbenzene. The anion-xchange moiety is a trialkylamino group.[2]]

Wash the column with 10 mL of 30% (v/v) methanol in water. Apply an appropriate volume (4.9 mL) of 30% (v/v) methanol in 0.1 N hydrochloric acid to the column. Collect the eluate in a 5-mL volumetric flask, containing 100 µL of 40% (w/v) sodium acetate in water, and dilute with 30% (v/v) methanol in 0.1 N hydrochloric acid to volume.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 200 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 2 mL/min

Injection volume: 100 µL

System suitability

Samples: *Standard solution* and a portion of *Standard solution* that has undergone *Solid-phase extraction*

Suitability requirements

Tailing factor: NMT 1.5, *Standard solution*

Relative standard deviation: NMT 2.0%, *Standard solution* and *Standard solution* that has undergone *Solid-phase extraction*

Recovery: 95%–100%, *Standard solution* that has undergone *Solid-phase extraction*

Analysis

Samples: *Standard solution* and *Sample solution* that have both undergone *Solid-phase extraction*

Measure the responses for the biotin peak. Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Biotin RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of biotin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

• **CYANOCOBALAMIN, *Method 1***

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Methanol and water (7:13)

Standard stock solution: 10 µg/mL of USP Cyanocobalamin RS in water. [NOTE—Store in a dark place, and discard after 1 week.]

Standard solution: 1 µg/mL of USP Cyanocobalamin RS from *Standard stock solution* diluted with water

Sample solution: Finely powder NLT 30 Tablets. Transfer a portion of the powder, equivalent to 100 µg of cyanocobalamin, to a 250-mL flask. Quantitatively add 100.0 mL of water, and carefully extract for 2 min. Filter 10 mL of the extract, and use the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: 550 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Flow rate: 0.5 mL/min

Injection volume: 200 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak responses for cyanocobalamin. Calculate the percentage of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of cyanocobalamin from the *Sample solution*

r_S = peak area of cyanocobalamin from the *Standard solution*

C_S = concentration of USP Cyanocobalamin RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cyanocobalamin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$)

Change to read:

• **CYANOCOBALAMIN, *Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Standard stock solution: 1.0 µg/mL of USP Cyanocobalamin RS in 25% alcohol. Store in a refrigerator.

Standard solution: Dilute a suitable volume of *Standard stock solution* with water to a measured volume such that

[2] A suitable cartridge is the Waters Oasis MAX Vac RC, particle size 30 µm, part #86000371.

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after the incubation period as described in the *Analysis*, the difference in transmittance between the inoculated blank and the 5.0-mL level of the *Standard solution* is NLT that which corresponds to a difference of 1.25 mg in dried cell weight. This concentration usually falls between 0.01 and 0.04 ng/mL of *Standard solution*. Prepare this solution fresh for each assay.

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powdered Tablets, equivalent to 1.0 µg of cyanocobalamin, to an appropriate vessel containing, for each gram of powdered Tablets taken, 25 mL of an aqueous extracting solution prepared just before use to contain, in each 100 mL, 1.29 g of dibasic sodium phosphate, 1.1 g of anhydrous citric acid, and 1.0 g of sodium metabisulfite. Autoclave the mixture at 121° for 10 min. Allow any undissolved particles of the extract to settle, and filter or centrifuge if necessary. Dilute an aliquot of the clear solution with water to obtain a final solution containing vitamin B_{12} activity approximately equivalent to that of the *Standard solution*.

Acid-hydrolyzed casein solution: Prepare as directed in *Biotin, Method 2*.

Asparagine solution: Dissolve 2.0 g of L-asparagine in water to make 200 mL. Store under toluene in a refrigerator.

Adenine–guanine–uracil solution: Prepare as directed in *Biotin, Method 2*.

Xanthine solution: Suspend 0.20 g of xanthine in 30–40 mL of water, heat to 70°, add 6.0 mL of 6 N ammonium hydroxide, and stir until the solid is dissolved. Cool, and dilute with water to 200 mL. Store under toluene in a refrigerator.

Salt solution A: Dissolve 10 g of monobasic potassium phosphate and 10 g of dibasic potassium phosphate in water to make 200 mL, and add 2 drops of hydrochloric acid. Store this solution under toluene.

Salt solution B: Dissolve 4.0 g of magnesium sulfate, 0.20 g of sodium chloride, 0.20 g of ferrous sulfate, and 0.20 g of manganese sulfate in water to make 200 mL. Add 2 drops of hydrochloric acid. Store this solution under toluene.

Polysorbate 80 solution: Dissolve 20 g of polysorbate 80 in alcohol to make 200 mL. Store in a refrigerator.

Vitamin solution A: Dissolve 10 mg of riboflavin, 10 mg of thiamine hydrochloride, 100 µg of biotin, and 20 mg of niacin in 0.02 N acetic acid to make 400 mL. Store under toluene, protected from light, in a refrigerator.

Vitamin solution B: Dissolve 20 mg of *p*-aminobenzoic acid, 10 mg of calcium pantothenate, 40 mg of pyridoxine hydrochloride, 40 mg of pyridoxal hydrochloride, 8 mg of pyridoxamine dihydrochloride, and 2 mg of folic acid in a mixture of water and neutralized alcohol (3:1) to make 400 mL. Store, protected from light, in a refrigerator.

Basal medium stock solution: Prepare the medium according to the following formula and directions. A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium comparable to that obtained from the formula given herein.

Add the ingredients in the order listed in *Table 2*, carefully dissolving cystine and tryptophan in the hydrochloric acid before adding the next eight solutions to the resulting solution. Add 100 mL of water, and dissolve dextrose, sodium acetate, and ascorbic acid. Filter, if necessary. Add the *Polysorbate 80 solution*, adjust with 1 N sodium hydroxide to a pH of 5.5–6.0, and dilute with Purified Water to 250 mL.

Table 2

L-Cystine	0.1 g
L-Tryptophan	0.05 g
1 N Hydrochloric acid	10 mL
Adenine–guanine–uracil solution	5 mL
Xanthine solution	5 mL
Vitamin solution A	10 mL
Vitamin solution B	10 mL
Salt solution A	5 mL
Salt solution B	5 mL
Asparagine solution	5 mL
Acid-hydrolyzed casein solution	25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Ascorbic acid	1 g
Polysorbate 80 solution	5 mL

Tomato juice preparation: Centrifuge commercially canned tomato juice so that most of the pulp is removed. Suspend 5 g/L of analytical filter aid in the supernatant, and pass, with the aid of reduced pressure, through a layer of the filter aid. Repeat, if necessary, until a clear, straw-colored filtrate is obtained. Store under toluene in a refrigerator.

Culture medium: [NOTE—A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium equivalent to that obtained from the formula given herein.] Dissolve 0.75 g of yeast extract, 0.75 g of dried peptone, 1.0 g of anhydrous dextrose, and 0.20 g of monobasic potassium phosphate in 60–70 mL of water. Add 10 mL of *Tomato juice preparation* and 1 mL of *Polysorbate 80 solution*. Adjust with 1 N sodium hydroxide to a pH of 6.8, and dilute with water to 100 mL. Place 10-mL portions of the solution in test tubes, and plug with cotton. Sterilize the tubes and contents in an autoclave at 121° for 15 min. Cool as rapidly as possible to avoid color formation resulting from overheating the medium.

Suspension medium: Dilute a measured volume of *Basal medium stock solution* with an equal volume of water. Place 10-mL portions of the diluted medium in test tubes. Sterilize, and cool as directed for *Culture medium*.

Stock culture of *Lactobacillus leichmannii*: To 100 mL of *Culture medium* add 1.0–1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Place 10-mL portions of the hot solution in test tubes, cover the tubes, sterilize at 121° for 15 min in an autoclave, and allow the tubes to cool in an upright position. Inoculate three or more of the tubes by stab transfer of a pure culture of *Lactobacillus leichmannii*.[3] [NOTE—Before first using a fresh culture in this assay, make NLT 10 successive transfers of the culture in a 2-week period.] Incubate for 16–24 h at a temperature between 30° and 40° held constant to within ±0.5°. Store in a refrigerator.

Prepare fresh stab cultures at least 3 times each week, and do not use them for preparing the *Inoculum* if more than 4 days old. The activity of the microorganism can be increased by daily or twice-daily transfer of the stab culture, to the point where definite turbidity in the liquid *Inoculum* can be observed 2–4 h after inoculation. A

[3] Pure cultures of *Lactobacillus leichmannii* (listed as *Lactobacillus delbrueckii*) may be obtained as #7830 from ATCC, 10801 University Blvd., Manassas, VA 20110-2209 (www.atcc.org).

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slow-growing culture seldom gives a suitable response curve and may lead to erratic results.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus leichmannii* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Transfer cells from the *Stock culture of Lactobacillus leichmannii* to two sterile tubes containing 10 mL each of the *Culture medium*. Incubate these cultures for 16–24 h at a temperature between 30° and 40° held constant to within ±0.5°. Under aseptic conditions centrifuge the cultures, and decant the supernatant. Suspend the cells from the culture in 5 mL of *Suspension medium*, and combine. Using sterile *Suspension medium*, adjust the volume so that a 1-in-20 dilution in saline TS produces 70% transmittance when read on a suitable spectrophotometer that has been set at a wavelength of 530 nm, equipped with a 10-mm cell, and read against saline TS set at 100% transmittance. Prepare a 1-in-400 dilution of the adjusted suspension using sterile *Basal medium stock solution*. [NOTE—This dilution may be altered, when necessary, to obtain the desired test response.] The cell suspension is the *Inoculum*.

Calibration of spectrophotometer: Check the wavelength of the spectrophotometer periodically, using a standard wavelength cell or other suitable device. Before reading any tests, calibrate the spectrophotometer for 0% and 100% transmittance, using water, the wavelength set at 530 nm.

Analysis

Samples: *Standard solution* and *Sample solution*

Because of the high sensitivity of the test organism to minute amounts of vitamin B_{12} activity and to traces of many cleansing agents, cleanse meticulously by suitable means, followed preferably by heating at 250° for 2 h, using hard-glass 20-mm × 150-mm test tubes and other necessary glassware.

To separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each of these tubes and to 4 similar but empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*. To each tube add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes to prevent bacterial contamination, and sterilize in an autoclave at 121° for 5 min, arranging to reach this temperature in NMT 10 min by preheating the autoclave if necessary. Cool as rapidly as possible to avoid color formation resulting from overheating the medium. Take precautions to maintain uniformity of sterilizing and cooling conditions throughout the assay, because packing the tubes too closely in the autoclave or overloading it may cause variation in the heating rate.

Aseptically add 0.5 mL of *Inoculum* to each tube so prepared, except 2 of the 4 containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 40°, held constant to within ±0.5°, for 16–24 h.

Terminate growth by heating to a temperature NLT 80° for 5 min. Cool to room temperature. After agitating contents, read the transmittance at 530 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of the inoculated blank. If the difference is greater than 5%, or if there is evidence of contamination with a foreign microorganism, disregard the results of the assay.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of each of the remaining tubes. Disregard the results of the assay if the slope of the standard curve indicates a problem with sensitivity.

Calculation: Prepare a standard concentration-response curve by the following procedure. Test for and replace any aberrant individual transmittances. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittances (Σ_S) as the difference, $y = 2.00 - \Sigma_S$. Plot this response on the ordinate of cross-section paper against the logarithm of the milliliter of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_U$, adding together the two transmittances (Σ_U) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X, for each dosage level. Average the values of X for each of three or more dosage levels to obtain $\overline{X}$, which equals the log-relative potency, M', of the *Sample solution*.

Determine the quantity, in µg, of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$) in the portion of Tablets taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = amount of cyanocobalamin assumed to be present in the portion of Tablets taken (µg)

Calculate the percentage of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$) in the portion of Tablets taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of cyanocobalamin in the portion of Tablets taken (µg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, $\overline{M}$, is the assayed log-potency of the test material (see ▲ (USP 1-May-2020) *Design and Analysis of Biological Assays* ⟨111⟩, *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$)

Change to read:

- **FOLIC ACID, *Method 1***

▲Proceed as directed in *Folic Acid Assay* ⟨411⟩, *Assay, Procedure 1*.▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–150.0% of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$)

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Change to read:

- **FOLIC ACID, *Method 2***

▲Proceed as directed in *Folic Acid Assay* ⟨411⟩, *Assay, Procedure 2*.▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–150.0% of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$)

- **CALCIUM PANTOTHENATE, *Method 1***

Mobile phase: Phosphoric acid and water (1:1000)

Internal standard solution: 80 mg of *p*-hydroxybenzoic acid in 3 mL of alcohol. Add 50 mL of water and 7.1 g of dibasic sodium phosphate, and dilute with water to 1000 mL. Adjust with phosphoric acid to a pH of 6.7.

Standard solution: 0.6 mg/mL of USP Calcium Pantothenate RS in *Internal standard solution*

Sample solution: Finely powder NLT 30 Tablets. Transfer a portion of the powder, equivalent to 15 mg of calcium pantothenate, to a centrifuge tube. Add 25.0 mL of the *Internal standard solution*, and shake vigorously for 10 min. Centrifuge, filter, and use the clear filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 210 nm

Column: 3.9-mm × 15-cm; packing L1

Flow rate: 1.5 mL/min

Injection volume: 10 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for calcium pantothenate and *p*-hydroxybenzoic acid are about 0.5 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for calcium pantothenate and the internal standard.

Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Tablets taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak area ratio of calcium pantothenate to *p*-hydroxybenzoic acid from the *Sample solution*

R_S = peak area ratio of calcium pantothenate to *p*-hydroxybenzoic acid from the *Standard solution*

C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

Change to read:

- **CALCIUM PANTOTHENATE, *Method 2***

Standard stock solution: Dissolve 50 mg of USP Calcium Pantothenate RS ▲▲ (USP 1-May-2020) in 500 mL of water in a 1000-mL volumetric flask. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), and dilute with water to volume to obtain a concentration of 50 µg/mL of USP Calcium Pantothenate RS. Store under toluene in a refrigerator.

Standard solution: On the day of the assay, dilute a volume of *Standard stock solution* with water to obtain a concentration of 0.01–0.04 µg/mL of calcium pantothenate, the exact concentration being such that the responses obtained as directed in the *Analysis*, 2.0 and 4.0 mL of the *Standard solution* being used, are within the linear portion of the log-concentration response curve.

Sample solution: Finely powder NLT 30 Tablets. Transfer a portion of the powder, equivalent to a nominal amount of 50 mg of calcium pantothenate, to a 1000-mL volumetric flask containing 500 mL of water. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), dilute with water to volume, and filter. Dilute a volume of this solution to obtain a solution with approximately the same concentration as that of the *Standard solution*.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and dilute with water to 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature NLT 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and dilute with water to 1000 mL. Store under toluene in a cool place at a temperature NLT 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and dilute with water to 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin–thiamine hydrochloride–biotin solution: 20 µg/mL of riboflavin, 10 µg/mL of thiamine hydrochloride, and 0.04 µg/mL of biotin in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

***p*-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution:** 10 µg/mL of *p*-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Basal medium stock solution: Dissolve the anhydrous dextrose and anhydrous sodium acetate in the solutions previously mixed according to *Table 3*, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Table 3

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g

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Table 3 *(continued)*

Adenine–guanine–uracil solution	5 mL
Riboflavin–thiamine hydrochloride–biotin solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water. Add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to the test tubes, close or cover the tubes, sterilize in an autoclave at 121° for 15 min, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*[1] incubating for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution* add 5.0 mL of water containing 0.2 µg of calcium pantothenate. Plug the tubes with cotton, sterilize in an autoclave at 121° for 15 min, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Transfer cells from the *Stock culture of Lactobacillus plantarum* to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. The cell suspension is the *Inoculum*.

Analysis

Samples: *Standard solution* and *Sample solution*

To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to 4 similar but empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool, and add 1 drop of *Inoculum* to each tube, except 2 of the 4 tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37°, held constant to within ±0.5° until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of Standard during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to an optical container if necessary. Read the transmittance between 540 and 660 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittance (Σ_S) as the difference, $y = 2.00 - \Sigma_S$. Plot this response on the ordinate of cross-section paper against the logarithm of the milliliter of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_U$, adding together the two transmittances (Σ_U) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that fall within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X, for each dosage level. Average the values of X for each of three or more dosage levels to obtain $\overline{X}$, which equals the log-relative potency, M', of the *Sample solution*.

Determine the quantity, in mg, of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Tablets taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = amount of calcium pantothenate assumed to be present in the portion of Tablets taken (mg)

Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Tablets taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of calcium pantothenate in the portion of Tablets taken (mg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, $\overline{M}$, is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* ⟨111⟩, *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• **CALCIUM PANTOTHENATE, *Method 3***

Buffer solution: Dissolve 10.0 g of monobasic potassium phosphate in 2000 mL of water, and adjust with phosphoric acid to a pH of 3.5.

Mobile phase: Methanol and *Buffer solution* (1:9)

Standard stock solution: 0.25 mg/mL of USP Calcium Pantothenate RS in water. Prepare fresh every 4 weeks. Store in a refrigerator.

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Standard solution: 40 µg/mL of USP Calcium Pantothenate RS from *Standard stock solution* diluted with water
Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to a nominal amount of 10 mg of calcium pantothenate, to a 250-mL volumetric flask. Add 10 mL of methanol, and swirl the flask to disperse. Dilute with water to volume, mix, and filter.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 205 nm
Column: 3.9-mm × 30-cm; 5-µm packing L1
Column temperature: 50°
Flow rate: 2 mL/min
Injection volume: 25 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 3.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas for calcium pantothenate. Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of calcium pantothenate from the *Sample solution*
r_S = peak area of calcium pantothenate from the *Standard solution*
C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

Change to read:

- **NIACIN OR NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, RIBOFLAVIN,** and **THIAMINE,** ***Method 1***

[NOTE—Use low-actinic glassware throughout this procedure.]
Diluent: Acetonitrile, glacial acetic acid, and water (5:1:94)
Mobile phase: A mixture of methanol, glacial acetic acid, and water (27:1:73) containing 140 mg of sodium 1-hexanesulfonate per 100 mL
Standard solution: [NOTE—Use USP Niacin RS in place of USP Niacinamide RS for formulations containing niacin.] Transfer 80 mg of USP Niacinamide RS, 20 mg of USP Pyridoxine Hydrochloride RS, 20 mg of USP Riboflavin RS, and 20 mg of USP Thiamine Hydrochloride RS to a 200-mL volumetric flask, and add 180 mL of *Diluent*. Immerse the flask in a hot water bath maintained at 65°–70° for 10 min with regular shaking or using a vortex mixer, until all the solid materials are dissolved. Chill rapidly in a cold water bath for 10 min to room temperature, and dilute with *Diluent* to volume.
Sample solution: Finely powder NLT 30 Tablets. Transfer a portion of the powder, equivalent to 10 mg of niacinamide and 2.5 mg each of pyridoxine hydrochloride, riboflavin, and thiamine hydrochloride, to a 50-mL centrifuge tube. Add 25.0 mL of *Diluent*, and mix using a vortex mixer for 30 s to completely suspend the powder. Immerse the centrifuge tube in a hot water bath maintained at 65°–70°, heat for 5 min, and mix using a vortex mixer for 30 s. Return the tube to the hot water bath, heat for another 5 min, and mix on a vortex mixer for 30 s. Filter a portion of the solution, cool to room temperature, and use the clear filtrate. [NOTE—Use the filtrate within 3 h of filtration.]
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 280 nm
Column: 3.9-mm × 30-cm; packing L1
Flow rate: 1 mL/min
Injection volume: 10 µL
System suitability
Sample: *Standard solution*
▲▲ (USP 1-May-2020)
Suitability requirements
Relative standard deviation: NMT 3.0%
Analysis
Samples: *Standard solution* and *Sample solution*
▲Calculate the percentage of the labeled amount of vitamin B_1 as thiamine ion ($C_{12}H_{17}N_4OS+$), vitamin B_2 as riboflavin ($C_{17}H_{20}N_4O_6$), vitamin B_3 as niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), vitamin B_6 as pyridoxine ($C_8H_{11}NO_3$), calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), and folic acid ($C_{19}H_{19}N_7O_6$), in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin from the corresponding *Sample solution*
r_S = peak area of the relevant vitamin from the corresponding *Standard solution*
C_S = concentration of the relevant vitamin Reference Standard in the corresponding *Standard solution* (µg/mL)
C_U = nominal concentration of the relevant vitamin in the corresponding *Sample solution* (µg/mL)▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–150.0% ▲of the labeled amount of each individual vitamin▲ (USP 1-May-2020)

Change to read:

- **NIACIN,** ***Method 2***

▲Proceed as directed in *Niacin or Niacinamide Assay* ⟨441⟩, *Assay, Chromatographic Methods, Procedure 2*.▲ (USP 1-May-2020)
Acceptance criteria: 90.0%–150.0% of the labeled amount of niacin ($C_6H_5NO_2$)

Change to read:

- **NIACINAMIDE,** ***Method 2***

▲Proceed as directed in *Niacin or Niacinamide Assay* ⟨441⟩, *Assay, Chromatographic Methods, Procedure 2*.▲ (USP 1-May-2020)
Acceptance criteria: 90.0%–150.0% of the labeled amount of niacinamide ($C_6H_6N_2O$)

- **PYRIDOXINE HYDROCHLORIDE,** ***Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]
Extraction solvent, Mobile phase, and **Sample solution:** Prepare as directed in *Niacin, Method 2*.
Standard stock solution: 0.1 mg/mL of USP Pyridoxine Hydrochloride RS in *Extraction solvent*

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Standard solution: Transfer 5.0 mL of *Standard stock solution* to a 25-mL volumetric flask, and dilute with *Extraction solvent* to volume.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; packing L1
Flow rate: 1 mL/min
Injection volume: 20 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 3.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas for pyridoxine.
Calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of pyridoxine from the *Sample solution*
r_S = peak area of pyridoxine from the *Standard solution*
C_S = concentration of USP Pyridoxine Hydrochloride RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of pyridoxine hydrochloride in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$)

Change to read:

- **RIBOFLAVIN,** ***Method 2***

▲Proceed as directed in *Riboflavin Assay* ⟨481⟩, *Assay, Chromatographic Methods, Procedure 2*.▲ (USP 1-May-2020)
Acceptance criteria: 90.0%–150.0% of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$)

Change to read:

- **THIAMINE,** ***Method 2***

▲Proceed as directed in *Thiamine Assay* ⟨531⟩, *Assay, Chromatographic Methods, Procedure 2*.▲ (USP 1-May-2020)
Acceptance criteria: 90.0%–150.0% of the labeled amount of thiamine ▲as thiamine ion ($C_{12}H_{17}N_4OS+$)▲ (USP 1-May-2020)

Change to read:

- **NIACIN OR NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, RIBOFLAVIN,** and **THIAMINE,** ***Method 3***

[NOTE—Use low-actinic glassware throughout this procedure.]
Reagent: 25 mg/mL of edetate disodium in water
Mobile phase: Transfer 0.4 mL of triethylamine, 15.0 mL of glacial acetic acid, and 350 mL of methanol to a 2000-mL volumetric flask. Dilute with 0.008 M sodium 1-hexanesulfonate to volume.
Standard stock solution: 1.5 mg/mL of USP Niacin RS or USP Niacinamide RS, 0.24 mg/mL of USP Pyridoxine Hydrochloride RS, 0.08 mg/mL of USP Riboflavin RS, and 0.24 mg/mL of USP Thiamine Hydrochloride RS in the *Reagent*, with heating if necessary
Standard solution: Transfer 5.0 mL of *Standard stock solution* to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1), and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few milliliters of the filtrate.
Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 7.5 mg of niacin or niacinamide, 1.2 mg of pyridoxine hydrochloride, 0.4 mg of riboflavin, and 1.2 mg of thiamine hydrochloride, to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few milliliters of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 270 nm
Column: 4.6-mm × 25-cm; packing L7
Column temperature: 50°
Flow rate: 2.0 mL/min
Injection volume: 5 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 2.0%
Analysis
Samples: *Standard solution* and *Sample solution*
▲Calculate the percentage of the labeled amount of vitamin B_1 as thiamine ion ($C_{12}H_{17}N_4OS+$), vitamin B_2 as riboflavin ($C_{17}H_{20}N_4O_6$), vitamin B_3 as niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), vitamin B_6 as pyridoxine ($C_8H_{11}NO_3$), calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), and folic acid ($C_{19}H_{19}N_7O_6$), in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin from the corresponding *Sample solution*
r_S = peak area of the relevant vitamin from the corresponding *Standard solution*
C_S = concentration of the relevant vitamin Reference Standard in the corresponding *Standard solution* (µg/mL)
C_U = nominal concentration of the relevant vitamin in the corresponding *Sample solution* (µg/mL)▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–150.0% ▲of the labeled amount of each individual vitamin▲ (USP 1-May-2020)

Add the following:

▲• **FOLIC ACID,** ***Method 3*****; ASCORBIC ACID, NIACIN OR NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, CALCIUM PANTOTHENATE, RIBOFLAVIN,** and **THIAMINE,** ***Method 4***

[NOTE—Use low-actinic glassware.]
Solution A: 0.56 g of edetate disodium and 2.04 g of potassium phosphate monobasic per 1000 mL of water. Adjust with phosphoric acid to a pH of 3.0.
Solution B: Acetonitrile; acetic acid, glacial; and water (5:1:94)
Solution C: Aqueous solution containing 2 mL of ammonium hydroxide and 1 g of sodium perchlorate per 100 mL
Solution D: Aqueous solution containing 2 mL of ammonium hydroxide, 1 g of sodium perchlorate, and 2 g of sodium ascorbate per 100 mL

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Mobile phase A: 0.02% trifluoroacetic acid in water
Mobile phase B: Acetonitrile
Mobile phase: See *Table 4*.

Table 4

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
0	100	0
1.0	100	0
6.0	95	5
15.0	83	17
17.0	80	20
19.0	80	20
19.1	100	0
25.0	100	0

Ascorbic acid standard stock solution: 1.0 mg/mL of ascorbic acid from USP Ascorbic Acid RS in *Solution A*
Thiamine standard stock solution: 0.2 mg/mL of thiamine from USP Thiamine Hydrochloride RS in *Solution B*. The solution can be placed on a steam bath for about 5–10 min with frequent swirling to assist in dissolution.
Riboflavin standard stock solution: 0.2 mg/mL of USP Riboflavin RS in *Solution B*. Heat the solution to 65°–70° for 10 min to assist in dissolution.
Niacin or niacinamide standard stock solution: 1.0 mg/mL of USP Niacin RS or USP Niacinamide RS in *Solution A*
Pyridoxine standard stock solution: 0.2 mg/mL of pyridoxine from USP Pyridoxine Hydrochloride RS in *Solution C*
Folic acid standard stock solution: 0.2 mg/mL of folic acid from USP Folic Acid RS in *Solution C*
Calcium Pantothenate standard stock solution: 1.0 mg/mL of calcium pantothenate from USP Calcium Pantothenate RS in *Mobile phase A*
System suitability solution: Transfer 0.5 mL of *Ascorbic acid standard stock solution*, 2.5 mL of *Thiamine standard stock solution*, and 0.5 mL of *Niacin or niacinamide standard stock solution* into 10-mL volumetric flask and dilute with *Mobile phase A* to volume.
Sample solution 1: Weigh and finely powder NLT 20 Tablets. Transfer a calculated amount of accurately weighed powder into a 100-mL volumetric flask to obtain a solution containing known nominal concentrations of thiamine, riboflavin, and calcium pantothenate in the ranges of 5–30 µg/mL for thiamine, 10–60 µg/mL for riboflavin, and 25–150 µg/mL for calcium pantothenate. Add *Solution B* to about half the volume and mix on a vortex mixer for 5 min. Heat the flask at 65°–70° for 5 min, swirling frequently, and sonicate additionally for 5 min. Cool to room temperature, dilute with *Mobile phase A* to volume, and mix well. Pass a portion of the solution through a suitable filter of 0.45-µm pore size and discard the first milliliter of the filtrate.
Sample solution 2: Transfer a portion of finely powdered Tablets into a 100-mL volumetric flask to obtain a solution containing known nominal concentrations of ascorbic acid and niacin or niacinamide in the ranges of 50–300 µg/mL for ascorbic acid and 12–65 µg/mL for niacin or niacinamide, add *Solution A* to about half the volume, and mix on a vortex mixer for 5 min. Sonicate for 10 min with occasional shaking. Cool to room temperature, dilute with *Solution A* to volume, and mix well. Pass a portion of the solution through a suitable filter of 0.45-µm pore size, and discard the first milliliter of the filtrate.
Sample solution 3: Transfer a portion of finely powdered Tablets into a 100-mL volumetric flask to obtain a solution containing known nominal concentrations of pyridoxine hydrochloride and folic acid in the ranges of 5–30 µg/mL for pyridoxine hydrochloride and 2–12 µg/mL for folic acid, add *Solution D* to about half the volume, heat in a water bath at 40° for 5 min, and sonicate for 10 min with occasional shaking. Cool to room temperature, dilute with *Solution D* to volume, and mix well. Pass a portion of the solution through a suitable filter of 0.45-µm pore size, and discard the first milliliter of the filtrate.
Standard solution 1: Transfer calculated volumes of *Thiamine standard stock solution*, *Riboflavin standard stock solution*, and *Calcium pantothenate standard stock solution* into a suitable volumetric flask to obtain a solution with final vitamin concentrations similar to the nominal concentrations in *Sample solution 1* for the corresponding vitamins. Dilute with *Mobile phase A* to volume, and mix well.
Standard solution 2: Transfer calculated volumes of *Ascorbic acid standard stock solution* and *Niacin or niacinamide standard stock solution* to a suitable volumetric flask to obtain a solution with final vitamin concentrations similar to the nominal concentrations in *Sample solution 2* for the corresponding vitamins. Dilute with *Mobile phase A* to volume, and mix well.
Standard solution 3: Transfer calculated volumes of *Pyridoxine standard stock solution* and *Folic acid standard stock solution* into a suitable volumetric flask to obtain a solution with final vitamin concentrations similar to the nominal concentrations in *Sample solution 3* for the corresponding vitamins. Dilute with *Solution C* to volume, and mix well.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detectors
For calcium pantothenate: UV 210 nm
For thiamine, ascorbic acid, niacin or niacinamide, and riboflavin: UV 254 nm
For pyridoxine hydrochloride and folic acid: UV 280 nm
Column: 4.6-mm × 15-cm; 5-µm packing L1
Column temperature: 25°
Flow rate: 1.0 mL/min
Injection volume: 20 µL
System suitability
Samples: *System suitability solution*, *Standard solution 1*, *Standard solution 2*, and *Standard solution 3*
Suitability requirements
Resolution: NLT 3.5 between the thiamine and ascorbic acid peaks; NLT 4.0 between the ascorbic acid and niacin or niacinamide peaks, *System suitability solution*
Relative standard deviation: NMT 2.0% for each individual peak, *Standard solution 1*, *Standard solution 2*, and *Standard solution 3*
Analysis
Samples: *Sample solutions 1–3* and *Standard solutions 1–3*
Calculate the percentage of the labeled amount of vitamin B_1 as thiamine ion ($C_{12}H_{17}N_4OS^+$), vitamin B_2 as riboflavin ($C_{17}H_{20}N_4O_6$), vitamin B_3 as niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), vitamin B_6 as pyridoxine ($C_8H_{11}NO_3$), calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), and folic acid ($C_{19}H_{19}N_7O_6$), in the portion of Tablets taken:

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$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin from the corresponding *Sample solution*

r_S = peak area of the relevant vitamin from the corresponding *Standard solution*

C_S = concentration of the relevant vitamin Reference Standard in the corresponding *Standard solution* (µg/mL)

C_U = nominal concentration of the relevant vitamin in the corresponding *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of each individual vitamin▲ (USP 1-May-2020)

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩, *Dissolution*: Meet the requirements
- **WEIGHT VARIATION** ⟨2091⟩: Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic microbial count does not exceed 3×10^3 cfu/g, and the combined molds and yeasts count does not exceed 3×10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures*, *Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meet the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** The label states that the product is Water-Soluble Vitamins Tablets. The label also states the quantity of each vitamin in terms of metric units per dosage unit, and where necessary, the salt form in which it is present. Where more than one assay method is given for a particular vitamin, the labeling states which assay method is used only if *Method 1* is not used.

Change to read:

- **USP REFERENCE STANDARDS** ⟨11⟩

▲USP Ascorbic Acid RS▲ (USP 1-May-2020)
USP Biotin RS
USP Calcium Pantothenate RS
USP Cyanocobalamin RS
USP Folic Acid RS
USP Niacin RS
USP Niacinamide RS
USP Pyridoxine Hydrochloride RS
USP Riboflavin RS
USP Thiamine Hydrochloride RS

Water-Soluble Vitamins with Minerals Capsules

DEFINITION

Water-Soluble Vitamins with Minerals Capsules contain one or more of the following water-soluble vitamins: Ascorbic Acid or its equivalent as Calcium Ascorbate or Sodium Ascorbate, Biotin, Cyanocobalamin, Folic Acid, Dexpanthenol or Panthenol, Niacin or Niacinamide, pantothenic acid (as Calcium Pantothenate or Racemic Calcium Pantothenate), Pyridoxine Hydrochloride, Riboflavin, and Thiamine Hydrochloride or Thiamine Mononitrate; and one or more minerals derived from substances generally recognized as safe, furnishing one or more of the following elements in ionizable form: boron, calcium, chromium, copper, fluorine, iodine, iron, magnesium, manganese, molybdenum, nickel, phosphorus, potassium, selenium, tin, vanadium, and zinc. Capsules contain NLT 90.0% and NMT 150.0% of the labeled amounts of ascorbic acid ($C_6H_8O_6$), or its salts as calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$) or sodium ascorbate ($C_6H_7NaO_6$), biotin ($C_{10}H_{16}N_2O_3S$), cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$), folic acid ($C_{19}H_{19}N_7O_6$), dexpanthenol or panthenol ($C_9H_{19}NO_4$), calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine ($C_{12}H_{17}ClN_4OS$) as thiamine hydrochloride or thiamine mononitrate; NLT 90.0% and NMT 125.0% of the labeled quantities of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), potassium (K), and zinc (Zn); and NLT 90.0% and NMT 160.0% of the labeled quantities of boron (B), chromium (Cr), fluorine (F), iodine (I), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), and vanadium (V).

They do not contain any form of Beta Carotene or Vitamin A, D, E, or K. They may contain other labeled added substances that are generally recognized as safe, in quantities that are unobjectionable.

STRENGTH

[NOTE—In the following assays, where more than one assay method is given for an individual ingredient, the requirements may be met by following any one of the specified methods, the method used being stated in the labeling only if *Method 1* is not used.]

- **ASCORBIC ACID, CALCIUM ASCORBATE,** and **SODIUM ASCORBATE**

(See *Vitamin C Assay* ⟨580⟩.)

[NOTE—For labeling purposes, consider *Method I—Titrimetric Method* as *Method 1*.]

Acceptance criteria: 90.0%–150.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$), or its salts as calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$), or sodium ascorbate ($C_6H_7NaO_6$)

- **BIOTIN, *Method 1***

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Mix 85 mL of acetonitrile, 1 g of sodium perchlorate, and 1 mL of phosphoric acid, and dilute with water to 1000 mL.

Standard stock solution: 0.333 mg/mL of USP Biotin RS in dimethyl sulfoxide

Standard solution: 5 µg/mL of USP Biotin RS prepared by diluting the *Standard stock solution* with water

Sample solution: Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without the loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing, if necessary, with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the average net weight per Capsule. Transfer a portion of the Capsule contents, equivalent to a nominal amount of 1 mg of biotin, to a 200-mL volumetric flask. Add 3 mL of dimethyl sulfoxide, and swirl to wet the contents. Place the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with water to volume, and filter.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 200 nm

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Column: 4.6-mm × 15-cm; 3-µm packing L7
Flow rate: 1.2 mL/min
Injection volume: 100 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 3.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the responses for the biotin peaks. Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP Biotin RS in the *Standard solution* (µg/mL)
C_U = nominal concentration of biotin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

• **BIOTIN, *Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Dehydrated mixtures yielding formulations similar to the media described herein may be used provided that, when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described herein.

Standard stock solution: 50 µg/mL of USP Biotin RS in 50% alcohol. Store this solution in a refrigerator.

Standard solution: On the day of the assay, dilute the *Standard stock solution* with water to a concentration of 0.1 ng/mL of USP Biotin RS.

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule". Transfer a portion of the Capsule contents, equivalent to a nominal amount of 100 µg of biotin, to a 200-mL volumetric flask. Add 3 mL of 50% alcohol, and swirl to wet the contents. Heat the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with diluted alcohol to volume, and filter. Dilute a volume of the filtrate quantitatively, and stepwise if necessary, with water to obtain a solution with a concentration of 0.1 ng/mL.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and dilute with water to 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature NLT 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water. Heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and dilute with water to 1000 mL. Store under toluene in a cool place at a temperature NLT 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and dilute with water to 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Calcium pantothenate solution: 10 µg/mL of calcium pantothenate in 50% alcohol. Store in a refrigerator.

Riboflavin–thiamine hydrochloride solution: 20 µg/mL of riboflavin and 10 µg/mL of thiamine hydrochloride in 0.02 N acetic acid. Store under toluene, protected from light, and in a refrigerator.

***p*-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution:** 10 µg/mL of *p*-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid, and mix. Store under toluene.

Basal medium stock solution: Dissolve the *anhydrous Dextrose* and *anhydrous Sodium acetate* in the solutions previously mixed according to *Table 1*, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Table 1

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Calcium pantothenate solution	5 mL
Riboflavin–thiamine hydrochloride solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water. Add 500 mg of *anhydrous Dextrose*, 500 mg of *anhydrous Sodium acetate*, and 1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to test tubes, close or cover the tubes, sterilize in an autoclave at 121°, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*,[1] incubating for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution* add 5.0 mL of water containing 0.5 ng of biotin. Plug the tubes with cotton, sterilize in an autoclave at 121°, and cool.

[1] ATCC No. 8014 is suitable. This strain was formerly known as *Lactobacillus arabinosus* 17-5.

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Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Make a transfer of cells from the *Stock culture of Lactobacillus plantarum* to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. The cell suspension so obtained is the *Inoculum*.

Analysis

Samples: *Standard solution* and *Sample solution*

To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool. Add 1 drop of *Inoculum* to each tube, except two of the four tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37° held constant to within ±0.5° until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of Standard during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to a spectrophotometer cell. Place the cell in a spectrophotometer that has been set at a specific wavelength 540–660 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittance (Σ) as the difference, $y = 2.00 - \Sigma$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, y, adding together the two transmittances for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that fall within the range of lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X, for each dosage level. Average the values of X for each of three or more dosage levels to obtain $\overline{X}$, which equals the log-relative potency, M', of the *Sample solution*.

Determine the quantity, in µg, of USP Biotin RS corresponding to the biotin in each mg of the portion of Capsules taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of µg of biotin assumed to be present in each mg of the portion of the Capsules taken

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, $\overline{M}$, is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* ⟨111⟩, *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

• **CYANOCOBALAMIN, *Method 1***

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Methanol and water (7:13)

Standard stock solution: 10 µg/mL of USP Cyanocobalamin (Crystalline) RS in water. [NOTE—Store this stock solution in a dark place, and discard after 1 week.]

Standard solution: 1 µg/mL of USP Cyanocobalamin (Crystalline) RS from *Standard stock solution* diluted with water

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." Transfer a portion of the Capsule contents, equivalent to a nominal amount of 100 µg of cyanocobalamin, to a 250-mL flask. Quantitatively add 100.0 mL of water, and carefully extract for 2 min. Filter 10 mL of the extract, and use the clear filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: 550 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Flow rate: 0.5 mL/min

Injection volume: 200 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak responses for cyanocobalamin. Calculate the percentage of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of cyanocobalamin from the *Sample solution*

r_S = peak response of cyanocobalamin from the *Standard solution*

C_S = concentration of USP Cyanocobalamin (Crystalline) RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cyanocobalamin in the *Sample solution* (µg/mL)

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Acceptance criteria: 90.0%–150.0% of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$)

• **CYANOCOBALAMIN, *Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Standard cyanocobalamin stock solution: 1.0 µg/mL of USP Cyanocobalamin (Crystalline) RS in 25% alcohol. Store in a refrigerator.

Standard solution: Dilute a suitable volume of *Standard cyanocobalamin stock solution* with water to a measured volume such that after the incubation period as described in the *Analysis*, the difference in transmittance between the inoculated blank and the 5.0-mL level of the *Standard solution* is NLT that which corresponds to a difference of 1.25 mg in dried cell weight. This concentration usually falls between 0.01 and 0.04 ng/mL of the *Standard solution*. Prepare this solution fresh for each assay.

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." Transfer a portion of the Capsule contents, equivalent to a nominal amount of 1.0 µg of cyanocobalamin, to an appropriate vessel containing, for each g of Capsule contents taken, 25 mL of an aqueous extracting solution prepared just before use to contain 12.9 mg/mL of dibasic sodium phosphate, 11.0 mg/mL of anhydrous citric acid, and 10 mg/mL of sodium metabisulfite. Autoclave the mixture at 121° for 10 min. Allow any undissolved particles of the extract to settle, and filter or centrifuge, if necessary. Dilute an aliquot of the clear solution with water to obtain a final solution containing vitamin B_{12} activity approximately equivalent to that of the *Standard solution*.

Acid-hydrolyzed casein solution: Prepare as directed in *Calcium Pantothenate, Method 2*.

Asparagine solution: Dissolve 2.0 g of L-asparagine in water to make 200 mL. Store under toluene in a refrigerator.

Adenine–guanine–uracil solution: Prepare as directed in *Calcium Pantothenate, Method 2*.

Xanthine solution: Suspend 0.20 g of xanthine in 30–40 mL of water, heat to 70°, add 6.0 mL of 6 N ammonium hydroxide, and stir until the solid is dissolved. Cool, and dilute with water to 200 mL. Store under toluene in a refrigerator.

Salt solution A: Dissolve 10 g of monobasic potassium phosphate and 10 g of dibasic potassium phosphate in water to make 200 mL, and add 2 drops of hydrochloric acid. Store this solution under toluene.

Salt solution B: Dissolve 4.0 g of magnesium sulfate, 0.20 g of sodium chloride, 0.20 g of ferrous sulfate, and 0.20 g of manganese sulfate in water to make 200 mL. Add 2 drops of hydrochloric acid. Store this solution under toluene.

Polysorbate 80 solution: Dissolve 20 g of polysorbate 80 in alcohol to make 200 mL. Store in a refrigerator.

Vitamin solution A: Dissolve 10 mg of riboflavin, 10 mg of thiamine hydrochloride, 100 µg of biotin, and 20 mg of niacin in 0.02 N glacial acetic acid to make 400 mL. Store under toluene, protected from light, in a refrigerator.

Vitamin solution B: Dissolve 20 mg of *p*-aminobenzoic acid, 10 mg of calcium pantothenate, 40 mg of pyridoxine hydrochloride, 40 mg of pyridoxal hydrochloride, 8 mg of pyridoxamine dihydrochloride, and 2 mg of folic acid in a mixture of water and neutralized alcohol (3:1) to make 400 mL. Store, protected from light, in a refrigerator.

Basal medium stock solution: Prepare the medium according to the following formula and directions. A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium comparable to that obtained from the formula given herein.

Add the ingredients in the order listed according to *Table 2*, carefully dissolving cystine and tryptophan in the hydrochloric acid before adding the next eight solutions in the resulting solution. Add 100 mL of water, and dissolve the dextrose, sodium acetate, and ascorbic acid. Filter, if necessary. Add the *Polysorbate 80 solution*, adjust with 1 N sodium hydroxide to a pH of between 5.5 and 6.0, and dilute with Purified Water to 250 mL.

Table 2

L-Cystine	0.1 g
L-Tryptophan	0.05 g
1 N hydrochloric acid	10 mL
Adenine–guanine–uracil solution	5 mL
Xanthine solution	5 mL
Vitamin solution A	10 mL
Vitamin solution B	10 mL
Salt solution A	5 mL
Salt solution B	5 mL
Asparagine solution	5 mL
Acid-hydrolyzed casein solution	25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Ascorbic acid	1 g
Polysorbate 80 solution	5 mL

Tomato juice preparation: Centrifuge commercially canned tomato juice so that most of the pulp is removed. Suspend 5 g/L of analytical filter aid in the supernatant, and pass, with the aid of reduced pressure, through a layer of the filter aid. Repeat, if necessary, until a clear, straw-colored filtrate is obtained. Store under toluene in a refrigerator.

Culture medium: [NOTE—A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium equivalent to that obtained from the formula given herein.] Dissolve 0.75 g of yeast extract, 0.75 g of dried peptone, 1.0 g of anhydrous dextrose, and 0.20 g of monobasic potassium phosphate in 60–70 mL of water. Add 10 mL of *Tomato juice preparation* and 1 mL of *Polysorbate 80 solution*. Adjust with 1 N sodium hydroxide to a pH of 6.8, and dilute with water to 100 mL. Place 10-mL portions of the solution in test tubes, and plug with cotton. Sterilize the tubes and contents in an autoclave at 121° for 15 min. Cool as rapidly as possible to avoid color formation resulting from overheating the medium.

Suspension medium: Dilute a measured volume of *Basal medium stock solution* with an equal volume of water. Place 10-mL portions of the diluted medium in test tubes. Sterilize, and cool as directed for *Culture medium*.

Stock culture of *Lactobacillus leichmannii*: To 100 mL of *Culture medium* add 1.0–1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Place 10-mL portions of the hot solution in test tubes, cover the tubes, sterilize at 121° for 15 min in an autoclave (exhaust line temperature), and allow the tubes to cool in an upright position. Inoculate three or more of the tubes by stab

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transfer of a pure culture of *Lactobacillus leichmannii*.[2] [NOTE—Before first using a fresh culture in this assay, make NLT 10 successive transfers of the culture in a 2-week period.] Incubate for 16–24 h at a temperature between 30° and 40° held constant to within ±0.5°. Store in a refrigerator.

Prepare fresh stab cultures at least three times each week, and do not use them for preparing the *Inoculum* if more than 4 days old. The activity of the microorganism can be increased by daily or twice-daily transfer of the stab culture, to the point where definite turbidity in the liquid *Inoculum* can be observed 2–4 h after inoculation. A slow-growing culture seldom gives a suitable response curve and may lead to erratic results.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus leichmannii* may be used as the stock culture provided it yields an *Inoculum* comparable to a fresh culture.] Make a transfer of cells from the *Stock culture of Lactobacillus leichmannii* to two sterile tubes each containing 10 mL of the *Culture medium*. Incubate these cultures for 16–24 h at a temperature between 30° and 40° held constant to within ±0.5°. Under aseptic conditions centrifuge the cultures, and decant the supernatant. Suspend the cells from the culture in 5 mL of sterile *Suspension medium*, and combine. Using sterile *Suspension medium*, adjust the volume so that a 1-in-20 dilution in saline TS produces 70% transmittance when read on a suitable spectrophotometer that has been set at a wavelength of 530 nm, equipped with a 10-mm cell, and read against saline TS set at 100% transmittance. Prepare a 1-in-400 dilution of the adjusted suspension using *Basal medium stock solution*. [NOTE—This dilution may be altered, when necessary, to obtain the desired test response.] The cell suspension so obtained is the *Inoculum*.

Calibration of spectrophotometer: Check the wavelength of the spectrophotometer periodically, using a standard wavelength cell or other suitable device. Before reading any tests, calibrate the spectrophotometer for 0% and 100% transmittance, using water and with the wavelength set at 530 nm.

Analysis

Samples: *Standard solution* and *Sample solution*

Because of the high sensitivity of the test organism to minute amounts of vitamin B_{12} activity and to traces of many cleansing agents, cleanse meticulously by suitable means, followed preferably by heating at 250° for 2 h using hard-glass 20-mm × 150-mm test tubes and other necessary glassware.

To separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each of these tubes and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*. To each tube add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes to prevent bacterial contamination, and sterilize in an autoclave at 121° for 5 min, arranging to reach this temperature in NMT 10 min by preheating the autoclave if necessary. Cool as rapidly as possible to avoid color formation resulting from overheating the medium. Take precautions to maintain uniformity of sterilizing and cooling conditions throughout the assay, because packing the tubes too closely in the autoclave or overloading it may cause variation in the heating rate.

Aseptically add 0.5 mL of *Inoculum* to each tube so prepared, except two of the four containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 40°, held constant to within ±0.5°, for 16–24 h.

Terminate growth by heating to a temperature NLT 80° for 5 min. Cool to room temperature. After agitating contents, place the container in a spectrophotometer that has been set at a wavelength of 530 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of the inoculated blank. If the difference is greater than 5% or if there is evidence of contamination with a foreign microorganism, disregard the results of the assay.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of each of the remaining tubes. Disregard the results of the assay if the slope of the standard curve indicates a problem with sensitivity.

Calculation: Prepare a standard concentration-response curve by the following procedure. Test for and replace any aberrant individual transmittances. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittances (Σ) as the difference, $y = 2.00 - \Sigma$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, y, adding together the two transmittances for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X, for each dosage level. Average the values of X for each of three or more dosage levels to obtain $\overline{X}$, which equals the log-relative potency, M', of the *Sample solution*.

Determine the quantity, in µg, of USP Cyanocobalamin (Crystalline) RS corresponding to the cyanocobalamin in each mg of the portion of Capsules taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of µg of cyanocobalamin assumed to be present in each mg of the portion of the Capsules taken

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, $\overline{M}$, is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* ⟨111⟩, *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

[2] Pure cultures of *Lactobacillus leichmannii* (listed as *Lactobacillus delbruekii*) may be obtained as No. 7830 from ATCC, 10801 University Blvd., Manassas, VA 20110-2209 (www.atcc.org).

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Acceptance criteria: 90.0%–150.0% of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$)

• **FOLIC ACID, *Method 1***

[NOTE—Use low-actinic glassware throughout this procedure.]

Reagent A: 25% solution of tetrabutylammonium hydroxide in methanol

Reagent B: Transfer 5.0 g of pentetic acid to a 50-mL volumetric flask. Using sonication if necessary, dissolve in and dilute with 1 N sodium hydroxide to volume.

Mobile phase: 2 g of monobasic potassium phosphate in 650 mL of water. Add 12.0 mL of *Reagent A*, 7.0 mL of 3 N phosphoric acid, and 240 mL of methanol. Cool to room temperature, adjust with phosphoric acid or ammonia TS to a pH of 7.0, dilute with water to 1000 mL, and filter. Recheck the pH before use by adding water or methanol to the prepared *Mobile phase* to obtain baseline separation of folic acid and the internal standard. The pH may be increased up to 7.15 to obtain better separation. [NOTE—The methanol and water content may be varied (between 1% and 3%).]

Internal standard solution: Transfer 40 mg of methylparaben to a 1000-mL volumetric flask, and add 220 mL of methanol to dissolve. Dissolve 2.0 g of monobasic potassium phosphate in 300 mL of water in a separate beaker, quantitatively transfer this solution to the flask containing the methylparaben solution, and add an additional 300 mL of water. Add 19 mL of *Reagent A*, 7 mL of 3 N phosphoric acid, and 30 mL of *Reagent B*. Adjust with ammonia TS to a pH of 9.8, bubble nitrogen through the solution for 30 min, dilute with water to volume, and mix.

Standard solution: 0.016 mg/mL of USP Folic Acid RS in *Internal standard solution*

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." Transfer an amount of Capsule contents to a suitable centrifuge tube, and add a volume of *Internal standard solution* to obtain a nominal concentration of 0.016 mg/mL of folic acid. Shake by mechanical means for 10 min, and centrifuge. Filter a portion of the clear supernatant, and use the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 3.9-mm × 30-cm; packing L1

Flow rate: 1 mL/min

Injection volume: 15 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for folic acid and methylparaben are about 0.8 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for folic acid and methylparaben. Calculate the percentage of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$) in the portion of Capsules taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak area ratio of folic acid to methylparaben from the *Sample solution*

R_S = peak area ratio of folic acid to methylparaben from the *Standard solution*

C_S = concentration of USP Folic Acid RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of folic acid in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$)

• **FOLIC ACID, *Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Reagent: Dissolve 7.5 g of edetate disodium, with stirring, in 500 mL of water containing 10 mL of ammonium hydroxide.

Diluent: 60 µg/mL of ammonium hydroxide

Mobile phase: Transfer 0.4 mL of triethylamine, 15 mL of glacial acetic acid, and 350 mL of methanol to a 2000-mL volumetric flask, and dilute with 0.008 M sodium 1-hexanesulfonate to volume.

Standard stock solution: 60 µg/mL of USP Folic Acid RS in *Diluent*. Prepare this solution fresh daily.

Standard solution: Mix 5.0 mL of *Standard stock solution* with 10.0 mL of methanol and 35.0 mL of *Reagent*. Shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." Transfer a portion of the Capsule contents, equivalent to a nominal amount of 0.3 mg of folic acid, to a 125-mL stoppered flask. Add 10.0 mL of methanol and 35.0 mL of *Reagent*. Shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 270 nm

Column: 4.6-mm × 25-cm; packing L7

Column temperature: 50°

Flow rate: 2 mL/min

Injection volume: 5 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the areas of the major peaks. Calculate the percentage of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of folic acid from the *Sample solution*

r_S = peak area of folic acid from the *Standard solution*

C_S = concentration of USP Folic Acid RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of folic acid in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$)

• **DEXPANTHENOL OR PANTHENOL**

[NOTE—The following procedure is applicable also to the determination of the dextrorotatory component of racemic panthenol in preparations containing panthenol.]

Dehydrated mixtures yielding formulations similar to the media described herein may be used provided that, when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described herein.

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Standard stock solution: 800 µg/mL of USP Dexpanthenol RS in water. Store in a refrigerator protected from light, and use within 30 days.

Standard solution: On the day of the assay, prepare a dilution of 1.2 µg/mL of dexpanthenol from *Standard stock solution* diluted with water.

Sample solution: Weigh NLT 30 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents as completely as possible to a beaker. Remove any contents adhering to the empty Capsule shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the average net weight per Capsule. Dissolve a portion of the Capsule contents, nominally equivalent to 1.2 mg of dexpanthenol or 2.4 mg of panthenol, in 100.0 mL of water. Quantitatively dilute a portion of this solution with water to obtain a nominal concentration of 1.2 µg/mL of dexpanthenol or 2.4 µg/mL of panthenol.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in about 500 mL of water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and dilute with water to 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature NLT 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine and 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to 75 ± 5°, and add hydrochloric acid solution (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and dilute with water to 1000 mL. Store under toluene in a cool place at a temperature NLT 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and dilute with water to 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin–thiamine hydrochloride–biotin solution: 20 µg/mL of riboflavin, 10 µg/mL of thiamine hydrochloride, and 0.04 µg/mL of biotin in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

***p*-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution:** 10 µg/mL of *p*-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in neutral 25% alcohol. Store in a refrigerator.

Salt solution A: Dissolve 50 mg/mL of monobasic potassium phosphate and 50 mg/mL of dibasic potassium phosphate in water. Add 10 drops of hydrochloric acid per L of solution. Store under toluene.

Salt solution B: Dissolve 20 mg/mL of magnesium sulfate, 1 mg/mL of sodium chloride, 1 mg/mL of ferrous sulfate, and 1 mg/mL of manganese sulfate in water. Add 10 drops of hydrochloric acid per L of the solution. Store under toluene.

Pyridoxal–calcium pantothenate solution: Dissolve 200 µg/mL of pyridoxal hydrochloride and 1.875 µg/mL of calcium pantothenate in 10% alcohol. Store in a refrigerator, and use within 30 days.

Polysorbate 40–oleic acid solution: Dissolve 50 mg/mL of polysorbate 40 and 0.5 mg/mL of oleic acid in 20% alcohol. Store in a refrigerator, and use within 30 days.

Modified pantothenate medium: Dissolve anhydrous dextrose and sodium acetate in the solutions previously mixed according to *Table 3*, and adjust with 1 N sodium hydroxide to a pH of 6.8. Finally, dilute with water to 250 mL.

Table 3

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Riboflavin–thiamine hydrochloride–biotin solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL
Pyridoxal–calcium pantothenate solution	5 mL
Polysorbate 40–oleic acid solution	5 mL

Double-strength modified pantothenate medium: Prepare as directed for the *Modified pantothenate medium*, but make the final dilution to 125 mL instead of 250 mL. Prepare fresh.

Stock culture of *Pediococcus acidilactici*: Dissolve in 800 mL of water, with the aid of heat, 6.0 g of peptone, 4.0 g of pancreatic digest of casein, 3.0 g of yeast extract, 1.5 g of beef extract, 1.0 g of dextrose, and 15.0 g of agar. Adjust with 0.1 N sodium hydroxide or 0.1 N hydrochloric acid to a pH of 6.5–6.6, and dilute with water to 1000 mL. Add 10-mL portions of the solution to culture tubes, place caps on the tubes, and sterilize in an autoclave at 121° for 15 min. Cool on a slant, and store in a refrigerator. Prepare a stock culture of *Pediococcus acidilactici*[3] on a slant of this medium. Incubate at 35° for 20–24 h, and store in a refrigerator. Maintain the stock culture by monthly transfer onto fresh slants.

Inoculum: Inoculate three 250-mL portions of *Modified pantothenate medium* from a stock culture slant, and incubate at 35° for 20–24 h. Centrifuge the suspension from the combined portions, and wash the cells with sterile *Modified pantothenate medium*. Resuspend the cells in sufficient *Modified pantothenate medium* so that a 1-in-50 dilution, when tested in a 13-mm diameter test tube, gives 80% light transmission at 530 nm. Transfer 1.2-mL portions of this stock suspension to sterile glass ampuls, seal, freeze in liquid nitrogen, and store in a freezer. On the day of the assay, allow the ampuls to reach room temperature, mix the contents, and dilute 1 mL of thawed culture with sterile saline TS to 150 mL. [NOTE—This dilution may be altered when necessary to obtain the desired test response.]

Analysis: Prepare in triplicate a series of eight culture tubes by adding the following quantities of water to the tubes within a set: 5.0, 4.5, 4.0, 3.5, 3.0, 2.0, 1.0, and 0.0 mL. To these same tubes and in the same order add 0.0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. Prepare in duplicate a series of five culture tubes by adding the following quantities of water to the tubes within a set: 4.0, 3.5, 3.0, 2.0, and 1.0 mL. To these same tubes and in the same order add 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*.

[3] ATCC No. 8042 is suitable.

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Add 5.0 mL of *Double-strength modified pantothenate medium* to each tube. Cover the tubes with metal caps, and sterilize in an autoclave at 121° for 5 min. Cool to room temperature in a chilled water bath, and inoculate each tube with 0.5 mL of the *Inoculum*. Allow to incubate at 37° for 16 h. Terminate growth by heating to a temperature NLT 80°, such as by steaming at atmospheric pressure in a sterilizer for 5–10 min. Cool, and determine the percentage transmittance of the suspensions, in cells of equal pathlength, on a suitable spectrophotometer at a wavelength of 530 nm.

Calculation: Draw a dose-response curve on arithmetic graph paper by plotting the average response, in percentage of transmittance, for each set of tubes of the standard curve against the Standard level concentrations. The curve is drawn by connecting each adjacent pair of points with a straight line. From this standard curve, determine by interpolation the potency, in terms of dexpanthenol, of each tube containing portions of the *Sample solution*. To obtain the individual responses, divide the potency of each tube by the amount of the *Sample solution* added to it. Calculate the mean response by averaging the individual responses that vary from their mean by NMT 15%, using NLT half the total number of tubes. Calculate the potency of the portion of the material taken for assay, in terms of dexpanthenol, by multiplying the mean response by the appropriate dilution factor.

Acceptance criteria: 90.0%–150.0% of the labeled amount of dexpanthenol or panthenol ($C_9H_{19}NO_4$)

• **CALCIUM PANTOTHENATE, *Method 1***

Mobile phase: Phosphoric acid and water (1:1000)

Internal standard solution: 80 mg of *p*-hydroxybenzoic acid in 3 mL of alcohol. Add 50 mL of water and 7.1 g of dibasic sodium phosphate, and dilute with water to 1000 mL. Adjust with phosphoric acid to a pH of 6.7.

Standard solution: 0.6 mg/mL of USP Calcium Pantothenate RS in *Internal standard solution*

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." Transfer the equivalent to 0.6 mg/mL of calcium pantothenate, from mixed Capsule contents, in *Internal standard solution*, and shake vigorously for 10 min. Centrifuge, filter, and use the clear filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 210 nm

Column: 3.9-mm × 15-cm; packing L1

Flow rate: 1.5 mL/min

Injection volume: 10 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for calcium pantothenate and *p*-hydroxybenzoic acid are about 0.5 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak responses for calcium pantothenate and the internal standard. Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Capsules taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of calcium pantothenate to *p*-hydroxybenzoic acid from the *Sample solution*

R_S = peak response ratio of calcium pantothenate to *p*-hydroxybenzoic acid from the *Standard solution*

C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• **CALCIUM PANTOTHENATE, *Method 2***

Standard stock solution: Dissolve 50 mg of USP Calcium Pantothenate RS, previously dried and stored in the dark over phosphorus pentoxide while protected from absorption of moisture during the weighing, in 500 mL of water in a 1000-mL volumetric flask. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), and dilute with water to volume to obtain a concentration of 50 µg/mL of USP Calcium Pantothenate RS. Store under toluene in a refrigerator.

Standard solution: On the day of the assay, dilute a volume of *Standard stock solution* with water to obtain a concentration of 0.01–0.04 µg/mL of calcium pantothenate, the exact concentration being such that the responses obtained as directed for *Analysis*, 2.0–4.0 mL of the *Standard solution* being used, are within the linear portion of the log-concentration response curve.

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." Transfer a portion of the Capsule contents equivalent to a nominal amount of 50 mg of calcium pantothenate to a 1000-mL volumetric flask containing 500 mL of water. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (16.66 mg/mL), dilute with water to volume, and filter. Dilute a volume of this solution to obtain a solution with approximately the same concentration as that of the *Standard solution*.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and dilute with water to 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature NLT 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine and 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and dilute with water to 1000 mL. Store under toluene in a cool place at a temperature NLT 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat in 10 mL of 4 N hydrochloric acid. Cool, and dilute with water to 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin–thiamine hydrochloride–biotin solution: 20 µg/mL of riboflavin, 10 µg/mL of thiamine hydrochloride, and 0.04 µg/mL of biotin in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

***p*-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution:** 10 µg/mL of *p*-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a

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mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Basal medium stock solution: Dissolve the *anhydrous Dextrose* and *anhydrous Sodium acetate* in the solutions previously mixed according to *Table 4*, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Table 4

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Riboflavin–thiamine hydrochloride–biotin solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water. Add 500 mg of *anhydrous Dextrose*, 500 mg of *anhydrous Sodium acetate*, and 1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to the test tubes, close or cover the tubes, sterilize in an autoclave at 121°, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*[1] incubating for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution* add 5.0 mL of water containing 0.2 µg of calcium pantothenate. Plug the tubes with cotton, sterilize in an autoclave at 121°, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Transfer cells from the *Stock culture of Lactobacillus plantarum* to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. The cell suspension so obtained is the *Inoculum*.

Analysis

Samples: *Standard solution* and *Sample solution*

To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution* including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool, and add 1 drop of *Inoculum* to each tube, except two of the four tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37°, held constant to within ±0.5° until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of Standard during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to an optical container if necessary. Place the container in a spectrophotometer that has been set at a specific wavelength between 540 and 660 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittance (Σ) as the difference, $y = 2.00 - \Sigma$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, *y*, adding together the two transmittances for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of *y* that fall within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, *X*, for each dosage level. Average the values of *X* for each of three or more dosage levels to obtain $\bar{X}$, which equals the log-relative potency, *M'*, of the *Sample solution*.

Determine the quantity, in mg, of USP Calcium Pantothenate RS corresponding to the calcium pantothenate in each mg of the portion of Capsules taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of mg of calcium pantothenate assumed to be present in each mg of the portion of the Capsules taken

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies *M* is NMT 0.08, their mean, $\bar{M}$, is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* ⟨111⟩, *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or

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more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• **CALCIUM PANTOTHENATE, *Method 3***

Buffer solution: Dissolve 10.0 g of monobasic potassium phosphate in 2000 mL of water, and adjust with phosphoric acid to a pH of 3.5.

Mobile phase: Methanol and *Buffer solution* (1:9)

Standard stock solution: 0.25 mg/mL of USP Calcium Pantothenate RS in water. Prepare fresh every 4 weeks. Store in a refrigerator.

Standard solution: 40 µg/mL of USP Calcium Pantothenate RS from the *Standard stock solution* diluted with water

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." Transfer a portion of the Capsule contents, equivalent to a nominal amount of 10 mg of calcium pantothenate, to a 250-mL volumetric flask. Add 10 mL of methanol, and swirl the flask to disperse the Capsules' contents. Dilute with water to volume, mix, and filter.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 205 nm

Column: 3.9-mm × 30-cm; 5-µm packing L1

Column temperature: 50°

Flow rate: 2 mL/min

Injection volume: 25 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for calcium pantothenate. Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• **NIACIN OR NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, RIBOFLAVIN,** and **THIAMINE, *Method 1***

[NOTE—Use low-actinic glassware throughout this procedure.]

Diluent: Acetonitrile, glacial acetic acid, and water (5:1:94)

Mobile phase: A mixture of methanol, glacial acetic acid, and water (27:1:73) containing 140 mg of sodium 1-hexanesulfonate per 100 mL

Standard solution: [NOTE—Use USP Niacin RS in place of USP Niacinamide RS for formulations containing Niacin.] Transfer 80 mg of USP Niacinamide RS, 20 mg of USP Pyridoxine Hydrochloride RS, 20 mg of USP Riboflavin RS, and 20 mg of USP Thiamine Hydrochloride RS to a 200-mL volumetric flask, and add 180 mL of *Diluent*. Immerse the flask in a hot water bath maintained at 65°–70° for 10 min with regular shaking or using a vortex mixer, until all the solid materials are dissolved. Chill rapidly in a cold water bath for 10 min to room temperature, and dilute with *Diluent* to volume.

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." Transfer a portion of the Capsule contents, equivalent to 10 mg of niacinamide and 2.5 mg each of pyridoxine hydrochloride, riboflavin, and thiamine hydrochloride, to a 50-mL centrifuge tube. Add 25.0 mL of *Diluent*, and mix using a vortex mixer for 30 s to completely suspend the powder. Immerse the centrifuge tube in a hot water bath maintained at 65°–70°, heat for 5 min, and mix on a vortex mixer for 30 s. Return the tube to the hot water bath, heat for another 5 min, and mix on a vortex mixer for 30 s. Filter a portion of the solution, cool to room temperature, and use the clear filtrate. [NOTE—Use the filtrate within 3 h of filtration.]

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 3.9-mm × 30-cm; packing L1

Flow rate: 1 mL/min

Injection volume: 10 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for niacinamide, pyridoxine, riboflavin, and thiamine are about 0.3, 0.5, 0.8, and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for niacin or niacinamide, pyridoxine, riboflavin, and thiamine. Calculate the percentage of the labeled amount of niacinamide ($C_6H_6N_2O$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacinamide from the *Sample solution*

r_S = peak area of niacinamide from the *Standard solution*

C_S = concentration of USP Niacinamide RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacinamide in the *Sample solution* (mg/mL)

For formulations containing niacin:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacin from the *Sample solution*

r_S = peak area of niacin from the *Standard solution*

C_S = concentration of USP Niacin RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacin in the *Sample solution* (mg/mL)

Separately calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the corresponding vitamin from the *Sample solution*

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r_S = peak area of the corresponding vitamin from the *Standard solution*
C_S = concentration of the relevant USP Reference Standard in the *Standard solution* (mg/mL)
C_U = nominal concentration of the corresponding vitamin in the *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of thiamine from the *Sample solution*
r_S = peak area of thiamine from the *Standard solution*
C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL)
M_{r1} = molecular weight of thiamine mononitrate, 327.36
M_{r2} = molecular weight of thiamine hydrochloride, 337.27

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacinamide ($C_6H_6N_2O$), niacin ($C_6H_5NO_2$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine as thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$)

• **NIACIN, *Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A: Transfer 1 mL of glacial acetic acid and 2.5 g of edetate disodium to a 100-mL volumetric flask. Dissolve in and dilute with water to volume.

Extraction solvent: *Solution A* and methanol (3:1)

Mobile phase: 0.1 M sodium acetate solution (13.6 mg/mL of sodium acetate in water). Adjust with acetic acid to a pH of 5.4. [NOTE—A small amount of methanol (up to 1%) may be added to the *Mobile phase* to improve resolution.]

Standard stock solution: 1 mg/mL of USP Niacin RS in *Extraction solvent*

Standard solution: Transfer 5.0 mL of *Standard stock solution* to a 25-mL volumetric flask, and dilute with *Extraction solvent* to volume.

Sample solution: [NOTE—This preparation is suitable for the determination of niacin or niacinamide, pyridoxine hydrochloride, and riboflavin, when present in the formulation.] Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents to a beaker. Remove any contents adhering to the shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to 0.02 mg/mL of riboflavin. If riboflavin is not present in the formulation, use a portion equivalent to 0.02 mg/mL of pyridoxine. If pyridoxine is not present in the formulation, use a portion equivalent to 0.2 mg/mL of niacin or niacinamide in *Extraction solvent*, and mix for 20 min using a wrist-action shaker. Immerse the flask in a water bath maintained at 70°–75°, and heat for 20 min. Mix on a vortex mixer for 30 s, cool to room temperature, and filter. Use the clear filtrate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; packing L1
Flow rate: 1 mL/min
Injection volume: 20 µL

System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 3.0%
[NOTE—If necessary, flush the column with methanol between injections.]

Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas for niacin. Calculate the percentage of the labeled amount of niacin ($C_6H_5NO_2$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacin from the *Sample solution*
r_S = peak area of niacin from the *Standard solution*
C_S = concentration of USP Niacin RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of niacin in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacin ($C_6H_5NO_2$)

• **NIACINAMIDE, *Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Extraction solvent, Mobile phase, Standard stock solution, Standard solution, Sample solution, and **Chromatographic system:** Using USP Niacinamide RS in place of USP Niacin RS, proceed as directed in *Niacin, Method 2*.

Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas for niacinamide. Calculate the percentage of the labeled amount of niacinamide ($C_6H_6N_2O$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacinamide from the *Sample solution*
r_S = peak area of niacinamide from the *Standard solution*
C_S = concentration of USP Niacinamide RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of niacinamide in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacinamide ($C_6H_6N_2O$)

• **PYRIDOXINE HYDROCHLORIDE, *Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Extraction solvent and **Mobile phase:** Prepare as directed in *Niacin, Method 2*.

Standard stock solution: 0.1 mg/mL of USP Pyridoxine Hydrochloride RS in *Extraction solvent*

Standard solution: 20 µg/mL of USP Pyridoxine Hydrochloride RS from *Standard stock solution* diluted with *Extraction solvent*

Sample solution: Prepare as directed in *Niacin, Method 2*.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; packing L1

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Flow rate: 1 mL/min
Injection volume: 20 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 3.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas for pyridoxine. Calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of pyridoxine hydrochloride from the *Sample solution*
r_S = peak area of pyridoxine hydrochloride from the *Standard solution*
C_S = concentration of USP Pyridoxine Hydrochloride RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of pyridoxine hydrochloride in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$)

• **RIBOFLAVIN, *Method 2***
[NOTE—Use low-actinic glassware throughout this procedure.]
Extraction solvent: Prepare as directed in *Niacin, Method 2*.
Solution A: 6.8 mg/mL of sodium acetate in water
Mobile phase: Prepare a mixture of *Solution A* and methanol (13:7). Add 2 mL of triethylamine per L of the mixture, and adjust with glacial acetic acid to a pH of 5.2.
Standard stock solution: Transfer 20 mg of USP Riboflavin RS to a 200-mL volumetric flask, and add 180 mL of *Extraction solvent*. Immerse the flask for 5 min in a water bath maintained at 65°–75°. Mix well, and repeat if necessary until dissolved. Chill rapidly in a cold water bath to room temperature, and dilute with *Extraction solvent* to volume.
Standard solution: Dilute 5.0 mL of *Standard stock solution* with *Extraction solvent* to 25.0 mL.
Sample solution: Prepare as directed in *Niacin, Method 2*.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; packing L1
Flow rate: 1 mL/min
Injection volume: 20 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 3.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas for riboflavin. Calculate the percentage of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of riboflavin from the *Sample solution*
r_S = peak area of riboflavin from the *Standard solution*
C_S = concentration of USP Riboflavin RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of riboflavin in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$)

• **THIAMINE, *Method 2***
[NOTE—Use low-actinic glassware throughout this procedure.]
Solution A: 1.88 mg/mL of sodium 1-hexanesulfonate in 0.1% phosphoric acid
Mobile phase: *Solution A* and acetonitrile (46:9)
Standard stock solution: 0.1 mg/mL of USP Thiamine Hydrochloride RS in 0.2 N hydrochloric acid
Standard solution: 0.02 mg/mL of USP Thiamine Hydrochloride RS from *Standard stock solution* diluted with 0.2 N hydrochloric acid
Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." Mix a portion of the Capsule contents with a volume of 0.2 N hydrochloric acid to obtain a nominal concentration of 0.02 mg/mL of thiamine. Shake for 15 min with a wrist-action shaker, and heat to boiling for 30 min. Cool to room temperature, and filter. Use the clear filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; packing L1
Flow rate: 2 mL/min
Injection volume: 20 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 3.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the areas for the major peaks. For products containing thiamine hydrochloride, calculate the percentage of the labeled amount of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of thiamine from the *Sample solution*
r_S = peak area of thiamine from the *Standard solution*
C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of thiamine hydrochloride in the *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of thiamine from the *Sample solution*
r_S = peak area of thiamine from the *Standard solution*
C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL)
M_{r1} = molecular weight of thiamine mononitrate, 327.36
M_{r2} = molecular weight of thiamine hydrochloride, 337.27

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Acceptance criteria: 90.0%–150.0% of the labeled amount of thiamine as thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$)

• **NIACIN OR NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, RIBOFLAVIN,** and **THIAMINE,** ***Method 3***

[NOTE—Use low-actinic glassware throughout this procedure.]

Reagent: 25 mg/mL of edetate disodium in water

Mobile phase: Transfer 0.4 mL of triethylamine, 15.0 mL of glacial acetic acid, and 350 mL of methanol to a 2000-mL volumetric flask. Dilute with 0.008 M sodium 1-hexanesulfonate to volume.

Standard stock solution: 1.5 mg/mL of USP Niacin RS or USP Niacinamide RS, 0.24 mg/mL of USP Pyridoxine Hydrochloride RS, 0.08 mg/mL of USP Riboflavin RS, and 0.24 mg/mL of USP Thiamine Hydrochloride RS in *Reagent*, with heating if necessary

Standard solution: Transfer 5.0 mL of *Standard stock solution* to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." Transfer a portion of the Capsule contents, equivalent to a nominal amount of 7.5 mg of niacin or niacinamide, 1.2 mg of pyridoxine hydrochloride, 0.4 mg of riboflavin, and 1.2 mg of thiamine hydrochloride, to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1), and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 270 nm

Column: 4.6-mm × 25-cm; packing L7

Column temperature: 50°

Flow rate: 2 mL/min

Injection volume: 5 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the areas of the peak responses. Calculate the percentage of the labeled amount of niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacin or niacinamide from the *Sample solution*

r_S = peak area of niacin or niacinamide from the *Standard solution*

C_S = concentration of USP Niacin RS or USP Niacinamide RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacin or niacinamide in the *Sample solution* (mg/mL)

Separately calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine ($C_{12}H_{17}ClN_4OS$) (for products containing thiamine hydrochloride) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the corresponding vitamin from the *Sample solution*

r_S = peak area of the corresponding vitamin from the *Standard solution*

C_S = concentration of the relevant USP Reference Standard in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding analyte in the *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of thiamine from the *Sample solution*

r_S = peak area of thiamine from the *Standard solution*

C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of thiamine mononitrate, 327.36

M_{r2} = molecular weight of thiamine hydrochloride, 337.27

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine as thiamine hydrochloride ($C_{12}H_{17}ClN_4OS$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S \cdot HCl$).

[NOTE—Commercially available atomic absorption standard solutions for the minerals, where applicable, may be used where preparation of a *Standard stock solution* is described in the following assays. Use deionized water where water is specified. Where atomic absorption spectrophotometry is specified in the assay, the *Standard solutions* and the *Sample solution* may be diluted quantitatively with the solvent specified, if necessary, to yield solutions of suitable concentrations adaptable to the linear or working range of the instrument.]

• **CALCIUM,** ***Method 1***

Lanthanum chloride solution: 267 mg/mL of lanthanum chloride heptahydrate in 0.125 N hydrochloric acid

Calcium standard solution: 400 µg/mL of calcium. Dissolve 1.001 g of calcium carbonate previously dried at 300° for 3 h and cooled in a desiccator for 2 h in 25 mL of 1 N hydrochloric acid. Boil to expel carbon dioxide, and dilute with water to 1000 mL.

Standard stock solution: 100 µg/mL of calcium from *Calcium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Into separate 100-mL volumetric flasks pipet 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, and dilute with water to volume to obtain concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 µg/mL of calcium.

Polysorbate 80 solution: Dilute polysorbate 80 with alcohol (1 in 10)

Sample solution: Transfer 5 Capsules to a 100-mL volumetric flask. [NOTE—For hard gelatin Capsules, weigh

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NLT 20 Capsules. Open the Capsules, without loss of shell material, and transfer the contents to a suitable container. Remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and allow the Capsule shells to dry. Weigh the empty Capsule shells, calculate the net weight of the Capsule contents, and transfer a portion of the Capsule contents, equivalent to 5 Capsules, to a 100-mL volumetric flask.] Add 15 mL of water, 10 mL of 6 N hydrochloric acid, and 1 mL of *Polysorbate 80 solution* to the flask. Heat on a hot plate or steam bath, with intermittent swirling, until the Capsules are completely disintegrated or the contents are dissolved. Boil gently for an additional 15 min. Cool, dilute with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute this solution with 0.125 N hydrochloric acid to obtain a concentration of 2 µg/mL of calcium, adding 1 mL of *Lanthanum chloride solution* per 100 mL of the final volume.

Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Lamp: Calcium hollow-cathode
Flame: Nitrous oxide–acetylene
Analytical wavelength: Calcium emission line at 422.7 nm
Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution* per 100 mL

Analysis
Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of calcium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of calcium in the *Sample solution*.
Calculate the percentage of the labeled amount of calcium (Ca) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of calcium in the *Sample solution* (µg/mL)
C_U = nominal concentration of calcium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of calcium (Ca)

• **CHROMIUM, *Method 1***
Chromium standard solution: 1000 µg/mL of chromium from potassium dichromate, previously dried at 120° for 4 h in water. Store in a polyethylene bottle.
Standard stock solution: 10 µg/mL of chromium from *Chromium standard solution* diluted with 6 N hydrochloric acid and water (1 in 20)
Standard solutions: Transfer 10.0 and 20.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks, and transfer 15.0 and 20.0 mL of the *Standard stock solution* to separate 50-mL volumetric flasks. Dilute the contents of each of the four flasks with 0.125 N hydrochloric acid to volume to obtain concentrations of 1.0, 2.0, 3.0, and 4.0 µg/mL of chromium.
Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain 1 µg/mL of chromium and omit the use of the *Lanthanum chloride solution*.
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Lamp: Chromium hollow-cathode
Flame: Air–acetylene
Analytical wavelength: Chromium emission line at 357.9 nm
Blank: 0.125 N hydrochloric acid

Analysis
Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of chromium, and draw the straight line best fitting the four plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of chromium in the *Sample solution*.
Calculate the percentage of the labeled amount of chromium (Cr) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of chromium in the *Sample solution* (µg/mL)
C_U = nominal concentration of chromium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of chromium (Cr)

• **COPPER, *Method 1***
Copper standard solution: Dissolve 1.00 g of copper foil in a minimum volume of a 50% (v/v) solution of nitric acid, and dilute with a 1% (v/v) solution of nitric acid to 1000 mL. This solution contains 1000 µg/mL of copper.
Standard stock solution: 100 µg/mL of copper from *Copper standard solution* diluted with 0.125 N hydrochloric acid
Standard solutions: To separate 200-mL volumetric flasks transfer 1.0, 2.0, 4.0, 6.0, and 8.0 mL of the *Standard stock solution*. Dilute with water to volume to obtain concentrations of 0.5, 1.0, 2.0, 3.0, and 4.0 µg/mL of copper.
Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain 2 µg/mL of copper and omit the use of the *Lanthanum chloride solution*.
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Lamp: Copper hollow-cathode
Flame: Air–acetylene
Analytical wavelength: Copper emission line at 324.7 nm
Blank: 0.125 N hydrochloric acid

Analysis
Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of copper, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of copper in the *Sample solution*.
Calculate the percentage of the labeled amount of copper (Cu) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of copper in the *Sample solution* (µg/mL)
C_U = nominal concentration of copper in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of copper (Cu)

• **FLUORIDE, *Method 1***
[NOTE—Store all solutions in plastic containers.]

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3 M sodium acetate solution: 408 mg/mL of sodium acetate in water. Adjust with a few drops of acetic acid to a pH of 7.0. [NOTE—Dissolve in a portion of water, and allow the solution to equilibrate to room temperature, then dilute with water to volume, and adjust the pH.]

Sodium citrate solution: Dissolve 222 g of sodium citrate in 250 mL of water in a 1000-mL volumetric flask. Add 28 mL of perchloric acid, and dilute with water to volume.

Fluoride standard stock solution: 500 µg/mL of fluoride from a quantity of sodium fluoride, previously dried at 100° for 4 h and cooled in a desiccator in water

Intermediate stock solution A: 100 µg/mL of fluoride from *Fluoride standard stock solution* diluted with water

Intermediate stock solution B: 10 µg/mL of fluoride from *Fluoride standard stock solution* diluted with water

Standard solutions: To five separate 100-mL volumetric flasks transfer 3.0, 5.0, and 10.0 mL of *Intermediate stock solution B* and 5.0 and 10.0 mL of *Intermediate stock solution A*. To each flask add 10.0 mL of 1 N hydrochloric acid, 25 mL of *3 M sodium acetate solution*, and 25.0 mL of *Sodium citrate solution*. Dilute the contents of each flask with water to volume to obtain concentrations of 0.3, 0.5, 1.0, 5.0, and 10.0 µg/mL of fluoride.

Sample solution: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the mixed Capsule contents, equivalent to a nominal amount of 200 mg of fluoride, to a 100-mL volumetric flask. Add 10.0 mL of 1 N hydrochloric acid, 25.0 mL of *3 M sodium acetate solution*, and 25.0 mL of *Sodium citrate solution*, and dilute with water to volume.

Analysis

Samples: *Standard solutions* and *Sample solution*

To separate plastic beakers, each containing a plastic-coated stirring bar, transfer 50.0 mL each of the *Standard solutions* and the *Sample solution*. Measure the potentials (see *pH* ⟨791⟩), in mV, of the *Standard solutions* and the *Sample solution* with a pH meter capable of a minimum reproducibility of ±0.2 mV and equipped with a fluoride-specific ion-indicating electrode and a calomel reference electrode. [NOTE—When taking measurements, immerse the electrodes in the solution, stir on a magnetic stirrer with an insulated top until equilibrium is attained (1–2 min), and record the potential. Rinse and dry the electrodes between measurements, taking care to avoid damaging the crystal of the specific-ion electrode.]

Plot the logarithms of fluoride concentrations, in µg/mL, of the *Standard solutions* versus the potential, in mV. From the standard response curve and the measured potential of the *Sample solution*, determine the concentration, *C*, in µg/mL, of fluoride in the *Sample solution*.

Calculate the percentage of the labeled amount of fluorine (F) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of fluoride in the *Sample solution* (µg/mL)

C_U = nominal concentration of fluorine in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (F)

• **FLUORIDE, *Method 2***

[NOTE—Use plastic containers and deionized water throughout this procedure.]

pH 10.0 buffer: Add 214 mL of 0.1 N sodium hydroxide to 1000 mL of 0.05 M sodium bicarbonate.

Mobile phase: Alcohol, 0.1 N sulfuric acid, and water (20:5:175)

Standard stock solution: 220 µg/mL of USP Sodium Fluoride RS in water. This solution contains 100 µg/mL of fluoride.

Standard solution

[NOTE—Condition the solid-phase extraction column specified for use in the *Standard solution* and the *Sample solution* in the following manner. Using a vacuum at a pressure not exceeding 5 mm of mercury, wash the column with 1 column volume of methanol followed by 1 column volume of *pH 10.0 buffer*. Do not allow the column top to dry. If the top of the column becomes dry, recondition the column.] Transfer 10.0 mL of the *Standard stock solution* to a 100-mL volumetric flask. Add 75 mL of water, and adjust with 0.1 N sodium hydroxide to a pH of 10.4 ± 0.1. Dilute with water to volume. Filter, discarding the first 15 mL of the filtrate. Transfer 25.0 mL of the filtrate to a 50-mL volumetric flask, and add 15.0 mL of water, and adjust with 0.1 N sodium hydroxide to a pH of 10.0. Dilute with *pH 10.0 buffer* to volume. Elute a portion of this solution through a 3-mL, solid-phase extraction column containing L1 packing that is connected through an adaptor to a second solid-phase extraction column containing sulfonylpropyl strong cation-exchange packing. Discard the first 3 mL of the eluate, and collect the rest of the eluate in a suitable flask for injection into the chromatograph.

Sample solution: Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents to a 100-mL container. If necessary, remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to a nominal amount of 1 mg of fluorine, to a 100-mL volumetric flask. Add 15 mL of water, and shake vigorously. Rinse the sides of the flask with 15 mL of water, and allow to stand for 10 min. Dilute with water to 85 mL, adjust with 1 N sodium hydroxide to a pH of 10.4 ± 0.1, and dilute with water to 100 mL. Proceed as directed for the *Standard solution* beginning with "Filter, discarding the first 15 mL of the filtrate."

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Conductivity

Columns

Guard: 4.6-mm × 3-cm; packing L17

Analytical: 7.8-mm × 30-cm; packing L17

Flow rate: 0.5 mL/min

Injection volume: 100 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for fluoride. Calculate the percentage of the labeled amount of fluorine (F) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of fluoride in the *Standard solution* (µg/mL)

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C_U = nominal concentration of fluorine in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (F)

• **IODIDE**

Bromine water: Bromine and water (1:5). Shake. Allow to stand for 30 min, and use the supernatant.

Analysis

Sample: Capsules

Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the contents, equivalent to 3 mg of iodine, to a nickel crucible. Add 5 g of sodium carbonate, 5 mL of 50% (w/v) sodium hydroxide solution, and 10 mL of alcohol, taking care that the entire specimen is moistened. Heat the crucible on a steam bath to evaporate the alcohol, then dry the crucible at 100° for 30 min to prevent spattering upon subsequent heating. Transfer the crucible with its contents to a furnace heated to 500°, and heat the crucible for 15 min. [NOTE—Heating at 500° is necessary to carbonize any organic matter present; a higher temperature may be used, if necessary, to ensure complete carbonization of all organic matter.] Cool the crucible, add 25 mL of water, cover the crucible with a watchglass, and boil gently for 10 min. Filter the solution, and wash the crucible with boiling water, collecting the filtrate and washings in a beaker. Add phosphoric acid until the solution is neutral to methyl orange, then add 1 mL excess of phosphoric acid. Add excess of *Bromine water*, and boil the solution gently until colorless, and then for 5 min longer. Add a few crystals of salicylic acid, and cool the solution to 20°. Add 1 mL of phosphoric acid and 0.5 g of potassium iodide, and titrate the liberated iodine with 0.005 N sodium thiosulfate VS, adding starch TS when the liberated iodine color has nearly disappeared.

Calculate the percentage of the labeled amount of iodine (I) in the portion of Capsules taken:

$$\text{Result} = V \times N_A \times F \times I_{me} \times (A_w/W) \times (100/L)$$

V = volume of sodium thiosulfate consumed (mL)
N_A = actual normality of the sodium thiosulfate solution used (meq/mL)
F = correction factor to convert mg to µg, 1000 µg/mg
I_{me} = milliequivalent of I, 21.16 mg/meq
A_w = average weight of the Capsules' contents
W = weight of the sample of Capsules' contents taken
L = labeled amount of iodine (µg/Capsule)

Acceptance criteria: 90.0%–160.0% of the labeled amount of iodine (I)

• **IRON, *Method 1***

Iron standard stock solution: Transfer 100 mg of iron powder to a 1000-mL volumetric flask. Dissolve in 25 mL of 6 N hydrochloric acid, dilute with water to volume, and mix.

Standard solutions: To separate 100-mL volumetric flasks transfer 2.0, 4.0, 5.0, 6.0, and 8.0 mL of *Iron standard stock solution*. Dilute the contents of each flask with water to volume to obtain concentrations of 2.0, 4.0, 5.0, 6.0, and 8.0 µg/mL of iron.

Polysorbate 80 solution: Prepare as directed in *Calcium, Method 1*.

Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain a nominal concentration of 5 µg/mL of iron and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Lamp: Iron hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Iron emission line at 248.3 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of iron, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C, in µg/mL, of iron in the *Sample solution*.

Calculate the percentage of the labeled amount of iron (Fe) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of iron in the *Sample solution* (µg/mL)
C_U = nominal concentration of iron in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of iron (Fe)

• **MAGNESIUM, *Method 1***

Lanthanum chloride solution: Prepare as directed in *Calcium, Method 1*.

Magnesium standard stock solution: 1000 µg/mL of magnesium from magnesium ribbon in 6 N hydrochloric acid and water (1:19)

Standard stock solution: 20 µg/mL of magnesium from *Magnesium standard stock solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, and dilute with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.2, 0.3, 0.4, 0.5, and 0.6 µg/mL of magnesium.

Polysorbate 80 solution: Prepare as directed in *Calcium, Method 1*.

Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain a nominal concentration of 0.4 µg/mL of magnesium.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Lamp: Magnesium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Magnesium emission line at 285.2 nm

Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution* per 100 mL

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of magnesium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C, in µg/mL, of magnesium in the *Sample solution*.

Calculate the percentage of the labeled amount of magnesium (Mg) in the portion of Capsules taken:

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$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of magnesium in the *Sample solution* (µg/mL)
C_U = nominal concentration of magnesium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of magnesium (Mg)

• **MANGANESE, *Method 1***

Manganese standard stock solution: 1000 µg/mL of manganese in 6 N hydrochloric acid and nitric acid (49:1). [NOTE—Dissolve the manganese in nitric acid, and dilute with 6 N hydrochloric acid to final volume.]

Standard stock solution: 50 µg/mL of manganese from *Manganese standard stock solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 3.0, and 4.0 mL of *Standard stock solution*. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions with concentrations of 0.5, 0.75, 1.0, 1.5, and 2.0 µg/mL of manganese.

Polysorbate 80 solution: Prepare as directed in *Calcium, Method 1*.

Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain a nominal concentration of 1 µg/mL of manganese and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Lamp: Manganese hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Manganese emission line at 279.5 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of manganese, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C, in µg/mL, of manganese in the *Sample solution*.

Calculate the percentage of the labeled amount of manganese (Mn) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of manganese in the *Sample solution* (µg/mL)
C_U = nominal concentration of manganese in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of manganese (Mn)

• **MOLYBDENUM, *Method 1***

Diluent: 20 mg/mL of ammonium chloride in water

Molybdenum standard solution: Transfer 1.0 g of molybdenum wire to a 1000-mL volumetric flask, and dissolve in 50 mL of nitric acid, warming if necessary. Dilute with water to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of molybdenum.

Standard stock solution: 100 µg/mL of molybdenum from *Molybdenum standard solution* diluted with water

Standard solutions: To separate 100-mL volumetric flasks transfer 5.0, 10.0, and 25.0 mL of the *Standard stock solution*, and add 5.0 mL of perchloric acid to each flask. Gently boil the solution in each flask for 15 min. Cool to room temperature, and dilute each with *Diluent* to volume to obtain concentrations of 5.0, 10.0, and 25.0 µg/mL of molybdenum.

Polysorbate 80 solution: Prepare as directed in *Calcium, Method 1*.

Sample solution: Proceed as directed in *Calcium, Method 1*, except take a number of Capsules or a portion of Capsule contents nominally equivalent to 1000 µg of molybdenum and make appropriate dilutions to obtain a final concentration of 10 µg/mL of molybdenum, omitting the addition of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Lamp: Molybdenum hollow-cathode

Flame: Nitrous oxide–acetylene

Analytical wavelength: Molybdenum emission line at 313.3 nm

Blank: *Diluent* and perchloric acid (20:1)

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of molybdenum, and draw the straight line best fitting the three plotted points. From the graph so obtained, determine the concentration, C, in µg/mL, of molybdenum in the *Sample solution*.

Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of molybdenum in the *Sample solution* (µg/mL)
C_U = nominal concentration of molybdenum in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

• **MOLYBDENUM, *Method 2***

Sodium fluoride solution: Add 200 mL of water to 10 g of sodium fluoride, stir until the solution is saturated, and filter. Store in a polyethylene bottle.

Ferrous sulfate solution: 4.98 mg/mL of ferrous sulfate in water

Potassium thiocyanate solution: 200 mg/mL of potassium thiocyanate in water

20% Stannous chloride solution: 400 mg/mL of stannous chloride in 6.5 N hydrochloric acid solution and water (1:4). [NOTE—Dissolve first in 6.5 N hydrochloric acid, and heat the solution until the stannous chloride is dissolved. Then dilute with water to volume.]

Diluted stannous chloride solution: *20% Stannous chloride solution* and water (1:24). Prepare this solution fresh at the time of use.

Standard solution: 20 µg/mL of molybdenum from ammonium molybdate in water

Sample: Remove the contents of a counted number of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the Capsule contents, equivalent to a nominal amount of 40 mg of molybdenum, to a 200-mL beaker.

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Mode: UV-Vis

Cell: 1 cm

Analytical wavelength: 465 nm

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Blank: Amyl alcohol

Analysis

Samples: *Standard solution* and *Sample*

Transfer the *Sample* and 2.0 mL of the *Standard solution* to separate 200-mL beakers. Add 20 mL of nitric acid to each beaker. Cover each beaker with a watchglass, and boil slowly on a hot plate for 45 min. Cool to room temperature. Add 6 mL of perchloric acid, cover the beakers with a watchglass, and continue the heating until digestion is complete, as indicated when the liquid becomes colorless or pale yellow. Evaporate the solutions in the beakers to dryness. Rinse the sides of the beakers and the watchglasses with water, and add more water to make 50 mL in each beaker. Gently boil the water solution for a few min. Cool to room temperature. Add 2 drops of methyl orange TS, and neutralize with ammonium hydroxide. Add 8.2 mL of hydrochloric acid. Quantitatively transfer the contents of the beakers to separate 100-mL volumetric flasks, rinse the beakers with water, transfer the rinsings to the corresponding volumetric flasks, and dilute with water to volume. Transfer 50.0 mL of each solution to separatory funnels. To each separatory funnel add 1.0 mL of *Sodium fluoride solution*, 0.5 mL of *Ferrous sulfate solution*, 4.0 mL of *Potassium thiocyanate solution*, 1.5 mL of *20% Stannous chloride solution*, and 15.0 mL of amyl alcohol, and shake the separatory funnel for 1 min. Allow the layers to separate, and discard the aqueous layers. Add 25 mL of *Diluted stannous chloride solution* to each separatory funnel, and shake gently for 15 s. Allow the layers to separate, and discard the aqueous layers. Transfer the organic layers from each separatory funnel to a centrifuge tube, and centrifuge at 2000 rpm for 10 min. Determine the absorbances of the organic phases obtained from the *Standard solution* and the *Sample*, and correct with the *Blank*.

Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Capsules taken:

$$\text{Result} = (A_U/A_S) \times [(V \times C_S)/M_U] \times 100$$

A_U = absorbance of the *Sample*
A_S = absorbance of the *Standard solution*
V = volume of the *Standard solution* analyzed, 2.0 mL
C_S = concentration of molybdenum in the *Standard solution* (µg/mL)
M_U = nominal amount of molybdenum in the *Sample* (µg)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

• **PHOSPHORUS, *Method 1***

Sulfuric acid solution: Cautiously add sulfuric acid to water (37.5: 100), and mix.

Ammonium molybdate solution: 50 mg/mL of ammonium molybdate in *Sulfuric acid solution* and water (2:3). [NOTE—Dissolve in water first, and then dilute with *Sulfuric acid solution* to volume.]

Hydroquinone solution: 5 mg/mL of hydroquinone in water. Add 1 drop of sulfuric acid per 100 mL of solution.

Sodium bisulfite solution: 200 mg/mL of sodium bisulfite in water

Phosphorus standard stock solution: Weigh 4.395 g of monobasic potassium phosphate, previously dried at 105° for 2 h and stored in a desiccator, and transfer to a 1000-mL volumetric flask. Dissolve in water, add 6 mL of sulfuric acid as a preservative, dilute with water to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of phosphorus.

Standard solution: 20 µg/mL of phosphorus from *Phosphorus standard stock solution* diluted with water

Sample solution: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the Capsule contents, equivalent to a nominal amount of 100 mg of phosphorus, to 25 mL of nitric acid, and digest on a hot plate for 30 min. Add 15 mL of hydrochloric acid, and continue the digestion to the cessation of brown fumes. Cool, and transfer the contents of the flask to a 500-mL volumetric flask with the aid of small portions of water. Dilute with water to volume. Transfer 10.0 mL of this solution to a 100-mL volumetric flask, and dilute with water to volume.

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Mode: UV-Vis

Cell: 1 cm

Analytical wavelength: 650 nm

Analysis

Samples: *Standard solution* and *Sample solution*

To three separate 25-mL volumetric flasks transfer 5.0 mL each of the *Standard solution*, the *Sample solution*, and water to provide the blank. To each of the three flasks add 1.0 mL each of *Ammonium molybdate solution*, *Hydroquinone solution*, and *Sodium bisulfite solution*, and swirl to mix. Dilute the contents of each flask with water to volume, and allow the flasks to stand for 30 min. Determine the absorbances of the solutions against the blank.

Calculate the percentage of the labeled amount of phosphorus (P) in the portion of Capsules taken:

$$\text{Result} = (A_U/A_S) \times (C_S/C_U) \times 100$$

A_U = absorbance of the *Sample solution*
A_S = absorbance of the *Standard solution*
C_S = concentration of phosphorus in the *Standard solution* (µg/mL)
C_U = nominal concentration of phosphorus in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of phosphorus (P)

• **POTASSIUM**

Potassium standard solution: 100 µg/mL of potassium from potassium chloride, previously dried at 105° for 2 h, in water

Standard stock solution: 10 µg/mL of potassium from *Potassium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Transfer 5.0, 10.0, 15.0, 20.0, and 25.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions containing 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of potassium.

Polysorbate 80 solution: Prepare as directed in *Calcium, Method 1*.

Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain a nominal amount of 1 µg/mL of potassium and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Lamp: Potassium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Potassium emission line at 766.5 nm

Blank: Water

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Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of potassium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of potassium in the *Sample solution*.

Calculate the percentage of the labeled amount of potassium (K) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of potassium in the *Sample solution* (µg/mL)

C_U = nominal concentration of potassium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of potassium (K)

• **SELENIUM, *Method 1***

Diluent: Prepare as directed in *Molybdenum, Method 1*.

Selenium standard solution: [**CAUTION**—Selenium is toxic; handle it with care.] Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness. Add 2 mL of water, and evaporate to dryness. Repeat the addition of water and the evaporation to dryness three times. Dissolve the residue in 3 N hydrochloric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume to obtain a concentration of 1000 µg/mL of selenium.

Standard stock solution: 100 µg/mL of selenium from *Selenium standard solution* diluted with water

Standard solutions: To separate 100-mL volumetric flasks transfer 5.0, 10.0, and 25.0 mL of the *Standard stock solution*, and add 5.0 mL of perchloric acid to each flask. Gently boil the solutions for 15 min, cool to room temperature, and dilute each with *Diluent* to volume to obtain solutions containing 5.0, 10.0, and 25.0 µg/mL of selenium.

Sample solution: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the Capsule contents, equivalent to a nominal amount of 1000 µg of selenium, to a suitable flask, and add 12 mL of nitric acid. [NOTE—The volume of nitric acid may be varied to ensure that the powder is uniformly dispersed.] Carefully swirl the flask to disperse the sample specimen. Sonicate for 10 min or until the sample specimen is completely dissolved. Gently boil the solution for 15 min, and cool to room temperature. Carefully add 8 mL of perchloric acid to the flask, heat the flask until perchloric acid fumes appear, and swirl the flask to dissipate the fumes. Repeat the heating and swirling until the fumes appear again. Cool to room temperature. Transfer the contents of the flask to a 50-mL volumetric flask with the aid of the *Diluent*, and dilute with *Diluent* to volume.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Lamp: Selenium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Selenium emission line at 196.0 nm

Blank: *Diluent* and perchloric acid (20:1)

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of selenium, and draw the straight line best fitting the three plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of selenium in the *Sample solution*.

Calculate the percentage of the labeled amount of selenium (Se) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of selenium in the *Sample solution* (µg/mL)

C_U = nominal concentration of selenium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of selenium (Se)

• **SELENIUM, *Method 2***

Hydrochloric acid solution: Hydrochloric acid diluted with water (1 in 10)

50% Ammonium hydroxide solution: Ammonium hydroxide diluted with water (1 in 2)

Reagent A: 9 mg/mL of edetate disodium and 25 mg/mL of hydroxylamine hydrochloride in water. [NOTE—Dissolve edetate disodium in a portion of water first, add hydroxylamine hydrochloride, then dilute with water to volume.]

Reagent B: Transfer 200 mg of 2,3-diaminonaphthalene to a 250-mL separatory funnel, and add 200 mL of 0.1 N hydrochloric acid. Wash the solution with three 40-mL portions of cyclohexane, and discard the cyclohexane layer. Filter the solution into a brown bottle, and cover the solution with a 1-cm layer of cyclohexane. This solution is stable for 1 week if stored in a refrigerator.

Standard stock solution: [**CAUTION**—Selenium is toxic; handle it with care.] Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness. Add 2 mL of water, and evaporate to dryness. Repeat the addition of water and evaporation to dryness three times. Dissolve the residue in 3 N hydrochoric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume to obtain a solution with a concentration of 1000 µg/mL of selenium. Dilute a volume of the solution with 0.125 N hydrochloric acid to obtain a concentration of 2.0 µg/mL of selenium.

Standard solution: Transfer 10.0 mL of *Standard stock solution* to a glass-stoppered flask. Add 1 mL of perchloric acid, and 1 mL of *Hydrochloric acid solution*, and dilute with water to 20 mL.

Sample solution: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the Capsule contents, equivalent to a nominal amount of 20 µg of selenium, to a suitable flask. Add 10 mL of nitric acid, and warm gently on a hot plate. Continue heating until the initial nitric acid reaction has subsided, then add 3 mL of perchloric acid. [**CAUTION**—Exercise care at this stage because perchloric acid reaction becomes vigorous.] Continue heating on the hot plate until the appearance of white fumes of perchloric acid or until the digest begins to darken. Add 0.5 mL of nitric acid, and resume heating, adding additional amounts of nitric acid if further darkening occurs. Digest for 10 min after the first appearance of perchloric acid fumes or until the digest becomes colorless. Cool the flask. Add 2.5 mL of *Hydrochloric acid solution*, and return the flask to the hot plate to expel residual nitric acid. Heat the mixture for 3 min after it begins to boil. Cool the flask to room temperature, and dilute with water to 20 mL.

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Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: UV
Cell: 1 cm
Analytical wavelength: 380 nm
Blank: 1 mL of perchloric acid and 1 mL of *Hydrochloric acid solution* diluted with water to 20 mL

Analysis
Samples: *Standard solution* and *Sample solution*
Treat the *Sample solution*, *Standard solution*, and *Blank* as follows. Add 5 mL of *Reagent A* to each flask, and swirl gently to mix. Adjust the solution in each flask with *50% Ammonium hydroxide solution* to a pH of 1.1 ± 0.1. Add 5 mL of *Reagent B* to each flask, and swirl gently to mix. Place the flasks in a water bath maintained at 50°, and equilibrate for 30 min, taking care that the flasks are covered to protect them from light. Cool to room temperature, and transfer the contents of each flask to separate separatory funnels. Transfer 10.0 mL of cyclohexane to each separatory funnel, and extract vigorously for 1 min. Discard the aqueous layer. Transfer the cyclohexane layer to a centrifuge tube, and centrifuge at 1000 rpm for 1 min to remove any remaining water. Determine the absorbances of the solutions obtained from the *Samples* against the solution obtained from the *Blank*.
Calculate the percentage of the labeled amount of selenium (Se) in the portion of Capsules taken:

$$\text{Result} = (A_U/A_S) \times [(V \times C_S)/M_U] \times 100$$

A_U = absorbance of the cyclohexane layer from the *Sample solution*
A_S = absorbance of the cyclohexane layer from the *Standard solution*
V = volume of the *Standard stock solution* used to prepare the *Standard solution*, 10 mL
C_S = concentration of selenium in the *Standard stock solution* (µg/mL)
M_U = nominal amount of selenium in the *Sample solution* (mg)

Acceptance criteria: 90.0%–160.0% of the labeled amount of selenium (Se)

• **ZINC, *Method 1***
Zinc standard solution: 1000 µg/mL of zinc from zinc oxide dissolved in 5 M hydrochloric acid (3.89 mg/mL), and diluted with water to final volume. [NOTE—Dissolve in 5 M hydrochloric acid by warming if necessary, cool, and then dilute to final volume.]
Standard stock solution: 50 µg/mL of zinc from *Zinc standard solution* diluted with 0.125 N hydrochloric acid
Standard solutions: Transfer 1.0, 2.0, 3.0, 4.0, and 5.0 mL of *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of zinc.
Polysorbate 80 solution: Prepare as directed in *Calcium, Method 1*.
Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain a nominal concentration of 2 µg/mL of zinc and omit the use of the *Lanthanum chloride solution*.
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Lamp: Zinc hollow-cathode
Flame: Air–acetylene
Analytical wavelength: Zinc emission line at 213.8 nm
Blank: 0.125 N hydrochloric acid

Analysis
Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of zinc, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C, in µg/mL, of zinc in the *Sample solution*.
Calculate the percentage of the labeled amount of zinc (Zn) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of zinc in the *Sample solution* (µg/mL)
C_U = nominal concentration of zinc in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of zinc (Zn)

• **BORON, NICKEL, TIN,** and **VANADIUM, *Method 1*; CALCIUM, CHROMIUM, COPPER, IRON, MAGNESIUM, MANGANESE, PHOSPHORUS,** and **ZINC, *Method 2*; MOLYBDENUM** and **SELENIUM, *Method 3***
Stock aqua regia solution: Prepare a mixture of hydrochloric acid and nitric acid (3:1) by adding the nitric acid to the hydrochloric acid. [NOTE—Periodically vent the solution in an appropriate fume hood.]
Diluent: Prepare a mixture of *Stock aqua regia solution* and water (1:9) by adding one volume of *Stock aqua regia solution* to two volumes of water. Dilute with additional water to volume, and mix well.
System suitability solution: Prepare a mixture of 1000 mg/L of yttrium in 5% (v/v) nitric acid solution, and 1000 mg/L of scandium in 5% (v/v) nitric acid solution with *Diluent* (1:1:198), and mix.
Standard stock solution 1 (Ca, Cu, Fe, Mg, Mn, P, and Zn): [NOTE—It is necessary to include only the minerals of interest in the solution.] Using commercially available element standard (single- or multi-element) solutions in 5% (v/v) nitric acid solution, pipet the appropriate amount of element standard solution into a volumetric flask, and dilute with 5% (v/v) nitric acid solution to obtain a solution with final concentrations of about 1000 mg/L of calcium, 100 mg/L of copper, 250 mg/L of iron, 500 mg/L of magnesium, 100 mg/L of manganese, 800 mg/L of phosphorus, and 250 mg/L of zinc.
Standard stock solution 2 (B, Cr, Mo, Ni, Se, Sn, and V): [NOTE—It is necessary to include only the minerals of interest in the solution.] Using commercially available element standard (single- or multi-element) solutions in 20% (v/v) hydrochloric acid solution, pipet the appropriate amount of element standard solution into a volumetric flask, and dilute with 20% (v/v) hydrochloric acid solution to obtain a solution with final concentrations of about 200 mg/L of boron and 100 mg/L each of chromium, molybdenum, nickel, selenium, tin, and vanadium.
Standard solutions: Prepare a mixture of *Standard stock solution 1* and *Standard stock solution 2* as required, in *Diluent*, to prepare a six-point calibration curve to bracket the concentration range of each mineral of interest.
Sample solution: Weigh, then transfer 5 Capsules to a 250-mL volumetric flask, and heat gently on a hot plate until the contents begin to release. Cautiously add 25 mL of *Stock aqua regia solution* in 5-mL increments, and swirl. Heat, continue to swirl until the Capsules dissolve into the acid, immediately remove from the heat source, and add 150 mL of water. Cool, and dilute with water to volume. Filter about 30 mL into a centrifuge tube, using a 5-µm pore

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size nylon syringe filter. If necessary, make any further adjustments using *Diluent*.

Instrumental conditions

(See *Plasma Spectrochemistry* ⟨730⟩.)

Mode: Inductively coupled plasma emission spectrometry, using a spectrometer set to measure the emission of each mineral of interest at about the corresponding wavelength. [NOTE—The operating conditions may be developed and optimized on the basis of the manufacturer's recommendation. The wavelengths selected should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision.]

System suitability

Sample: *System suitability solution*

[NOTE—Analyze the *System suitability solution* and obtain the response as directed for *Analysis*.]

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the emission of each mineral of interest in the *Standard solutions* and *Sample solution* with an inductively coupled plasma system using the *Diluent* as the blank. Plot the emission of the *Standard solutions* versus the concentration, in mg/L, of the minerals of interest, and draw the straight line best fitting the plotted points. From the graph so obtained, determine the concentration, *C*, in mg/L, for each mineral of interest in the *Sample solution*.

Calculate the percentage of the labeled amount for each mineral:

$$\text{Result} = C \times (V/W) \times F \times (C_W/L) \times 100$$

C = measured concentration of the relevant element in the *Sample solution* (mg/L)
V = volume of the *Sample solution* (L)
W = sample weight (mg)
F = dilution factor of the *Sample solution*
C_W = average Capsule weight (mg)
L = labeled amount of the relevant element/Capsule (mg/Capsule)

Acceptance criteria: 90.0%–125.0% of the labeled amount of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), potassium (K), and zinc (Zn); and 90.0%–160.0% of the labeled amounts of boron (B), chromium (Cr), fluorine (F), iodine (I), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), and vanadium (V)

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS,** *Dissolution* ⟨2040⟩**:** Meet the requirements for *Dissolution*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩**:** Meet the requirements

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 3×10^3 cfu/g, and the combined molds and yeasts count does not exceed 3×10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meet the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** The label states that the product is Water-Soluble Vitamins with Minerals Capsules. The label also states the quantity of each vitamin and mineral in terms of metric units per dosage unit and, where necessary, the chemical form in which a vitamin is present, and also states the salt form of the mineral used as the source of each element. Where more than one assay method is given for a particular vitamin or mineral, the labeling states with which assay method the product complies only if *Method 1* is not used.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Biotin RS
USP Calcium Pantothenate RS
USP Cyanocobalamin (Crystalline) RS
USP Dexpanthenol RS
USP Folic Acid RS
USP Niacin RS
USP Niacinamide RS
USP Pyridoxine Hydrochloride RS
USP Riboflavin RS
USP Sodium Fluoride RS
USP Thiamine Hydrochloride RS

Water-Soluble Vitamins with Minerals Oral Solution

DEFINITION

Water-Soluble Vitamins with Minerals Oral Solution contains one or more of the following water-soluble vitamins: Cyanocobalamin, Niacin or Niacinamide, Dexpanthenol or Panthenol, Pantothenic Acid (as Calcium Pantothenate or Racemic Calcium Pantothenate), Pyridoxine Hydrochloride, Riboflavin or Riboflavin-5′-Phosphate Sodium, and Thiamine Hydrochloride or Thiamine Mononitrate; and one or more minerals derived from substances generally recognized as safe, furnishing one or more of the following elements in ionizable form: iodine, iron, magnesium, manganese, and zinc. Oral Solution contains NLT 90.0% and NMT 250.0% of the labeled amount of thiamine ($C_{12}H_{17}ClN_4OS$) as thiamine hydrochloride or thiamine mononitrate; NLT 90.0% and NMT 150.0% of the labeled amounts of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), dexpanthenol or panthenol ($C_9H_{19}NO_4$), niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), and riboflavin ($C_{17}H_{20}N_4O_6$) or riboflavin-5′-phosphate sodium ($C_{17}H_{20}N_4NaO_9P$); NLT 90.0% and NMT 450.0% of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$); NLT 90.0% and NMT 160.0% of the labeled amount of iodine (I); and NLT 90.0% and NMT 125.0% of the labeled amounts of iron (Fe), magnesium (Mg), manganese (Mn), and zinc (Zn).

STRENGTH

[NOTE—In the following assays, where more than one assay method is given for an individual ingredient, the requirements may be met by following any one of the specified methods, the method used being stated in the labeling only if *Method 1* is not used.]

- **CYANOCOBALAMIN**

[NOTE—Use low-actinic glassware throughout this procedure.]

Standard stock solution: 1.0 µg/mL from USP Cyanocobalamin (Crystalline) RS in 25% alcohol. Store in a refrigerator.

Standard solution: Dilute a suitable volume of *Standard stock solution* with water to a measured volume such that after the incubation period as described in *Analysis*, the difference in transmittance between the inoculated blank

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and the 5.0-mL level of the *Standard solution* is NLT that which corresponds to a difference of 1.25 mg in dried cell weight. This concentration usually falls between 0.01 ng/mL and 0.04 ng/mL of *Standard solution*. Prepare this solution fresh for each assay.

Sample solution: Transfer an accurately measured volume of Oral Solution, assumed to contain 1.0 µg of cyanocobalamin, to an appropriate vessel containing, for each mL of the Oral Solution taken, 25 mL of an aqueous extracting solution prepared just before use to contain, in each 100 mL, 1.29 g of dibasic sodium phosphate, 1.1 g of anhydrous citric acid, and 1.0 g of sodium metabisulfite. Autoclave the mixture at 121° for 10 min. Allow any undissolved particles of the extract to settle, and filter or centrifuge if necessary. Dilute an aliquot of the clear solution with water to obtain a final solution containing vitamin B_{12} activity approximately equivalent to that of the *Standard solution*.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and dilute with water to 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature NLT 10°. Filter the solution if a precipitate forms during storage.

Asparagine solution: Dissolve 2.0 g of L-asparagine in water to make 200 mL. Store under toluene in a refrigerator.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat in 10 mL of 4 N hydrochloric acid. Cool, and dilute with water to 200 mL. Store under toluene in a refrigerator.

Xanthine solution: Suspend 0.20 g of xanthine in 30–40 mL of water, heat to 70°, add 6.0 mL of 6 N ammonium hydroxide, and stir until the solid is dissolved. Cool, and dilute with water to 200 mL. Store under toluene in a refrigerator.

Salt solution A: Dissolve 10 g of monobasic potassium phosphate and 10 g of dibasic potassium phosphate in water to make 200 mL, and add 2 drops of hydrochloric acid. Store this solution under toluene.

Salt solution B: Dissolve 4.0 g of magnesium sulfate, 0.20 g of sodium chloride, 0.20 g of ferrous sulfate, and 0.20 g of manganese sulfate in water to make 200 mL, and add 2 drops of hydrochloric acid. Store this solution under toluene.

Polysorbate 80 solution: 20 g of polysorbate 80 in alcohol to make 200 mL. Store in a refrigerator.

Vitamin solution A: 10 mg of riboflavin, 10 mg of thiamine hydrochloride, 100 µg of biotin, and 20 mg of niacin in 0.02 N acetic acid to make 400 mL. Store under toluene protected from light in a refrigerator.

Vitamin solution B: 20 mg of *p*-aminobenzoic acid, 10 mg of calcium pantothenate, 40 mg of pyridoxine hydrochloride, 40 mg of pyridoxal hydrochloride, 8 mg of pyridoxamine dihydrochloride, and 2 mg of folic acid in a mixture of water and neutralized alcohol (3:1) to make 400 mL. Store, protected from light, in a refrigerator.

Basal medium stock solution: Prepare the medium according to the following formula and directions. A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium comparable to that obtained from the formula given herein.

Add the ingredients in the order listed in *Table 1*, carefully dissolving *L-Cystine* and *L-Tryptophan* in the hydrochloric acid before adding the next eight solutions. To the resulting solution add 100 mL of water, and dissolve *Dextrose*, *Sodium acetate*, and *Ascorbic acid*. Filter if necessary. Add the *Polysorbate 80 solution*, adjust with 1 N sodium hydroxide to a pH of 5.5–6.0, and dilute with Purified Water to 250 mL.

Table 1

L-Cystine	0.1 g
L-Tryptophan	0.05 g
1 N hydrochloric acid	10 mL
Adenine–guanine–uracil solution	5 mL
Xanthine solution	5 mL
Vitamin solution A	10 mL
Vitamin solution B	10 mL
Salt solution A	5 mL
Salt solution B	5 mL
Asparagine solution	5 mL
Acid-hydrolyzed casein solution	25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Ascorbic acid	1 g
Polysorbate 80 solution	5 mL

Tomato juice preparation: Centrifuge commercially canned tomato juice so that most of the pulp is removed. Suspend 5 g/L of analytical filter aid in the supernatant, and filter with the aid of reduced pressure through a layer of the filter aid. Repeat, if necessary, until a clear, straw-colored filtrate is obtained. Store under toluene in a refrigerator.

Culture medium: [NOTE—A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium equivalent to that obtained from the formula given herein.] Dissolve 0.75 g of yeast extract, 0.75 g of dried peptone, 1.0 g of *anhydrous Dextrose*, and 0.20 g of monobasic potassium phosphate in 60–70 mL of water. Add 10 mL of *Tomato juice preparation* and 1 mL of *Polysorbate 80 solution*. Adjust with 1 N sodium hydroxide to a pH of 6.8, and dilute with water to 100 mL. Place 10-mL portions of the solution in test tubes, and plug with cotton. Sterilize the tubes and contents in an autoclave at 121° for 15 min. Cool as rapidly as possible to avoid color formation resulting from overheating the medium.

Suspension medium: Dilute a measured volume of the *Basal medium stock solution* with an equal volume of water. Place 10-mL portions of the diluted medium in test tubes. Sterilize, and cool as directed in *Culture medium*.

Stock culture of *Lactobacillus leichmannii*: To 100 mL of the *Culture medium* add 1.0–1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Place 10-mL portions of the hot solution in test tubes, cover the tubes, sterilize at 121° for 15 min in an autoclave, and allow the tubes to cool in an upright position. Inoculate three or more of the tubes by stab transfer of a pure culture of *Lactobacillus leichmannii*.[1] [NOTE—Before first using a fresh

[1] Pure cultures of *Lactobacillus leichmannii* (listed as *Lactobacillus delbrueckii*) may be obtained as No. 7830 from ATCC, 10801 University Blvd., Manassas, VA 20110-2209 (www.atcc.org).

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culture in this assay make NLT 10 successive transfers of the culture in a 2-week period.]

Incubate for 16–24 h at a temperature between 30° and 40° held constant to within ±0.5°. Store in a refrigerator.

Prepare fresh stab cultures at least three times each week, and do not use them for preparing the *Inoculum* if more than 4 days old. The activity of the microorganism can be increased by daily or twice-daily transfer of the stab culture to the point where definite turbidity in the liquid *Inoculum* can be observed 2–4 h after inoculation. A slow-growing culture seldom gives a suitable response curve and may lead to erratic results.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus leichmannii* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Transfer cells from the *Stock culture of Lactobacillus leichmannii* to two sterile tubes each containing 10 mL of the *Culture medium*. Incubate these cultures for 16–24 h at a temperature between 30° and 40° held constant to within ±0.5°. Under aseptic conditions centrifuge the cultures, and decant the supernatant. Suspend the cells from the culture in 5 mL of sterile *Suspension medium*, and combine. Using sterile *Suspension medium*, adjust the volume so that a 1-in-20 dilution in saline TS produces 70% transmittance when read on a suitable spectrophotometer that has been set at a wavelength of 530 nm, equipped with a 10-mm cell, and read against saline TS set at 100% transmittance. Prepare a 1-in-400 dilution of the adjusted suspension using sterile *Basal medium stock solution*. [NOTE—This dilution may be altered, when necessary, to obtain the desired test response.] The cell suspension so obtained is the *Inoculum*.

Calibration of spectrophotometer: Check the wavelength of the spectrophotometer periodically using a standard wavelength cell or other suitable device. Before reading any tests, calibrate the spectrophotometer for 0% and 100% transmittance using water and with the wavelength set at 530 nm.

Analysis

Samples: *Standard solution* and *Sample solution*

Because of the high sensitivity of the test organism to minimum amounts of vitamin B_{12} activity and to traces of many cleansing agents, cleanse meticulously by suitable means followed preferably by heating at 250° for 2 h using hard-glass 20-mm × 150-mm test tubes and other necessary glassware.

To separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each of these tubes and to four similar empty tubes add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes to prevent bacterial contamination, and sterilize in an autoclave at 121° for 5 min, arranging to reach this temperature in NMT 10 min by preheating the autoclave if necessary. Cool as rapidly as possible to avoid color formation resulting from overheating the medium. Take precautions to maintain uniformity of sterilizing and cooling conditions throughout the assay, because packing the tubes too closely in the autoclave or overloading it may cause variation in the heating rate.

Aseptically add 0.5 mL of the *Inoculum* to each tube so prepared, except two of the four containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 40°, held constant to within ±0.5° for 16–24 h.

Terminate growth by heating to a temperature NLT 80° for 5 min. Cool to room temperature. After agitating contents read the transmittance at 530 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of the inoculated blank. If the difference is greater than 5% or if there is evidence of contamination with a foreign microorganism, disregard the results of the assay.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of each of the remaining tubes. Disregard the results of the assay if the slope of the standard curve indicates a problem with sensitivity.

Calculation: Prepare a standard concentration-response curve by the following procedure. Test for and replace any aberrant individual transmittances. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittances (Σ_S) as the difference, $y = 2.00 - \Sigma_S$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of the *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_U$, adding together the two transmittances (Σ_U) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X, for each dosage level. Average the values of X for each of three or more dosage levels to obtain $\overline{X}$, which equals the log-relative potency, M', of the *Sample solution*.

Determine the quantity, in µg, of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$) in the portion of Oral Solution taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = µg of cyanocobalamin assumed to be present in the portion of Oral Solution taken

Calculate the percentage of the labeled amount of cyanocobalamin in the portion of Oral Solution taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of cyanocobalamin in the portion of Oral Solution taken

Replication: Repeat the entire determination at least once using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, $\overline{M}$, is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* ⟨111⟩, *The Confidence Interval and Limits of Potency, Vitamin B_{12} Activity*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not

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differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–450.0% of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$)

• **CALCIUM PANTOTHENATE, *Method 1***

Mobile phase: Methanol and 0.2 M monobasic sodium phosphate (3:97). Adjust with 1.7 M phosphoric acid to a pH of 3.2 ± 0.1.

Standard solution: 80 µg/mL of USP Calcium Pantothenate RS in *Mobile phase*

System suitability solution: 80 µg/mL of USP Racemic Panthenol RS in *Mobile phase*. Mix the resulting solution and *Standard solution* (1:1).

Sample solution: Equivalent to 80 µg/mL of calcium pantothenate from Oral Solution in the *Mobile phase*

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 210 nm

Column: 4.0-mm × 10-cm; packing L1

Flow rate: 1 mL/min

Injection volume: 20 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 1.5 between panthenol and calcium pantothenate, *System suitability solution*

Tailing factor: NMT 2.0 for both the calcium pantothenate and the panthenol peaks, *System suitability solution*

Relative standard deviation: NMT 2.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of calcium pantothenate from the *Sample solution*

r_S = peak area of calcium pantothenate from the *Standard solution*

C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• **CALCIUM PANTOTHENATE, *Method 2***

Standard stock solution: Dissolve 50 mg of USP Calcium Pantothenate RS, previously dried and stored in the dark over phosphorus pentoxide and protected from absorption of moisture while weighing, in 500 mL of water in a 1000-mL volumetric flask. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), and dilute with water to volume to obtain a concentration of 50 µg/mL of USP Calcium Pantothenate RS. Store under toluene in a refrigerator.

Standard solution: On the day of the assay, dilute a volume of the *Standard stock solution* with water to obtain a concentration of 0.01–0.04 µg/mL of calcium pantothenate, the exact concentration being such that the responses obtained as directed in *Analysis*, 2.0 and 4.0 mL of the *Standard solution* being used, are within the linear portion of the log-concentration response curve.

Sample solution: Transfer an accurately measured volume of Oral Solution, equivalent to 50 mg of calcium pantothenate, to a 1000-mL volumetric flask containing 500 mL of water. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), dilute with water to volume, and filter. Dilute a measured volume of this solution quantitatively, and stepwise if necessary, with water to obtain a solution with about the same concentration as that of the *Standard solution*.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and dilute with water to 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a refrigerator at a temperature NLT 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and dilute with water to 1000 mL. Store under toluene in a refrigerator at a temperature NLT 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat in 10 mL of 4 N hydrochloric acid. Cool, and dilute with water to 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin–thiamine hydrochloride–biotin solution: 20 µg/mL of riboflavin, 10 µg/mL of thiamine hydrochloride, and 0.04 µg/mL of biotin in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

***p*-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution:** 10 µg/mL of *p*-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Basal medium stock solution: Dissolve the *anhydrous Dextrose* and *anhydrous Sodium acetate* in the solutions previously mixed according to *Table 2*, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Table 2

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Riboflavin–thiamine hydrochloride–biotin solution	5 mL

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Table 2 *(continued)*

p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water; add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar; and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to the test tubes, close or cover the tubes, sterilize in an autoclave at 121° for 15 min, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes using a pure culture of *Lactobacillus plantarum*,[2] incubating for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for the *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of the *Basal medium stock solution* add 5.0 mL of water containing 0.2 µg of calcium pantothenate. Plug the tubes with cotton, sterilize in an autoclave at 121° for 15 min, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Transfer cells from the *Stock culture of Lactobacillus plantarum* to a sterile tube containing 10 mL of the *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. The cell suspension so obtained is the *Inoculum*.

Analysis

Samples: *Standard solution* and *Sample solution*

To similar separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to four similar empty tubes add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution* including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool, add 1 drop of the *Inoculum* to each tube, except two of the four tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37°, held constant to within ±0.5°, following 16–24 h of incubation, until there has been no substantial increase in turbidity in the tubes containing the highest level of Standard during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to an optical container if necessary. Read the transmittance between 540 and 660 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the Standard calculate the response from the sum of the duplicate values of the transmittance (Σ_S) as the difference, $y = 2.00 - \Sigma_S$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of the *Standard solution* per tube on the abscissa using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_U$, adding together the two transmittances (Σ_U) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X, for each dosage level. Average the values of X for each of three or more dosage levels to obtain $\overline{X}$, which equals the log-relative potency, M', of the *Sample solution*.

Determine the quantity, in mg, of USP Calcium Pantothenate RS corresponding to the calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Oral Solution taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of mg of calcium pantothenate assumed to be present in the portion of Oral Solution taken

Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Oral Solution taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of calcium pantothenate in the portion of Oral Solution (mg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, $\overline{M}$, is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* ⟨111⟩, *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• **DEXPANTHENOL OR PANTHENOL, *Method 1***

Mobile phase and **Chromatographic system:** Proceed as directed in *Calcium Pantothenate, Method 1*.

Standard solution: 80 µg/mL of USP Dexpanthenol RS or USP Racemic Panthenol RS in the *Mobile phase*. [NOTE—Use USP Dexpanthenol RS to analyze Oral Solution that

[2] ATCC No. 8014 is suitable. This strain was formerly known as *Lactobacillus arabinosus* 17-5.

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contains dexpanthenol, and use USP Racemic Panthenol RS to analyze Oral Solution that contains panthenol.]

System suitability solution: 80 µg/mL of USP Calcium Pantothenate RS in the *Mobile phase*. Mix the resulting solution and the *Standard solution* (1:1).

Sample solution: Equivalent to 80 µg/mL of dexpanthenol or panthenol from Oral Solution in the *Mobile phase*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of dexpanthenol or panthenol ($C_9H_{19}NO_4$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of dexpanthenol or panthenol from the *Sample solution*

r_S = peak area of dexpanthenol or panthenol from the *Standard solution*

C_S = concentration of USP Dexpanthenol RS or USP Racemic Panthenol RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of dexpanthenol or panthenol ($C_9H_{19}NO_4$)

• **DEXPANTHENOL OR PANTHENOL, *Method 2***

[NOTE—The following procedure is applicable also to the determination of the dextrorotatory component of racemic panthenol in preparations containing panthenol.]

Dehydrated mixtures yielding formulations similar to the media described herein may be used provided that, when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described herein.

Standard stock solution: 800 µg/mL of USP Dexpanthenol RS or 1600 µg/mL of USP Racemic Panthenol RS in water. Store in a refrigerator, protected from light, and use within 30 days. [NOTE—Use USP Dexpanthenol RS to analyze Oral Solution that contains dexpanthenol, and use USP Racemic Panthenol RS to analyze Oral Solution that contains panthenol.]

Standard solution: On the day of the assay, prepare 1.2 µg/mL of dexpanthenol or 2.4 µg/mL of racemic panthenol from the *Standard stock solution* with water.

Sample solution: 1.2 µg/mL of dexpanthenol or 2.4 µg/mL of panthenol from Oral Solution in water

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid (1 in 2), and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in about 500 mL of water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and dilute with water to 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a refrigerator at a temperature NLT 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to 75 ± 5°, and add 6 M hydrochloric acid dropwise, with stirring, until the solids are dissolved. Cool, dilute with water to 1000 mL, and mix. Store under toluene in a refrigerator at a temperature NLT 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, add dilute with to 200 mL, and mix. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin–thiamine hydrochloride–biotin solution: Prepare a solution of riboflavin, thiamine hydrochloride, and biotin in 0.02 N acetic acid containing 20 µg/mL of riboflavin, 10 µg/mL of thiamine hydrochloride, and 0.04 µg/mL of biotin. Store under toluene protected from light in a refrigerator.

***p*-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution:** Prepare a solution in neutral 25% alcohol containing 10 µg/mL of *p*-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride. Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid, and mix. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid, and mix. Store under toluene.

Pyridoxal–calcium pantothenate solution: Dissolve 40 mg of pyridoxal hydrochloride and 375 µg of calcium pantothenate in 10% alcohol to make 200 mL, and mix. Store in a refrigerator, and use within 30 days.

Polysorbate 40–oleic acid solution: Dissolve 25 g of polysorbate 40 and 0.25 g of oleic acid in 20% alcohol to make 500 mL, and mix. Store in a refrigerator, and use within 30 days.

Modified pantothenate medium: Dissolve *anhydrous Dextrose* and *Sodium acetate* in the solutions previously mixed according to *Table 3*, and adjust with 1 N sodium hydroxide to a pH of 6.8. Finally, dilute with water to 250 mL, and mix.

Table 3

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Riboflavin–thiamine hydrochloride–biotin solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution 1	5 mL
Salt solution 2	5 mL
Pyridoxal–calcium pantothenate solution	5 mL
Polysorbate 40–oleic acid solution	5 mL

Double-strength modified pantothenate medium: Prepare as directed in *Modified pantothenate medium*, but make the final dilution to 125 mL instead of 250 mL. Prepare fresh.

Stock culture of *Pediococcus acidilactici*: Dissolve in 800 mL of water, with the aid of heat, 6.0 g of peptone, 4.0 g of pancreatic digest of casein, 3.0 g of yeast extract, 1.5 g of beef extract, 1.0 g of dextrose, and 15.0 g of agar. Adjust with 0.1 N sodium hydroxide or 0.1 N hydrochloric acid to a pH between 6.5 and 6.6, dilute with water to 1000 mL, and mix. Add 10-mL portions of the solution to culture

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tubes, place caps on the tubes, and sterilize in an autoclave at 121° for 15 min. Cool on a slant, and store in a refrigerator. Prepare a stock culture of *Pediococcus acidilactici*[3] on a slant of this medium. Incubate at 35° for 20–24 h, and store in a refrigerator. Maintain the stock culture by monthly transfer onto fresh slants.

Inoculum: Inoculate three 250-mL portions of sterile *Modified pantothenate medium* from a stock culture slant, and incubate at 35° for 20–24 h. Centrifuge the suspension from the combined portions, and wash the cells with the *Modified pantothenate medium*. Resuspend the cells in sufficient sterile *Modified pantothenate medium* so that a 1-in-50 dilution, when tested in a 13-mm diameter test tube, gives 80% light transmission at 530 nm. Transfer 1.2-mL portions of this stock suspension to sterile glass ampuls, seal, freeze in liquid nitrogen, and store in a freezer. On the day of the assay, allow the ampuls to reach room temperature, mix the contents, and dilute 1 mL of thawed culture with sterile saline TS to 150 mL. [NOTE—This dilution may be altered when necessary to obtain the desired test response.]

Analysis: Prepare in triplicate a series of eight culture tubes by adding the following quantities of water to the tubes within a set: 5.0, 4.5, 4.0, 3.5, 3.0, 2.0, 1.0, and 0.0 mL. To these same tubes, and in the same order, add 0.0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. Prepare in duplicate a series of five culture tubes by adding the following quantities of water to the tubes within a set: 4.0, 3.5, 3.0, 2.0, and 1.0 mL. To these same tubes, and in the same order, add 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*.

Add 5.0 mL of *Double-strength modified pantothenate medium* to each tube, and mix. Cover the tubes with metal caps, and sterilize in an autoclave at 121° for 5 min. Cool to room temperature in a chilled water bath, and inoculate each tube with 0.5 mL of the *Inoculum*. Allow to incubate at 37° for 16 h. Terminate growth by heating to a temperature NLT 80°, such as by steaming at atmospheric pressure in a sterilizer for 5–10 min. Cool, and concomitantly determine the percentage transmittance of the suspensions, in cells of equal pathlength, on a suitable spectrophotometer, at a wavelength of 530 nm.

Calculation: Draw a dose-response curve on arithmetic graph paper by plotting the average response, in percent transmittance, for each set of tubes of the standard curve against the standard level concentrations. The curve is drawn by connecting each adjacent pair of points with a straight line. From this standard curve, determine by interpolation the potency, in terms of dexpanthenol, of each tube containing portions of the *Sample solution*. Divide the potency of each tube by the amount of the *Sample solution* added to it to obtain the individual responses. Calculate the mean response by averaging the individual responses that vary from their mean by NMT 15%, using NLT half the total number of tubes. Calculate the potency of the portion of the Oral Solution taken for assay, by multiplying the mean response by the appropriate dilution factor.

Calculate the percentage of the labeled amount of dexpanthenol or panthenol in the portion of Oral Solution taken:

$$\text{Result} = (P/N) \times 100$$

P = calculated potency of dexpanthenol or panthenol in the portion of Oral Solution taken (mg)

N = nominal amount of dexpanthenol or panthenol in the portion of Oral Solution taken (mg)

Acceptance criteria: 90.0%–150.0% of the labeled amount of dexpanthenol or panthenol ($C_9H_{19}NO_4$)

• **NIACIN OR NIACINAMIDE**

[NOTE—Use low-actinic glassware throughout this procedure.]

Diluent: 25 mg/mL of edetate disodium in water

Mobile phase: Methanol, glacial acetic acid, triethylamine, and 0.008 M sodium 1-hexanesulfonate (350: 15: 0.4: 1634.6)

Standard solution: 0.10 mg/mL of USP Niacin RS or USP Niacinamide RS in the *Diluent*. [NOTE—Use USP Niacin RS for Oral Solution that contains niacin, and use USP Niacinamide RS for Oral Solution that contains niacinamide.]

Sample solution: Dilute an accurately measured volume of Oral Solution with *Diluent* to obtain a solution with a concentration of 0.1 mg/mL of niacin or niacinamide.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 270 nm

Column: 4.6-mm × 25-cm; packing L7

Flow rate: 2 mL/min

Injection volume: 5 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacin or niacinamide from the *Sample solution*

r_S = peak area of niacin or niacinamide from the *Standard solution*

C_S = concentration of USP Niacin RS or USP Niacinamide RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacin or niacinamide in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$)

• **PYRIDOXINE HYDROCHLORIDE**

Diluent, Mobile phase, and **Chromatographic system:** Proceed as directed in *Niacin or Niacinamide*.

Standard solution: Equivalent to 24 µg/mL of USP Pyridoxine Hydrochloride RS in the *Diluent*

Sample solution: Equivalent to 24 µg/mL of pyridoxine hydrochloride from Oral Solution in the *Diluent*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of pyridoxine hydrochloride from the *Sample solution*

[3] ATCC No. 8042 is suitable.

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r_S = peak area of pyridoxine hydrochloride from the *Standard solution*
C_S = concentration of USP Pyridoxine Hydrochloride RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of pyridoxine hydrochloride in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$)

• **RIBOFLAVIN OR RIBOFLAVIN-5′-PHOSPHATE SODIUM, *Method 1***

[NOTE—Riboflavin-5′-phosphate sodium is quantitated against USP Riboflavin RS in this procedure. In the chromatogram of the *Sample solution*, the riboflavin-5′-phosphate peak is the only peak measured for calculation.]

Diluent, Mobile phase, and **Chromatographic system:** Proceed as directed in *Niacin or Niacinamide*.

[NOTE—The relative retention times for riboflavin-5′-phosphate and riboflavin are about 0.18 and 1.0, respectively.]

Standard solution: Equivalent to 8 µg/mL of USP Riboflavin RS in the *Diluent*, by heating if necessary

Sample solution: Equivalent to 8 µg/mL of riboflavin from Oral Solution in the *Diluent*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of riboflavin-5′-phosphate from the *Sample solution*
r_S = peak area of riboflavin from the *Standard solution*
C_S = concentration of USP Riboflavin RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of riboflavin in the *Sample solution* (mg/mL)
F = factor for converting the response obtained for riboflavin-5′-phosphate to riboflavin, 1.493. [NOTE—Riboflavin Phosphate Sodium is a mixture of isomeric monophosphates and diphosphates containing an average amount of 67% of riboflavin-5′-monophosphate, which separates in this chromatographic system. The factor 1.493 assumes 67% of riboflavin-5′-monophosphate.]

Acceptance criteria: 90.0%–150.0% of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$)

• **RIBOFLAVIN OR RIBOFLAVIN-5′-PHOSPHATE SODIUM, *Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Solvent blank: 1 N hydrochloric acid, 2.5 M sodium acetate, and water (1:2:97)

Riboflavin stock solution: Transfer 0.16 mg/mL of USP Riboflavin RS in 1 N hydrochloric acid, 2.5 M sodium acetate, and water (1:2:97). Mix the resulting solution and water (1:9).

Standard solution: *Riboflavin stock solution*, 1 N hydrochloric acid, 2.5 M sodium acetate, and water (1:1:2:96)

Sample solution: Transfer the equivalent to 0.8 mg of riboflavin from the Oral Solution to a 100-mL volumetric flask, and dilute with water to volume. Mix the resulting solution, 1 N hydrochloric acid, 2.5 M sodium acetate, and water (2:1:2:95).

Instrumental conditions

(See *Fluorescence Spectroscopy* ⟨853⟩.)

Mode: Fluorescence

Analytical wavelengths

Excitation: 440 nm

Emission: 530 nm

Blank: *Solvent blank*

Analysis

Samples: *Standard solution* and *Sample solution*

Determine the maximum fluorescence intensities, I_S and I_U, of the *Standard solution* and the *Sample solution*, respectively.

Calculate the percentage of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$) in the portion of Oral Solution taken:

$$\text{Result} = (I_U/I_S) \times (C_S/C_U) \times 100$$

I_U = fluorescence intensity from the *Sample solution*
I_S = fluorescence intensity from the *Standard solution*
C_S = concentration of USP Riboflavin RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of riboflavin in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$)

• **THIAMINE**

Diluent, Mobile phase, and **Chromatographic system:** Proceed as directed in *Niacin or Niacinamide*.

Standard solution: Equivalent to 24 µg/mL of USP Thiamine Hydrochloride RS in the *Diluent*

Sample solution: Equivalent to 24 µg/mL of thiamine hydrochloride from Oral Solution in the *Diluent*

Analysis

Samples: *Standard solution* and *Sample solution*

For products containing thiamine hydrochloride, calculate the percentage of the labeled amount of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of thiamine from the *Sample solution*
r_S = peak area of thiamine from the *Standard solution*
C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of thiamine hydrochloride in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–250.0% of the labeled amount of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$)

• **IODIDE**

Mobile phase: Dissolve 5.15 g of tetrabutylammonium bromide in 320 mL of acetonitrile. Dilute with water to 2000 mL.

Standard stock solution: 1.3 mg/mL of potassium iodide in the *Mobile phase*. This solution has a concentration of 1 mg/mL of iodide.

Standard solution: 2.5 µg/mL of iodide from the *Standard stock solution* in the *Mobile phase*

System suitability solution: Transfer 0.13 g of potassium iodide and 0.5 g of potassium iodate to a 100-mL volumetric flask. Dissolve in the *Mobile phase*, using sonication if necessary, dilute with the *Mobile phase* to volume, and mix. Transfer 1.0 mL of this solution to a 100-mL volumetric flask, dilute with the *Mobile phase* to

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volume, and mix. Transfer 25.0 mL of this solution to a 100-mL volumetric flask, and dilute with the *Mobile phase* to volume.

Sample solution: Dilute an accurately measured volume of Oral Solution to obtain a solution with a concentration of 2.5 µg/mL of iodine in the *Mobile phase.*

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability.*)

Mode: LC

Detector: UV 225 nm

Column: 4.6-mm × 15-cm; packing L1

Flow rate: 1.5 mL/min

Injection volume: 30 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

[NOTE—The relative retention times for iodate and iodide are about 0.32 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 2.5 between iodate and iodide, *System suitability solution*

Relative standard deviation: NMT 2.0% for the iodide peak, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of iodine (I) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of iodide from the *Sample solution*

r_S = peak area of iodide from the *Standard solution*

C_S = concentration of iodide in the *Standard solution* (µg/mL)

C_U = nominal concentration of iodine in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of iodine (I)

• **IRON**

Iron standard stock solution: Transfer 100 mg of iron powder to a 1000-mL volumetric flask, dissolve in 6 N hydrochloric acid, and dilute with water to volume.

Standard solutions: To separate 100-mL volumetric flasks, transfer 2.0, 4.0, 5.0, 6.0, and 8.0 mL of the *Iron standard stock solution.* Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 2.0, 4.0, 5.0, 6.0, and 8.0 µg/mL of iron.

Sample solution: 6 µg/mL of iron from Oral Solution in 0.125 N hydrochloric acid

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometer

Lamp: Iron hollow-cathode

Flame: Air–acetylene

Analytical wavelength: 248.3 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank.* Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of iron, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of iron in the *Sample solution.*

Calculate the percentage of the labeled amount of iron (Fe) in the portion of Oral Solution taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of iron in the *Sample solution* from the graph (µg/mL)

C_U = nominal concentration of iron in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of iron (Fe)

• **MAGNESIUM**

Magnesium standard solution: Transfer 1.00 g of magnesium ribbon to a 1000-mL volumetric flask. Dissolve in 50 mL of 6 N hydrochloric acid, and dilute with water to volume.

Standard stock solution: 20 µg/mL of magnesium from the *Magnesium standard solution* in 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks, transfer 5.0, 7.5, 10.0, 12.5, and 15.0 mL of the *Standard stock solution.* Dilute with 0.125 N hydrochloric acid to volume to obtain concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 µg/mL of magnesium.

Sample solution: 2.5 µg/mL of magnesium from Oral Solution in 0.125 N hydrochloric acid

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometer

Lamp: Magnesium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: 285.2 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank.* Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of magnesium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of magnesium in the *Sample solution.*

Calculate the percentage of the labeled amount of magnesium (Mg) in the portion of Oral Solution taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of magnesium in the *Sample solution* from the graph (µg/mL)

C_U = nominal concentration of magnesium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of magnesium (Mg)

• **MANGANESE**

Manganese standard stock solution: Transfer 1.0 g of manganese to a 1000-mL volumetric flask. Dissolve in 20 mL of nitric acid, and dilute with 6 N hydrochloric acid to volume.

Standard stock solution: 50 µg/mL of manganese from the *Manganese standard stock solution* in 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Standard stock solution.* Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions with known concentrations of 0.5, 0.75, 1.0, 1.5, and 2.0 µg/mL of manganese.

Sample solution: Dilute an accurately measured volume of Oral Solution to obtain a solution with a concentration of 1.5 µg/mL of manganese in 0.125 N hydrochloric acid.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometer

Lamp: Manganese hollow-cathode

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Flame: Air–acetylene
Analytical wavelength: 279.5 nm
Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of manganese, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of manganese in the *Sample solution*.

Calculate the percentage of the labeled amount of manganese (Mn) in the portion of Oral Solution taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of manganese in the *Sample solution* from the graph (µg/mL)
C_U = nominal concentration of manganese in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% labeled amount of manganese (Mn)

- **ZINC**

Zinc standard solution: Transfer 311 mg of zinc oxide to a 250-mL volumetric flask, and add 80 mL of 6 N hydrochloric acid, warming if necessary to dissolve. Cool, dilute with water to volume, and mix to obtain a solution with a known concentration of 1000 µg/mL of zinc.

Standard stock solution: 50 µg/mL of zinc from the *Zinc standard solution* in 0.125 N hydrochloric acid

Standard solutions: Transfer 1.0, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of zinc.

Sample solution: Dilute an accurately measured volume of Oral Solution to obtain a solution with a concentration of 1 µg/mL of zinc in 0.125 N hydrochloric acid.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometer
Lamp: Zinc hollow-cathode
Flame: Air–acetylene
Analytical wavelength: 213.8 nm
Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of zinc, and draw the straight line best fitting the five plotted points. From the graph so obtained determine the concentration, *C*, in µg/mL, of zinc in the *Sample solution*.

Calculate the percentage of the labeled amount of zinc (Zn) in the portion of Oral Solution taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of zinc in the *Sample solution* from the graph (µg/mL)
C_U = nominal concentration of zinc in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of zinc (Zn)

OTHER COMPONENTS

- **ALCOHOL DETERMINATION,** *Method I* ⟨611⟩ (if present): 90.0%–120.0% of the labeled amount of alcohol (C_2H_5OH)

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 3×10^3 cfu/mL, and the combined molds and yeasts count does not exceed 3×10^2 cfu/mL.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, under an inert gas or with a minimum of headspace.
- **LABELING:** The label states that the product is Water-Soluble Vitamins with Minerals Oral Solution. The label states the quantity of each vitamin and mineral present in terms of metric units in a given volume of the Oral Solution and, where necessary, the chemical form in which a vitamin is present, and also states the salt form of the mineral used as the source of each element. Where products are labeled to contain panthenol, the label states the equivalent content of dexpanthenol. Where more than one assay method is given for a particular vitamin or mineral, the labeling states with which assay method the product complies only if *Method 1* is not used.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Calcium Pantothenate RS
  USP Cyanocobalamin (Crystalline) RS
  USP Dexpanthenol RS
  USP Niacin RS
  USP Niacinamide RS
  USP Pyridoxine Hydrochloride RS
  USP Racemic Panthenol RS
  USP Riboflavin RS
  USP Thiamine Hydrochloride RS

Water-Soluble Vitamins with Minerals Tablets

DEFINITION

Change to read:

Water-Soluble Vitamins with Minerals Tablets contain one or more of the following water-soluble vitamins: Ascorbic Acid or its equivalent as Calcium Ascorbate or Sodium Ascorbate, Biotin, Cyanocobalamin, Folic Acid, Niacin or Niacinamide, pantothenic acid (as Calcium Pantothenate or Racemic Calcium Pantothenate), Pyridoxine Hydrochloride, Riboflavin, and Thiamine Hydrochloride or Thiamine Mononitrate; and one or more minerals derived from substances generally recognized as safe, furnishing one or more of the following elements in ionizable form: boron, calcium, chromium, copper, fluorine, iodine, iron, magnesium, manganese, molybdenum, nickel, phosphorus, potassium, selenium, tin, vanadium, and zinc. Tablets contain NLT 90.0% and NMT 150.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$), biotin ($C_{10}H_{16}N_2O_3S$), cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$), folic acid ($C_{19}H_{19}N_7O_6$), calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), pyridoxine

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▲▲ (USP 1-May-2020) ($C_8H_{11}NO_3 \cdot$ ▲▲ (USP 1-May-2020)), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine ▲as thiamine ion ($C_{12}H_{17}N_4OS^+$);▲ (USP 1-May-2020) NLT 90.0% and NMT 125.0% of the labeled amount of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), potassium (K), and zinc (Zn); and NLT 90.0% and NMT 160.0% of the labeled amount of boron (B), chromium (Cr), fluorine (F), iodine (I), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), and vanadium (V).

They do not contain any form of Beta Carotene or vitamin A, D, E, or K. They may contain other labeled added substances that are generally recognized as safe, in quantities that are unobjectionable.

IDENTIFICATION

Add the following:

▲• **A.** Characteristic absorption of each mineral as obtained in the tests in *Strength*▲ (USP 1-May-2020)

Add the following:

▲• **B.** The retention times of the vitamin peaks of the *Sample solutions* correspond to those of the corresponding vitamin peaks of the *Standard solutions* as obtained in the tests for *Strength*.▲ (USP 1-May-2020)

STRENGTH

[NOTE—In the following assays, where more than one assay method is given for an individual ingredient, the requirements may be met by following any one of the specified methods, with the method used being stated in the labeling only if *Method 1* is not used.]

Change to read:

• **ASCORBIC ACID, CALCIUM ASCORBATE,** and **SODIUM ASCORBATE**

(See *Vitamin C Assay* ⟨580⟩.)

[NOTE—For labeling purposes, consider ⟨580⟩, *Method I—Titrimetric Method* as *Method 1*; ▲⟨580⟩, *Method II—Chromatographic Method, Procedure 1* as *Method 2*; and ⟨580⟩, *Method II—Chromatographic Method, Procedure 2* as *Method 3*.▲ (USP 1-May-2020)]

Acceptance criteria: 90.0%–150.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$)

• **BIOTIN,** ***Method 1***

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Mix 85 mL of acetonitrile, 1 g of sodium perchlorate, and 1 mL of phosphoric acid, and dilute with water to 1000 mL.

Standard stock solution: 0.333 mg/mL of USP Biotin RS in dimethyl sulfoxide

Standard solution: 5 µg/mL of USP Biotin RS prepared by diluting the *Standard stock solution* in water

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of powder, equivalent to a nominal amount of 1 mg of biotin, to a 200-mL volumetric flask. Add 3 mL of dimethyl sulfoxide, and swirl to wet the contents. Place the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with water to volume, and filter.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 200 nm

Column: 4.6-mm × 15-cm; 3-µm packing L7

Flow rate: 1.2 mL/min

Injection volume: 100 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the responses for the biotin peaks. Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Biotin RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of biotin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

• **BIOTIN,** ***Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Dehydrated mixtures yielding formulations similar to the media described herein may be used provided that when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described herein.

Standard stock solution: 50 µg/mL of USP Biotin RS in 50% alcohol. Store in a refrigerator.

Standard solution: On the day of the assay, dilute the *Standard stock solution* with water to a concentration of 0.1 ng/mL of USP Biotin RS.

Sample solution: Finely powder NLT 30 Tablets. Transfer a portion of the powder, equivalent to a nominal amount of 100 µg of biotin, to a 200-mL volumetric flask. Add 3 mL of 50% alcohol, and swirl to wet the contents. Heat the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with diluted alcohol to volume, and filter. Dilute a volume of the filtrate quantitatively, and stepwise if necessary, with water to obtain a solution with a concentration of 0.1 ng/mL.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and dilute with water to 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature NLT 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water. Heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and dilute with water to 1000 mL. Store under toluene in a cool place at a temperature NLT 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and dilute with water to 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

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Calcium pantothenate solution: 10 µg/mL of calcium pantothenate in 50% alcohol. Store in a refrigerator.

Riboflavin–thiamine hydrochloride solution: 20 µg/mL of riboflavin and 10 µg/mL of thiamine hydrochloride in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

***p*-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution:** 10 µg/mL of *p*-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid, and mix. Store under toluene.

Basal medium stock solution: Dissolve the anhydrous dextrose and anhydrous sodium acetate in the solutions previously mixed according to *Table 1*, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Table 1

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Calcium pantothenate solution	5 mL
Riboflavin–thiamine hydrochloride solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water. Add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to test tubes, close or cover the tubes, sterilize in an autoclave at 121°, and allow the tubes to cool in an upright position. Prepare stab cultures in 3 or more of the tubes, using a pure culture of *Lactobacillus plantarum*,[1] incubating for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution* add 5.0 mL of water containing 0.5 ng of biotin. Plug the tubes with cotton, sterilize in an autoclave at 121°, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Transfer cells from the *Stock culture of Lactobacillus plantarum* to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. The cell suspension is the *Inoculum*.

Analysis

Samples: *Standard solution* and *Sample solution*

To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to 4 similar but empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar test tubes add, in duplicate, volumes of the *Sample solution* corresponding to 3 or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool. Add 1 drop of *Inoculum* to each tube, except 2 of the 4 tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37° held constant to within ±0.5° until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of Standard during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to a spectrophotometer cell. Place the cell in a spectrophotometer that has been set at a specific wavelength of 540–660 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittance (Σ) as the difference, $y = 2.00 - \Sigma$. Plot this response on the ordinate of cross-section paper against the logarithm of the milliliter of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, y, adding together the two transmittances for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that fall within the range of lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X, for each dosage level. Average the values of X for each of 3 or more dosage levels to obtain $\bar{X}$, which equals the log-relative potency, M', of the *Sample solution*.

Determine the quantity, in µg, of USP Biotin RS corresponding to the biotin in each mg of the portion of Tablets taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

[1] ATCC #8014 is suitable. This strain was formerly known as *Lactobacillus arabinosus* 17-5.

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R = amount of biotin assumed to be present in each mg in the portion of Tablets taken (µg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, $\overline{M}$, is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* ⟨111⟩, *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of 2 or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

• **BIOTIN, *Method 3***

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A: Transfer 800 mL of water and 100 mL of triethylamine to a 1000-mL volumetric flask. Add 80 mL of 85% phosphoric acid, and dilute with water to volume.

Mobile phase: Transfer 80 mL of acetonitrile and 10 mL of *Solution A* to a 1000-mL volumetric flask. Dilute with water to volume.

Standard solution: 0.6 µg/mL of USP Biotin RS in water. [NOTE—A portion of the *Standard solution* will be used to determine the percent recovery of biotin from the *Solid-phase extraction* procedure.]

Sample solution: Finely powder NLT 20 Tablets. Transfer an amount of powdered Tablets to a volumetric flask to obtain a nominal concentration of 0.6 µg/mL of biotin. Add water up to 80% of the flask capacity, and sonicate for 30–40 min, with occasional mixing, to dissolve. Dilute with water to volume, and filter. Adjust the pH of the solution with either diluted acetic acid or 0.1 N sodium hydroxide to 6.0–7.0.

Solid-phase extraction: [NOTE—Condition the extraction column specified in this procedure in the following manner. Wash the column with a 2-mL portion of methanol. Equilibrate with a 2-mL portion of water.] Separately pipet 5.0 mL of the *Sample solution* and *Standard solution* into freshly conditioned solid-phase extraction columns consisting of a mixed-mode packing with a sorbent mass of 60 mg. [NOTE—The mixed-mode packing consists of anion-exchange and reversed-phase sorbents. The reverse-phase component is a copolymer *N*-vinylpyrrolidone and divinylbenzene. The anion exchange moiety is a trialkylamino group.[2]]

Wash the column with 10 mL of 30% (v/v) methanol in water. Apply an appropriate volume (4.9 mL) of 30% (v/v) methanol in 0.1 N hydrochloric acid to the column. Collect the eluate in a 5-mL volumetric flask, containing 100 µL of 40% (w/v) sodium acetate in water, and dilute with 30% (v/v) methanol in 0.1 N hydrochloric acid to volume.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 200 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 2 mL/min

Injection volume: 100 µL

System suitability

Samples: *Standard solution* and a portion of *Standard solution* that has undergone *Solid-phase extraction*

Suitability requirements

Tailing factor: NMT 1.5, *Standard solution*

Relative standard deviation: NMT 2.0%, *Standard solution* and *Standard solution* that has undergone *Solid-phase extraction*

Recovery: 95%–100%, *Standard solution* that has undergone *Solid-phase extraction*

Analysis

Samples: *Standard solution* and *Sample solution* that have both undergone *Solid-phase extraction*

Measure the responses for the biotin peak.

Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Biotin RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of biotin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

• **CYANOCOBALAMIN, *Method 1***

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Methanol and water (7:13)

Standard stock solution: 10 µg/mL of USP Cyanocobalamin RS in water. [NOTE—Store in a dark place, and discard after 1 week.]

Standard solution: 1 µg/mL of USP Cyanocobalamin RS from the *Standard stock solution* diluted with water

Sample solution: Finely powder NLT 30 Tablets. Transfer a portion of the powder, equivalent to a nominal amount of 100 µg of cyanocobalamin, to a 250-mL flask. Quantitatively add 100.0 mL of water, and carefully extract for 2 min. Filter 10 mL of the extract, and use the filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: 550 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Flow rate: 0.5 mL/min

Injection volume: 200 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak responses for cyanocobalamin. Calculate the percentage of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of cyanocobalamin from the *Sample solution*

r_S = peak response of cyanocobalamin from the *Standard solution*

C_S = concentration of USP Cyanocobalamin RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cyanocobalamin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$)

[2] A suitable cartridge is the Waters Oasis MAX Vac RC cartridge, particle size 30 µm, part #186000371.

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• **CYANOCOBALAMIN, *Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]

Standard cyanocobalamin stock solution: 1.0 µg/mL of USP Cyanocobalamin RS in 25% alcohol. Store in a refrigerator.

Standard solution: Dilute a suitable volume of *Standard cyanocobalamin stock solution* with water to a measured volume such that after the incubation period as directed in the *Analysis*, the difference in transmittance between the inoculated blank and the 5.0-mL level of the *Standard solution* is NLT that which corresponds to a difference of 1.25 mg in dried cell weight. This concentration usually falls between 0.01 and 0.04 ng/mL of *Standard solution*. Prepare this solution fresh for each assay.

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powdered Tablets, equivalent to a nominal amount of 1.0 µg of cyanocobalamin, to an appropriate vessel containing, for each gram of powdered Tablets taken, 25 mL of an aqueous extracting solution prepared just before use to contain, in each 100 mL, 1.29 g of dibasic sodium phosphate, 1.1 g of anhydrous citric acid, and 1.0 g of sodium metabisulfite. Autoclave the mixture at 121° for 10 min. Allow any undissolved particles of the extract to settle, and filter or centrifuge if necessary. Dilute an aliquot of the clear solution with water to obtain a final solution containing vitamin B_{12} activity equivalent to the nominal activity of the *Standard solution*.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and dilute with water to 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature NLT 10°. Filter the solution if a precipitate forms during storage.

Asparagine solution: Dissolve 2.0 g of L-asparagine in water to make 200 mL. Store under toluene in a refrigerator.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and dilute with water to 200 mL. Store under toluene in a refrigerator.

Xanthine solution: Suspend 0.20 g of xanthine in 30–40 mL of water, heat to 70°, add 6.0 mL of 6 N ammonium hydroxide, and stir until the solid is dissolved. Cool, and dilute with water to 200 mL. Store under toluene in a refrigerator.

Salt solution A: Dissolve 10 g of monobasic potassium phosphate and 10 g of dibasic potassium phosphate in water to make 200 mL, and add 2 drops of hydrochloric acid. Store this solution under toluene.

Salt solution B: Dissolve 4.0 g of magnesium sulfate, 0.20 g of sodium chloride, 0.20 g of ferrous sulfate, and 0.20 g of manganese sulfate in water to make 200 mL. Add 2 drops of hydrochloric acid. Store this solution under toluene.

Polysorbate 80 solution: Dissolve 20 g of polysorbate 80 in alcohol to make 200 mL. Store in a refrigerator.

Vitamin solution A: Dissolve 10 mg of riboflavin, 10 mg of thiamine hydrochloride, 100 µg of biotin, and 20 mg of niacin in 0.02 N glacial acetic acid to make 400 mL. Store under toluene, protected from light, in a refrigerator.

Vitamin solution B: Dissolve 20 mg of *p*-aminobenzoic acid, 10 mg of calcium pantothenate, 40 mg of pyridoxine hydrochloride, 40 mg of pyridoxal hydrochloride, 8 mg of pyridoxamine dihydrochloride, and 2 mg of folic acid in a mixture of water and neutralized alcohol (3:1) to make 400 mL. Store, protected from light, in a refrigerator.

Basal medium stock solution: Prepare the medium according to the following formula and directions. A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium comparable to that obtained from the formula given herein.

Add the ingredients in the order listed in *Table 2*, carefully dissolving cystine and tryptophan in the hydrochloric acid before adding the next 8 solutions in the resulting solution. Add 100 mL of water, and dissolve the dextrose, sodium acetate, and ascorbic acid. Filter, if necessary. Add the *Polysorbate 80 solution*, adjust with 1 N sodium hydroxide to a pH of between 5.5 and 6.0, and dilute with Purified Water to 250 mL.

Table 2

L-Cystine	0.1 g
L-Tryptophan	0.05 g
1 N hydrochloric acid	10 mL
Adenine–guanine–uracil solution	5 mL
Xanthine solution	5 mL
Vitamin solution A	10 mL
Vitamin solution B	10 mL
Salt solution A	5 mL
Salt solution B	5 mL
Asparagine solution	5 mL
Acid-hydrolyzed casein solution	25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Ascorbic acid	1 g
Polysorbate 80 solution	5 mL

Tomato juice preparation: Centrifuge commercially canned tomato juice so that most of the pulp is removed. Suspend 5 g/L of analytical filter aid in the supernatant and pass, with the aid of reduced pressure, through a layer of the filter aid. Repeat, if necessary, until a clear, straw-colored filtrate is obtained. Store under toluene in a refrigerator.

Culture medium: [NOTE—A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium equivalent to that obtained from the formula given herein.] Dissolve 0.75 g of yeast extract, 0.75 g of dried peptone, 1.0 g of anhydrous dextrose, and 0.20 g of monobasic potassium phosphate in 60–70 mL of water. Add 10 mL of *Tomato juice preparation* and 1 mL of *Polysorbate 80 solution*. Adjust with 1 N sodium hydroxide to a pH of 6.8, and dilute with water to 100 mL. Place 10-mL portions of the solution in test tubes, and plug with cotton. Sterilize the tubes and contents in an autoclave at 121° for 15 min. Cool as rapidly as possible to avoid color formation resulting from overheating the medium.

Suspension medium: Dilute a measured volume of *Basal medium stock solution* with an equal volume of water. Place 10-mL portions of the diluted medium in test tubes. Sterilize, and cool as directed for *Culture medium*.

Stock culture of *Lactobacillus leichmannii*: To 100 mL of *Culture medium* add 1.0–1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Place

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10-mL portions of the hot solution into test tubes, cover the tubes, sterilize at 121° for 15 min in an autoclave (exhaust line temperature), and allow the tubes to cool in an upright position. Inoculate 3 or more of the tubes by stab transfer of a pure culture of *Lactobacillus leichmannii*.[3] [NOTE—Before first using a fresh culture in this assay, make NLT 10 successive transfers of the culture in a 2-week period.] Incubate for 16–24 h at a temperature between 30° and 40° held constant to within ±0.5°. Store in a refrigerator.

Prepare fresh stab cultures at least 3 times each week, and do not use them for preparing the *Inoculum* if more than 4 days old. The activity of the microorganism can be increased by daily or twice-daily transfer of the stab culture, to the point where definite turbidity in the liquid *Inoculum* can be observed 2–4 h after inoculation. A slow-growing culture seldom gives a suitable response curve and may lead to erratic results.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus leichmannii* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Transfer cells from the *Stock culture of Lactobacillus leichmannii* to 2 sterile tubes each containing 10 mL of the *Culture medium*. Incubate these cultures for 16–24 h at a temperature between 30° and 40° held constant to within ±0.5°. Under aseptic conditions centrifuge the cultures, and decant the supernatant. Suspend the cells from the culture in 5 mL of sterile *Suspension medium*, and combine. Using sterile *Suspension medium*, adjust the volume so that a 1-in-20 dilution in saline TS produces 70% transmittance when read on a suitable spectrophotometer that has been set at a wavelength of 530 nm, equipped with a 10-mm cell, and read against saline TS set at 100% transmittance. Prepare a 1-in-400 dilution of the adjusted suspension using *Basal medium stock solution*. [NOTE—This dilution may be altered, when necessary, to obtain the desired test response.] The cell suspension is the *Inoculum*.

Calibration of spectrophotometer: Check the wavelength of the spectrophotometer periodically, using a standard wavelength cell or other suitable device. Before reading any tests, calibrate the spectrophotometer for 0% and 100% transmittance, using water and with the wavelength set at 530 nm.

Analysis

Samples: *Standard solution* and *Sample solution*

Because of the high sensitivity of the test organism to minute amounts of vitamin B_{12} activity and to traces of many cleansing agents, cleanse meticulously by suitable means, followed preferably by heating at 250° for 2 h, using hard-glass 20-mm × 150-mm test tubes and other necessary glassware.

To separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each of these tubes and to 4 similar but empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*. To each tube add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes to prevent bacterial contamination, and sterilize in an autoclave at 121° for 5 min, arranging to reach this temperature in NMT 10 min by preheating the autoclave if necessary. Cool as rapidly as possible to avoid color formation resulting from overheating the medium. Take precautions to maintain uniformity of sterilizing and cooling conditions throughout the assay, because packing the tubes too closely in the autoclave or overloading it may cause variation in the heating rate.

Aseptically add 0.5 mL of *Inoculum* to each tube so prepared, except 2 of the 4 containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 40°, held constant to within ±0.5°, for 16–24 h.

Terminate growth by heating to a temperature NLT 80° for 5 min. Cool to room temperature. After agitating contents, place the container in a spectrophotometer that has been set at a wavelength of 530 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of the inoculated blank. If the difference is greater than 5%, or if there is evidence of contamination with a foreign microorganism, disregard the results of the assay.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of each of the remaining tubes. Disregard the results of the assay if the slope of the standard curve indicates a problem with sensitivity.

Calculation: Prepare a standard concentration-response curve by the following procedure. Test for and replace any aberrant individual transmittances. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittances (Σ) as the difference, $y = 2.00 - \Sigma$. Plot this response on the ordinate of cross-section paper against the logarithm of the milliliter of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, y, adding together the two transmittances for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X, for each dosage level. Average the values of X for each of 3 or more dosage levels to obtain $\overline{X}$, which equals the log-relative potency, M', of the *Sample solution*.

Determine the quantity, in µg, of USP Cyanocobalamin RS corresponding to the cyanocobalamin in each mg of the portion of Tablets taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = amount of cyanocobalamin assumed to be present in each mg in the portion of Tablets taken (µg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, $\overline{M}$, is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* ⟨111⟩, *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of 2 or more values of M that do not differ by more than 0.15,

[3] Pure cultures of *Lactobacillus leichmannii* (listed as *Lactobacillus delbruekii*) may be obtained as No. 7830 from ATCC, 10801 University Blvd., Manassas, VA 20110-2209 (www.atcc.org).

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compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$)

Change to read:

- **FOLIC ACID, *Method 1***

▲Proceed as directed in *Folic Acid Assay* ⟨411⟩, *Assay, Procedure 1*.▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–150.0% of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$)

Change to read:

- **FOLIC ACID, *Method 2***

▲Proceed as directed in *Folic Acid Assay* ⟨411⟩, *Assay, Procedure 2*.▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–150.0% of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$)

- **CALCIUM PANTOTHENATE, *Method 1***

Mobile phase: Phosphoric acid and water (1:1000)

Internal standard solution: 80 mg of *p*-hydroxybenzoic acid in 3 mL of alcohol. Add 50 mL of water and 7.1 g of dibasic sodium phosphate, and dilute with water to 1000 mL. Adjust with phosphoric acid to a pH of 6.7.

Standard solution: 0.6 mg/mL of USP Calcium Pantothenate RS in *Internal standard solution*

Sample solution: Finely powder NLT 30 Tablets. Transfer a portion of the powder, equivalent to a nominal amount of 15 mg of calcium pantothenate, to a centrifuge tube. Add 25.0 mL of the *Internal standard solution*, and shake vigorously for 10 min. Centrifuge, filter, and use the clear filtrate.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 210 nm

Column: 3.9-mm × 15-cm; packing L1

Flow rate: 1.5 mL/min

Injection volume: 10 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for calcium pantothenate and *p*-hydroxybenzoic acid are about 0.5 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak responses for calcium pantothenate and the internal standard. Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Tablets taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of calcium pantothenate to *p*-hydroxybenzoic acid from the *Sample solution*

R_S = peak response ratio of calcium pantothenate to *p*-hydroxybenzoic acid from the *Standard solution*

C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

Change to read:

- **CALCIUM PANTOTHENATE, *Method 2***

Standard stock solution: Dissolve 50 mg of USP Calcium Pantothenate RS▲ (USP 1-May-2020)▲ in 500 mL of water in a 1000-mL volumetric flask. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), and dilute with water to volume to obtain a concentration of 50 µg/mL of USP Calcium Pantothenate RS. Store under toluene in a refrigerator.

Standard solution: On the day of the assay, dilute a volume of *Standard stock solution* with water to obtain a concentration of 0.01–0.04 µg/mL of calcium pantothenate, the exact concentration being such that the responses obtained as directed in *Analysis*, 2.0–4.0 mL of the *Standard solution* being used, are within the linear portion of the log-concentration response curve.

Sample solution: Finely powder NLT 30 Tablets. Transfer a portion of the powder, equivalent to a nominal amount of 50 mg of calcium pantothenate, to a 1000-mL volumetric flask containing 500 mL of water. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (16.66 mg/mL), dilute with water to volume, and filter. Dilute a volume of this solution to obtain a solution with approximately the same concentration as that of the *Standard solution*.

Acid-hydrolyzed casein solution: Prepare as directed in *Cyanocobalamin, Method 2*.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine and 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and dilute with water to 1000 mL. Store under toluene in a cool place at a temperature NLT 10°.

Adenine–guanine–uracil solution: Prepare as directed in *Cyanocobalamin, Method 2*.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin–thiamine hydrochloride–biotin solution: 20 µg/mL of riboflavin, 10 µg/mL of thiamine hydrochloride, and 0.04 µg/mL of biotin in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

***p*-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution:** 10 µg/mL of *p*-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Basal medium stock solution: Dissolve the anhydrous dextrose and anhydrous sodium acetate in the solutions previously mixed according to *Table 3*, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Table 3

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g

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Table 3 *(continued)*

Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Riboflavin–thiamine hydrochloride–biotin solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water; add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar; and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to the test tubes, close or cover the tubes, sterilize in an autoclave at 121°, and allow the tubes to cool in an upright position. Prepare stab cultures in 3 or more of the tubes, using a pure culture of *Lactobacillus plantarum*,[1] incubating for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution* add 5.0 mL of water containing 0.2 µg of calcium pantothenate. Plug the tubes with cotton, sterilize in an autoclave at 121°, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Transfer cells from the *Stock culture of Lactobacillus plantarum* to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. The cell suspension is the *Inoculum*.

Analysis

Samples: *Standard solution* and *Sample solution*

To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to 4 similar but empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, volumes of the *Sample solution* corresponding to 3 or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool, and add 1 drop of *Inoculum* to each tube, except 2 of the 4 tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37°, held constant to within ±0.5° until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of Standard during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to an optical container if necessary. Place the container in a spectrophotometer that has been set at a specific wavelength between 540 and 660 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittance (Σ) as the difference, $y = 2.00 - \Sigma$. Plot this response on the ordinate of cross-section paper against the logarithm of the milliliter of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, y, adding together the two transmittances for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that fall within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X, for each dosage level. Average the values of X for each of 3 or more dosage levels to obtain $\bar{X}$, which equals the log-relative potency, M', of the *Sample solution*.

Determine the quantity, in mg, of USP Calcium Pantothenate RS corresponding to the calcium pantothenate in each mg of the portion of Tablets taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = amount of calcium pantothenate assumed to be present in each mg in the portion of Tablets taken (mg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, $\bar{M}$, is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* ⟨111⟩, *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of 2 or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• **CALCIUM PANTOTHENATE, *Method 3***

Buffer solution: 5.0 mg/mL of monobasic potassium phosphate in water. Adjust with phosphoric acid to a pH of 3.5.

Mobile phase: Methanol and *Buffer solution* (1:9)

Standard stock solution: 0.25 mg/mL of USP Calcium Pantothenate RS in water. Prepare fresh every 4 weeks. Store in a refrigerator.

Standard solution: 40 µg/mL of USP Calcium Pantothenate RS from *Standard stock solution* diluted with water

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to a nominal amount of 10 mg of calcium pantothenate, to a 250-mL volumetric flask. Add 10 mL of methanol, and swirl the flask to disperse. Dilute with water to volume, mix, and filter.

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Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 205 nm
Column: 3.9-mm × 30-cm; 5-µm packing L1
Column temperature: 50°
Flow rate: 2 mL/min
Injection volume: 25 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 3.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas for calcium pantothenate. Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*
r_S = peak area from the *Standard solution*
C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

Change to read:

- **NIACIN OR NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, RIBOFLAVIN,** and **THIAMINE,** ***Method 1***

[NOTE—Use low-actinic glassware throughout this procedure.]
Diluent: Acetonitrile, glacial acetic acid, and water (5:1:94)
Mobile phase: A mixture of methanol, glacial acetic acid, and water (27:1:73) containing 140 mg of sodium 1-hexanesulfonate per 100 mL
Standard solution: [NOTE—Use USP Niacin RS in place of USP Niacinamide RS for formulations containing niacin.] Transfer 80 mg of USP Niacinamide RS, 20 mg of USP Pyridoxine Hydrochloride RS, 20 mg of USP Riboflavin RS, and 20 mg of USP Thiamine Hydrochloride RS to a 200-mL volumetric flask, and add 180 mL of *Diluent*. Immerse the flask in a hot water bath maintained at 65°–70° for 10 min with regular shaking, or using a vortex mixer, until all the solid materials are dissolved. Chill rapidly in a cold water bath for 10 min to room temperature, and dilute with *Diluent* to volume.
Sample solution: Finely powder NLT 30 Tablets. Transfer a portion of the powder, equivalent to a nominal amount of 10 mg of niacinamide and 2.5 mg each of pyridoxine hydrochloride, riboflavin, and thiamine hydrochloride, to a 50-mL centrifuge tube. Add 25.0 mL of *Diluent*, and mix using a vortex mixer for 30 s to completely suspend the powder. Immerse the centrifuge tube in a hot water bath maintained at 65°–70°, heat for 5 min, and mix on a vortex mixer for 30 s. Return the tube to the hot water bath, heat for another 5 min, and mix on a vortex mixer for 30 s. Filter a portion of the solution, cool to room temperature, and use the clear filtrate. [NOTE—Use the filtrate within 3 h of filtration.]
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 280 nm
Column: 3.9-mm × 30-cm; packing L1
Flow rate: 1 mL/min
Injection volume: 10 µL
System suitability
Sample: *Standard solution*
▲▲ (USP 1-May-2020)
Suitability requirements
Relative standard deviation: NMT 3.0%
Analysis
Samples: *Standard solution* and *Sample solution*
▲Calculate the percentage of the labeled amount of vitamin B_1 as thiamine ion ($C_{12}H_{17}N_4OS+$), vitamin B_2 as riboflavin ($C_{17}H_{20}N_4O_6$), vitamin B_3 as niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), vitamin B_6 as pyridoxine ($C_8H_{11}NO_3$), calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), and folic acid ($C_{19}H_{19}N_7O_6$), in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin from the corresponding *Sample solution*
r_S = peak area of the relevant vitamin from the corresponding *Standard solution*
C_S = concentration of the relevant vitamin Reference Standard in the corresponding *Standard solution* (µg/mL)
C_U = nominal concentration of the relevant vitamin in the corresponding *Sample solution* (µg/mL)▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–150.0% of the labeled amount of ▲each individual vitamin▲ (USP 1-May-2020)

Change to read:

- **NIACIN,** ***Method 2***

▲Proceed as directed in *Niacin or Niacinamide Assay* ⟨441⟩, *Assay, Chromatographic Methods, Procedure 2*.▲ (USP 1-May-2020)
Acceptance criteria: 90.0%–150.0% of the labeled amount of niacin ($C_6H_5NO_2$)

Change to read:

- **NIACINAMIDE,** ***Method 2***

▲Proceed as directed in *Niacin or Niacinamide Assay* ⟨441⟩, *Assay, Chromatographic Methods, Procedure 2*.▲ (USP 1-May-2020)
Acceptance criteria: 90.0%–150.0% of the labeled amount of niacinamide ($C_6H_6N_2O$)

- **PYRIDOXINE HYDROCHLORIDE,** ***Method 2***

[NOTE—Use low-actinic glassware throughout this procedure.]
Extraction solvent, Mobile phase, and **Sample solution:** Prepare as directed in *Niacin, Method 2*.
Standard stock solution: 0.1 mg/mL of USP Pyridoxine Hydrochloride RS in *Extraction solvent*
Standard solution: Transfer 5.0 mL of *Standard stock solution* to a 25-mL volumetric flask, and dilute with *Extraction solvent* to volume.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; packing L1
Flow rate: 1 mL/min
Injection volume: 20 µL

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System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 3.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas for pyridoxine. Calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of pyridoxine from the *Sample solution*
r_S = peak area of pyridoxine from the *Standard solution*
C_S = concentration of USP Pyridoxine Hydrochloride RS in the *Standard solution* (mg/mL)
C_U = nominal concentration of pyridoxine hydrochloride in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$)

Change to read:

- **RIBOFLAVIN,** ***Method 2***

▲Proceed as directed in *Riboflavin Assay* ⟨481⟩, *Assay, Chromatographic Methods, Procedure 2.*▲ (USP 1-May-2020)
Acceptance criteria: 90.0%–150.0% of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$)

Change to read:

- **THIAMINE,** ***Method 2***

▲Proceed as directed in *Thiamine Assay* ⟨531⟩, *Assay, Chromatographic Methods, Procedure 2.*▲ (USP 1-May-2020)
Acceptance criteria: 90.0%–150.0% of the labeled amount of thiamine ▲as thiamine ion ($C_{12}H_{17}N_4OS+$)▲ (USP 1-May-2020)

Change to read:

- **NIACIN OR NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, RIBOFLAVIN,** and **THIAMINE,** ***Method 3***

[NOTE—Use low-actinic glassware throughout this procedure.]
Reagent: 25 mg/mL of edetate disodium in water
Mobile phase: Transfer 0.4 mL of triethylamine, 15.0 mL of glacial acetic acid, and 350 mL of methanol to a 2000-mL volumetric flask. Dilute with 0.008 M sodium 1-hexanesulfonate to volume.
Standard stock solution: 1.5 mg/mL of USP Niacin RS or USP Niacinamide RS, 0.24 mg/mL of USP Pyridoxine Hydrochloride RS, 0.08 mg/mL of USP Riboflavin RS, and 0.24 mg/mL of USP Thiamine Hydrochloride RS in *Reagent*, with heating if necessary
Standard solution: Transfer 5.0 mL of *Standard stock solution* to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few milliliters of the filtrate.
Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to a nominal amount of 7.5 mg of niacin or niacinamide, 1.2 mg of pyridoxine hydrochloride, 0.4 mg of riboflavin, and 1.2 mg of thiamine hydrochloride, to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few milliliters of the filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 270 nm
Column: 4.6-mm × 25-cm; packing L7
Column temperature: 50°
Flow rate: 2 mL/min
Injection volume: 5 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 2.0%
Analysis
Samples: *Standard solution* and *Sample solution*
▲Calculate the percentage of the labeled amount of vitamin B_1 as thiamine ion ($C_{12}H_{17}N_4OS+$), vitamin B_2 as riboflavin ($C_{17}H_{20}N_4O_6$), vitamin B_3 as niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), vitamin B_6 as pyridoxine ($C_8H_{11}NO_3$), calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), and folic acid ($C_{19}H_{19}N_7O_6$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin from the corresponding *Sample solution*
r_S = peak area of the relevant vitamin from the corresponding *Standard solution*
C_S = concentration of the relevant vitamin Reference Standard in the corresponding *Standard solution* (µg/mL)
C_U = nominal concentration of the relevant vitamin in the corresponding *Sample solution* (µg/mL)▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–150.0% of the labeled amount of ▲each individual vitamin▲ (USP 1-May-2020)

Add the following:

▲• **FOLIC ACID,** ***Method 3*****; ASCORBIC ACID, NIACIN OR NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, CALCIUM PANTOTHENATE, RIBOFLAVIN,** and **THIAMINE,** ***Method 4***

[NOTE—Use low-actinic glassware.]
Solution A: 0.56 g of edetate disodium and 2.04 g of potassium phosphate monobasic per 1000 mL of water. Adjust with phosphoric acid to a pH of 3.0.
Solution B: Acetonitrile; acetic acid, glacial; and water (5:1:94)
Solution C: Aqueous solution containing 2 mL of ammonium hydroxide and 1 g of sodium perchlorate per 100 mL
Solution D: Aqueous solution containing 2 mL of ammonium hydroxide, 1 g of sodium perchlorate, and 2 g of sodium ascorbate per 100 mL
Mobile phase A: 0.02% trifluoroacetic acid in water
Mobile phase B: Acetonitrile
Mobile phase: See *Table 4*.

Table 4

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
0	100	0

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Table 4 *(continued)*

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
1.0	100	0
6.0	95	5
15.0	83	17
17.0	80	20
19.0	80	20
19.1	100	0
25.0	100	0

Ascorbic acid standard stock solution: 1.0 mg/mL of ascorbic acid from USP Ascorbic Acid RS in *Solution A*

Thiamine standard stock solution: 0.2 mg/mL of thiamine from USP Thiamine Hydrochloride RS in *Solution B*. The solution can be placed on a steam bath for about 5–10 min with frequent swirling to assist in dissolution.

Riboflavin standard stock solution: 0.2 mg/mL of USP Riboflavin RS in *Solution B*. Heat the solution to 65°–70° for 10 min to assist in dissolution.

Niacin or niacinamide standard stock solution: 1.0 mg/mL of USP Niacin RS or USP Niacinamide RS in *Solution A*

Pyridoxine standard stock solution: 0.2 mg/mL of pyridoxine from USP Pyridoxine Hydrochloride RS in *Solution C*

Folic acid standard stock solution: 0.2 mg/mL of folic acid from USP Folic Acid RS in *Solution C*

Calcium pantothenate standard stock solution: 1.0 mg/mL of calcium pantothenate from USP Calcium Pantothenate RS in *Mobile phase A*

System suitability solution: Transfer 0.5 mL of *Ascorbic acid standard stock solution*, 2.5 mL of *Thiamine standard stock solution*, and 0.5 mL of *Niacin or niacinamide standard stock solution* to a 10-mL volumetric flask and dilute with *Mobile phase A* to volume.

Sample solution 1: Weigh and finely powder NLT 20 Tablets. Transfer a calculated amount of accurately weighed powder to a 100-mL volumetric flask to obtain a solution containing known nominal concentrations of thiamine, riboflavin, and calcium pantothenate in the ranges of 5–30 µg/mL for thiamine, 10–60 µg/mL for riboflavin, and 25–150 µg/mL for calcium pantothenate. Add *Solution B* to about half the volume and mix on a vortex mixer for 5 min. Heat the flask at 65°–70° for 5 min, swirl frequently, and sonicate additionally for 5 min. Cool to room temperature, dilute with *Mobile phase A* to volume, and mix well. Pass a portion of the solution through a suitable filter of 0.45-µm pore size, and discard the first milliliter of the filtrate.

Sample solution 2: Transfer a portion of finely powdered Tablets to a 100-mL volumetric flask to obtain a solution containing known nominal concentrations of ascorbic acid and niacin or niacinamide in the ranges of 50–300 µg/mL for ascorbic acid and 12–65 µg/mL for niacin or niacinamide, add *Solution A* to about half the volume, and vortex for 5 min, and sonicate for 10 min with occasional shaking. Cool to room temperature, dilute with *Solution A* to volume, and mix well. Pass a portion of the solution through a suitable filter of 0.45-µm pore size, and discard the first milliliter of the filtrate.

Sample solution 3: Transfer a portion of finely powdered Tablets to a 100-mL volumetric flask to obtain a solution containing known nominal concentrations of pyridoxine hydrochloride and folic acid in the ranges of 5–30 µg/mL for pyridoxine hydrochloride and 2–12 µg/mL for folic acid, add *Solution D* to about half the volume, heat in a water bath at 40° for 5 min and sonicate for 10 min with occasional shaking. Cool to room temperature, dilute with *Solution D* to volume, and mix well. Pass a portion of the solution through a suitable filter of 0.45-µm pore size, and discard the first milliliter of the filtrate.

Standard solution 1: Transfer calculated volumes of *Thiamine standard stock solution*, *Riboflavin standard stock solution*, and *Calcium pantothenate standard stock solution* into a suitable volumetric flask to obtain a solution with final vitamin concentrations similar to the nominal concentrations in *Sample solution 1* for the corresponding vitamins. Dilute with *Mobile phase A* to volume, and mix well.

Standard solution 2: Transfer calculated volumes of *Ascorbic acid standard stock solution* and *Niacin or niacinamide standard stock solution* into a suitable volumetric flask to obtain a solution with final vitamin concentrations similar to the nominal concentrations in *Sample solution 2* for the corresponding vitamins. Dilute with *Mobile phase A* to volume, and mix well.

Standard solution 3: Transfer calculated volumes of *Pyridoxine standard stock solution* and *Folic acid standard stock solution* into a suitable volumetric flask to obtain a solution with final vitamin concentrations similar to the nominal concentrations in *Sample solution 3* for the corresponding vitamins. Dilute with *Solution C* to volume, and mix well.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector

For calcium pantothenate: UV 210 nm

For thiamine, ascorbic acid, niacin or niacinamide, and riboflavin: UV 254 nm

For pyridoxine hydrochloride and folic acid: UV 280 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Column temperature: 25°

Flow rate: 1.0 mL/min

Injection volume: 20 µL

System suitability

Samples: *System suitability solution*, *Standard solution 1*, *Standard solution 2*, and *Standard solution 3*

Suitability requirements

Resolution: NLT 3.5 between the thiamine and ascorbic acid peaks; NLT 4.0 between the ascorbic acid and niacin or niacinamide peaks, *System suitability solution*

Relative standard deviation: NMT 2.0% for each individual peak, *Standard solution 1*, *Standard solution 2*, and *Standard solution 3*

Analysis

Samples: *Sample solutions 1–3* and *Standard solutions 1–3*

Calculate the percentage of the labeled amount of vitamin B_1 as thiamine ion ($C_{12}H_{17}N_4OS^+$), vitamin B_2 as riboflavin ($C_{17}H_{20}N_4O_6$), vitamin B_3 as niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), vitamin B_6 as pyridoxine ($C_8H_{11}NO_3$), calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), and folic acid ($C_{19}H_{19}N_7O_6$), in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin from the corresponding *Sample solution*

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r_S = peak area of the relevant vitamin from the corresponding *Standard solution*

C_S = concentration of the relevant vitamin Reference Standard in the corresponding *Standard solution* (µg/mL)

C_U = nominal concentration of the relevant vitamin in the corresponding *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of each individual vitamin▲ (USP 1-May-2020)

[NOTE—Commercially available atomic absorption standard solutions for the minerals, where applicable, may be used where preparation of a *Standard stock solution* is described in the following assays. Use deionized water where water is specified. Where atomic absorption spectrophotometry is specified in the assay, the *Standard solutions* and the *Sample solution* may be diluted quantitatively with the solvent specified, if necessary, to yield solutions of suitable concentrations adaptable to the linear or working range of the instrument.]

• **CALCIUM, *Method 1***

Lanthanum chloride solution: 267 mg/mL of lanthanum chloride heptahydrate in 0.125 N hydrochloric acid

Calcium standard solution: 400 µg/mL of calcium. Dissolve 1.001 g of calcium carbonate, previously dried at 300° for 3 h and cooled in a desiccator for 2 h, in 25 mL of 1 N hydrochloric acid. Boil to expel carbon dioxide, and dilute with water to 1000 mL.

Standard stock solution: 100 µg/mL of calcium from *Calcium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Into separate 100-mL volumetric flasks pipet 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, and dilute with water to volume to obtain concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 µg/mL of calcium.

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 5 Tablets, to a porcelain crucible. Heat the crucible in a muffle furnace maintained at 550° for 6–12 h, and cool. Add 60 mL of hydrochloric acid, and boil gently on a hot plate or steam bath for 30 min, intermittently rinsing the inner surface of the crucible with 6 N hydrochloric acid. Cool, and quantitatively transfer the contents of the crucible to a 100-mL volumetric flask. Rinse the crucible with small portions of 6 N hydrochloric acid, and add the rinsings to the flask. Dilute with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute this solution, quantitatively, with 0.125 N hydrochloric acid to obtain a nominal concentration of 2 µg/mL of calcium, adding 1 mL of *Lanthanum chloride solution* per 100 mL of the final volume.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Lamp: Calcium hollow-cathode

Flame: Nitrous oxide–acetylene

Analytical wavelength: Calcium emission line at 422.7 nm

Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution* per 100 mL

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of calcium, and draw the straight line best fitting the 5 plotted points. From the graph, determine the concentration (C), in µg/mL of calcium in the *Sample solution*.

Calculate the percentage of the labeled amount of calcium (Ca) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of calcium in the *Sample solution* (µg/mL)

C_U = nominal concentration of calcium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of calcium (Ca)

• **CHROMIUM, *Method 1***

Chromium standard solution: 1000 µg/mL of chromium from potassium dichromate, previously dried at 120° for 4 h, in water. Store in a polyethylene bottle.

Standard stock solution: 10 µg/mL of chromium from *Chromium standard solution* diluted with 6 N hydrochloric acid and water (1 in 20)

Standard solutions: Transfer 10.0 and 20.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks, and transfer 15.0 and 20.0 mL of the *Standard stock solution* to separate 50-mL volumetric flasks. Dilute the contents of each of the 4 flasks with 0.125 N hydrochloric acid to volume to obtain concentrations of 1.0, 2.0, 3.0, and 4.0 µg/mL of chromium.

Sample solution: Prepare as directed in *Calcium, Method 1*, except obtain 1 µg/mL of chromium and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Lamp: Chromium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Chromium emission line at 357.9 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of chromium, and draw the straight line best fitting the 4 plotted points. From the graph, determine the concentration (C), in µg/mL of chromium in the *Sample solution*.

Calculate the percentage of the labeled amount of chromium (Cr) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of chromium in the *Sample solution* (µg/mL)

C_U = nominal concentration of chromium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of chromium (Cr)

• **COPPER, *Method 1***

Copper standard solution: Dissolve 1.00 g of copper foil in a minimum volume of a 50% (v/v) solution of nitric acid, and dilute with a 1% (v/v) solution of nitric acid to 1000 mL. This solution contains 1000 µg/mL of copper.

Standard stock solution: 100 µg/mL of copper from *Copper standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 200-mL volumetric flasks transfer 1.0, 2.0, 4.0, 6.0, and 8.0 mL of the *Standard stock solution*. Dilute with water to volume to obtain

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concentrations of 0.5, 1.0, 2.0, 3.0, and 4.0 µg/mL of copper.

Sample solution: Prepare as directed in *Calcium, Method 1*, except obtain 2 µg/mL of copper and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Lamp: Copper hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Copper emission line at 324.7 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of copper, and draw the straight line best fitting the 5 plotted points. From the graph, determine the concentration (*C*), in µg/mL of copper in the *Sample solution*.

Calculate the percentage of the labeled amount of copper (Cu) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of copper in the *Sample solution* (µg/mL)

C_U = nominal concentration of copper in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of copper (Cu)

• **FLUORIDE, *Method 1***

[NOTE—Store all solutions in plastic containers.]

3 M sodium acetate solution: Dissolve 408 g of sodium acetate in 600 mL of water in a 1000-mL volumetric flask. Allow the solution to equilibrate to room temperature, and dilute with water to volume. Adjust with a few drops of acetic acid to a pH of 7.0.

Sodium citrate solution: Dissolve 222 g of sodium citrate in 250 mL of water in a 1000-mL volumetric flask. Add 28 mL of perchloric acid, and dilute with water to volume.

Fluoride standard stock solution: 500 µg/mL of fluoride from a quantity of sodium fluoride, previously dried at 100° for 4 h and cooled in a desiccator in water

Intermediate stock solution A: 100 µg/mL of fluoride from *Fluoride standard stock solution* diluted with water

Intermediate stock solution B: 10 µg/mL of fluoride from *Fluoride standard stock solution* diluted with water

Standard solutions: To 5 separate 100-mL volumetric flasks transfer 3.0, 5.0, and 10.0 mL of *Intermediate stock solution B* and 5.0 and 10.0 mL of *Intermediate stock solution A*. To each flask add 10.0 mL of 1 N hydrochloric acid, 25 mL of *3 M sodium acetate solution*, and 25.0 mL of *Sodium citrate solution*. Dilute the contents of each flask with water to volume to obtain concentrations of 0.3, 0.5, 1.0, 5.0, and 10.0 µg/mL of fluoride.

Sample solution: Transfer a quantity of the finely powdered Tablets, equivalent to a nominal amount of 200 µg of fluoride, to a 100-mL volumetric flask. Add 10.0 mL of 1 N hydrochloric acid, 25.0 mL of *3 M sodium acetate solution*, and 25.0 mL of *Sodium citrate solution*, and dilute with water to 100 mL.

Analysis

Samples: *Standard solutions* and *Sample solution*

To separate plastic beakers, each containing a plastic-coated stirring bar, transfer 50.0 mL each of the *Standard solutions* and the *Sample solution*. Measure the potentials (see *pH* ⟨791⟩), in mV, of the *Standard solutions* and the *Sample solution* with a pH meter capable of a minimum reproducibility of ±0.2 mV and equipped with a fluoride-specific ion-indicating electrode and a calomel reference electrode. [NOTE—When taking measurements, immerse the electrodes in the solution, stir on a magnetic stirrer with an insulated top until equilibrium is attained (1–2 min), and record the potential. Rinse and dry the electrodes between measurements, taking care to avoid damaging the crystal of the specific-ion electrode.]

Plot the logarithms of fluoride concentrations, in µg/mL, of the *Standard solutions* versus the potential, in mV. From the standard response curve and the measured potential of the *Sample solution*, determine the concentration (*C*), in µg/mL, of fluoride in the *Sample solution*.

Calculate the percentage of the labeled amount of fluorine (F) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of fluoride in the *Sample solution* (µg/mL)

C_U = nominal concentration of fluorine in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (F)

• **FLUORIDE, *Method 2***

[NOTE—Use plastic containers and deionized water throughout this procedure.]

pH 10.0 buffer: Add 214 mL of 0.1 N sodium hydroxide to 1000 mL of 0.05 M sodium bicarbonate.

Mobile phase: Alcohol, 0.1 N sulfuric acid, and water (20:5:175)

Standard stock solution: 220 µg/mL of USP Sodium Fluoride RS in water. This solution contains 100 µg/mL of fluoride.

Standard solution: [NOTE—Condition the solid-phase extraction column specified for use in the *Standard solution* and the *Sample solution* in the following manner. Using a vacuum at a pressure not exceeding 5 mm of mercury, wash the column with 1 column volume of methanol followed by 1 column volume of *pH 10.0 buffer*. Do not allow the column top to dry. If the top of the column becomes dry, recondition the column.] Transfer 10.0 mL of the *Standard stock solution* to a 100-mL volumetric flask. Add 75 mL of water, and adjust with 0.1 N sodium hydroxide to a pH of 10.4 ± 0.1. Dilute with water to volume. Filter, discarding the first 15 mL of the filtrate. Transfer 25.0 mL of the filtrate to a 50-mL volumetric flask, add 15.0 mL of water, and adjust with 0.1 N sodium hydroxide to a pH of 10.0. Dilute with *pH 10.0 buffer* to volume. Elute a portion of this solution through a 3-mL solid-phase extraction column containing L1 packing that is connected through an adaptor to a second solid-phase extraction column containing sulfonylpropyl strong cation-exchange packing. Discard the first 3 mL of the eluate, and collect the rest of the eluate in a suitable flask for injection into the chromatograph.

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of powdered Tablets, equivalent to a nominal amount of 1 mg of fluorine, to 15 mL of water, and shake vigorously. Rinse the sides of the flask with 15 mL of water to a 100-mL volumetric flask. Add 15 mL of water, and allow to stand for 10 min. Dilute with water to 85 mL, adjust with 1 N sodium hydroxide to a pH of 10.4 ± 0.1, and dilute with water to 100 mL. Prepare as directed for the *Standard solution*, beginning with "Filter, discarding the first 15 mL of the filtrate."

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

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Mode: LC
Detector: Conductivity
Columns
Guard: 4.6-mm × 3-cm; packing L17
Analytical: 7.8-mm × 30-cm; packing L17
Flow rate: 0.5 mL/min
Injection volume: 100 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 2.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the peak areas for fluoride. Calculate the percentage of the labeled amount of fluorine (F) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of fluorine from the *Sample solution*
r_S = peak area of fluorine from the *Standard solution*
C_S = concentration of fluoride in the *Standard solution* (µg/mL)
C_U = nominal concentration of fluorine in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (F)

• **IODIDE**

Bromine water: To 20 mL of bromine in a glass-stoppered bottle add 100 mL of water. Insert the stopper into the bottle, and shake. Allow to stand for 30 min, and use the supernatant.

Analysis: Transfer a portion of finely powdered Tablets, equivalent to a nominal amount of 3 mg of iodide, to a nickel crucible. Add 5 g of sodium carbonate, 5 mL of 50% (w/v) sodium hydroxide solution, and 10 mL of alcohol, taking care that the entire specimen is moistened. Heat the crucible on a steam bath to evaporate the alcohol, then dry the crucible at 100° for 30 min to prevent spattering upon subsequent heating. Transfer the crucible with its contents to a furnace heated to 500°, and heat the crucible for 15 min. [NOTE—Heating at 500° is necessary to carbonize any organic matter present; a higher temperature may be used, if necessary, to ensure complete carbonization of all organic matter.] Cool the crucible, add 25 mL of water, cover the crucible with a watchglass, and boil gently for 10 min. Filter the solution, and wash the crucible with boiling water, collecting the filtrate and washings in a beaker. Add phosphoric acid until the solution is neutral to methyl orange, then add 1 mL excess of phosphoric acid. Add excess of *Bromine water*, and boil the solution gently until colorless and then for 5 min longer. Add a few crystals of salicylic acid, and cool the solution to 20°. Add 1 mL of phosphoric acid and 0.5 g of potassium iodide, and titrate the liberated iodine with 0.005 N sodium thiosulfate VS, adding starch TS when the liberated iodine color has nearly disappeared.
Calculate the percentage of the labeled amount of iodine (I) in the portion of Tablets taken:

$$\text{Result} = V \times N_A \times F \times I_{me} \times (A_w/W) \times (100/L)$$

V = volume of sodium thiosulfate consumed (mL)
N_A = actual normality of the sodium thiosulfate solution used (meq/mL)
F = correction factor to convert mg to µg, 1000 µg/mg
I_{me} = milliequivalent weight of iodine, 21 mg/meq
A_w = average weight of the Tablets
W = weight of the portion of Tablets taken
L = labeled amount of iodine (µg/Tablet)

Acceptance criteria: 90.0%–160.0% of the labeled amount of iodine (I)

• **IRON, *Method 1***

Iron standard stock solution: Transfer 100 mg of iron powder to a 1000-mL volumetric flask. Dissolve in 25 mL of 6 N hydrochloric acid, dilute with water to volume, and mix.
Standard solutions: To separate 100-mL volumetric flasks transfer 2.0, 4.0, 5.0, 6.0, and 8.0 mL of *Iron standard stock solution*. Dilute the contents of each flask with water to volume to obtain concentrations of 2.0, 4.0, 5.0, 6.0, and 8.0 µg/mL of iron.
Sample solution: Proceed as directed in *Calcium, Method 1*, except obtain a nominal concentration of 5 µg/mL of iron and omit the use of the *Lanthanum chloride solution*.
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Lamp: Iron hollow-cathode
Flame: Air–acetylene
Analytical wavelength: Iron emission line at 248.3 nm
Blank: 0.125 N hydrochloric acid
Analysis
Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of iron, and draw the straight line best fitting the 5 plotted points. From the graph, determine the concentration (C), in µg/mL, of iron in the *Sample solution*.
Calculate the percentage of the labeled amount of iron (Fe) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of iron in the *Sample solution* (µg/mL)
C_U = nominal concentration of iron in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of iron (Fe)

• **MAGNESIUM, *Method 1***

Lanthanum chloride solution: Prepare as directed in *Calcium, Method 1*.
Magnesium standard solution: Transfer 1.0 g of magnesium ribbon to a 1000-mL volumetric flask, dissolve in 50 mL of 6 N hydrochloric acid, dilute with water to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of magnesium.
Standard stock solution: 20 µg/mL of magnesium from *Magnesium standard solution* diluted with 0.125 N hydrochloric acid
Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 2.5, and 3.0 mL of *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, and dilute with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.2, 0.3, 0.4, 0.5, and 0.6 µg/mL of magnesium.
Sample solution: Proceed as directed in *Calcium, Method 1*, except obtain a nominal concentration of 0.4 µg/mL of magnesium.
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry

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Lamp: Magnesium hollow-cathode
Flame: Air–acetylene
Analytical wavelength: Magnesium emission line at 285.2 nm
Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution* per 100 mL

Analysis
Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of magnesium, and draw the straight line best fitting the 5 plotted points. From the graph, determine the concentration (C), in µg/mL, of magnesium in the *Sample solution*.
Calculate the percentage of the labeled amount of magnesium (Mg) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of magnesium in the *Sample solution* (µg/mL)
C_U = nominal concentration of magnesium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of magnesium (Mg)

• **MANGANESE, *Method 1***
Manganese standard stock solution: Transfer a weighed amount of 1.00 g of manganese to a 1000-mL volumetric flask. Dissolve in 20 mL of nitric acid, dilute with 6 N hydrochloric acid to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of manganese.
Standard stock solution: 50 µg/mL of manganese from *Manganese standard stock solution* diluted with 0.125 N hydrochloric acid
Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 3.0, and 4.0 mL of *Standard stock solution*. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions with concentrations of 0.5, 0.75, 1.0, 1.5, and 2.0 µg/mL of manganese.
Sample solution: Prepare as directed in *Calcium, Method 1*, except obtain a nominal concentration of 1 µg/mL of manganese and omit the use of the *Lanthanum chloride solution*.
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Lamp: Manganese hollow-cathode
Flame: Air–acetylene
Analytical wavelength: Manganese emission line at 279.5 nm
Blank: 0.125 N hydrochloric acid
Analysis
Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of manganese, and draw the straight line best fitting the 5 plotted points. From the graph, determine the concentration (C), in µg/mL, of manganese (Mn) in the *Sample solution*.
Calculate the percentage of the labeled amount of manganese (Mn) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of manganese in the *Sample solution* (µg/mL)
C_U = nominal concentration of manganese in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of manganese (Mn)

• **MOLYBDENUM, *Method 1***
Diluent: 20 mg/mL of ammonium chloride in water
Molybdenum standard solution: Transfer 1.0 g of molybdenum wire to a 1000-mL volumetric flask, and dissolve in 50 mL of nitric acid, warming if necessary. Dilute with water to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of molybdenum.
Standard stock solution: 100 µg/mL of molybdenum from *Molybdenum standard solution* diluted with water
Standard solutions: To separate 100-mL volumetric flasks transfer 2.0, 10.0, and 25.0 mL of the *Standard stock solution*, and add 5.0 mL of perchloric acid to each flask. Gently boil the solution in each flask for 15 min, cool to room temperature, and dilute each with *Diluent* to volume to obtain concentrations of 5.0, 10.0, and 25.0 µg/mL of molybdenum.
Sample solution: Transfer a portion of the powder, equivalent to a nominal amount of 1000 µg of molybdenum, to a suitable flask, and add 12 mL of nitric acid. [NOTE—The volume of nitric acid may be varied to ensure that the powder is uniformly dispersed.] Carefully swirl the flask to disperse the test specimen. Sonicate for 10 min, or until the test specimen is completely dissolved. Gently boil the solution for 15 min, and cool to room temperature. Carefully add 8 mL of perchloric acid, heat until perchloric acid fumes appear, and swirl the flask to dissipate the fumes. Repeat the heating and swirling until no fumes are present. Cool to room temperature. Quantitatively transfer the contents of the flask to a 100-mL volumetric flask with the aid of *Diluent*, and dilute with *Diluent* to volume.
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Lamp: Molybdenum hollow-cathode
Flame: Nitrous oxide–acetylene
Analytical wavelength: Molybdenum emission line at 313.3 nm
Blank: *Diluent* and perchloric acid (20:1)
Analysis
Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of molybdenum, and draw the straight line best fitting the 3 plotted points. From the graph, determine the concentration (C), in µg/mL, of molybdenum in the *Sample solution*.
Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of molybdenum in the *Sample solution* (µg/mL)
C_U = nominal concentration of molybdenum in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

• **MOLYBDENUM, *Method 2***
Sodium fluoride solution: Add 200 mL of water to 10 g of sodium fluoride, stir until the solution is saturated, and filter. Store in a polyethylene bottle.
Ferrous sulfate solution: 4.98 mg/mL of ferrous sulfate in water
Potassium thiocyanate solution: 200 mg/mL of potassium thiocyanate in water

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20% stannous chloride solution: Transfer 40 g of stannous chloride to a beaker, add 20 mL 6.5 N hydrochloric acid, and heat the solution until the stannous chloride is dissolved. Cool, and dilute with water to 100 mL.
Diluted stannous chloride solution: *20% stannous chloride solution* diluted with water (1 in 25). Prepare this solution fresh at the time of use.
Standard solution: 20 µg/mL of molybdenum in water
Sample: A portion of finely powdered Tablets, equivalent to a nominal amount of 40 µg of molybdenum
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: UV-Vis
Cell: 1 cm
Analytical wavelength: 465 nm
Blank: Amyl alcohol
Analysis
Samples: *Standard solution* and *Sample*
Transfer the *Sample* and 2.0 mL of the *Standard solution* to separate 200-mL beakers. Add 20 mL of nitric acid to each beaker. Cover each beaker with a watchglass, and boil slowly on a hot plate for 45 min. Cool to room temperature. Add 6 mL of perchloric acid, cover the beakers with a watchglass, and continue the heating until digestion is complete, as indicated when the liquid becomes colorless or pale yellow. Evaporate the solutions in the beakers to dryness. Rinse the sides of the beakers and the watchglasses with water, and add more water to make 50 mL in each beaker. Gently boil the water solution for a few min. Cool to room temperature. Add 2 drops of methyl orange TS, and neutralize with ammonium hydroxide. Add 8.2 mL of hydrochloric acid. Quantitatively transfer the contents of the beakers to separate 100-mL volumetric flasks, rinse the beakers with water, transfer the rinsings to the corresponding volumetric flasks, and dilute with water to volume. Transfer 50.0 mL of each solution to separatory funnels. To each separatory funnel add 1.0 mL of *Sodium fluoride solution*, 0.5 mL of *Ferrous sulfate solution*, 4.0 mL of *Potassium thiocyanate solution*, 1.5 mL of *20% stannous chloride solution*, and 15.0 mL of amyl alcohol, and shake the separatory funnels for 1 min. Allow the layers to separate, and discard the aqueous layers. Add 25 mL of *Diluted stannous chloride solution* to each separatory funnel, and shake gently for 15 s. Allow the layers to separate, and discard the aqueous layers. Transfer the organic layers from each separatory funnel to a centrifuge tube, and centrifuge at 2000 rpm for 10 min. Determine the absorbances of the organic phases from the *Standard solution* and the *Sample*, and correct with the *Blank*.
Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Tablets taken:

$$\text{Result} = (A_U/A_S) \times [(V \times C_S)/M_U] \times 100$$

A_U = absorbance of the solution from the Tablets
A_S = absorbance of the solution from the *Standard solution*
V = volume of the *Standard solution* analyzed, 2.0 mL
C_S = concentration of molybdenum in the *Standard solution* (µg/mL)
M_U = nominal amount of molybdenum in the *Sample* (µg)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

• **PHOSPHORUS, *Method 1***
Sulfuric acid solution: Cautiously add sulfuric acid to water (37.5: 100), and mix.
Ammonium molybdate solution: 50 mg/mL of ammonium molybdate in *Sulfuric acid solution* and water (2:3). [NOTE—Dissolve in water first, then dilute with *Sulfuric acid solution* to volume.]
Hydroquinone solution: 5 mg/mL of hydroquinone in water. Add 1 drop of sulfuric acid per 100 mL of solution.
Sodium bisulfite solution: 200 mg/mL of sodium bisulfite in water
Phosphorus standard stock solution: Weigh 4.395 g of monobasic potassium phosphate, previously dried at 105° for 2 h and stored in a desiccator, and transfer to a 1000-mL volumetric flask. Dissolve in water, add 6 mL of sulfuric acid as a preservative, dilute with water to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of phosphorus.
Standard solution: 20 µg/mL of phosphorus from *Phosphorus standard stock solution* diluted with water
Sample solution: [NOTE—Finely powder and weigh a counted number of Tablets.] Transfer a portion of the powder, equivalent to a nominal amount of 100 mg of phosphorus, to 25 mL of nitric acid, and digest on a hot plate for 30 min. Add 15 mL of hydrochloric acid, and continue the digestion to the cessation of brown fumes. Cool, and transfer the contents of the flask to a 500-mL volumetric flask with the aid of small portions of water. Dilute with water to volume. Transfer 10.0 mL of this solution to a 100-mL volumetric flask, and dilute with water to volume.
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: UV-Vis
Cell: 1 cm
Analytical wavelength: 650 nm
Analysis
Samples: *Standard solution* and *Sample solution*
To 3 separate 25-mL volumetric flasks transfer 5.0 mL each of the *Standard solution*, the *Sample solution*, and water to provide the blank. To each of the 3 flasks add 1.0 mL each of *Ammonium molybdate solution*, *Hydroquinone solution*, and *Sodium bisulfite solution*, and swirl to mix. Dilute the contents of each flask with water to volume, and allow the flasks to stand for 30 min. Determine the absorbances of the solutions against the blank.
Calculate the percentage of the labeled amount of phosphorus (P) in the portion of Tablets taken:

$$\text{Result} = (A_U/A_S) \times (C_S/C_U) \times 100$$

A_U = absorbance of the *Sample solution*
A_S = absorbance of the *Standard solution*
C_S = concentration of phosphorus in the *Standard solution* (µg/mL)
C_U = nominal concentration of phosphorus in the *Sample solution* (µg/mL)

Acceptance criteria: 90%–125% of the labeled amount of phosphorus (P)

• **POTASSIUM**
Potassium standard solution: 100 µg/mL of potassium from potassium chloride, previously dried at 105° for 2 h, in water
Standard stock solution: 10 µg/mL of potassium from *Potassium standard solution* diluted with 0.125 N hydrochloric acid
Standard solutions: Transfer 5.0, 10.0, 15.0, 20.0, and 25.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions containing 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of potassium.

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Sample solution: Prepare as directed in *Calcium, Method 1*, except obtain a nominal amount of 1 µg/mL of potassium and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Lamp: Potassium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Potassium emission line at 766.5 nm

Blank: Water

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of potassium, and draw the straight line best fitting the 5 plotted points. From the graph, determine the concentration (C), in µg/mL, of potassium in the *Sample solution*.

Calculate the percentage of the labeled amount of potassium (K) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of potassium in the *Sample solution* (µg/mL)

C_U = nominal concentration of potassium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of potassium (K)

• **SELENIUM, *Method 1***

Diluent: Prepare as directed in *Molybdenum, Method 1*.

Selenium standard solution: [**CAUTION**—Selenium is toxic; handle it with care.] Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness. Add 2 mL of water, and evaporate to dryness. Repeat the addition of water and the evaporation to dryness 3 times. Dissolve the residue in 3 N hydrochloric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume to obtain a concentration of 1000 µg/mL of selenium.

Standard stock solution: 100 µg/mL of selenium from *Selenium standard solution* diluted with water

Standard solutions: To separate 100-mL volumetric flasks transfer 5.0, 10.0, and 25.0 mL of the *Standard stock solution*, and add 5.0 mL of perchloric acid to each flask. Gently boil the solutions for 15 min, cool to room temperature, and dilute each with *Diluent* to volume to obtain solutions with concentrations of 5.0, 10.0, and 25.0 µg/mL of selenium.

Sample solution: Transfer a portion of powder, equivalent to a nominal amount of 1000 µg of selenium, to a suitable flask, and add 12 mL of nitric acid. [NOTE—The volume of nitric acid may be varied to ensure that the powder is uniformly dispersed.] Carefully swirl the flask to disperse the test specimen. Sonicate for 10 min or until the test specimen is completely dissolved. Gently boil the solution for 15 min, and cool to room temperature. Carefully add 8 mL of perchloric acid to the flask, heat the flask until perchloric acid fumes appear, and swirl the flask to dissipate the fumes. Repeat the heating and swirling until the fumes appear again. Cool to room temperature. Transfer the contents of the flask to a 50-mL volumetric flask with the aid of *Diluent*, and dilute with *Diluent* to volume.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Lamp: Selenium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Selenium emission line at 196.0 nm

Blank: *Diluent* and perchloric acid (20:1)

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of selenium, and draw the straight line best fitting the 3 plotted points. From the graph, determine the concentration (C), in µg/mL, of selenium in the *Sample solution*.

Calculate the percentage of the labeled amount of selenium (Se) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of selenium in the *Sample solution* (µg/mL)

C_U = nominal concentration of selenium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of selenium (Se)

• **SELENIUM, *Method 2***

Hydrochloric acid solution: Hydrochloric acid diluted with water (1 in 10)

50% ammonium hydroxide solution: Ammonium hydroxide diluted with water (1 in 2)

Reagent A: 9 mg/mL of edetate disodium and 25 mg/mL of hydroxylamine hydrochloride in water. [NOTE—Dissolve edetate disodium in a portion of water first, add hydroxylamine hydrochloride, then dilute with water to volume.]

Reagent B: Transfer 200 mg of 2,3-diaminonaphthalene to a 250-mL separatory funnel, and add 200 mL of 0.1 N hydrochloric acid. Wash the solution with 3 40-mL portions of cyclohexane, and discard the cyclohexane layer. Filter the solution into a brown bottle, and cover the solution with a 1-cm layer of cyclohexane. This solution is stable for 1 week if stored in a refrigerator.

Standard stock solution: [**CAUTION**—Selenium is toxic; handle it with care.] Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness. Add 2 mL of water, and evaporate to dryness. Repeat the addition of water and evaporation to dryness 3 times. Dissolve the residue in 3 N hydrochoric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume to obtain a solution with a concentration of 1000 µg/mL of selenium. Dilute a volume of the solution with 0.125 N hydrochloric acid to obtain a concentration of 2.0 µg/mL of selenium.

Standard solution: Transfer 10.0 mL of the *Standard stock solution* to a glass-stoppered flask. Add 1 mL of perchloric acid and 1 mL of *Hydrochloric acid solution*, and dilute with water to 20 mL.

Sample solution: Transfer a portion of finely powdered Tablets, equivalent to a nominal amount of 20 µg of selenium, to a suitable flask. Add 10 mL of nitric acid, and warm gently on a hot plate. Continue heating until the initial nitric acid reaction has subsided, then add 3 mL of perchloric acid. [**CAUTION**—Exercise care at this stage, because the perchloric acid reaction becomes vigorous.] Continue heating on the hot plate until the appearance of white fumes of perchloric acid or until the digest begins to darken. Add 0.5 mL of nitric acid, and resume heating, adding additional amounts of nitric acid if further darkening occurs. Digest for 10 min after the first appearance of perchloric acid fumes or until the digest becomes colorless. Cool the flask. Add 2.5 mL of *Hydrochloric acid solution*, and

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return the flask to the hot plate to expel residual nitric acid. Heat the mixture for 3 min after it begins to boil. Cool the flask to room temperature, and dilute with water to 20 mL.

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Mode: UV

Cell: 1 cm

Analytical wavelength: 380 nm

Blank: 1 mL of perchloric acid and 1 mL of *Hydrochloric acid solution* diluted with water to 20 mL

Analysis

Samples: *Standard solution* and *Sample solution*

Treat the *Sample solution*, *Standard solution*, and *Blank* as follows. Add 5 mL of *Reagent A* to each flask, and swirl gently to mix. Adjust the solution in each flask with *50% ammonium hydroxide solution* to a pH of 1.1 ± 0.1. Add 5 mL of *Reagent B* to each flask, and swirl gently to mix. Place the flasks in a water bath maintained at 50°, and equilibrate for 30 min, taking care that the flasks are covered to protect them from light. Cool to room temperature, and transfer the contents of each flask to separate separatory funnels. Transfer 10.0 mL of cyclohexane to each separatory funnel, and extract vigorously for 1 min. Discard the aqueous layer. Transfer the cyclohexane layer to a centrifuge tube, and centrifuge at 1000 rpm for 1 min to remove any remaining water. Determine the absorbances of the solutions from the *Samples* against the solution from the *Blank*.

Calculate the percentage of the labeled amount of selenium (Se) in the portion of Tablets taken:

$$\text{Result} = (A_U/A_S) \times [(V \times C_S)/M_U] \times 100$$

A_U = absorbance of the cyclohexane layer from the *Sample solution*

A_S = absorbance of the cyclohexane layer from the *Standard solution*

V = volume of the *Standard stock solution* used to prepare the *Standard solution*, 10 mL

C_S = concentration of selenium in the *Standard stock solution* (µg/mL)

M_U = nominal amount of selenium in the *Sample solution* (µg)

Acceptance criteria: 90.0%–160.0% of the labeled amount of selenium (Se)

• **ZINC, *Method 1***

Zinc standard stock solution: 1000 µg/mL of zinc from zinc oxide dissolved in 5 M hydrochloric acid (3.89 mg/mL) and diluted with water to final volume. [NOTE—Dissolve in 5 M hydrochloric acid by warming if necessary, cool, then dilute to final volume.]

Standard stock solution: 50 µg/mL of zinc from *Zinc standard stock solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Transfer 1.0, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of zinc.

Sample solution: Prepare as directed in *Calcium, Method 1*, obtain a nominal concentration of 2 µg/mL of zinc and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Lamp: Zinc hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Zinc emission line at 213.8 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of zinc, and draw the straight line best fitting the 5 plotted points. From the graph, determine the concentration (C), in µg/mL, of zinc in the *Sample solution*.

Calculate the percentage of the labeled amount of zinc (Zn) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of zinc in the *Sample solution* (µg/mL)

C_U = nominal concentration of zinc in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of zinc (Zn)

• **BORON, NICKEL, TIN,** and **VANADIUM, *Method 1*; CALCIUM, CHROMIUM, COPPER, IRON, MAGNESIUM, MANGANESE, PHOSPHORUS,** and **ZINC, *Method 2*; MOLYBDENUM** and **SELENIUM, *Method 3***

Stock aqua regia solution: Prepare a mixture of hydrochloric acid and nitric acid (3:1) by adding the nitric acid to the hydrochloric acid. [NOTE—Periodically vent the solution in an appropriate fume hood.]

Diluent: Prepare a mixture of *Stock aqua regia solution* and water (1:9) by adding 1 volume of *Stock aqua regia solution* to 2 volumes of water. Dilute with additional water to volume, and mix well.

System suitability solution: Prepare a mixture of 1000 mg/L of yttrium in 5% (v/v) nitric acid solution and 1000 mg/L of scandium in 5% (v/v) nitric acid solution with *Diluent* (1:1:198), and mix.

Standard stock solution 1 (Ca, Cu, Fe, Mg, Mn, P, and Zn): [NOTE—It is necessary to include only the minerals of interest in the solution.] Using commercially available element standard (single- or multi-element) solutions in 5% (v/v) nitric acid solution, pipet the appropriate amount of element standard solution into a volumetric flask, and dilute with 5% (v/v) nitric acid solution to obtain a solution with final concentrations of about 1000 mg/L of calcium, 100 mg/L of copper, 250 mg/L of iron, 500 mg/L of magnesium, 100 mg/L of manganese, 800 mg/L of phosphorus, and 250 mg/mL of zinc.

Standard stock solution 2 (B, Cr, Mo, Ni, Se, Sn, and V): [NOTE—It is necessary to include only the minerals of interest in the solution.] Using commercially available element standard (single- or multi-element) solutions in 20% (v/v) hydrochloric acid solution, pipet the appropriate amount of element standard solution into a volumetric flask, and dilute with 20% (v/v) hydrochloric acid solution to obtain a solution with final concentrations of about 200 mg/L of boron and 100 mg/L each of chromium, molybdenum, nickel, selenium, tin, and vanadium.

Standard solutions: Prepare a mixture of *Standard stock solution 1* and *Standard stock solution 2* as required, in *Diluent*, to prepare a6-point calibration curve to bracket the concentration range of each mineral of interest.

Sample solution 1 (for Tablets containing minerals in ***Standard stock solution 1*** and ***Standard stock solution 2***): Weigh and finely powder NLT than 20 Tablets. Transfer a portion, equal to 3.5 times the average Tablet weight, to a 250-mL volumetric flask. Slowly add 25 mL of *Stock aqua regia solution* in 5-mL increments, followed by mixing. [NOTE—If the sample contains a carbonate, bubbling will occur. Wait until bubbling ends to proceed.] Bring the

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solution to a boil on a hot plate. Continue to heat gently until fumes cease (about 1 h). [NOTE—If the sample contains selenium, digest for NMT 15 min.] Remove from heat, cool, and dilute with water to volume. Pass about 30 mL into a centrifuge tube, using a nylon syringe filter of 5-µm pore size. If necessary, make any further dilutions using *Diluent*.

Sample solution 2 (for Tablets containing minerals only in ***Standard stock solution 2***): Weigh and finely powder NLT 20 Tablets. Transfer a portion, equal to 3.5 times the average Tablet weight, to a 250-mL volumetric flask. Slowly add 25 mL of *Stock aqua regia solution* in 5-mL increments, followed by mixing. [NOTE—If the sample contains a carbonate, bubbling will occur. Wait until bubbling ends to proceed.] Bring the solution to a boil on a hot plate. Continue to heat gently until fumes cease (about 1 h). [NOTE—If the sample contains selenium, digest for NMT 15 min.] Remove from heat, cool, and dilute with water to volume. Pass about 30 mL into a centrifuge tube using a nylon syringe filter of 5-µm pore size. If necessary, make any further dilutions using *Diluent*.

Sample solution 3 (for Tablets containing minerals only in ***Standard stock solution 1***): Weigh and finely powder NLT 20 Tablets. Transfer a portion, equal to the average Tablet weight, to a 250-mL volumetric flask. Slowly add 25 mL of *Stock aqua regia solution* in 5-mL increments, followed by mixing. [NOTE—If the sample contains a carbonate, bubbling will occur. Wait until bubbling ends to proceed.] Bring the solution to a boil on a hot plate. Continue to heat gently (about 1 h) until fumes cease. Remove from heat, cool, and dilute with water to volume. Pass about 30 mL into a centrifuge tube, using a nylon syringe filter of 5-µm pore size. If necessary, make any further dilutions using *Diluent*.

Instrumental conditions

(See *Plasma Spectrochemistry* ⟨730⟩.)

Mode: Inductively coupled plasma emission spectrometry, using a spectrometer set to measure the emission of each mineral of interest at about the corresponding wavelength. [NOTE—The operating conditions may be developed and optimized on the basis of the manufacturer's recommendation. The wavelengths selected should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision.]

System suitability

Sample: *System suitability solution* [NOTE—Analyze the *System suitability solution*, and obtain the response as directed in *Analysis*.]

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the emission of each mineral of interest in the *Standard solutions* and *Sample solution* with an inductively coupled plasma system using *Diluent* as the blank. Plot the emission of the *Standard solutions* versus the concentration, in mg/L, of the minerals of interest, and draw the straight line best fitting the plotted points. From the graph, determine the concentration (C), in mg/L, for each mineral of interest in the *Sample solution*.

Calculate the percentage of the labeled amount for each mineral:

$$\text{Result} = C \times (V/W) \times F \times (C_W/L) \times 100$$

C = measured concentration of the relevant element in the *Sample solution* (mg/L)
V = volume of the *Sample solution* (L)
W = sample weight (mg)
F = dilution factor of the *Sample solution*
C_W = average Tablet weight (mg)
L = labeled amount/Tablet (mg)

Acceptance criteria: 90.0%–125.0% of the labeled amount of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), and zinc (Zn); and 90.0%–160.0% of the labeled amount of boron (B), chromium (Cr), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), and vanadium (V).

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩, *Dissolution*: Meet the requirements
- **WEIGHT VARIATION** ⟨2091⟩: Meet the requirements

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic microbial count does not exceed 3×10^3 cfu/g, and the combined molds and yeasts count does not exceed 3×10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩, *Test Procedures, Test for Absence of Salmonella Species* and *Test for Absence of Escherichia coli*: Meet the requirements

ADDITIONAL REQUIREMENTS

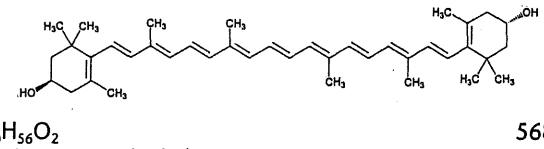
- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** The label states that the product is Water-Soluble Vitamins with Minerals Tablets. The label also states the quantity of each vitamin and mineral in terms of metric units per dosage unit and, where necessary, the chemical form in which a vitamin is present, and also states the salt form of the mineral used as the source of each element. Where more than one assay method is given for a particular vitamin or mineral, the labeling states with which assay method the product complies only if *Method 1* is not used.

Change to read:

- **USP REFERENCE STANDARDS** ⟨11⟩

▲USP Ascorbic Acid RS▲ (USP 1-May-2020)
USP Biotin RS
USP Calcium Pantothenate RS
USP Cyanocobalamin RS
USP Folic Acid RS
USP Niacin RS
USP Niacinamide RS
USP Pyridoxine Hydrochloride RS
USP Riboflavin RS
USP Sodium Fluoride RS
USP Thiamine Hydrochloride RS

meso-Zeaxanthin



$C_{40}H_{56}O_2$ 568.88

β,β-Carotene-3,3′-diol (3*R*,3′*S*)-; (3*R*,3′*S*-*meso*)-Zeaxanthin [31272-50-1].

DEFINITION

meso-Zeaxanthin consists chiefly of the 3*R*,3′*S*-isomer of zeaxanthin. It contains NLT 80.0% of total carotenoids

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calculated as zeaxanthin ($C_{40}H_{56}O_2$) and NLT 74.0% of zeaxanthin ($C_{40}H_{56}O_2$) on the anhydrous basis.

IDENTIFICATION

• **A.**

Sample solution: Use the *Sample solution* of the test for *Content of Total Carotenoids*.

Analysis: Record the UV-Vis spectrum from 300 to 600 nm.

Acceptance criteria: The *Sample solution* shows a shoulder at about 427 nm and a maximum absorption at about 453 nm and at about 480 nm.

• **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Zeaxanthin*.

• **C.** The retention time of the major peak of the *Sample solution* corresponds to that of (3*R*,3′*S*-*meso*)-zeaxanthin from the *Standard solution*, as obtained in the test for *Stereoisomeric Composition*.

COMPOSITION

• **CONTENT OF TOTAL CAROTENOIDS**

[NOTE—Use low-actinic glassware.]

Sample stock solution: Transfer 25.0 mg of *meso*-Zeaxanthin to a 100-mL volumetric flask, add 20 mL of chloroform, and place the flask in an ultrasonic bath at 30° for 2–5 min to obtain a clear solution. Dilute with cyclohexane to volume to obtain a solution containing 250 µg/mL.

Sample solution: 2.5 µg/mL of the *Sample stock solution* in cyclohexane

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Analytical wavelength: 453 nm

Cell: 1 cm

Blank: Cyclohexane

Analysis

Sample: *Sample solution*

Calculate the percentage of total carotenoids as zeaxanthin ($C_{40}H_{56}O_2$) in the portion of *meso*-Zeaxanthin taken:

$$\text{Result} = A/(C \times F)$$

A = absorbance of the *Sample solution*

C = concentration of the *Sample solution* (g/mL)

F = coefficient of extinction ($E^{1\%}$) of zeaxanthin in cyclohexane (100 mL · g^{-1} · cm^{-1}), 2540

Acceptance criteria: NLT 80.0% of total carotenoids (T) as zeaxanthin ($C_{40}H_{56}O_2$) on the anhydrous basis

• **CONTENT OF ZEAXANTHIN**

[NOTE—Use low-actinic glassware.]

Mobile phase: Hexane and ethyl acetate (75:25)

Standard solution: 150 µg/mL of USP *meso*-Zeaxanthin RS prepared as follows. Dissolve 15.0 mg of USP *meso*-Zeaxanthin RS in 10 mL of chloroform, swirling briefly, and dilute with *Mobile phase* to 100 mL.

Sample solution: Transfer 15.0 mg of sample to a 100-mL volumetric flask, add 10 mL of chloroform, and place the flask in an ultrasonic bath at 30° for 2–5 min to obtain a clear solution. Dilute with *Mobile phase* to volume.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: 453 nm

Column: 4.6-mm × 25-cm; 3-µm packing L3

Column temperature: 25°

Flow rate: 1.5 mL/min

Injection volume: 10 µL

System suitability

Sample: *Standard solution*

[NOTE—The approximate relative retention times for lutein and zeaxanthin are 0.95 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 1.0 between zeaxanthin and lutein

Tailing factor: NMT 2.0

Relative standard deviation: NMT 2.0%

Analysis: Inject the *Sample solution*, and measure the peak areas. [NOTE—The peak response of zeaxanthin is NLT 90.0% of the sum of all the peak responses.]

Calculate the percentage of zeaxanthin ($C_{40}H_{56}O_2$) in the sample taken:

$$\text{Result} = (r_U/r_T) \times T$$

r_U = peak response of zeaxanthin

r_T = sum of all the peak responses

T = percentage of total carotenoids as determined in the test for *Content of Total Carotenoids*

Acceptance criteria: NLT 74.0% of zeaxanthin ($C_{40}H_{56}O_2$) on the anhydrous basis

• **LUTEIN AND OTHER RELATED COMPOUNDS**

[NOTE—Use low-actinic glassware.]

Mobile phase, Standard solution, Sample solution, and **Chromatographic system:** Proceed as directed in the test for *Content of Zeaxanthin*.

Analysis

Sample: *Sample solution*

[NOTE—The peak response of lutein is NMT 9.0% of the sum of all the peak responses.]

Calculate the percentage of lutein in the portion of *meso*-Zeaxanthin taken:

$$\text{Result} = (r_U/r_T) \times T$$

r_U = peak response of lutein

r_T = sum of all the peak responses

T = percentage of total carotenoids as determined in the test for *Content of Total Carotenoids*

Calculate the percentage of other related compounds in the portion of *meso*-Zeaxanthin taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = individual peak response of any other peak (excluding zeaxanthin and lutein)

r_T = sum of all the peak responses

Acceptance criteria

Lutein: NMT 8.5%

Other related compounds: NMT 1.0% of any other individual related compound

• **STEREOISOMERIC COMPOSITION**

[NOTE—Use low-actinic glassware.]

Mobile phase: Gradient elution (see *Table 1*)

Table 1

Time (min)	*n*-Hexane (%)	2-Propanol (%)
0.0	95	5
50	95	5
55	50	50
63	50	50
65	95	5
75	95	5

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Standard solution: Transfer 2.5 mg of USP *meso*-Zeaxanthin RS to a 50-mL volumetric flask, add 25 mL of dehydrated alcohol, and sonicate at 60° for 2–5 min to dissolve the substance. Cool the flask to room temperature and dilute with *n*-hexane to volume. Transfer 1.0 mL of the resultant solution to a 15-mL test tube, and evaporate with a stream of nitrogen to dryness. Dissolve the residue in a 10.0-mL mixture of 2-propanol and *n*-hexane (5:95). Pass through a membrane filter of 0.45-µm pore size.

Sample solution: Transfer 2.5 mg of *meso*-Zeaxanthin to a 50-mL volumetric flask, add 25 mL of dehydrated alcohol, and sonicate at 60° for 2–5 min to dissolve the substance. Cool the flask to room temperature and dilute with *n*-hexane to volume. Transfer 1.0 mL of the resultant solution to a 15-mL test tube, and evaporate with a stream of nitrogen to dryness. Dissolve the residue in a 10.0-mL mixture of 2-propanol and *n*-hexane (5:95). Pass through a membrane filter of 0.45-µm pore size.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: HPLC
Detector: 453 nm
Column: 4.6-mm × 25-cm; 5-µm packing L51
Column temperature: 30°
Flow rate: 0.8 mL/min
Injection volume: 20 µL

System suitability
Sample: *Standard solution*
[NOTE—The approximate relative retention times for (3*S*,3′*S*)-zeaxanthin, (3*R*,3′*S-meso*)-zeaxanthin, (3*R*,3′*R*)-zeaxanthin, and (3*R*,3′*R*,6′*R*)-lutein are 0.94, 1.00, 1.06, and 1.11, respectively.]
Suitability requirements
Resolution: NLT 1.0 between each pair of peaks due to (3*S*,3′*S*)-zeaxanthin, (3*R*,3′*S-meso*)-zeaxanthin, (3*R*,3′*R*)-zeaxanthin, and (3*R*,3′*R*,6′*R*)-lutein
Chromatogram similarity: The chromatogram from the *Standard solution* is similar to the reference chromatogram provided with the lot of USP *meso*-Zeaxanthin RS being used.

Analysis
Samples: *Standard solution* and *Sample solution*
Identify the peaks of the relevant analytes from the *Standard solution* by comparison with the reference chromatogram provided with the USP Reference Standard being used.
Calculate the percentages of (3*S*,3′*S*)-zeaxanthin, (3*R*,3′*S-meso*)-zeaxanthin, and (3*R*,3′*R*)-zeaxanthin:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of the corresponding analyte
r_T = sum of all the peak responses

Acceptance criteria
(3*R*,3′*S-meso*)-Zeaxanthin: NLT 85.0%
(3*R*,3′*R*)-Zeaxanthin: NMT 15.0%
(3*S*,3′*S*)-Zeaxanthin: NMT 1.0%

IMPURITIES
- **LEAD** ⟨251⟩**:** NMT 1 ppm
- **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 1.0%

SPECIFIC TESTS
- **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 1.0%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP *meso*-Zeaxanthin RS
β,β-Carotene-3,3′-diol (3*R*,3′*S*)-; (3*R*,3′*S-meso*)-Zeaxanthin.
$C_{40}H_{56}O_2$ 568.88

meso-Zeaxanthin Preparation

DEFINITION
meso-Zeaxanthin Preparation is a combination of *meso*-Zeaxanthin with one or more inert substances. It may be in a solid or a liquid form. It contains NLT 95.0% and NMT 130.0% of the labeled amount of total carotenoids, calculated as zeaxanthin ($C_{40}H_{56}O_2$) on the anhydrous basis. It contains NLT 85.0% of zeaxanthin and NMT 9.0% of lutein of the total carotenoid content.

IDENTIFICATION
- **A.**
Sample solution: Prepare as directed for *Sample solution A* or *Sample solution B* in the test for *Content of Total Carotenoids*.
Analysis: Record the UV-Vis spectrum from 300 to 600 nm.
Acceptance criteria: The *Sample solution* shows a shoulder at about 427 nm, an absorption maximum at about 453 nm, and another maximum at about 480 nm.
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Zeaxanthin*.
- **C.** The retention time of the major peak of the *Sample solution* corresponds to that of (3*R*,3′*S-meso*)-zeaxanthin from the *Standard solution*, as obtained in the test for *Stereoisomeric Composition*.

COMPOSITION
- **CONTENT OF TOTAL CAROTENOIDS**
Sample stock solution A (for solid Preparations)**:** Transfer an amount of Preparation equivalent to 7.5 mg of zeaxanthin to a 100-mL low-actinic volumetric flask, add 5 mL of water, and sonicate at 60° for 5 min. Add 50 mL of alcohol, and dilute with methylene chloride to volume. Shake thoroughly to obtain a fine dispersion, transfer 10 mL of this dispersion to a centrifuge tube, and centrifuge. Discard the foremost 2 mL of the supernatant. Use the clear solution.
Sample solution A: Transfer 1.0 mL of *Sample stock solution A* to a 50-mL low-actinic flask, and dilute with a mixture of cyclohexane and alcohol (9:1) to volume.
Sample stock solution B (for liquid Preparations)**:** Transfer an amount of Preparation equivalent to 7.5 mg of *meso*-zeaxanthin to a 100-mL low-actinic volumetric flask, add 20 mL of chloroform, and sonicate for 5 min. Cool the solution to room temperature, and dilute with cyclohexane to volume. Shake thoroughly to obtain a fine dispersion, transfer 10 mL of this dispersion to a centrifuge tube, and centrifuge.
Sample solution B: Transfer 1.0 mL of *Sample stock solution B* to a 50-mL low-actinic flask, and dilute with cyclohexane to volume.
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Analytical wavelength: 453 nm
Cell: 1 cm
Blank: Cyclohexane
Analysis
Sample: *Sample solution*
Calculate the percentage of total carotenoids (*T*) as zeaxanthin ($C_{40}H_{56}O_2$) in the portion of Preparation taken:

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$$\text{Result} = A/(C \times F)$$

A = absorbance of the *Sample solution*
C = concentration of Preparation in *Sample solution A* or *Sample solution B* (g/mL)
F = coefficient of extinction ($E^{1\%}$) of zeaxanthin in cyclohexane (100 mL · g^{-1} · cm^{-1}), 2540

Calculate the percentage of the labeled amount of total carotenoids:

$$\text{Result} = (T/L) \times 100$$

T = percentage of total carotenoids found as calculated above
L = labeled amount of total carotenoids (%)

Acceptance criteria: 95.0%–130.0% of the labeled amount of total carotenoids as zeaxanthin ($C_{40}H_{56}O_2$) on the anhydrous basis

• **CONTENT OF ZEAXANTHIN**

[NOTE—Use low-actinic glassware.]

Mobile phase: Hexane and ethyl acetate (75:25)

Standard solution: 75 µg/mL of USP *meso*-Zeaxanthin RS in *Mobile phase*

Sample solution: Proceed as directed in *Sample stock solution A* or *Sample stock solution B* in the test for *Content of Total Carotenoids*. Pass through a membrane filter of 0.45-µm pore size.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 453 nm
Column: 4.6-mm × 25-cm; 3-µm packing L3
Flow rate: 1.5 mL/min
Injection volume: 10 µL

System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for zeaxanthin and lutein are about 1.0 and 0.95, respectively.]
Suitability requirements
Resolution: NLT 1.0 between zeaxanthin and lutein
Tailing factor: NMT 2.0
Relative standard deviation: NMT 2.0%

Analysis
Sample: *Sample solution*
Calculate the percentage of zeaxanthin relative to total carotenoids in the portion of Preparation taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = individual peak response of zeaxanthin
r_T = sum of all the peak responses

Calculate the percentage of zeaxanthin in the portion of Preparation taken:

$$\text{Result} = (r_U/r_T) \times T$$

r_U = individual peak response of *meso*-zeaxanthin in the *Sample solution*
r_T = sum of all the peak responses
T = percentage of total carotenoids as determined in the test for *Content of Total Carotenoids*

Acceptance criteria: NLT 85.0% of zeaxanthin in the total carotenoid content

• **LUTEIN AND OTHER RELATED COMPOUNDS**

Mobile phase, Standard solution, Sample solution, and **Chromatographic system:** Proceed as directed in the test for *Content of Zeaxanthin*.
Injection volume: 10 µL

Analysis
Sample: *Sample solution*
Calculate the percentage of lutein and other related compounds relative to total carotenoids in the portion of Preparation taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of lutein or other individual related compounds
r_T = sum of all the peak areas

Acceptance criteria
Lutein: NMT 9.0%
Any other individual related compound: NMT 1.0%
Total related compounds (including lutein): NMT 15.0%

• **STEREOISOMERIC COMPOSITION**

[NOTE—Use low-actinic glassware.]

Mobile phase: Gradient elution (see *Table 1*)

Table 1

Time (min)	*n*-Hexane (%)	2-Propanol (%)
0.0	95	5
50	95	5
55	50	50
63	50	50
65	95	5
75	95	5

Standard solution: Transfer 2.5 mg of USP *meso*-Zeaxanthin RS to a 50-mL volumetric flask, add 25 mL of dehydrated alcohol, and sonicate at 60° for 2–5 min to dissolve the substance. Cool the flask to room temperature and dilute with *n*-hexane to volume. Transfer 1.0 mL of the resultant solution to a 15-mL test tube, and evaporate with a stream of nitrogen to dryness. Dissolve the residue in a 10.0-mL mixture of 2-propanol and *n*-hexane (5:95). Pass through a membrane filter of 0.45-µm pore size.

Sample solution: Proceed as directed for *Sample stock solution A* or *Sample stock solution B* in the test for *Content of Total Carotenoids*. Transfer 1.0 mL of *Sample stock solution A* or *Sample stock solution B* to a 15-mL test tube, and evaporate with a stream of nitrogen to dryness. Dissolve the residue in a 10.0-mL mixture of 2-propanol and *n*-hexane (5:95). Pass through a membrane filter of 0.45-µm pore size.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: HPLC
Detector: 453 nm
Column: 4.6-mm × 25-cm; 5-µm packing L51
Column temperature: 30°
Flow rate: 0.8 mL/min
Injection volume: 20 µL

System suitability
Sample: *Standard solution*
[NOTE—The approximate relative retention times for (3*S*,3′*S*)-zeaxanthin, (3*R*,3′*S-meso*)-zeaxanthin, (3*R*,3′*R*)-zeaxanthin, and (3*R*,3′*R*,6′*R*)-lutein are 0.94, 1.00, 1.06, and 1.11, respectively.]

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Suitability requirements
Resolution: NLT 1.0 between each pair of peaks due to (3*S*,3′*S*)-zeaxanthin, (3*R*,3′*S-meso*)-zeaxanthin, (3*R*,3′*R*)-zeaxanthin, and (3*R*,3′*R*,6′*R*)-lutein
Chromatogram similarity: The chromatogram from the *Standard solution* is similar to the reference chromatogram provided with the lot of USP *meso*-Zeaxanthin RS being used.

Analysis
Samples: *Standard solution* and *Sample solution*
Identify the peaks of the relevant analytes in the chromatogram of the *Standard solution* by comparison with the reference chromatogram provided with the USP Reference Standard being used.
Calculate the percentage of (3*S*,3′*S*)-zeaxanthin, (3*R*,3′*S-meso*)-zeaxanthin, and (3*R*,3′*R*)-zeaxanthin:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of the corresponding analyte
r_T = sum of all the peak responses

Acceptance criteria
(3 *R*,3′ *S-meso*)-Zeaxanthin: NLT 85.0%
(3 *R*,3′ *R*)-Zeaxanthin: NMT 15.0%
(3 *S*,3′ *S*)-Zeaxanthin: NMT 1.0%

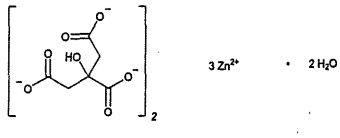
IMPURITIES
• **Residue on Ignition** ⟨281⟩: NMT 2.0%

SPECIFIC TESTS
• **Water Determination,** *Method I* ⟨921⟩: NMT 8.0% for solid Preparations; NMT 1.0% for liquid Preparations

ADDITIONAL REQUIREMENTS
• **Packaging and Storage:** Preserve in tightly sealed, light- and oxygen-resistant containers. Store in a cool place.
• **Labeling:** The label states the name and content of any carriers and antioxidants added to the formulation, and the content of total carotenoids as zeaxanthin.
• **USP Reference Standards** ⟨11⟩
USP *meso*-Zeaxanthin RS
β,β-Carotene-3,3′-diol (3*R*,3′*S*)-;
(3*R*,3′*S-meso*)-Zeaxanthin.
$C_{40}H_{56}O_2$ 568.88

Zinc Acetate Oral Solution
—*see Zinc Acetate Oral Solution General Monographs*

Zinc Citrate



$C_{12}H_{10}O_{14}Zn_3 \cdot 2H_2O$ 610.36
2-Hydroxy-1,2,3-propanetricarboxylic acid zinc salt, dihydrate [5990-32-9].
Anhydrous [546-46-3].

DEFINITION
Zinc Citrate contains NLT 31.3% of zinc (Zn), calculated on the dried basis.

IDENTIFICATION
• **A. Identification Tests—General,** *Zinc* ⟨191⟩: A solution (1 in 10) meets the requirements.
• **B. Identification Tests—General,** *Citrate* ⟨191⟩: A solution (1 in 10) meets the requirements.

ASSAY
• **Procedure**
Sample: 350 mg of Zinc Citrate, previously dried at 105° for 2 h
Blank: 60 mL of water
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.05 M edetate disodium VS
Endpoint detection: Visual
Analysis: Dissolve the *Sample* in 60 mL of water. Add 10 mL of ammonia–ammonium chloride buffer TS and 0.1 mL of eriochrome black TS. Titrate with the *Titrant* to a blue endpoint. Perform a blank determination.
Calculate the percentage of zinc (Zn) in the portion of Zinc Citrate taken:

$$\text{Result} = [(V - B) \times M \times F \times 100]/W$$

V = sample titrant volume (mL)
B = blank titrant volume (mL)
M = titrant molarity (mM/mL)
F = equivalency factor, 65.4 mg/mM
W = sample weight (mg)

Acceptance criteria: NLT 31.3% on the dried basis

IMPURITIES
• **Chloride and Sulfate,** *Chloride* ⟨221⟩: A 1.0-g portion shows no more chloride than corresponds to 0.7 mL of 0.020 N hydrochloric acid (NMT 0.05%).
• **Chloride and Sulfate,** *Sulfate* ⟨221⟩: A 1.8-g portion shows no more sulfate than corresponds to 0.5 mL of 0.020 N sulfuric acid (NMT 0.05%).
• **Limit of Arsenic, Cadmium, and Lead**
Arsenic standard solution: 1.0 µg/mL in 1% nitric acid, prepared from an arsenic standard solution (10 mg/L)
Cadmium standard solution: 1.0 µg/mL in 1% nitric acid, prepared from a cadmium standard solution (10 mg/L)
Lead standard solution: 1.0 µg/mL in 1% nitric acid, prepared from a lead standard solution (10 mg/L)
Multi-element standard solution: 10 µg/L of lead, 5 µg/L of cadmium, and 3 µg/L of arsenic in 1% nitric acid, prepared from the *Lead standard solution*, *Cadmium standard solution*, and *Arsenic standard solution*, respectively
Sample solution: 2 mg/mL of Zinc Citrate in 1% nitric acid
Instrumental conditions
(See *Plasma Spectrochemistry* ⟨730⟩.)
Mode: ICP-MS
Radio frequency: 1350 Watts
Nebulizer flow rate: 0.9 L/min
[Note—The radio frequency and nebulizer flow rate settings may be developed and optimized based on the manufacturer's recommendation.]
Detection atomic masses: As, Cd, and Pb
Blank: 1% nitric acid solution
Analysis
Samples: *Multi-element standard solution*, *Sample solution*, and *Blank*
Determine the responses of the *Multi-element standard solution*, *Sample solution*, and *Blank* at the masses indicated above.
Calculate the content of each element, in µg/g, in the portion of Zinc Citrate taken:

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$$\text{Result} = (r_U/r_S) \times (C_S/C_U)$$

r_U = peak response of the corresponding element from the *Sample solution*
r_S = peak response of the corresponding element from the *Multi-element standard solution*
C_S = concentration of the corresponding element in the *Multi-element standard solution* (µg/L)
C_U = concentration of Zinc Citrate in the *Sample solution* (g/L)

Acceptance criteria
Arsenic: NMT 3 µg/g
Cadmium: NMT 5 µg/g
Lead: NMT 10 µg/g

SPECIFIC TESTS
- **Loss on Drying** ⟨731⟩**:** Dry a sample at 105° for 2 h: it loses NMT 1.0% of its weight.
- **Microbial Enumeration Tests** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^3 cfu/g. The total combined yeasts and molds count does not exceed 10^2 cfu/g.
- **Microbiological Procedures for Absence of Specified Microorganisms** ⟨2022⟩**:** It meets the requirements of the test for absence of *Escherichia coli*.

ADDITIONAL REQUIREMENTS
- **Packaging and Storage:** Preserve in well-closed containers.

Zinc Citrate Tablets

DEFINITION
Zinc Citrate Tablets contain NLT 90.0% and NMT 110.0% of the labeled amount of zinc (Zn).

IDENTIFICATION
- **A.** The *Sample solution* for *Strength* produces line emissions or absorptions at the characteristic wavelengths for zinc.
- **B. Identification Tests—General,** *Citrate* ⟨191⟩
Sample solution: Transfer a quantity of powdered Tablets, equivalent to about 15 mg of zinc, to a centrifuge tube. Add 2–5 mL of water, sonicate for 1 min, shake, and centrifuge.
Acceptance criteria: Meets the requirements

STRENGTH
- **Content of Zinc**
Method 1
[Note—A standard stock solution is commercially available at different zinc concentrations, which may be used for preparation of the *Standard stock solution*. Necessary volumetric adjustment can be made in the *Standard solution*. Concentrations of the *Standard solution* and the *Sample solution* may be modified to fit the linear or working range of the instrument.]
Standard stock solution: Dissolve 625 mg of zinc oxide, weighed, and previously ignited to constant weight, in 10 mL of nitric acid, and add water to make 500.0 mL. This solution contains 1000 mg/L of zinc.
Standard solution: To a 500-mL volumetric flask add 200 mL of water and 10 mL of nitric acid, and mix thoroughly. Pipette 10.0 mL of the *Standard stock solution* into the volumetric flask, and dilute with water to volume to obtain a solution having a known concentration of about 20 mg/L of zinc.
Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer weighed portion of the powdered Tablets, equivalent to about 0.1 g of zinc, to a 50-mL flask. Add 10 mL of nitric acid, and heat the solution on a hot plate to boil gently, during which process fuming evolves. Boil the solution for an additional 30 min with constant swirling, during which no fuming should be observed. Cool the solution to room temperature, quantitatively transfer all of the solution to a 500-mL volumetric flask, dilute with water to volume, and mix. Pipette 25.0 mL of this solution into a 250-mL volumetric flask, add 5 mL of nitric acid, dilute with water to volume, mix, and filter.
Inductively coupled plasma system
(See *Plasma Spectrochemistry* ⟨730⟩.)
Mode: Atomic emission spectroscopy
Analytical wavelength: 206.20 nm. [Note—The operating conditions may be developed and optimized based on the manufacturer's recommendation. A typical setting includes radio frequency (RF) power of about 1300 watts, argon torch flow of about 15 L/min, argon auxiliary flow of about 0.2 L/min, and a nebulizer flow rate of about 0.8 L/min.]
Blank: 2% nitric acid solution
Analysis
Samples: *Standard solutions*, *Sample solution*, and *Blank*
Calculate the percentage of the labeled amount of zinc (Zn) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = response from the *Sample solution*
r_S = response from the *Standard solution*
C_S = concentration of the *Standard solution* (mg/L)
C_U = nominal concentration of zinc in the *Sample solution* (mg/L)

Acceptance criteria: 90.0%–110.0%
Method 2
Standard stock solution A: 1000 µg/mL of zinc from zinc oxide in 5 M hydrochloric acid (3.89 mg/mL) and diluted with water to final volume. [Note—Dissolve in 5 M hydrochloric acid by warming, if necessary. Cool, and then dilute to final volume.]
Standard stock solution B: 50 µg/mL of zinc from *Standard stock solution A* diluted with 0.125 N hydrochloric acid
Standard solutions: Transfer 1.0, 2.0, 3.0, 4.0, and 5.0 mL of *Standard stock solution B* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of zinc.
Sample solution: Finely powder NLT 20 tablets. Transfer an equivalent to 5 tablets to a porcelain crucible. Heat the crucible in a muffle furnace maintained at 550° for 6–12 h, and cool. Add 60 mL of hydrochloric acid, and boil gently on a hot plate or steam bath for 30 min, intermittently rinsing the inner surface of the crucible with 6 N hydrochloric acid. Cool, and quantitatively transfer the contents of the crucible to a 100-mL volumetric flask. Rinse the crucible with small portions of 6 N hydrochloric acid, and add the rinsings to the flask. Dilute with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute this solution quantitatively, with 0.125 N hydrochloric acid to obtain a nominal concentration of 2 µg/mL of zinc.
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Analytical wavelength: 213.8 nm
Lamp: Zinc hollow-cathode
Flame: Air–acetylene

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Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of zinc, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C, in µg/mL, of zinc in the *Sample solution*.

Calculate the percentage of the labeled amount of zinc (Zn) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = determined concentration of zinc in the *Sample solution* (µg/mL)

C_U = nominal concentration of zinc in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–110.0%

PERFORMANCE TESTS

• **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩

Medium: Water; 900 mL

Apparatus 2: 75 rpm

Time: 60 min

Analysis: Proceed as directed in *Method 1* or *Method 2* for *Strength*, making any necessary volumetric adjustments.

Sample solution: If *Method 1* is used, pipet 10.0 mL of the filtered pooled solution under test to a 50-mL volumetric flask, and dilute with 2% nitric acid solution to 50 mL. If *Method 2* is used, dilute the filtered pooled solution under test with 0.125 N hydrochloric acid to a concentration falling within the range of the *Standard solutions*.

Calculate the percentage of the labeled amount of zinc (Zn) dissolved:

$$\text{Result} = C \times (V_M/a) \times (D/L) \times 100$$

C = concentration of zinc in the *Sample solution* (mg/L)

V_M = volume of *Medium*, 900 mL

a = aliquot of solution under test (mL)

D = dilution factor to prepare the *Sample solution* from the aliquot taken

L = label claim (mg/Tablet)

Tolerances: NLT 75% of the labeled amount of zinc is dissolved.

• **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩**:** Meet the requirements

SPECIFIC TESTS

• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined yeast and mold count does not exceed 10^2 cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the test for absence of *Escherichia coli*.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

• **LABELING:** The label states the quantity of zinc in terms of mg/Tablet.

Zinc Gluconate

—see Zinc Gluconate General Monographs

Zinc and Vitamin C Lozenges

DEFINITION

Zinc and Vitamin C Lozenges contain NLT 90.0% and NMT 110.0% of the labeled amount of zinc (Zn) derived from substances generally recognized as safe and furnishing an ionizable form of zinc; and NLT 90.0% and NMT 120.0% of the labeled amount of vitamin C, as ascorbic acid ($C_6H_8O_6$), sodium ascorbate ($C_6H_7NaO_6$), or calcium ascorbate dihydrate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$). It contains no other vitamins or minerals for which nutritional value is claimed. It may contain other labeled added substances or additional ingredients in amounts that are unobjectionable.

IDENTIFICATION

• **A.**

Analysis: Proceed as directed in *Strength* for *Content of Zinc*.

Acceptance criteria: The *Sample solution* produces line emissions or absorptions at the characteristic wavelengths for zinc.

• **B.**

Analysis: Triturate a quantity of finely powdered Lozenges with sufficient alcohol to obtain a solution containing the equivalent of 20 mg/mL of ascorbic acid, sodium ascorbate, or calcium ascorbate dihydrate, and filter. Add 1 mL of 0.1 N hydrochloric acid to 4 mL of the filtrate from Lozenges containing sodium ascorbate or calcium ascorbate.

Acceptance criteria: A portion of the filtrate reduces alkaline cupric tartrate TS slowly at room temperature but more readily upon heating.

STRENGTH

• **CONTENT OF ZINC**

Procedure 1

[NOTE—A standard stock solution is commercially available at different zinc concentrations, which may be used for preparation of *Standard stock solution*. Necessary volumetric adjustment can be made in the *Standard solution*. Concentrations of the *Standard solution* and the *Sample solution* may be modified to fit the linear or working range of the instrument.]

Standard stock solution: Dissolve 625 mg of zinc oxide, weighed, and previously ignited to constant weight, in 10 mL of nitric acid, and add water to make 500.0 mL. This solution contains 1000 µg/mL of zinc.

Standard solution: To a 500-mL volumetric flask add 200 mL of water and 10 mL of nitric acid, and mix thoroughly. Pipet 10.0 mL of the *Standard stock solution* into the volumetric flask, and dilute with water to volume to obtain a solution having a known concentration of about 20 µg/mL of zinc.

Sample solution: Weigh and finely powder NLT 20 Lozenges. Transfer an accurately weighed portion of the powdered Lozenges, equivalent to about 0.1 g of zinc, to a 50-mL flask. Add 10 mL of nitric acid, and heat the solution on a hot plate to boil gently, during which process fuming evolves. Boil the solution for an additional 30 min with constant swirling, during which no fuming should be observed. Cool the solution to room temperature, quantitatively transfer all of the solution to a 500-mL volumetric flask, dilute with water to volume, and mix. Pipet 25.0 mL of this solution into a 250-mL volumetric flask, add 5 mL of nitric acid, dilute with water to volume, mix, and filter.

Inductively coupled plasma system

(See *Plasma Spectrochemistry* ⟨730⟩.)

Mode: Atomic emission spectroscopy

Analytical wavelength: 206.20 nm

DS Monographs

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[NOTE—The operating conditions may be developed and optimized based on the manufacturer's recommendation. A typical setting includes radio frequency (RF) power of about 1300 watts, argon torch flow of about 15 L/min, argon auxiliary flow of about 0.2 L/min, and a nebulizer flow rate of about 0.8 L/min.]

Blank: 2% nitric acid solution

Analysis

Samples: *Standard solutions*, *Sample solution*, and *Blank*

Calculate the percentage of the labeled amount of zinc (Zn) in the portion of Lozenges taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = response from the *Sample solution*
r_S = response from the *Standard solution*
C_S = concentration of zinc in the *Standard solution* (µg/mL)
C_U = nominal concentration of zinc in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–110.0% of the labeled amount of zinc

Procedure 2

Standard stock solution A: Dissolve zinc oxide in 5 M hydrochloric acid with warming, if necessary, to obtain a solution with a concentration of 3.89 mg/mL. Dilute with water to obtain a solution with a concentration of 1000 µg/mL of zinc.

Standard stock solution B: 50 µg/mL of zinc from *Standard stock solution A* in 0.125 N hydrochloric acid

Standard solutions: Transfer 1.0, 2.0, 3.0, 4.0, and 5.0 mL of *Standard stock solution B* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions containing 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of zinc.

Sample solution: Finely powder NLT 20 Lozenges. Transfer an equivalent to 5 Lozenges to a porcelain crucible. Heat the crucible in a muffle furnace maintained at 550° for 6–12 h, and cool. Add 60 mL of hydrochloric acid, and boil gently on a hot plate or steam bath for 30 min, intermittently rinsing the inner surface of the crucible with 6 N hydrochloric acid. Cool, and quantitatively transfer the contents of the crucible to a 100-mL volumetric flask. Rinse the crucible with small portions of 6 N hydrochloric acid, and add the rinsings to the flask. Dilute with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute this solution quantitatively with 0.125 N hydrochloric acid to obtain a nominal concentration of 2 µg/mL of zinc.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: 213.8 nm

Lamp: Zinc hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of zinc, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C, in µg/mL, of zinc in the *Sample solution*.

Calculate the percentage of the labeled amount of zinc (Zn) in the portion of Lozenges taken:

$$\text{Result} = (C/C_U) \times 100$$

C = determined concentration of zinc in the *Sample solution* (µg/mL)
C_U = nominal concentration of zinc in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–110.0% of the label claim

• **CONTENT OF VITAMIN C**

Sample solution: Transfer NLT 20 Lozenges to a 1000-mL volumetric flask containing 250 mL of metaphosphoric–acetic acids TS. Insert the stopper in the flask, and shake by mechanical means for 30 min or until the lozenges have disintegrated completely. Dilute with water to volume. Transfer a portion of the solution to a centrifuge tube, and centrifuge until a clear supernatant is obtained. Quantitatively dilute the clear supernatant with water, if necessary, to obtain a solution containing 0.5 mg/mL of ascorbic acid.

Blank: A mixture of 5.5 mL of metaphosphoric– acetic acids TS and 15 mL of water

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Direct titration

Titrant: Standard dichlorophenol–indophenol VS

Endpoint detection: Visual, a rose-pink color that persists for at least 5 s

Analysis: Transfer a volume of the *Sample solution*, equivalent to 2 mg of ascorbic acid, into a 50-mL conical flask. Add 5 mL of metaphosphoric–acetic acids TS, and titrate with *Titrant*. Correct for the volume of the *Titrant* consumed by the *Blank*.

Calculate the percentage of the labeled amount of ascorbic acid ($C_6H_8O_6$) in the portion of the Lozenges taken:

$$\text{Result} = \{[(V_S - V_B) \times F]/W\} \times 100$$

V_S = *Titrant* volume consumed by the *Sample solution* (mL)
V_B = *Titrant* volume consumed by the *Blank* (mL)
F = ascorbic acid equivalent of the *Titrant* (mg/mL)
W = nominal weight of ascorbic acid taken for *Analysis* (mg)

Acceptance criteria: 90.0%–120.0% of the labeled amount

SPECIFIC TESTS

• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩**:** The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined yeasts and molds count does not exceed 10^2 cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩**:** It meets the requirements of the test for absence of *Escherichia coli*.

PERFORMANCE TESTS

• **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩

Medium: 0.1 N hydrochloric acid; 900 mL

Apparatus 2: 75 rpm

Time: 60 min

Analysis: Determine the amount of zinc (Zn) and vitamin C dissolved, using the procedures in *Strength* for *Content of Zinc* and *Content of Vitamin C*, making any necessary volumetric adjustments. [NOTE—Proceed without delay in the vitamin C determination.]

Calculate the percentage of the labeled amount of zinc (Zn) dissolved:

$$\text{Result} = C \times (V_M/a) \times (D/L) \times 100$$

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C = measured concentration of Zinc in the *Sample solution* (mg/mL)
V_M = volume of *Medium*, 900 mL
a = aliquot of solution under test taken (mL)
D = dilution factor to prepare the *Sample solution* from the aliquot taken
L = labeled amount of zinc (mg/Tablet)

Calculate the percentage of the labeled amount of vitamin C, as ascorbic acid ($C_6H_8O_6$), dissolved:

$$\text{Result} = (V_S - V_B) \times F \times [(V_M/a)/L] \times 100$$

V_S = *Titrant* volume consumed by the *Sample solution* (mL)
V_B = *Titrant* volume consumed by the *Blank* (mL)
F = concentration of the *Titrant* in terms of the equivalent of ascorbic acid (mg/mL)
V_M = volume of *Medium*, 900 mL
a = volume of the aliquot taken for *Analysis*
L = labeled amount of ascorbic acid (mg/Tablet)

Tolerances: NLT 75% of the labeled amount of zinc (Zn) and vitamin C is dissolved.

- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** ⟨2091⟩**:** Meet the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **LABELING:** The label states the quantity of zinc and vitamin C as ascorbic acid in mg per Lozenge, and the salt form of zinc and the chemical form of vitamin C present in the Lozenges.

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NF 38

THE NATIONAL FORMULARY

Official from May 1, 2020

THIRTY-EIGHTH EDITION

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NF Admissions

Articles Admitted to *NF 38*

First Supplement (August 1, 2019)

Star Anise Oil

Second Supplement (December 1, 2019)

Neohesperidin Dihydrochalcone

New Articles Appearing in *NF 38* That Were Not Included in *NF 37* Including Supplements

[NOTE—The articles included in this list are noted in the book with the following symbols ▲▲(NF 1-May-2020). This applies to new articles as well as sections of existing items that have been revised.]

NEW ARTICLES APPEARING IN *NF 38*

Glyceryl Mono and Dicaprylate
Glyceryl Mono and Dicaprylocaprate
Polypropylene Glycol 11 Stearyl Ether

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NF Annotated List

General Notices, Monographs, General Chapters, Reagents, and Tables Affected by Changes Appearing in NF 38

Page citations refer to the pages of NF 38. Note—In the list below, if a section is new or if a subsection is added to or deleted from an existing section, it is labeled as such in parentheses after the section or subsection name. Items on this list that appear without the designation "new", "added", or "deleted" are items in which changes have been made to existing official text.

Excipients (USP and NF), Listed by Category

Emollient; Solvent; Vehicle
- Polypropylene Glycol 11 Stearyl Ether (new)

Lubricant; Emulsifying Agent
- Glyceryl Mono and Dicaprylate (new)
- Glyceryl Mono and Dicaprylocaprate (new)

Sweetening Agent; Flavors and Fragrance
- Anise Oil (new)
- Neohesperidin Dihydrochalcone (new)

Monographs (NF 38)

Alginic Acid, 5608
- DEFINITION
- IDENTIFICATION
- ADDITIONAL REQUIREMENTS
  - *USP Reference Standards* (added)

Low-Substituted Carboxymethylcellulose Sodium, 5682
- IDENTIFICATION
- ADDITIONAL REQUIREMENTS
- *USP Reference Standards* (added)

Gelatin (Stage 4 Harmonization official May 1,2020), 5783
- DEFINITION
- IDENTIFICATION
- SPECIFIC TESTS
  - *Sulfur Dioxide*

Glyceryl Mono and Dicaprylate (new), 5794

Glyceryl Mono and Dicaprylocaprate (new), 5796

Glyceryl Monocaprylate, 5799
- TITLE
- DEFINITION
- IDENTIFICATION
  - *Fats and Fixed Oils*
- ASSAY
- ADDITIONAL REQUIREMENTS
  - *Labeling* (deleted)

Glyceryl Monocaprylocaprate, 5802
- TITLE
- CHEMICAL INFORMATION
- DEFINITION
- IDENTIFICATION
  - *Fats and Fixed Oils*
- ASSAY
- ADDITIONAL REQUIREMENTS
  - *USP Reference Standards*
  - *Labeling* (deleted)

Hexylene Glycol, 5814
- CHEMICAL INFORMATION
- DEFINITION
- IDENTIFICATION
  - *Test A* and *Test B* (added)
- ASSAY (added)
- IMPURITIES (added)
- ADDITIONAL REQUIREMENTS
  - *USP Reference Standards*

Polypropylene Glycol 11 Stearyl Ether (new), 5963

Potassium Alginate, 5975
- DEFINITION
- IDENTIFICATION
- ADDITIONAL REQUIREMENTS
  - *USP Reference Standards* (added)

Potassium Sorbate, 5980
- CHEMICAL INFORMATION
- DEFINITION
- IDENTIFICATION
- ASSAY
- IMPURITIES (added)
- ADDITIONAL REQUIREMENTS
  - *USP Reference Standards* (added)

Sodium Alginate, 6011
- DEFINITION
- IDENTIFICATION
- ADDITIONAL REQUIREMENTS
  - *USP Reference Standards* (added)

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Medium-Chain Triglycerides, 6095
IDENTIFICATION
IMPURITIES
Alkaline Impurities
SPECIFIC TESTS
Fats and Fixed Oils, Unsaponifiable Matter (deleted)
Fats and Fixed Oils, Saponification Value (deleted)
Refractive Index (deleted)
ADDITIONAL REQUIREMENTS
Labeling

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Excipients

USP and NF Excipients, Listed by Functional Category

In the following reference table, the grouping of excipients by functional category is intended to summarize commonly identified purposes that these excipients serve in drug product formulations. The association of a functional category with a particular dosage form in this table is not absolute and does not limit the use of an excipient to a single type of dosage form or delivery system.

Adhesive

Dosage Form: Transdermals and "Patches"

- Dimethicone
- Polyisobutylene

Air Displacement

- Carbon Dioxide
- Nitrogen

Alcohol Denaturant

- Denatonium Benzoate
- Methyl Isobutyl Ketone
- Sucrose Octaacetate

Antifoaming Agent

- Dimethicone
- Lauric Acid
- Myristic Acid
- Palmitic Acid
- Simethicone

Antimicrobial Preservative

Dosage Form: Oral Liquids

- Alcohol
- Benzalkonium Chloride
- Benzalkonium Chloride Solution
- Benzethonium Chloride
- Benzoic Acid
- Benzyl Alcohol
- Boric Acid
- Butylparaben
- Calcium Acetate
- Calcium Chloride
- Calcium Lactate
- Calcium Propionate
- Cetrimonium Bromide
- Cetylpyridinium Chloride
- Chlorobutanol
- Chlorocresol
- Chloroxylenol
- Cresol
- Dehydroacetic Acid
- Erythorbic Acid
- Ethylparaben
- Ethylparaben Sodium
- Glycerin
- Imidurea
- Mandelic Acid
- Methylparaben
- Methylparaben Sodium
- Monothioglycerol
- Pentetic Acid
- Phenol
- Phenoxyethanol
- Phenylethyl Alcohol
- Phenylmercuric Acetate
- Phenylmercuric Nitrate
- Potassium Benzoate
- Potassium Metabisulfite
- Potassium Sorbate
- Propionic Acid
- Propylene Glycol
- Propylparaben
- Propylparaben Sodium
- Sodium Acetate
- Sodium Benzoate
- Sodium Borate
- Sodium Dehydroacetate
- Sodium Lactate Solution
- Sodium Metabisulfite
- Sodium Propionate
- Sodium Sulfite
- Sorbic Acid
- Sulfur Dioxide
- Thimerosal
- Thymol
- Zinc Oxide

Dosage Form: Ophthalmic Preparations

- Benzalkonium Chloride

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Benzyl Alcohol
Chlorobutanol
Propylparaben
Sorbic Acid

Antioxidant

Dosage Form: Oral Liquids
Ascorbic Acid
Ascorbyl Palmitate
Butylated Hydroxyanisole
Butylated Hydroxytoluene
Citric Acid Monohydrate
Erythorbic Acid
Fumaric Acid
Hypophosphorous Acid
Lactobionic Acid
Malic Acid
Methionine
Monothioglycerol
Potassium Metabisulfite
Propionic Acid
Propyl Gallate
Racemethionine
Sodium Ascorbate
Sodium Bisulfite
Sodium Formaldehyde Sulfoxylate
Sodium Metabisulfite
Sodium Sulfite
Sodium Thiosulfate
Stannous Chloride
Sulfur Dioxide
Thymol
Tocopherol
Tocopherols Excipient
Vitamin E
Vitamin E Polyethylene Glycol Succinate

Bulking Agent

Alpha-Lactalbumin
Polydextrose
Polydextrose, Hydrogenated
Pullulan

Dosage Form: Parenterals
Creatinine
Glycine
Mannitol
Trehalose

Capsule Shell

Dosage Form: Tablets and Capsules
Gelatin
Hypromellose
Pullulan

Dosage Form: Dry Powder Inhalers
Gelatin
Hypromellose

Carrier

Dosage Form: Dry Powder Inhalers
Lactose, Anhydrous
Lactose, Monohydrate

Chelating and/or Complexing Agent

Dosage Form: Oral Liquids
Alfadex
Betadex
Betadex Sulfobutyl Ether Sodium
Citric Acid Monohydrate
Edetate Calcium Disodium
Edetate Disodium
Edetic Acid
Galactose
Gamma Cyclodextrin
Hydroxypropyl Betadex
Alpha-Lactalbumin
Malic Acid
Oxyquinoline Sulfate
Pentetic Acid
Potassium Citrate
Sodium Phosphate, Dibasic
Sodium Phosphate, Monobasic

Coating Agent

Dosage Form: Tablets and Capsules
Amino Methacrylate Copolymer
Ammonio Methacrylate Copolymer
Ammonio Methacrylate Copolymer Dispersion
Calcium Carbonate
Carboxymethylcellulose Calcium
Carboxymethylcellulose Sodium
Carboxymethylcellulose Sodium, Enzymatically-Hydrolyzed
Cellaburate
Cellacefate
Cellulose Acetate
Cetyl Alcohol
Chitosan
Coconut Oil
Coconut Oil, Hydrogenated
Copovidone
Corn Syrup Solids
Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion
Ethylcellulose
Ethylcellulose Aqueous Dispersion
Ethylcellulose Dispersion Type B
Ethylene Glycol and Vinyl Alcohol Graft Copolymer
Gelatin
Glaze, Pharmaceutical
Glucose, Liquid
Glyceryl Behenate
[(Title for this monograph—not to change until December 1, 2019.) (Prior to December 1, 2019, the current practice of labeling the article of commerce with the name Glyceryl Behenate may be continued. Use of the name Glyceryl Dibehenate will be permitted as of December 1, 2014; however, the use

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of this name will not be mandatory until December 1, 2019. The 60-month extension will provide the time needed by manufacturers and users to make necessary changes.)]
Glyceryl Dibehenate
Hydroxyethyl Cellulose
Hydroxypropyl Cellulose
Hypromellose
Hypromellose Acetate Succinate
Hypromellose Phthalate
Isomalt
Alpha-Lactalbumin
Maltitol
Maltodextrin
Methacrylic Acid and Ethyl Acrylate Copolymer
Methacrylic Acid and Ethyl Acrylate Copolymer Dispersion
Methacrylic Acid and Ethyl Acrylate Copolymer, Partially-Neutralized
Methacrylic Acid and Methyl Methacrylate Copolymer
Methylcellulose
Palm Kernel Oil
Palm Oil
Palm Oil, Hydrogenated
Polydextrose
Polydextrose, Hydrogenated
Polyethylene Glycol
Polyethylene Glycol 3350
Polyethylene Oxide
Polyvinyl Acetate
Polyvinyl Acetate Dispersion
Polyvinyl Acetate Phthalate
Polyvinyl Alcohol
Pullulan
Rapeseed Oil, Fully Hydrogenated
Rapeseed Oil, Superglycerinated Fully Hydrogenated
Shellac
Starch, Pregelatinized Modified
Sucrose
Sugar, Confectioner's
Sunflower Oil
Titanium Dioxide
Wax, Carnauba
Wax, Microcrystalline
Xylitol
Zein
Zinc Oxide

Colloid Stabilizing Agent

Dosage Form: Radiopharmaceuticals
Gelatin

Coloring Agent

Caramel
Ferric Oxide
Ferrosoferric Oxide

Dosage Form: Tablets and Capsules
Aluminum Oxide

Desiccant

Calcium Chloride
Calcium Sulfate
Polyvinyl Acetate
Silicon Dioxide

Diluent

Dosage Form: Tablets and Capsules
Amino Methacrylate Copolymer
Ammonio Methacrylate Copolymer
Ammonio Methacrylate Copolymer Dispersion
Calcium Carbonate
Calcium Phosphate, Dibasic, Anhydrous
Calcium Phosphate, Dibasic, Dihydrate
Calcium Phosphate, Tribasic
Calcium Sulfate
Cellaburate
Cellulose, Microcrystalline
Cellulose, Silicified Microcrystalline
Cellulose, Powdered
Cellulose Acetate
Corn Syrup
Corn Syrup Solids
Dextrates
Dextrin
Dextrose
Dextrose Excipient
Erythritol
Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion
Fructose
Invert Sugar
Isomalt
Kaolin
Alpha-Lactalbumin
Lactitol
Lactose, Anhydrous
Lactose, Monohydrate
Magnesium Carbonate
Magnesium Oxide
Maltitol
Maltodextrin
Maltose
Mannitol
Methacrylic Acid and Ethyl Acrylate Copolymer
Methacrylic Acid and Ethyl Acrylate Copolymer Dispersion
Methacrylic Acid and Methyl Methacrylate Copolymer
Polydextrose
Polyethylene Glycol
Polyethylene Glycol 3350
Propylene Glycol Monocaprylate
Pullulan
Simethicone
Sodium Chloride
Sorbitol
Starch, Pregelatinized
Starch, Pregelatinized Modified
Starch, Corn

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Starch, Hydroxypropyl Corn
Starch, Pregelatinized Hydroxypropyl Corn
Starch, Pea
Starch, Hydroxypropyl Pea
Starch, Pregelatinized Hydroxypropyl Pea
Starch, Potato
Starch, Hydroxypropyl Potato
Starch, Pregelatinized Hydroxypropyl Potato
Starch, Tapioca
Starch, Wheat
Starch Hydrolysate, Hydrogenated
Sucrose
Sugar, Compressible
Sugar, Confectioner's
Sugar Spheres
Sunflower Oil
Talc
Trehalose
Xylitol

Disintegrant

Dosage Form: Tablets and Capsules

Alginic Acid
Carboxymethylcellulose Calcium
Carboxymethylcellulose Sodium
Cellulose, Microcrystalline
Cellulose, Silicified Microcrystalline
Cellulose, Powdered
Croscarmellose Sodium
Crospovidone
Glycine
Guar Gum
Hydroxypropyl Cellulose, Low-Substituted
Magnesium Aluminum Silicate
Maltose
Methylcellulose
Polacrilin Potassium
Pullulan
Silicon Dioxide, Colloidal
Sodium Alginate
Sodium Starch Glycolate
Starch, Pregelatinized Modified
Starch, Corn
Starch, Hydroxypropyl Corn
Starch, Pregelatinized Hydroxypropyl Corn
Starch, Pea
Starch, Hydroxypropyl Pea
Starch, Pregelatinized Hydroxypropyl Pea
Starch, Potato
Starch, Hydroxypropyl Potato
Starch, Pregelatinized Hydroxypropyl Potato
Starch, Tapioca
Starch, Wheat
Trehalose

Change to read:

Emollient

Dosage Form: Semisolids, Topicals, and Suppositories

Alkyl (C12-15) Benzoate
Almond Oil
Aluminum Monostearate
Canola Oil
Castor Oil
Cetostearyl Alcohol
Cholesterol
Coconut Oil
Cyclomethicone
Dimethicone
Ethylene Glycol Stearates
Glycerin
Glyceryl Monooleate
Glyceryl Monostearate
Isopropyl Isostearate
Isopropyl Myristate
Isopropyl Palmitate
Isostearyl Isostearate
Hydrogenated Lanolin
Lecithin
Mineral Oil
Mineral Oil, Light
Myristyl Alcohol
Octyldodecanol
Oleyl Alcohol
Oleyl Oleate
Petrolatum
Polydecene, Hydrogenated
▲Polypropylene Glycol 11 Stearyl Ether▲ (NF 1-May-2020)
Propylene Glycol Dilaurate
Propylene Glycol Monolaurate
Safflower Oil
Soybean Oil, Hydrogenated
Sunflower Oil
Wax, Cetyl Esters
Xylitol
Zinc Acetate

Change to read:

Emulsifying Agent

Dosage Form: Oral Liquids

Acacia
Agar
Behenoyl Polyoxylglycerides
Benzalkonium Chloride
Benzyl Benzoate
Caprylic Acid
Caprylocaproyl Polyoxylglycerides
Carbomer Copolymer
Carbomer Homopolymer
Carbomer Interpolymer
Carboxymethylcellulose Calcium
Cetostearyl Alcohol
Cetyl Alcohol
Cetylpyridinium Chloride
Cholesterol
Coconut Oil
Desoxycholic Acid

[(Title for this monograph—not to change until December 1, 2021.) (Prior to December 1, 2021, the

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current practice of labeling the article of commerce with the name Desoxycholic Acid may be continued. Use of the name Deoxycholic Acid will be permitted as of December 1, 2016; however, the use of this name will not be mandatory until December 1, 2021. The 60-month extension will provide the time needed by manufacturers and users to make necessary changes.)]

Deoxycholic Acid
Diethanolamine (Adjunct)
Diethylene Glycol Monoethyl Ether
Diethylene Glycol Stearates
Egg Phospholipids
Ethylene Glycol Stearates
Glyceryl Distearate
▲Glyceryl Mono and Dicaprylate
Glyceryl Mono and Dicaprylocaprate▲ (NF 1-May-2020)
Glyceryl Monocaprylate
Glyceryl Monocaprylocaprate
Glyceryl Monolinoleate
Glyceryl Monooleate
Glyceryl Monostearate
Glyceryl Tricaprylate
Glyceryl Tristearate
Hydroxypropyl Cellulose
Hypromellose
Isopropyl Isostearate
Isostearyl Isostearate
Alpha-Lactalbumin
Lanolin
Hydrogenated Lanolin
Lanolin Alcohols
Lauric Acid
Lauroyl Polyoxylglycerides
Lecithin
Linoleoyl Polyoxylglycerides
Magnesium Oxide
Medium-chain Triglycerides
Methylcellulose
Mono- and Di-glycerides
Monoethanolamine (Adjunct)
Myristic Acid
Octyldodecanol
Oleic Acid (Adjunct)
Oleoyl Polyoxylglycerides
Oleyl Alcohol (Stabilizer)
Oleyl Oleate
Palm Kernel Oil
Palm Oil
Palmitic Acid
Pectin
Poloxamer
Polycarbophil
Polyglyceryl 3 Diisostearate
Polyglyceryl Dioleate
Polyoxyl 10 Oleyl Ether
Polyoxyl 15 Hydroxystearate
Polyoxyl 20 Cetostearyl Ether
Polyoxyl 35 Castor Oil
Polyoxyl 40 Castor Oil, Hydrogenated
Polyoxyl 40 Stearate
Polyoxyl Lauryl Ether
Polyoxyl Stearate
Polyoxyl Stearyl Ether
Polysorbate 20
Polysorbate 40
Polysorbate 60
Polysorbate 80
Potassium Alginate
Propylene Glycol Alginate
Propylene Glycol Diacetate
Propylene Glycol Dicaprylate/Dicaprate
Propylene Glycol Dilaurate
Propylene Glycol Monocaprylate
Propylene Glycol Monolaurate
Propylene Glycol Monostearate
Rapeseed Oil, Superglycerinated Fully Hydrogenated
Sodium Borate
Sodium Cetostearyl Sulfate
Sodium Lauryl Sulfate
Sodium Stearate
Sorbitan Monolaurate
Sorbitan Monooleate
Sorbitan Monopalmitate
Sorbitan Monostearate
Sorbitan Sesquioleate
Sorbitan Trioleate
Stannous Chloride
Starch, Hydroxypropyl Corn
Starch, Hydroxypropyl Pea
Starch, Hydroxypropyl Potato
Stearic Acid
Stearoyl Polyoxylglycerides
Sucrose Palmitate
Sucrose Stearate
Sunflower Oil
Trolamine
Vitamin E Polyethylene Glycol Succinate
Wax, Emulsifying

Film-Forming Agent

Dosage Form: Tablets and Capsules

Alginic Acid
Amino Methacrylate Copolymer
Ammonio Methacrylate Copolymer
Ammonio Methacrylate Copolymer Dispersion
Carboxymethylcellulose Calcium
Carboxymethylcellulose Sodium
Carboxymethylcellulose Sodium, Enzymatically-Hydrolyzed
Cellaburate
Cellacefate
Cellulose Acetate
Chitosan
Copovidone
Dibutyl Phthalate
Diethyl Phthalate
Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion
Ethylcellulose
Ethylcellulose Aqueous Dispersion

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Ethylcellulose Dispersion Type B
Ethylene Glycol and Vinyl Alcohol Grafted Copolymer
Gelatin
Glaze, Pharmaceutical
Hydroxyethyl Cellulose
Hydroxypropyl Cellulose
Hypromellose
Hypromellose Acetate Succinate
Hypromellose Phthalate
Methacrylic Acid and Ethyl Acrylate Copolymer
Methacrylic Acid and Ethyl Acrylate Copolymer Dispersion
Methacrylic Acid and Ethyl Acrylate Copolymer, Partially-Neutralized
Methacrylic Acid and Methyl Methacrylate Copolymer
Methylcellulose
Polyethylene Glycol 3350
Polyvinyl Acetate
Polyvinyl Acetate Dispersion
Polyvinyl Acetate Phthalate
Polyvinyl Alcohol
Pullulan
Pyroxylin
Shellac
Sodium Alginate

Dosage Form: Transdermals and "Patches"

Chitosan
Dextrin
Gelatin
Hydroxyethyl Cellulose
Hypromellose
Pectin
Polyethylene Glycol
Polyvinyl Alcohol
Pullulan
Sodium Alginate
Xanthan Gum

Filtering Aid

Cellulose, Powdered
Siliceous Earth, Purified

Change to read:

Flavors and Fragrance

▲Anise Oil▲ (NF 1-May-2020)
Eucalyptus Oil
Isobutyl Alcohol
▲Neohesperidin Dihydrochalcone▲ (NF 1-May-2020)
Sodium Succinate
▲Star Anise Oil▲ (NF 1-Dec-2019)

Dosage Form: Tablets and Capsules

Adipic Acid
Almond Oil
Anethole
Benzaldehyde
Denatonium Benzoate
Ethyl Acetate
Ethyl Maltol
Ethyl Vanillin
Ethylcellulose
Fructose
Fumaric Acid
L-Glutamic Acid, Hydrochloride
Lactitol
Leucine
Malic Acid
Maltol
Menthol
Methionine
Methyl Salicylate
Monosodium Glutamate
Peppermint
Peppermint Oil
Peppermint Spirit
Racemethionine
Rose Oil
Rose Water, Stronger
Sodium Acetate
Sodium Lactate Solution
Tartaric Acid
Thymol
Vanillin

Free Radical Scavenger

Dosage Form: Radiopharmaceuticals

Aminobenzoic Acid
Methylene Blue

Glidant and/or Anticaking Agent

Dosage Form: Tablets and Capsules

Calcium Phosphate, Tribasic
Calcium Silicate
Cellulose, Powdered
Magnesium Oxide
Magnesium Silicate
Magnesium Trisilicate
Silica, Dental-Type
Silica, Hydrophobic Colloidal
Silicon Dioxide, Colloidal
Sodium Stearate
Talc

Humectant

Corn Syrup Solids
Cyclomethicone
Erythritol
Glycerin
Hexylene Glycol
Inositol
Hydrogenated Lanolin
Maltitol
Polydextrose
Polydextrose, Hydrogenated
Propylene Glycol
Sodium Lactate Solution
Sorbitol
Sorbitol Sorbitan Solution
Starch Hydrolysate, Hydrogenated
Tagatose

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Triacetin
Xylitol

Change to read:

Lubricant

Dosage Form: Tablets and Capsules

Behenoyl Polyoxylglycerides
Calcium Stearate
Castor Oil, Hydrogenated
Coconut Oil, Hydrogenated
Glyceryl Behenate
[(Title for this monograph—not to change until December 1, 2019.) (Prior to December 1, 2019, the current practice of labeling the article of commerce with the name Glyceryl Behenate may be continued. Use of the name Glyceryl Dibehenate will be permitted as of December 1, 2014; however, the use of this name will not be mandatory until December 1, 2019. The 60-month extension will provide the time needed by manufacturers and users to make necessary changes.)]
Glyceryl Dibehenate
▲Glyceryl Mono and Dicaprylate
Glyceryl Mono and Dicaprylocaprate▲ (NF 1-May-2020)
Glyceryl Monocaprylate
Glyceryl Monocaprylocaprate
Glyceryl Monostearate
Glyceryl Tricaprylate
Glyceryl Tristearate
Lauric Acid
Magnesium Stearate
Mineral Oil, Light
Myristic Acid
Palm Oil, Hydrogenated
Palmitic Acid
Poloxamer
Polyethylene Glycol
Polyethylene Glycol 3350
Polyoxyl 10 Oleyl Ether
Polyoxyl 15 Hydroxystearate
Polyoxyl 20 Cetostearyl Ether
Polyoxyl 35 Castor Oil
Polyoxyl 40 Castor Oil, Hydrogenated
Polyoxyl 40 Stearate
Polysorbate 20
Polysorbate 40
Polysorbate 60
Polysorbate 80
Potassium Benzoate
Sodium Benzoate
Sodium Lauryl Sulfate
Sodium Stearate
Sodium Stearyl Fumarate
Sorbitan Monolaurate
Sorbitan Monooleate
Sorbitan Monopalmitate
Sorbitan Monostearate
Sorbitan Sesquioleate
Sorbitan Trioleate
Stearic Acid
Stearic Acid, Purified
Sucrose Stearate
Talc
Vegetable Oil, Hydrogenated, Type I
Zinc Stearate

Ointment Base

Dosage Form: Semisolids, Topicals, and Suppositories

Caprylocaproyl Polyoxylglycerides
Coconut Oil
Diethylene Glycol Monoethyl Ether
Lanolin
Hydrogenated Lanolin
Lanolin Alcohols
Lauroyl Polyoxylglycerides
Linoleoyl Polyoxylglycerides
Ointment, Hydrophilic
Ointment, White
Ointment, Yellow
Oleoyl Polyoxylglycerides
Paraffin
Petrolatum
Petrolatum, Hydrophilic
Petrolatum, White
Polydecene, Hydrogenated
Polyethylene Glycol
Polyethylene Glycol 3350
Polyethylene Glycol Monomethyl Ether
Polyglyceryl 3 Diisostearate
Rose Water Ointment
Squalane
Stearoyl Polyoxylglycerides
Vegetable Oil, Hydrogenated, Type II
Vitamin E Polyethylene Glycol Succinate

Pharmaceutical Water

Dosage Form: Parenterals

Water for Injection
Water for Injection, Bacteriostatic
Water for Injection, Sterile
Water for Irrigation, Sterile
Water Purified
Water Purified, Sterile

pH Modifier (Acidifying Agent/ Alkalizing Agent/Buffering Agent)

Sodium Succinate

Dosage Form: Oral Liquids

Acetic Acid
Acetic Acid, Glacial
Adipic Acid
Ammonia Solution, Strong
Ammonium Carbonate
Ammonium Chloride
Ammonium Phosphate
Boric Acid
Calcium Carbonate
Calcium Hydroxide
Calcium Lactate

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Calcium Phosphate, Tribasic
Citric Acid Monohydrate
Citric Acid, Anhydrous
Diethanolamine
Fumaric Acid
Glycine
Hydrochloric Acid
Hydrochloric Acid, Diluted
Alpha-Lactalbumin
Lactic Acid
Lysine Hydrochloride
Maleic Acid
Malic Acid
Methionine
Monoethanolamine
Monosodium Glutamate
Nitric Acid
Phosphoric Acid
Phosphoric Acid, Diluted
Potassium Bicarbonate
Potassium Citrate
Potassium Hydroxide
Potassium Metaphosphate
Potassium Phosphate, Dibasic
Potassium Phosphate, Monobasic
Propionic Acid
Racemethionine
Sodium Acetate
Sodium Bicarbonate
Sodium Borate
Sodium Carbonate
Sodium Citrate
Sodium Hydroxide
Sodium Lactate Solution
Sodium Phosphate, Dibasic
Sodium Phosphate, Monobasic
Succinic Acid
Sulfuric Acid
Tartaric Acid
Trolamine

Plasticizer

Dosage Form: Tablets and Capsules
Acetyltributyl Citrate
Acetyltriethyl Citrate
Benzyl Benzoate
Castor Oil
Chlorobutanol
Diacetylated Monoglycerides
Dibutyl Sebacate
Diethyl Phthalate
Glycerin
Mannitol
Polyethylene Glycol
Polyethylene Glycol 3350
Polyethylene Glycol Monomethyl Ether
Propylene Glycol
Pullulan
Sorbitol
Sorbitol Sorbitan Solution
Triacetin
Tributyl Citrate
Triethyl Citrate
Vitamin E

Polymer Membrane

Dosage Form: Tablets and Capsules
Amino Methacrylate Copolymer
Ammonio Methacrylate Copolymer
Ammonio Methacrylate Copolymer Dispersion
Cellaburate
Cellulose Acetate
Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion
Ethylcellulose
Ethylcellulose Aqueous Dispersion
Ethylcellulose Dispersion Type B
Pullulan

Polymers for Ophthalmic Use

Dosage Form: Ophthalmic Preparations
Carbomer Copolymer
Carbomer Homopolymer
Carbomer Interpolymer
Carmellose
Guar Gum
Hydroxyethyl Cellulose
Hypromellose
Polyvinyl Alcohol
Povidone
Xanthan Gum

Propellant

Dosage Form: Aerosols
Butane
Carbon Dioxide
Dichlorodifluoromethane
Dichlorotetrafluoroethane
Isobutane
Nitrogen
Nitrous Oxide
Propane
Trichloromonofluoromethane

Reducing Agent

Dosage Form: Radiopharmaceuticals
Stannous Chloride
Stannous Fluoride

Release-Modifying Agent

Dosage Form: Tablets and Capsules
Alginic Acid
Carbomer Copolymer
Carbomer Homopolymer
Carbomer Interpolymer
Carboxymethylcellulose Sodium
Carrageenan
Cellaburate
Ethylcellulose

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Ethylcellulose Aqueous Dispersion
Ethylcellulose Dispersion Type B
Glyceryl Monooleate
Glyceryl Monostearate
Guar Gum
Hydroxypropyl Betadex
Hydroxypropyl Cellulose
Hypromellose
Polyethylene Oxide
Polyvinyl Acetate Dispersion
Shellac
Sodium Alginate
Starch, Pregelatinized
Starch, Pregelatinized Modified
Xanthan Gum

Sequestering Agent

Dosage Form: Oral Liquids

Betadex
Betadex Sulfobutyl Ether Sodium
Calcium Acetate
Cyclodextrin, Gamma
Hydroxypropyl Betadex
Pentetic Acid
Pullulan
Sodium Citrate
Sodium Tartrate
Tartaric Acid

Change to read:

Solvent

Acetone
Alcohol
Alcohol, Diluted
Almond Oil
Amylene Hydrate
Benzyl Alcohol
Benzyl Benzoate
Butyl Alcohol
Butylene Glycol
Canola Oil
Caprylocaproyl Polyoxylglycerides
Castor Oil
Corn Oil
Cottonseed Oil
Dibutyl Phthalate
Diethyl Phthalate
Diethylene Glycol Monoethyl Ether
Dimethyl Sulfoxide
Ethyl Acetate
Ethyl Oleate
Glycerin
Hexylene Glycol
Isobutyl Alcohol
Isopropyl Alcohol
Isopropyl Myristate
Isopropyl Palmitate
Lauroyl Polyoxylglycerides
Linoleoyl Polyoxylglycerides
Medium-chain Triglycerides
Methyl Alcohol
Methyl Isobutyl Ketone
Methylene Chloride
Methylpyrrolidone
Mineral Oil
Mineral Oil, Light
Oleoyl Polyoxylglycerides
Peanut Oil
Polydecene, Hydrogenated
Polyethylene Glycol
Polyethylene Glycol 3350
Polyethylene Glycol Monomethyl Ether
▲Polypropylene Glycol 11 Stearyl Ether▲ (NF 1-May-2020)
Propylene Carbonate
Propylene Glycol
Propylene Glycol Diacetate
Safflower Oil
Sesame Oil
Soybean Oil
Stearoyl Polyoxylglycerides
Sunflower Oil
Triacetin
Triethyl Citrate
Water for Injection
Water for Injection, Sterile
Water for Irrigation, Sterile
Water, Purified

Sorbent

Cellulose, Powdered
Charcoal, Activated
Siliceous Earth, Purified

Sorbent, Carbon Dioxide

Barium Hydroxide Lime
Soda Lime

Stiffening Agent

Dosage Form: Semisolids, Topicals, and Suppositories

Castor Oil, Hydrogenated
Cetostearyl Alcohol
Cetyl Alcohol
Cetyl Palmitate
Dextrin
Hard Fat
Alpha-Lactalbumin
Paraffin
Paraffin, Synthetic
Rapeseed Oil, Fully Hydrogenated
Rapeseed Oil, Superglycerinated Fully Hydrogenated
Sodium Stearate
Stearyl Alcohol
Wax, Cetyl Esters
Wax, Emulsifying
Wax, Microcrystalline
Wax, White
Wax, Yellow

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Suppository Base

Dosage Form: Semisolids, Topicals, and Suppositories

Agar
Cocoa Butter
Hard Fat
Palm Kernel Oil
Polyethylene Glycol
Polyethylene Glycol 3350

Suspending and/or Viscosity-Increasing Agent

Dosage Form: Semisolids, Topicals, and Suppositories

Acacia
Agar
Alamic Acid
Alginic Acid
Aluminum Monostearate
Attapulgite, Activated
Attapulgite, Colloidal Activated
Bentonite
Bentonite, Purified
Bentonite Magma
Carbomer 910
Carbomer 934
Carbomer 934P
Carbomer 940
Carbomer 941
Carbomer 1342
Carbomer Copolymer
Carbomer Homopolymer
Carbomer Interpolymer
Carboxymethylcellulose Calcium
Carboxymethylcellulose Sodium
Carboxymethylcellulose Sodium 12
Carboxymethylcellulose Sodium, Enzymatically-Hydrolyzed
Carmellose
Carrageenan
Cellulose, Microcrystalline
Cellulose, Microcrystalline, and Carboxymethylcellulose Sodium
Cellulose, Powdered
Cetostearyl Alcohol
Chitosan
Corn Syrup
Corn Syrup Solids
Cyclomethicone
Dextrin
Egg Phospholipids
Ethylcellulose
Gelatin
Gellan Gum
Glyceryl Behenate
[(Title for this monograph—not to change until December 1, 2019.) (Prior to December 1, 2019, the current practice of labeling the article of commerce with the name Glyceryl Behenate may be continued. Use of the name Glyceryl Dibehenate will be permitted as of December 1, 2014; however, the use of this name will not be mandatory until December 1, 2019. The 60-month extension will provide the time needed by manufacturers and users to make necessary changes.)]
Glyceryl Dibehenate
Guar Gum
Hydroxyethyl Cellulose
Hydroxypropyl Cellulose
Hypromellose
Isomalt
Alpha-Lactalbumin
Kaolin
Magnesium Aluminum Silicate
Maltitol Solution
Maltodextrin
Medium-chain Triglycerides
Methylcellulose
Pectin
Polycarbophil
Polydextrose
Polydextrose, Hydrogenated
Polyethylene Oxide
Polysorbate 20
Polysorbate 40
Polysorbate 60
Polysorbate 80
Polyvinyl Alcohol
Potassium Alginate
Povidone
Propylene Glycol Alginate
Pullulan
Silica, Dental-Type
Silica, Hydrophobic Colloidal
Silicon Dioxide
Silicon Dioxide, Colloidal
Sodium Alginate
Sorbitan Monolaurate
Sorbitan Monooleate
Sorbitan Monopalmitate
Sorbitan Monostearate
Sorbitan Sesquioleate
Sorbitan Trioleate
Starch, Corn
Starch, Hydroxypropyl Corn
Starch, Pregelatinized Hydroxypropyl Corn
Starch, Pea
Starch, Hydroxypropyl Pea
Starch, Pregelatinized Hydroxypropyl Pea
Starch, Potato
Starch, Hydroxypropyl Potato
Starch, Pregelatinized Hydroxypropyl Potato
Starch, Tapioca
Starch, Wheat
Sucrose
Sucrose Palmitate
Tragacanth
Vitamin E Polyethylene Glycol Succinate
Xanthan Gum

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Change to read:

Sweetening Agent

Dosage Form: Oral Liquids

Acesulfame Potassium
Aspartame
Aspartame Acesulfame
Corn Syrup
Corn Syrup, High Fructose
Corn Syrup Solids
Dextrates
Dextrose
Dextrose Excipient
Erythritol
Fructose
Galactose
Glucose, Liquid
Glycerin
Inulin
Invert Sugar
Isomalt
Lactitol
Maltitol
Maltitol Solution
Maltose
Mannitol
Neotame
▲Neohesperidin Dihydrochalcone▲ (NF 1-May-2020)
Saccharin
Saccharin Calcium
Saccharin Sodium
Sorbitol
Sorbitol Solution
Starch Hydrolysate, Hydrogenated
Sucralose
Sucrose
Sugar, Compressible
Sugar, Confectioner's
Syrup
Tagatose
Trehalose
Xylitol

Tonicity Agent

Dosage Form: Parenterals

Corn Syrup
Corn Syrup Solids
Dextrose
Glycerin
Mannitol
Potassium Chloride
Sodium Chloride

Transfer Ligand

Dosage Form: Radiopharmaceuticals

Edetate Disodium
Sodium Acetate
Sodium Citrate
Sodium Gluconate
Sodium Tartrate

Change to read:

Vehicle

▲Polypropylene Glycol 11 Stearyl Ether▲ (NF 1-May-2020)

Dosage Form: Oral Liquids

FLAVORED AND/OR SWEETENED

Aromatic Elixir
Benzaldehyde Elixir, Compound
Corn Syrup Solids
Dextrose
Ethyl Maltol
Peppermint Water
Sorbitol Solution
Syrup
Trehalose

OLEAGINOUS

Alkyl (C12–15) Benzoate
Almond Oil
Canola Oil
Castor Oil
Corn Oil
Cottonseed Oil
Ethyl Oleate
Isopropyl Myristate
Isopropyl Palmitate
Mineral Oil
Mineral Oil, Light
Myristyl Alcohol
Octyldodecanol
Olive Oil
Peanut Oil
Polydecene, Hydrogenated
Polyoxyl 15 Hydroxystearate
Safflower Oil
Sesame Oil
Soybean Oil
Squalane
Sunflower Oil

SOLID CARRIER

Chitosan
Corn Syrup Solids
Alpha-Lactalbumin
Glyceryl Tricaprylate
Propylene Glycol Dicaprylate/Dicaprate
Propylene Glycol Monocaprylate
Sugar Spheres

STERILE

rAlbumin Human
Sodium Chloride Injection, Bacteriostatic
Water for Injection, Bacteriostatic

Water-Repelling Agent

Cyclomethicone
Dimethicone
Simethicone

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Wet Binder

Dosage Form: Tablets and Capsules

Acacia
Agar
Alginic Acid
Amino Methacrylate Copolymer
Ammonio Methacrylate Copolymer
Ammonio Methacrylate Copolymer Dispersion
Calcium Carbonate
Calcium Lactate
Carbomer Copolymer
Carbomer Homopolymer
Carbomer Interpolymer
Carboxymethylcellulose Sodium
Cellulose, Microcrystalline
Cellulose, Silicified Microcrystalline
Coconut Oil, Hydrogenated
Copovidone
Corn Syrup
Corn Syrup Solids
Dextrates
Dextrin
Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion
Ethylcellulose
Ethylene Glycol and Vinyl Alcohol Graft Copolymer
Gelatin
Glucose, Liquid
Glyceryl Behenate
[(Title for this monograph—not to change until December 1, 2019.) (Prior to December 1, 2019, the current practice of labeling the article of commerce with the name Glyceryl Behenate may be continued. Use of the name Glyceryl Dibehenate will be permitted as of December 1, 2014; however, the use of this name will not be mandatory until December 1, 2019. The 60-month extension will provide the time needed by manufacturers and users to make necessary changes.)]
Glyceryl Dibehenate
Guar Gum
Hydroxyethyl Cellulose
Hydroxypropyl Cellulose
Hydroxypropyl Cellulose, Low-Substituted
Hypromellose
Hypromellose Acetate Succinate
Inulin
Invert Sugar
Alpha-Lactalbumin
Lactose, Monohydrate
Maltodextrin
Maltose
Methacrylic Acid and Ethyl Acrylate Copolymer
Methacrylic Acid and Ethyl Acrylate Copolymer Dispersion
Methacrylic Acid and Methyl Methacrylate Copolymer
Methylcellulose
Palm Oil, Hydrogenated
Polycarbophil
Polydextrose, Hydrogenated
Polyethylene Oxide
Polyvinyl Acetate
Povidone
Pullulan
Sodium Alginate
Starch, Pregelatinized
Starch, Pregelatinized Modified
Starch, Corn
Starch, Hydroxypropyl Corn
Starch, Pregelatinized Hydroxypropyl Corn
Starch, Pea
Starch, Hydroxypropyl Pea
Starch, Pregelatinized Hydroxypropyl Pea
Starch, Potato
Starch, Hydroxypropyl Potato
Starch, Pregelatinized Hydroxypropyl Potato
Starch, Tapioca
Starch, Wheat
Starch Hydrolysate, Hydrogenated
Sucrose
Sunflower Oil
Syrup
Trehalose
Vegetable Oil, Hydrogenated
Vitamin E Polyethylene Glycol Succinate
Zein

Wetting and/or Solubilizing Agent

Betadex Sulfobutyl Ether Sodium

Dosage Form: Oral Liquids

Behenoyl Polyoxylglycerides
Benzalkonium Chloride
Benzethonium Chloride
Butylene Glycol
Caprylocaproyl Polyoxylglycerides
Cetylpyridinium Chloride
Docusate Sodium
Egg Phospholipids
Glycine
Lauroyl Polyoxylglycerides
Linoleoyl Polyoxylglycerides
Nonoxynol 9
Octoxynol 9
Oleoyl Polyoxylglycerides
Poloxamer
Polyoxyl 10 Oleyl Ether
Polyoxyl 15 Hydroxystearate
Polyoxyl 20 Cetostearyl Ether
Polyoxyl 35 Castor Oil
Polyoxyl 40 Castor Oil, Hydrogenated
Polyoxyl 40 Stearate
Polyoxyl Lauryl Ether
Polyoxyl Stearate
Polyoxyl Stearyl Ether
Polysorbate 20
Polysorbate 40
Polysorbate 60
Polysorbate 80
Pullulan
Sodium Lauryl Sulfate
Sorbitan Monolaurate

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Sorbitan Monooleate
Sorbitan Monopalmitate
Sorbitan Monostearate
Sorbitan Sesquioleate
Sorbitan Trioleate
Stearoyl Polyoxylglycerides
Tyloxapol
Wax, Emulsifying

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Official Monographs for NF 38

Acacia

DEFINITION
Acacia is the dried gummy exudate from the stems and branches of *Acacia senegal* (L.) Willd. or of other related African species of *Acacia* (Fam. Leguminosae).

IDENTIFICATION
• **A.**
Analysis: To 10 mL of a cold solution (1 in 50) add 0.2 mL of diluted lead subacetate TS.
Acceptance criteria: A flocculent, or curdy, white precipitate is formed immediately.

IMPURITIES
• **ARSENIC,** *Method II* ⟨211⟩**:** NMT 3 ppm
• **LEAD** ⟨251⟩**:** NMT 10 ppm

SPECIFIC TESTS
• **BOTANIC CHARACTERISTICS**
Acacia: Spheroidal tears up to 32 mm in diameter or in angular fragments of white to yellowish white color. It is translucent or somewhat opaque from the presence of numerous minute fissures; very brittle, the fractured surface glassy and occasionally iridescent. It is almost odorless and produces a mucilaginous sensation on the tongue.
Flake Acacia: White to yellowish white, thin flakes, appearing under the microscope as colorless, striated fragments
Powdered Acacia: White to yellowish white, angular microscopic fragments with only traces of starch or vegetable tissues present
Granular Acacia: White to pale yellowish white, fine granules. Under the microscope it appears as colorless, glassy, irregularly angular fragments up to 100 µm in thickness, some of which exhibit parallel linear streaks.
Spray-dried Acacia: White to off-white compacted microscopic fragments or whole spheres
• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** Meets the requirements of the tests for absence of *Salmonella* species
• **WATER DETERMINATION,** *Method III (Gravimetric)* ⟨921⟩
Analysis: For unground Acacia, crush in a mortar until it passes through a No. 40 sieve, and mix the ground material before weighing the test specimen. Dry a sample at 105° for 5 h.
Acceptance criteria: NMT 15.0% of its weight
• **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩**:** NMT 0.5%
• **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 4.0%
• **INSOLUBLE RESIDUE**
Sample solution: Dissolve 5.0 g of powdered or finely ground Acacia in 100 mL of water. Add 10 mL of 3 N hydrochloric acid.
Analysis: Boil the *Sample solution* gently for 15 min. Pass by suction, while hot, through a tared filtering crucible. Wash thoroughly with hot water, dry at 105° for 1 h, and weigh.
Acceptance criteria: The weight of the residue thus obtained does not exceed 50 mg.
• **STARCH OR DEXTRIN**
Sample solution: A solution (1 in 50)
Analysis: Boil the *Sample solution* cool, and add iodine TS.
Acceptance criteria: No bluish or reddish color is produced.
• **SOLUBILITY AND REACTION**
Sample: 1 g
Analysis: Dissolve the *Sample* in 2 mL of water.
Acceptance criteria: The resulting solution flows readily and is acid to litmus.
• **TANNIN-BEARING GUMS**
Sample solution: A solution (1 in 50)
Analysis: To 10 mL of the *Sample solution* add 0.1 mL of ferric chloride TS.
Acceptance criteria: No blackish coloration or blackish precipitate is produced.

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight containers.

Acacia Syrup

DEFINITION
Prepare Acacia Syrup as follows (see *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩).

Acacia, granular or powdered	100 g
Sodium Benzoate	1 g
Vanilla Tincture	5 mL
Sucrose	800 g
Purified Water, a sufficient quantity to make	1000 mL

Mix *Acacia*, *Sodium Benzoate*, and *Sucrose*. Add 425 mL of *Purified Water*, and mix. Heat the mixture on a steam bath until dissolved. When cool, remove the scum, add *Vanilla Tincture* and sufficient *Purified Water* to make the product measure 1000 mL, and strain, if necessary.

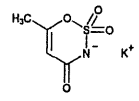
SPECIFIC TESTS
• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** Meets the requirements of the test for absence of *Salmonella* species

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Package in tight containers, and prevent exposure to excessive heat.

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• **LABELING:** The label states the Latin binomial name and, following the official name, the part of the plant source from which the article was derived.

Acesulfame Potassium



$C_4H_4NO_4SK$ 201.24

6-Methyl-1,2,3-oxathiazine-4(3*H*)-one-2,2-dioxide potassium salt;
3,4-Dihydro-6-methyl-1,2,3-oxathiazine-4-one-2,2-dioxide potassium salt [55589-62-3].

DEFINITION

Acesulfame Potassium contains NLT 99.0% and NMT 101.0% of $C_4H_4NO_4SK$, calculated on the dried basis.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

• **B. IDENTIFICATION TESTS—GENERAL,** *Potassium* ⟨191⟩
Sample solution: 100 mg/mL
Acceptance criteria: Meets the requirements

ASSAY

• **PROCEDURE**
Sample: 150 mg
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.1 N perchloric acid VS
Blank: 50 mL of glacial acetic acid
Endpoint detection: Potentiometric
Analysis: Dissolve the *Sample* in 50 mL of glacial acetic acid. Titrate with 0.1 N perchloric acid VS. Perform a blank determination.
Calculate the percentage of acesulfame potassium ($C_4H_4NO_4SK$) in the *Sample*:

$$\text{Result} = [(V - B) \times N \times F \times 100]/W$$

V = titrant volume consumed by the *Sample* (mL)
B = titrant volume consumed by the *Blank* (mL)
N = titrant actual normality (mEq/mL)
F = equivalency factor, 201.2 mg/mEq
W = weight of *Sample* (mg)

Acceptance criteria: 99.0%–101.0% on the dried basis

IMPURITIES

• **LIMIT OF FLUORIDE**
[NOTE—Use plasticware throughout this test.]
Solution A: Dissolve 210 g of citric acid monohydrate in 400 mL of water. Adjust with concentrated ammonia to a pH of 7.0, and dilute with water to 1000 mL.
Solution B: 132 mg/mL of dibasic ammonium phosphate
Solution C: To a suspension of 292 g of edetic acid in 500 mL of water, add 200 mL of ammonium hydroxide, adjust with ammonium hydroxide to a pH between 6 and 7, and dilute with water to make 1000 mL.
Buffer solution: Mix equal volumes of *Solution A*, *Solution B*, and *Solution C*, and adjust with ammonium hydroxide to a pH of 7.5.
Standard stock solution: Weigh 0.442 g of sodium fluoride, previously dried at 300° for 12 h, into a 1-L volumetric flask, and dilute with water to volume. Store the solution in a closed plastic container. Immediately before use, pipet 5 mL of this solution into a 100-mL volumetric flask, and dilute with water to volume. Each mL of this solution contains 10 µg of fluoride ion.
Standard solution A: Mix 0.5 mL of *Standard stock solution* and 15.0 mL of *Buffer solution*, and dilute with water to 50 mL.
Standard solution B: Mix 1.0 mL of *Standard stock solution* and 15.0 mL of *Buffer solution*, and dilute with water to 50 mL.
Standard solution C: Mix 1.5 mL of *Standard stock solution* and 15.0 mL of *Buffer solution*, and dilute with water to 50 mL.
Standard solution D: Mix 3.0 mL of *Standard stock solution* and 15.0 mL of *Buffer solution*, and dilute with water to 50 mL.
Sample solution: To a 50-mL volumetric flask add 3 g of Acesulfame Potassium. Dissolve in water, add 15.0 mL of *Buffer solution*, and dilute with water to volume.
Analysis
Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, *Standard solution D*, and *Sample solution*
Concomitantly measure the potential (see *Titrimetry* ⟨541⟩), in mV, of the *Standard solutions* and the *Sample solution*, with a suitable pH meter equipped with a fluoride-specific ion electrode and a silver–silver chloride reference electrode. When taking the measurements, transfer the solution to a 25-mL beaker, and immerse the electrodes. Insert a polytef-coated stirring bar into the beaker, place the beaker on a magnetic stirrer having an insulated top, and allow to stir until equilibrium is attained (1–2 min). Rinse, and dry the electrodes between measurements, taking care not to scratch the crystal in the fluoride-specific ion electrode. Measure the potential of each *Standard solution*, and plot the fluoride concentration, in µg/mL, versus the potential, in mV, on semilogarithmic paper. Measure the potential of the *Sample solution*, and determine the fluoride concentration from the standard curve, in µg/mL.
Calculate the content, in ppm, of fluoride in the portion of Acesulfame Potassium taken:

$$\text{Result} = (V \times C/W)$$

V = volume of the *Sample solution* (mL)
C = concentration of fluoride in the *Sample solution*, from the standard curve (µg/mL)
W = weight of Acesulfame Potassium taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 3 ppm

• **CHROMATOGRAPHIC PURITY**
Solution A: 3.3 mg/mL of tetrabutylammonium hydrogen sulfate
Mobile phase: Acetonitrile and *Solution A* (2:3)
System suitability solution: 2 µg/mL each of USP Acesulfame Potassium RS and ethylparaben
Standard solution: 0.2 µg/mL of USP Acesulfame Potassium RS
Sample solution: 10 mg/mL
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 227 nm

Supplement 1

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Column: 4.6-mm × 25-cm; 5-µm packing L1
Flow rate: 1 mL/min
Injection size: 20 µL
System suitability
Sample: *System suitability solution*
Suitability requirements
Resolution: NLT 2 between acesulfame potassium and ethylparaben
Analysis
Samples: *Standard solution* and *Sample solution*
Record the chromatograms for a run time NLT 3 times the retention time of the acesulfame potassium peak, and measure the area responses of the peaks.
Acceptance criteria: The response of any peak at a retention time other than that of acesulfame potassium from the *Sample solution* does not exceed the response of the acesulfame potassium peak from the *Standard solution* (0.002%).

SPECIFIC TESTS
• **ACIDITY OR ALKALINITY**
Sample solution: 4.0 g in 20 mL of carbon dioxide-free water
Analysis: Add 0.1 mL of bromothymol blue TS. If the solution is yellow, titrate with 0.01 N sodium hydroxide to produce a blue color. If the solution is blue, titrate with 0.01 N hydrochloride acid to produce a yellow color.
Acceptance criteria: NMT 0.2 mL of 0.01 N sodium hydroxide or NMT 0.2 mL of 0.01 N hydrochloric acid is required.
• **LOSS ON DRYING** ⟨731⟩**:** Dry a sample at 105° for 3 h: it loses NMT 1.0% of its weight.

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in a well-closed container, and protect from light. Store at room temperature.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Acesulfame Potassium RS

Acetic Acid

Acetic acid;
Acetic acid [64-19-7].

DEFINITION
Acetic Acid is a solution containing NLT 36.0% and NMT 37.0%, by weight, of $C_2H_4O_2$.

IDENTIFICATION
• **A. IDENTIFICATION TESTS—GENERAL,** *Acetate* ⟨191⟩**:** Meets the requirements

ASSAY
• **PROCEDURE**
Analysis: Place 6 mL in a tared, glass-stoppered flask, and weigh. Add 40 mL of water, then add phenolphthalein TS. Titrate with 1 N sodium hydroxide VS. Each mL of 1 N sodium hydroxide is equivalent to 60.05 mg of $C_2H_4O_2$.
Acceptance criteria: 36.0%–37.0%

IMPURITIES
• **NONVOLATILE RESIDUE**
Analysis: Evaporate 20 mL in a tared porcelain dish on a steam bath, and dry at 105° for 1 h.
Acceptance criteria: The weight of the residue does not exceed 1.0 mg (0.005%).
• **CHLORIDE**
Sample solution: Acetic acid (1 in 10) in water
Analysis: To 10 mL of the *Sample solution* add 5 drops of silver nitrate TS.
Acceptance criteria: No opalescence is produced.
• **SULFATE**
Sample solution: Acetic acid (1 in 10) in water
Analysis: To 10 mL of the *Sample solution* add 5 drops of barium chloride TS.
Acceptance criteria: No turbidity is produced.
• **READILY OXIDIZABLE SUBSTANCES**
Analysis: Dilute 4.0 mL in a glass-stoppered vessel with 20 mL of water, and add 0.30 mL of 0.10 N potassium permanganate.
Acceptance criteria: The pink color is not changed to brown at once, and the liquid does not become entirely brown or free from a pink tint in less than 30 s.

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight containers.

Diluted Acetic Acid

DEFINITION
Diluted Acetic Acid is a solution containing, in each 100 mL, NLT 5.7 g and NMT 6.3 g of acetic acid ($C_2H_4O_2$).
Prepare Diluted Acetic Acid as follows (see *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩).

Acetic Acid	158 mL
Purified Water, a sufficient quantity to make	1000 mL

Mix the ingredients.

IDENTIFICATION
• **A. IDENTIFICATION TESTS—GENERAL,** *Acetate* ⟨191⟩**:** Meets the requirements

ASSAY
• **PROCEDURE**
Sample: 25 mL
Analysis: To the *Sample* add 15 mL of carbon dioxide-free water. Add phenolphthalein TS, and titrate with 1 N sodium hydroxide VS. Each mL of 1 N sodium hydroxide is equivalent to 60.05 mg of acetic acid ($C_2H_4O_2$).
Acceptance criteria: 5.7–6.3 g of acetic acid per 100 mL of Diluted Acetic Acid

IMPURITIES
• **LIMIT OF CHLORIDE**
Sample solution: A solution of Diluted Acetic Acid in water (6 in 10)
Analysis: Add 5 drops of silver nitrate TS to 10 mL of the *Sample solution*.
Acceptance criteria: No opalescence is found.
• **LIMIT OF SULFATE**
Sample solution: A solution of Diluted Acetic Acid in water (6 in 10)
Analysis: Add 5 drops of barium chloride TS to 10 mL of the *Sample solution*.
Acceptance criteria: No turbidity is produced.
• **LIMIT OF NONVOLATILE RESIDUE**
Sample: 20 mL
Analysis: Evaporate the *Sample* in a tared porcelain dish on a steam bath, and dry it at 105° for 1 h.
Acceptance criteria: The weight of the residue does not exceed 1.0 mg (NMT 0.005%).

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SPECIFIC TESTS
• **READILY OXIDIZABLE SUBSTANCES**
Sample: 20 mL in a glass-stoppered flask
Analysis: Add 0.30 mL of 0.10 N potassium permanganate to the *Sample*.
Acceptance criteria: The pink color is not changed to brown immediately, and the liquid does not become entirely brown or free from a pink tint in less than 30 s.

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight containers.

Acetic Acid, Glacial—*see Glacial Acetic Acid General Monographs*

Acetone



C_3H_6O 58.08
2-Propanone;
Acetone [67-64-1].

DEFINITION
Acetone contains NLT 99.0% of C_3H_6O, calculated on the anhydrous basis.
[**CAUTION**—Acetone is very flammable. Do not use where it may be ignited.]

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F**▲ (CN 1-May-2020)
• **B.** The retention time of the *Sample* corresponds to that of USP Acetone RS, as obtained in the *Assay*.

ASSAY
• **PROCEDURE**
Sample: Acetone
System suitability solution: Dilute 1.0 mL of USP Methyl Alcohol RS and 1.0 mL of USP Acetone RS with tetrahydrofuran to 50 mL.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.32-mm × 30-m fused-silica capillary; 1.8-µm of phase G43
Temperature
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
40	0	40	5
40	20	240	—

Injector: 200°
Detector: 280°
Carrier gas: Helium
Flow rate: 35 cm/s (linear velocity)
Split ratio: 400:1
Injection volume: 1 µL
System suitability
Sample: *System suitability solution*
[NOTE—The relative retention times for methyl alcohol, acetone, and tetrahydrofuran are about 0.6, 1.0, and 1.9, respectively.]
Suitability requirements
Resolution: NLT 15 between the methyl alcohol and acetone peaks
Analysis
Sample: *Sample*
Calculate the percentage of acetone (C_3H_6O) in the portion of Acetone taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak area due to the acetone peak in the *Sample*
r_T = sum of the areas of all the peaks in the *Sample*

[NOTE—No separate correction is applied for water content, because water does not respond to the flame-ionization detector.]
Acceptance criteria: NLT 99.0% on the anhydrous basis

SPECIFIC TESTS
• **SPECIFIC GRAVITY** ⟨841⟩**:** NMT 0.789
• **NONVOLATILE RESIDUE:** Evaporate 50 mL in a tared porcelain dish on a steam bath, and dry at 105° for 1 h.
Acceptance criteria: The weight of the residue does not exceed 2 mg (0.004%).
• **WATER**
Sample: Acetone
Standard solution: Transfer 0.50 mL of water to a dry 100-mL volumetric flask, dilute with dehydrated isopropyl alcohol to volume, and mix.
Blank: Dehydrated isopropyl alcohol
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Thermal conductivity
Column: 0.32-mm × 50-m capillary; 5.0-µm layer of support S2
Temperature
Column: See *Table 2*.

Table 2

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
100	25	190	—

Injector: 250°
Detector: 250°
Carrier gas: Helium
Flow rate: 11 mL/min
Split ratio: 4.5:1
Injection size: 1.0 µL
Analysis
Samples: Acetone, *Standard solution*, and *Blank*
[NOTE—Identify the peaks based on their relative retention times, which are 1.0 for water and about 1.9 for isopropyl alcohol.]
Acceptance criteria: The area of the water peak for Acetone is NMT that from the *Standard solution*, corrected for the area of the water peak from the *Blank* (0.5%).

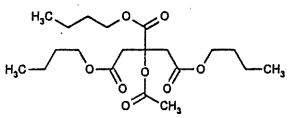
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• **READILY OXIDIZABLE SUBSTANCES**
Analysis: Mix 20 mL with 0.10 mL of 0.10 N potassium permanganate in a glass-stoppered bottle.
Acceptance criteria: The permanganate color of the mixture does not completely disappear within 15 min.

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight containers, remote from fire.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Acetone RS
USP Methyl Alcohol RS

Acetylcysteine—*see Acetylcysteine General Monographs*

Acetyltributyl Citrate



$C_{20}H_{34}O_8$ 402.48

DEFINITION
Acetyltributyl Citrate contains NLT 99.0% of $C_{20}H_{34}O_8$, calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

• **A.** ▲**SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F**▲ (CN 1-May-2020)
• **B.** The retention time of the *Sample solution* corresponds to that of a similar preparation of USP Acetyltributyl Citrate RS, as obtained in the *Assay*.

ASSAY
• **PROCEDURE**
System suitability solution: 30 mg/mL each of USP Acetyltributyl Citrate RS and USP Tributyl Citrate RS in toluene
Sample solution: 30 mg/mL of Acetyltributyl Citrate in toluene
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.32-mm × 30-m, bonded with a 0.5-µm layer of phase G42
Temperature
Injector: 240°
Detector: 280°
Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
80	—	80	0
80	20	230	15

Flow rate: 1.9 mL/min
Carrier gas: Helium
Injection type: Split, 30:1
Injection size: 1 µL
System suitability
Sample: *System suitability solution*
[NOTE—The relative retention times for tributyl citrate and acetyl tributyl citrate are 0.9 and 1.0, respectively.]
Suitability requirements
Resolution: NLT 1.5 between tributyl citrate and acetyl tributyl citrate
Relative standard deviation: NMT 2.0% determined from both the tributyl citrate and acetyl tributyl citrate peaks, based on area percent calculation
Analysis
Sample: *Sample solution*
[NOTE—Measure all of the peak areas, excluding the solvent peak.]
Calculate the percentage of $C_{20}H_{34}O_8$ in the portion of Acetyltributyl Citrate taken:

$$\text{Result} = (r_U/r_T) \times 100$$

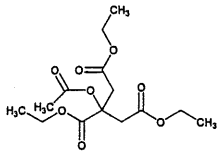
r_U = peak area of the *Sample solution*
r_T = sum of all the peak areas

Acceptance criteria: NLT 99.0% on the anhydrous basis

SPECIFIC TESTS
• **SPECIFIC GRAVITY** ⟨841⟩: 1.045–1.055
• **REFRACTIVE INDEX** ⟨831⟩: 1.4410–1.4425
• **ACIDITY**
Neutralized isopropyl alcohol: To a suitable quantity of isopropyl alcohol add 2–3 drops of bromothymol blue TS and just sufficient 0.10 N sodium hydroxide dropwise to produce a faint blue color. [NOTE—Prepare *Neutralized isopropyl alcohol* just before use.]
Sample solution: 32.0 g of Acetyltributyl Citrate in 30 mL of *Neutralized isopropyl alcohol*
Analysis: Add bromothymol blue TS. Titrate with 0.10 N sodium hydroxide to a faint blue endpoint.
Acceptance criteria: NMT 1.0 mL of 0.10 N sodium hydroxide is required.
• **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 0.25%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight containers.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Acetyltributyl Citrate RS
USP Tributyl Citrate RS

Acetyltriethyl Citrate



$C_{14}H_{22}O_8$ 318.32

DEFINITION
Acetyltriethyl Citrate contains NLT 99.0% of acetyltriethyl citrate ($C_{14}H_{22}O_8$), calculated on the anhydrous basis.

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IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F▲** (CN 1-May-2020)
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *System suitability solution*, as obtained in the *Assay*.

ASSAY

- **PROCEDURE**

System suitability solution: 30 mg/mL each of USP Acetyltriethyl Citrate RS and USP Triethyl Citrate RS in toluene

Sample solution: 30 mg/mL in toluene

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC, equipped with an on-column, temperature-programmable injector

Detector: Flame ionization

Column: 0.32-mm × 30-m, bonded with a 0.5-µm layer of phase G42

Temperatures

Injector: See *Table 1*.

Detector: 275°

Column: See *Table 2*.

Table 1

Start Temperature (°)	Ramp (°)	End Temperature (°)	Hold Time (min)
85	—	85	0.5
85	20	225	10

Table 2

Start Temperature (°)	Ramp (°)	End Temperature (°)	Hold Time (min)
80	—	80	0.5
80	20	220	10

Flow rate: 2.3 mL/min

Carrier gas: Helium

Injection volume: 1 µL

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times of triethyl citrate and acetyltriethyl citrate are 0.9 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 1.5 between triethyl citrate and acetyltriethyl citrate

Relative standard deviation: NMT 2.0% determined from both the triethyl citrate and acetyltriethyl citrate peaks

Analysis

Sample: *Sample solution*

Calculate the percentage of acetyltriethyl citrate ($C_{14}H_{22}O_8$) in the portion of sample taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak area of acetyltriethyl citrate from the *Sample solution*

r_T = sum of all the peaks excluding the solvent peak

Acceptance criteria: NLT 99.0% on the anhydrous basis

SPECIFIC TESTS

- **SPECIFIC GRAVITY** ⟨841⟩: 1.135–1.139
- **REFRACTIVE INDEX** ⟨831⟩: 1.432–1.441
- **ACIDITY**

Sample: 32.0 g

Analysis: Dissolve the *Sample* in 30 mL of isopropyl alcohol, previously neutralized to bromothymol blue. Add bromothymol blue TS, and titrate with 0.10 N sodium hydroxide to a faint blue endpoint.

Acceptance criteria: NMT 1.0 mL of 0.10 N sodium hydroxide is required.

- **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 0.3%

ADDITIONAL REQUIREMENTS

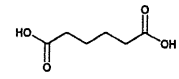
- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Acetyltriethyl Citrate RS

USP Triethyl Citrate RS

Charcoal, Activated —*see Activated Charcoal General Monographs*

Adipic Acid



$C_6H_{10}O_4$ 146.14

Hexanedioic acid;
1,4-Butanedicarboxylic acid [124-04-9].

DEFINITION

Adipic Acid contains NLT 99.0% and NMT 101.0% of $C_6H_{10}O_4$, calculated on the dried basis.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K▲** (CN 1-May-2020)

ASSAY

- **PROCEDURE**

Sample: 60 mg

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Direct titration

Titrant: 0.1 N sodium hydroxide VS

Blank: 50.0 mL of water

Endpoint detection: Colorimetric

Analysis: Dissolve the *Sample* in 50 mL of water. Add 0.2 mL of phenolphthalein TS, and titrate with 0.1 N sodium hydroxide VS to a permanent pale pink endpoint. Perform a blank determination. Calculate the percentage of adipic acid ($C_6H_{10}O_4$) in the *Sample* taken:

$$\text{Result} = [(V - B) \times N \times F \times 100]/W$$

V = titrant volume consumed by the *Sample* (mL)

B = titrant volume consumed by the *Blank* (mL)

N = titrant actual normality (mEq/mL)

F = equivalency factor, 73.1 mg/mEq

W = weight of the *Sample* (mg)

Supplement 1

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Acceptance criteria: 99.0%–101.0% on the dried basis

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%
- **LIMIT OF NITRATES**

Standard stock solution: 1.63 mg/mL of potassium nitrate

Standard solution: Dilute 1 mL of the *Standard stock solution* with water to 10 mL. Dilute 1 mL of this solution with water to 50 mL to obtain a solution containing 2 µg/mL of nitrate.

Sample solution: Transfer 5 g of Adipic Acid to a 50-mL volumetric flask. Dissolve in water, with heating, and dilute with water to volume. Allow to cool and crystallize, then pass through a sintered-glass filter. Wash the filter with water, and collect the filtrate and washings until a volume of 50 mL is obtained. [NOTE—This solution is also to be used for *Chloride*, *Sulfate*, and *Iron*.]

Control: 2 mg/L of potassium permanganate

Analysis: Transfer 1.0 mL of the *Sample solution*, 1.5 mL of the *Standard solution*, and 1 mL of water (blank) to three separate flasks. To each flask add 2 mL of concentrated ammonia, 0.5 mL of 10 mg/mL manganese sulfate, and 1 mL of 10 mg/mL sulfanilamide, and dilute each solution with water to 20 mL. Add 100 mg of zinc powder to each of the three flasks, and cool in an ice bath for 30 min, shaking the solutions periodically. Separately filter 10 mL of each solution, cool in an ice bath, and then add 2.5 mL of hydrochloric acid and 1 mL of 10 mg/mL of naphthylethylenediamine dihydrochloride. Allow the solutions to stand at room temperature for 15 min.

System suitability: The test is invalid if the concomitantly prepared blank solution is darker than the *Control*.

Acceptance criteria: The color of the solution containing the *Sample solution* is not darker than the concomitantly prepared solution containing the *Standard solution* (NMT 30 ppm).

- **CHLORIDE AND SULFATE,** *Chloride* ⟨221⟩

Sample: A 5-mL portion of the *Sample solution* from *Limit of Nitrates*

Analysis: Proceed as directed in the chapter.

Acceptance criteria: The *Sample* shows no more chloride than a corresponding 0.14-mL portion of 0.020 N hydrochloric acid (NMT 0.02%).

- **CHLORIDE AND SULFATE,** *Sulfate* ⟨221⟩

Sample: A 5-mL portion of the *Sample solution* from *Limit of Nitrates*

Analysis: Proceed as directed in the chapter.

Acceptance criteria: The *Sample* shows no more sulfate than a corresponding 0.26-mL portion of 0.020 N sulfuric acid (NMT 0.05%).

- **IRON** ⟨241⟩

Sample: A 10-mL portion of the *Sample solution* from *Limit of Nitrates*

Analysis: Proceed as directed in the chapter.

Acceptance criteria: NMT 10 ppm

SPECIFIC TESTS

- **MELTING RANGE OR TEMPERATURE** ⟨741⟩: 151°–154°
- **LOSS ON DRYING** ⟨731⟩: Dry a sample at 105° to constant weight; it loses NMT 0.2% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in a tight containers. No storage requirements specified.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Adipic Acid RS

Agar

[9002-18-0].

DEFINITION

Agar is the dried, hydrophilic, colloidal substance consisting of the polysaccharides extracted from *Gelidium cartilagineum* (L.) Gaillon (Fam. Gelidiaceae), *Gracilaria confervoides* (L.) Greville (Fam. Sphaerococcaceae), and related red algae (Class Rhodophyceae).

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
- **B.** Iodine TS colors some of the fragments of Agar bluish black, with some areas reddish to violet.
- **C.**

Analysis: Boil a sample with 65 times its weight of water for 10 min, with constant stirring, and subsequently adjust with hot water to a concentration of 1.5%, by weight.

Acceptance criteria: Agar forms a clear liquid that congeals at 30°–39° to form a firm resilient gel, which does not liquefy below 80°.

IMPURITIES

INORGANIC IMPURITIES

- **Arsenic,** *Method II* ⟨211⟩: NMT 3 ppm
- **Lead** ⟨251⟩: NMT 10 ppm
- **ARTICLES OF BOTANICAL ORIGIN,** *Acid-Insoluble Ash* ⟨561⟩: NMT 0.5%, on a dry-weight basis

ORGANIC IMPURITIES

- **Procedure 1: Limit of Gelatin**

Sample solution: Dissolve 1 g of sample in 100 mL of boiling water. Allow to cool to about 50°.

Analysis: To 5 mL of the *Sample solution* add 2–3 drops of a mixture of 0.2 M potassium dichromate solution and 3 N hydrochloric acid (4:1).

Acceptance criteria: No yellow precipitate is formed.

- **Procedure 2: Limit of Foreign Starch**

Sample solution: Boil 0.10 g in 100 mL of water.

Acceptance criteria: The *Sample solution* does not, upon cooling, produce a blue color upon the addition of iodine TS.

- **Procedure 3: Limit of Foreign Insoluble Matter**

Sample dispersion: Add sufficient water to 7.5 g of sample to make 500 g, boil for 15 min, and readjust to the original 500 g.

Analysis: To 100 g of the uniformly mixed *Sample dispersion* add hot water to make 200 mL. Heat almost to boiling, filter while hot through a tared filtering crucible. Rinse the container with several portions of hot water, and pass these rinsings through the crucible. Dry the crucible and its contents at 105° to a constant weight.

Acceptance criteria: NMT 15 mg (1.0%) remains in the crucible.

- **Procedure 4: Articles of Botanical Origin,** *Foreign Organic Matter* ⟨561⟩: NMT 1.0%

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩: The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g. It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.
- **WATER DETERMINATION,** *Method III* ⟨921⟩

Analysis: If necessary, cut a sample into pieces from a 2- to 5-mm square, and dry at 105° for 5 h.

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Acceptance criteria: The sample loses NMT 20.0% of its weight.

- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 6.5%, on a dry-weight basis
- **WATER ABSORPTION**

Sample: 5.0 g

Analysis: Place the *Sample* in a 100-mL graduated cylinder, fill to the mark with water, mix, and allow to stand at 25° for 24 h. Pour the contents of the cylinder through moistened glass wool, allowing the water to drain into a second 100-mL graduated cylinder.

Acceptance criteria: NMT 75 mL of water is obtained.

- **BOTANIC CHARACTERISTICS**

Agar: Usually occurs in bundles consisting of thin, membranous, agglutinated strips or in cut, flaked, or granulated forms. It may be colored weak yellowish orange, yellowish gray to pale yellow, or colorless. It is tough when damp, brittle when dry.

Histology: When mounted in water, Agar appears granular and somewhat filamentous; a few fragments of the spicules of sponges and a few frustules of diatoms may be present. In Japanese Agar, the frustules of *Arachnoidiscus ehrenbergii* Baillon often occur, being disk-shaped and 100–300 µm in diameter.

Powdered agar: White to yellowish white or pale yellow; in chloral hydrate TS, its fragments are transparent, more or less granular, striated, and angular, and occasionally they contain frustules of diatoms.

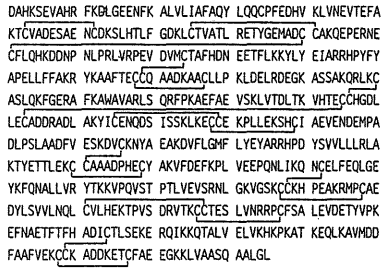
ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements are specified.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Agar RS

Albumin Human—*see Albumin Human General Monographs*

rAlbumin Human



$C_{2936}H_{4624}N_{786}O_{889}S_{41}$ 66,438 Da

DEFINITION

Recombinant Albumin Human (rAlbumin Human or rHA) is produced by recombinant DNA expression in *Saccharomyces cerevisiae*. Structural equivalence (primary, secondary, and tertiary) between rHA and human serum albumin (HSA) has been demonstrated. It consists of three domains composed of 585 amino acids containing a single tryptophan (Trp_{214}), one free thiol (Cys_{34}), and 17 disulfide bridges. It is presented as a sterile and nonpyrogenic aqueous liquid consisting of a 10% (w/v) or 20% (w/v) solution in Water for Injection. No human- or animal-derived raw material is involved in its manufacture. It contains NLT 95% and NMT 105% of the labeled amount and NLT 99% of its total protein is albumin. It contains no added antimicrobial agents, but it may contain appropriate stabilizing agents. The presence of process-related impurities, host cell DNA, and host cell proteins is process specific; suitable limits should be determined by appropriately validated methods. However, the limit for host cell proteins should be NMT 0.15 µg/g.

IDENTIFICATION

- **A. PEPTIDE MAPPING**

[NOTE—See *Biotechnology-Derived Articles—Peptide Mapping* ⟨1055⟩ for guidance.]

Tris buffer: 0.1 M tris(hydroxymethyl)aminomethane. Adjust with hydrochloric acid to a pH of 8.0.

Dilute Tris buffer: *Tris buffer* and water (50:50)

Solution A: Trifluoroacetic acid and water (1:1000)

Solution B: To 350 mL of acetonitrile add 150 mL of water and 425 µL of trifluoroacetic acid.

Dithiothreitol solution: 0.1 M dithiothreitol

Iodoacetamide solution: 0.1 M iodoacetamide in *Tris buffer*

Trypsin solution: 1 mg/mL of trypsin in 10 mM hydrochloric acid

EDTA solution: 0.1 M ethylenediaminetetraacetic acid (EDTA) in water

Diluent: To 5.76 g of guanidine hydrochloride add 5 mL of *Dilute Tris buffer* and 200 µL of *EDTA solution*. Dilute with *Dilute Tris buffer* to a final volume of 10 mL.

Standard solution: Add 20 µL of USP rAlbumin Human RS to 80 µL of *Diluent*. Add 5 µL of *Dithiothreitol solution*, and incubate at 37° for 75 min. Add 10 µL of *Iodoacetamide solution*, and incubate for an additional 75 min at 37° in the dark. Add 100 µL of *Dilute Tris buffer*, 400 µL of water, and 10 µL of *Trypsin solution*, and incubate at 37° with shaking for 24 h. Centrifuge, and dilute a portion of the supernatant in *Solution A* (50:50).

Sample solution: 50 mg/mL of rAlbumin Human in water. To 20 µL of this solution add 80 µL of *Diluent*. Add 5 µL of *Dithiothreitol solution*, and incubate at 37° for 75 min. Add 10 µL of *Iodoacetamide solution*, and incubate for an additional 75 min at 37° in the dark. Add 100 µL of *Dilute Tris buffer*, 400 µL of water, and 10 µL of *Trypsin solution*, and incubate at 37° with shaking for 24 h. Pulse centrifuge, and dilute a portion of the supernatant in *Solution A* (50:50).

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)	Flow Rate (mL/min)
0	95	5	0.5
5	95	5	0.5
75	60	40	0.5
100	40	60	0.5
104	0	100	0.5
108	0	100	0.5
109	95	5	1.0
115	95	5	1.0
116	95	5	0.5
120	95	5	0.5

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

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Detector: UV 214 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 35°
Flow rate: See *Table 1*.
Injection volume: 100 µL
Analysis
Samples: *Standard solution* and *Sample solution*
Acceptance criteria: The peptide map chromatographic profiles of the *Sample solution* are similar to those of the *Standard solution*.

• **B. ELECTROSPRAY MASS SPECTROMETRY**
Solution A: Trifluoroacetic acid and water (1:1000)
Solution B: To 140 mL of acetonitrile add 60 mL of water and 180 µL of trifluoroacetic acid.
Solution C: Acetonitrile and water (50:50)
Solution D: To 5 mL of *Solution C* add 10 µL of formic acid.
System suitability solution: Dissolve 2 mg of horse heart myoglobin in 589 µL of Water for Injection. Dilute 25 µL of this solution with 475 µL of *Solution D*.
Sample solution: 10 mg/mL of rAlbumin Human in water
Mobile phase: See *Table 2*.

Table 2

Time (min)	Solution A (%)	Solution B (%)
0	95	5
5	95	5
10	0	100
15	0	100

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 280 nm
Column: 2.1-mm × 3-cm; desalting cartridge, equilibrated with *Solution C*[1]
Flow rate: 0.2 mL/min
Injection volume: 20 µL of the *Sample solution*
Analysis: Desalt the *Sample solution*, and collect the eluate. Ensure that a single protein peak elutes.
Spectrometric system
(See *Mass Spectrometry* ⟨736⟩.)
Mode: LC/MS (using infusion pump)
[NOTE—The infusion system flow rate can be adjusted as needed. To assist in nebulization, the infusion system can contain a sheathing gas fluid.]
Mobile phase: *Solution C*
Detector: Electrospray in the positive ion mode
Injection volume: 50 µL of desalted *Sample solution*
System suitability
Sample: *System suitability solution*
Suitability requirements
Peak position: A single peak in the 16,949–16,953 Da range is found.
Analysis: Obtain and transform the spectrogram for the desalted *Sample solution*.
Acceptance criteria: The mass is within 20 Da of the theoretical mass.

[1] A suitable reverse-phase desalting column is available from Perkin Elmer (No. 0711-0056).

ASSAY

• **ALBUMIN CONTENT**
Stock sample buffer:[2] Mix 4 mL of 0.5 M Tris hydrochloride pH 8.6, 0.5 mL of 0.1% bromophenol blue, 2.0 mL of glycerol, and dilute with water to 1000 mL.
Diluted sample buffer: *Stock sample buffer* and water (1:1)
Native stock running buffer:[3] 29 mg/mL of Tris base and 144 mg/mL of glycerol
Running buffer: *Native stock running buffer* and water (1:9)
Gel-staining solution: A suitable Coomassie G-250-based solution[4]
Native PAGE gel: Prepare a 14% Tris-Glycine gel.[5]
Sample solution: 4 mg/mL of rAlbumin Human in water. Dilute this solution with *Stock sample buffer* to 2 mg/mL.
Calibration solutions: Dilute the *Sample solution* quantitatively, and stepwise if necessary, with *Diluted sample buffer* to 100, 20, 15, 10, 5, 2, and 1 µg/mL of rAlbumin Human.
Electrophoretic system
Run buffer: *Running buffer*
Voltage: 125 V
Amperage: 35 mA
Wattage: 5.0 W
Run time: Approximately 2 h
Loading volume: 10 µL
Analysis
Samples: *Sample solution* and *Calibration solutions*
Gel loading scheme
Lane 1: 1 µg/mL *Calibration solution*
Lane 2: 2 µg/mL *Calibration solution*
Lane 3: 5 µg/mL *Calibration solution*
Lane 4: 10 µg/mL *Calibration solution*
Lane 5: 15 µg/mL *Calibration solution*
Lane 6: 20 µg/mL *Calibration solution*
Lane 7: *Diluted sample buffer*
Lane 8: *Sample solution*
Lane 9: *Sample solution*
Lane 10: *Diluted sample buffer*
Gel staining: Place the gel in 100 mL of water, and shake gently with circumgyration for about 30 min. Pour approximately 50 mL of *Gel-staining solution* into a staining container. Place the gel into the staining container, and allow the stain to completely cover the gel. Place the staining container on an orbital shaker, and stain the gel for 120 min with gentle shaking.
Destaining: Drain the *Gel-staining solution*, and add 100 mL of water to the container to cover the gel. Place the container on an orbital shaker, and shake at low speed for about 60 min. Change the water, and repeat for a total of two washes.
Gel scan procedure: Set up a gel scanner according to the manufacturer's instructions. Place the gel in the detector, and obtain a single image of all 10 lanes of the gel.
Data analysis: Perform image analysis of *Lanes 1–6* to generate a linear calibration curve. Determine the linear regression equation of the standards by the least-squares method, with standard concentrations, in ng, as the dependent variable (x), and the sample band intensity (optical density) as the independent variable (y). Record the linear regression equation and the correlation

[2] Available from Invitrogen (No. LC2673).
[3] Available from Invitrogen (No. LC2672).
[4] A suitable Coomassie stain is available from Pierce (No. 24890 or No. 24592).
[5] Alternatively, a precast 14% Tris-Glycine gel is available from Invitrogen (No. EC6485).

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coefficient, *r*. A suitable system is one that yields a line having an r^2 of NLT 0.990.

Examine *Lanes 8* and *9* (the *Sample solution* lanes) for the presence of bands below the main albumin band. If bands are present below the main albumin band in either or both lanes, quantify the relative amount, in ng, of protein present in each band against the calibration curve. Convert the quantified value to a contaminant level in percentage by dividing the quantified value by a factor of 200.

Calculate the purity of the *Sample solution*:

$$\text{Result} = 100 - C_I$$

C_I = mean of the percentages of contaminant levels found in *Lanes 8* and *9* (all the bands other than the albumin band), disregarding any band due to the *Diluted sample buffer*

Acceptance criteria: *Sample solution* purity is NLT 99.0%. [NOTE—The main albumin band is not quantitated. See the test for *Total Protein*.]

• **TOTAL PROTEIN**

Sodium chloride solution: 0.15 M sodium chloride in water

Copper sulfate solution: 60 mg/mL of copper sulfate pentahydrate and 600 mg/mL of potassium sulfate[6] in sulfuric acid low in nitrogen

Sample solution: Dilute 0.5 g of rAlbumin Human with 2.5 mL of *Sodium chloride solution* (equivalent to about 3.3 mg/mL of total protein).

Blank: 33.3 mg/mL of glycine in *Sodium chloride solution*

Analysis: To 3.0 mL of the *Sample solution* and the *Blank*, in suitable distillation tubes, add 5 mL of *Copper sulfate solution*. Incubate at 420°, for a minimum of 2 h, or until the residues appear white. When the solutions are cool, transfer the residues quantitatively with a minimum quantity of water to a micro-Kjeldahl flask, and determine the residues, using *Nitrogen Determination* ⟨461⟩, *Method II*. Multiply the result, corrected for the *Blank* and for the specific gravity of the *Sample solution*, by 6.25 to calculate the quantity of protein.

Acceptance criteria: 95%–105% of the quantity of protein stated on the label

OTHER COMPONENTS

• **SODIUM CONTENT**

Diluent: 1.0 mg/mL of cesium chloride in water

Standard solutions: Prepare 0.5, 1.00, 1.50, and 2.00 mg/mL of sodium chloride in *Diluent*.

Sample solution: 80 µg/mL of rAlbumin Human in *Diluent*

Apparatus

Mode: Atomic absorption

Emission wavelength: 589 nm

Analysis: [NOTE—Use peak area measurements for quantitation.]

Samples: *Diluent* (as blank), *Standard solutions*, and *Sample solution*

Introduce a blank solution (*Diluent*) into the atomic generator, and adjust the instrument reading to zero. Determinations are made by comparison with the *Standard solutions* of known concentration. If the *Sample solution* emission exceeds that of the *Standard solutions* with the highest concentration, dilute the *Sample solution* with *Diluent*. Introduce the most concentrated *Standard solution* into the instrument, and adjust the sensitivity to obtain a suitable reading. Introduce the *Sample solution* and *Standard solutions* into the instrument at least three times, and record the steady reading. Rinse the apparatus with blank solution each time, and ascertain that the reading returns to its initial blank value. Plot the mean of the readings obtained for the *Standard solutions* against their respective sodium concentrations. From the standard curve, calculate the sodium concentration content in the *Sample solution*, and adjust for the specific gravity of the rAlbumin Human (see *Total Protein*).

Acceptance criteria: 120–160 mM sodium

IMPURITIES

• **LIMIT OF HIGH MOLECULAR WEIGHT PROTEINS**

Solution A: 200 mg/mL of sodium azide

Buffer: Dissolve 54.2 g of dibasic sodium phosphate dihydrate, 30.0 g of monobasic sodium phosphate dihydrate, and 284.0 g of anhydrous sodium sulfate in 1600 mL of water. Add 50 mL of *Solution A*, and dilute with water to 2000 mL.

Mobile phase: *Buffer* and water (10:90)

Sample solution: 40 mg/mL of rAlbumin Human

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 7.8-mm × 30-cm; 5-µm packing L59

Flow rate: 1.0 mL/min

Injection volume: 50 µL. [NOTE—The peak due to high molecular weight impurities, such as the polymer of albumin, appears in the void volume of the chromatogram.]

Analysis

Sample: *Sample solution*

Calculate the percentage of albumin polymer in the sample:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of albumin polymer

r_T = sum of all rAlbumin Human related peak responses

Acceptance criteria

Individual impurities: NMT 1.0%

SPECIFIC TESTS

• **pH** ⟨791⟩

Sample solution: 1% (w/v) protein solution diluted with 0.9% (w/v) sodium chloride

Acceptance criteria: 6.4–7.4

• **STERILITY TESTS** ⟨71⟩**:** Meets the requirements

• **BACTERIAL ENDOTOXINS TEST** ⟨85⟩**:** NMT 0.5 USP Endotoxin Unit/mL of rAlbumin Human

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight glass containers, and store at 2°–8°. Do not freeze.

• **LABELING:** Label to indicate that the material is of recombinant DNA origin.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP rAlbumin Human RS

Alcohol—*see Alcohol General Monographs*

[6] Copper sulfate pentahydrate and potassium sulfate tablets (each tablet with 1.5 g of K_2SO_4 + 0.15 g of $CuSO_4 \cdot 5H_2O$) are available from Foss (No. 15270054).

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Diluted Alcohol

DEFINITION

Diluted Alcohol is a mixture of Alcohol and water containing NLT 41.0% and NMT 42.0% by weight, corresponding to NLT 48.4% and NMT 49.5% by volume, at 15.56°, of C_2H_5OH.

Diluted Alcohol may be prepared as follows.

Alcohol	500 mL
Purified Water	500 mL

Measure the *Alcohol* and the *Purified Water* separately at the same temperature, and mix. If the water and the Alcohol and the resulting mixture are measured at 25°, the volume of the mixture will be 970 mL.

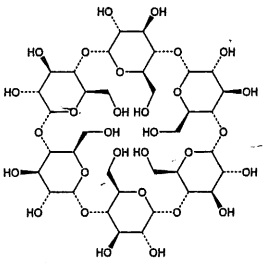
SPECIFIC TESTS

- **SPECIFIC GRAVITY** ⟨841⟩: 0.935–0.937 at 15.56°, indicating 41.0%–42.0% by weight, or between 48.4% and 49.5% by volume, of C_2H_5OH
- **OTHER REQUIREMENTS:** In other respects, it meets the requirements in *Alcohol*, allowance being made for the difference in alcohol concentration.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, remote from fire.

Alfadex



$(C_6H_{10}O_5)_6$ 972.84

Alpha cyclodextrin [10016-20-3].

DEFINITION

Alfadex is composed of six alpha-(1-4) linked D-glucopyranosyl units. It contains NLT 98.0% and NMT 101.0% of alfadex $(C_6H_{10}O_5)_6$, calculated on the anhydrous basis.

IDENTIFICATION

- **A.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.
- **B.** It meets the requirements of the test for *Optical Rotation* ⟨781S⟩, *Specific Rotation*.
- **C.**

Sample: 0.2 g

Analysis: Mix the *Sample* with 2 mL of iodine TS, warm in a water bath to dissolve, and allow to stand at room temperature.

Acceptance criteria: A yellow-brown precipitate is formed.

ASSAY

- **PROCEDURE**

Mobile phase: Methanol and water (7:93)

System suitability solution: 0.5 mg/mL of USP Alpha Cyclodextrin RS, 0.5 mg/mL of USP Beta Cyclodextrin RS, and 0.5 mg/mL of USP Gamma Cyclodextrin RS

Standard solution: 1.0 mg/mL of USP Alpha Cyclodextrin RS

Sample stock solution: Transfer 250 mg of Alfadex to a 25-mL volumetric flask, and dissolve in water with the aid of heat. Cool, and dilute with water to volume.

Sample solution: 1.0 mg/mL of Alfadex, diluted from the *Sample stock solution*

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 4.6-mm × 15-cm; 5-µm packing L1

Temperature

Column: 30°

Detector: 40°

Flow rate: 1.5 mL/min

Injection volume: 50 µL

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for gamma cyclodextrin, alpha cyclodextrin, and beta cyclodextrin are 0.8, 1.0, and 2.0, respectively.]

Suitability requirements

Resolution: NLT 1.5 between the gamma cyclodextrin and alpha cyclodextrin peaks

Tailing factor: 0.8–2.0 for the three cyclodextrins

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of alfadex $(C_6H_{10}O_5)_6$ in the portion of the sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of alpha cyclodextrin from the *Sample solution*

r_S = peak response of alpha cyclodextrin from the *Standard solution*

C_S = concentration of alpha cyclodextrin in the *Standard solution* (mg/mL)

C_U = concentration of alpha cyclodextrin in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–101.0% on the anhydrous basis

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩

Sample: 1.0 g

Acceptance criteria: NMT 0.1%

- **LIMIT OF LIGHT-ABSORBING SUBSTANCES**

Sample solution: 10 mg/mL of Alfadex, calculated on the anhydrous basis, in water that has been previously boiled and cooled to room temperature. Pass through a filter of 0.2-µm pore size.

Analysis: Determine the absorbance of the *Sample solution* in a 1-cm cell with a suitable spectrophotometer, after correcting for the blank.

Acceptance criteria: Between 230 and 350 nm, the absorbance is NMT 0.10; and between 350 and 750 nm, the absorbance is NMT 0.05.

- **LIMIT OF BETADEX, GAMMA CYCLODEXTRIN, AND OTHER RELATED SUBSTANCES**

System suitability solution, Chromatographic system, and **System suitability:** Proceed as directed in the *Assay*.

Standard solution: *System suitability solution* and water (1:9)

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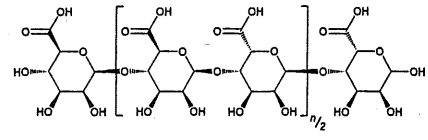
Sample solution: Use the *Sample stock solution* prepared as directed in the *Assay*.
Analysis
Samples: *Standard solution* and *Sample solution*
Acceptance criteria
Beta cyclodextrin: The area of any peak corresponding to beta cyclodextrin is NMT 0.5 times the area of the corresponding peak from the *Standard solution* (0.25%).
Gamma cyclodextrin: The area of any peak corresponding to gamma cyclodextrin is NMT 0.5 times the area of the corresponding peak from the *Standard solution* (0.25%).
Other related substances: The sum of all of the peak areas, excluding the alpha cyclodextrin, beta cyclodextrin, and gamma cyclodextrin is NMT the area of the peak due to alpha cyclodextrin from the *Standard solution* (0.5%).

• **LIMIT OF REDUCING SUGARS**
Cupric solution: 150 mg/mL of cupric sulfate
Tartrate solution: 25 mg/mL of anhydrous sodium carbonate, 25 mg/mL of potassium sodium tartrate, 20 mg/mL of sodium bicarbonate, and 200 mg/mL of anhydrous sodium sulfate
Cupric–tartaric solution: Immediately before use, mix *Cupric solution* with *Tartrate solution* (1:25).
Ammonium molybdate reagent: Mix 10 mL of disodium arsenate solution (6 in 100), 50 mL of a solution of ammonium molybdate (1 in 10), and 90 mL of diluted sulfuric acid, and dilute with water to 200 mL.
Sample solution: 1.0 g of Alfadex, calculated on the anhydrous basis, in 100 mL of water that has been previously boiled and cooled to room temperature. To 1 mL of this solution add 1 mL of *Cupric–tartaric solution*. Heat on a water bath for 10 min, then cool to room temperature. Add 10 mL of *Ammonium molybdate reagent*, and allow to stand for 15 min.
Standard stock solution: 20 mg/L of USP Dextrose RS
Standard solution: Prepare as directed for the *Sample solution*, at the same time, except use 1 mL of *Standard stock solution* in place of 1 mL of 10 mg/mL of Alfadex solution.
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: Vis
Analytical wavelength: 740 nm
Blank: Water
Analysis
Samples: *Sample solution* and *Standard solution*
Measure the *Samples* at the analytical wavelengths against the *Blank*.
Acceptance criteria: The absorbance of the *Sample solution* is NMT that of the *Standard solution* (0.2%).

SPECIFIC TESTS
• **CLARITY OF SOLUTION**
Sample: 10 mg/mL in previously boiled and cooled water
Acceptance criteria: The resulting solution is clear.
• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic bacterial count does not exceed 10^3 cfu/g, the total combined molds and yeasts count does not exceed 10^2 cfu/g, and it meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.
• **OPTICAL ROTATION,** *Specific Rotation* ⟨781S⟩
Sample solution: 10 mg/mL
Acceptance criteria: +147° to +152°, determined at 20°
• **pH** ⟨791⟩
Diluent: 224 g/L of potassium chloride
Sample solution: 30 mL of its aqueous solution (1 in 100) and 1 mL of *Diluent*
Acceptance criteria: 5.0–8.0
• **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 11.0%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Alpha Cyclodextrin RS
USP Beta Cyclodextrin RS
USP Dextrose RS
USP Gamma Cyclodextrin RS

Alginic Acid



$(C_6H_8O_6)_n$
Alginic acid [9005-32-7].

DEFINITION

Change to read:

Alginic Acid is a hydrophilic colloidal carbohydrate extracted with dilute alkali from various species of brown seaweeds (Phaeophyceae). ▲Alginic Acid is a linear, unbranched copolymer of β-D-mannuronic acid (M) and α-L-guluronic acid (G) linked to each other by 1→4 glycosidic bonds. The M and G units in the Alginic Acid may be randomly or non-randomly arrayed as heterogeneous or homogeneous sequences. The typical average molecular weight for Alginic Acid is in the range of 10,000–600,000 g/mol.▲ (NF 1-May-2020)

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: ⟨197K⟩ or ⟨197A⟩▲ (NF 1-May-2020)

Change to read:

• **B.**
▲Analysis 1: To 5 mL of a 1-in-150 solution in 0.1 N sodium hydroxide add 1 mL of calcium chloride TS.
Acceptance criteria 1: A voluminous, gelatinous precipitate is formed.▲ (NF 1-May-2020)
Analysis ▲2:▲ (NF 1-May-2020) To 5 mL of a 1-in-150 solution in 0.1 N sodium hydroxide add 1 mL of 4 N sulfuric acid.
Acceptance criteria ▲2:▲ (NF 1-May-2020) A heavy, gelatinous precipitate is formed.
• **C.**
Analysis: To 5 mg in a test tube add 5 mL of water, 1 mL of a freshly prepared 1-in-100 solution of 1,3-naphthalenediol in alcohol, and 5 mL of hydrochloric acid. Heat the mixture to boiling, boil gently for 3 min, then cool to 15°. Transfer the contents of the test tube to a 30-mL separator with the aid of 5 mL of water, and extract with 15 mL of isopropyl ether.
Acceptance criteria: The isopropyl ether extract exhibits a deeper purplish hue than that from a blank, similarly prepared.

IMPURITIES
• **ARSENIC** ⟨211⟩, *Procedures, Method II*: NMT 3 ppm

NF Monographs

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• **LEAD** ⟨251⟩
Standard solution: 5 mL of *Diluted standard lead solution*
Test preparation: Add 1.0 g to 20 mL of nitric acid in a 250-mL conical flask, mix, and heat carefully until the Alginic Acid is dissolved. Continue heating until the volume is reduced to 7 mL. Cool rapidly to room temperature, transfer to a 100-mL volumetric flask, and dilute with water to volume.
Analysis: Proceed as directed in the chapter, using 15 mL of ammonium citrate solution, 3 mL of potassium cyanide solution, and 0.5 mL of hydroxylamine hydrochloride solution. After the first dithizone extraction, wash the combined chloroform layers with 5 mL of water, discarding the water layer and continuing in the usual manner by extracting with 20 mL of 0.2 N nitric acid.
Acceptance criteria: A 50.0-mL portion of the *Test preparation* contains NMT 5 µg of lead (corresponding to NMT 10 ppm).

SPECIFIC TESTS
• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic microbial count does not exceed 2×10^2 cfu/g. The tests for *Salmonella* species and *Escherichia coli* are negative.
• **pH** ⟨791⟩**:** 1.5–3.5, in a 3-in-100 dispersion in water
• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry at 105° for 4 h.
Acceptance criteria: NMT 15.0%
• **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Total Ash*
Sample: 4 g of Alginic Acid
Analysis: Proceed as directed in the chapter, carefully igniting the *Sample* in a tared platinum dish, until the residue is thoroughly carbonized (5 min). Then ignite in a muffle furnace at a temperature of 800 ± 25° until the carbon is completely burned off (20–35 min).
Acceptance criteria: NMT 4.0% of ash
• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Acid Value*
Sample: 1 g of Alginic Acid
Analysis: Suspend the *Sample* in a mixture of 50 mL of water and 30.0 mL of calcium acetate solution (11 in 250). Shake thoroughly, allow the mixture to stand for 1 h, and add phenolphthalein TS. Titrate the liberated acetic acid with 0.1 N sodium hydroxide VS.
Perform a blank determination, and calculate the *Acid Value*:

$$\text{Result} = [(V_S - V_B) \times N \times F]/W$$

V_S = volume of 0.1 N sodium hydroxide VS consumed in the titration of the *Sample* (mL)
V_B = volume of 0.1 N sodium hydroxide VS consumed in the titration of the blank (mL)
N = normality of sodium hydroxide VS (mEq/mL)
F = equivalency factor of potassium hydroxide, 56.11 (mg/mEq)
W = weight of Alginic Acid taken (g)

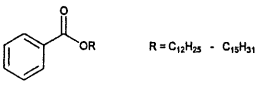
Acceptance criteria: NLT 230 on the dried basis

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Add the following:

▲• **USP REFERENCE STANDARDS** ⟨11⟩
USP Alginic Acid RS▲ (NF 1-May-2020)

Alkyl (C12–15) Benzoate



$C_{20}H_{32}O_2$ 304.47 (average)
Benzoic acid, C12–15 alkyl ester [68411-27-8].

DEFINITION
Alkyl (C12–15) Benzoate consists of esters of a mixture of C12 to C15 primary and branched alcohols and benzoic acid.

IDENTIFICATION
• **A.** Infrared absorption spectrum, obtained by spreading a capillary film of it between sodium chloride plates, exhibits maxima at 2800 cm^{-1} (broad); 2950 cm^{-1} (broad); 1730 cm^{-1} (medium, sharp); 1270 cm^{-1} (medium, sharp); and 710 cm^{-1} (medium, sharp).

IMPURITIES
• **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 0.5%

SPECIFIC TESTS
• **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 0.3%
• **SPECIFIC GRAVITY** ⟨841⟩**:** 0.915–0.935
• **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩**:** NMT 0.5
• **REFRACTIVE INDEX** ⟨831⟩**:** 1.483–1.487 at 20°
• **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩
Sample: 2 g of Alkyl (C12–15) Benzoate
Analysis: Transfer the *Sample* to a 200-mL flask. Proceed as directed in the chapter, except use 50.0 mL of 0.5 N alcoholic potassium hydroxide VS instead of 25.0 mL, maintaining reflux for 2 h instead of 30 min, and rinsing the reflux condenser with 25 mL of water after the reflux period.
Acceptance criteria: 169–182
• **VISCOSITY—ROTATIONAL METHODS** ⟨912⟩
Sample: 500 mL
Analysis: Transfer the *Sample* to a 600-mL tall-form beaker, and adjust the temperature to 25 ± 0.1°. Using a suitable rotational viscometer with a spindle having a cylinder 5.63 cm in diameter and 2.25 cm in height attached to a shaft 0.32 cm in diameter, the distance from the top of the cylinder to the lower tip of the shaft being 2.91 cm, and the immersion depth being 6.11 cm (No. 1 spindle), operate the viscometer at 100 rpm for 60 s, accurately timed, and record the scale reading. Convert the scale reading to centipoises by multiplying by the constant for the spindle and speed used.
Acceptance criteria: The average of three viscosities obtained is NMT 100 centipoises.

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

Almond Oil

Almond Oil [8007-69-0].

DEFINITION
Almond Oil is the refined fixed oil obtained by expression from the kernels of varieties of *Prunus dulcis* (Miller) D.A. Webb (formerly known as *Prunus amygdalus* Batsch) (Fam. Rosaceae), except for *Prunus dulcis* (Miller) D.A. Webb var. amara (De Candolle) Focke. It may contain suitable antioxidants.

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IDENTIFICATION

• **A. Identity by Fatty Acid Composition**

Analysis: Proceed as directed in the test for *Fats and Fixed Oils* ⟨401⟩, *Procedures, Fatty Acid Composition.*

Acceptance criteria: Meets the composition profile of fatty acids in *Table 1*

• **B. Identity by Triglyceride Profile**

Analysis: Proceed as directed in *Identification of Fixed Oils by Thin-Layer Chromatography* ⟨202⟩, *Identification, Method I* or *Method II.*

Acceptance criteria: Meets the requirements in the chapter

SPECIFIC TESTS

• **Fats and Fixed Oils** ⟨401⟩, *Procedures, Acid Value*: NMT 0.5

• **Fats and Fixed Oils** ⟨401⟩, *Procedures, Fatty Acid Composition*: Almond Oil exhibits the composition profiles of fatty acids in *Table 1*.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
<16	0	≤0.1
16	0	4.0–9.0
17	0	≤0.2
18	0	≤3.0
20	0	≤0.2
22	0	≤0.2
24	0	≤0.2
16	1	≤0.8
17	1	≤0.2
18	1	62.0–76.0
18	2	20.0–30.0
18	3	≤0.4
20	1	≤0.3
22	1	≤0.1

• **Fats and Fixed Oils** ⟨401⟩, *Procedures, Peroxide Value*: NMT 5.0

• **Fats and Fixed Oils** ⟨401⟩, *Procedures, Unsaponifiable Matter*: NMT 0.9%

• **Specific Gravity** ⟨841⟩: 0.910–0.915

• **Sterol Composition**

Separation of the sterols fraction

Reference solution A: 5% (w/v) of cholesterol in chloroform

Developing solvent system: Toluene and acetone (19:1) or hexane and ether (13:7)

Sample solution A: Weigh 5 g of Almond Oil into a 250-mL flask. Add 50 mL of 2 N alcoholic potassium hydroxide, and heat to gentle boiling with continuous vigorous stirring until saponification takes place (the solution becomes clear). Continue heating for an additional 20 min, and add 50 mL of water from the top of the condenser. Cool the flask to approximately 30°. Transfer the contents of the flask to a 500-mL separating funnel with several rinses of water, amounting in all to 50 mL. Add approximately 80 mL of ether, shake vigorously for approximately 30 s, and allow to settle. [Note—Any emulsion can be destroyed by adding small quantities of ethyl or methyl alcohol by means of a spray.]

Separate the lower aqueous phase, and collect it into a second separating funnel. Perform two further extractions on the water–alcohol phase in the same way, using 60–70 mL of ether on each occasion. Pool the ether extracts into a single separating funnel, and wash with water, 50 mL at a time, until the wash water is no longer alkaline to phenolphthalein. Dry the ether phase with anhydrous sodium sulfate, and filter on anhydrous sodium sulfate into a previously weighed 250-mL flask, washing the funnel and filter with small quantities of ether. Distill the ether down to a few milliliters, and bring to dryness under a slight vacuum or in a stream of nitrogen. Completely dry at 100° for approximately 15 min, and then weigh after cooling in a desiccator. Dissolve the unsaponifiables so obtained in chloroform to prepare a solution having a concentration of approximately 5%.

Sample solution B: Treat 5 g of canola oil in the same way as prescribed for Almond Oil in *Sample solution A*, beginning with "Add 50 mL of 2 N alcoholic potassium hydroxide".

Sample solution C: Treat 5 g of sunflower oil in the same way as prescribed for Almond Oil in *Sample solution A*, beginning with "Add 50 mL of 2 N alcoholic potassium hydroxide".

Analysis: Precondition the thin-layer chromatographic plate (see *Chromatography* ⟨621⟩), 20-cm × 20-cm silica gel on polyester with a layer thickness of 200 µm and particle size of 5–17 µm, by immersing completely in the 0.2 N alcoholic potassium hydroxide for 10 s, then allow to dry in a fume cupboard for 2 h, and finally place at 100° for 1 h.

Remove from the validated heating device, and keep the plate in a desiccator until required for use. The plates must be used within 15 days. [Note—Thin-layer chromatographic plates without requiring the preconditioning are also commercially available.] Use a separate plate for each *Sample solution.*

Place the *Developing solvent system* in the chamber to a depth of approximately 1 cm. Close the chamber with the appropriate cover, and leave for at least 30 min. Strips of filter paper dipping into the eluant may be placed on the internal surfaces of the chamber. The *Developing solvent system* should be replaced for every test to ensure reproducible elution conditions.

Apply 0.3 mL of *Sample solution A* approximately 2 cm from the lower edge in a streak that is as thin and as uniform as possible. In line with the streak, place 2–3 µL of *Reference solution A* at one end of the plate. Develop the chromatograms in an equilibrated chamber with a *Developing solvent system* until the solvent front reaches approximately 1 cm from the upper edge of the plate. Remove the plate from the developing chamber, and evaporate the solvent under a current of hot air (avoid excessive heat), or by leaving the plate for a short while under a hood. Spray the plate with a 0.2% alcoholic solution of 2,7-dichlorofluorescein, and examine in UV light at 254 nm.

[Note—The plates pretreated with UV indicator are also commercially available and used equivalently.]

In each of the plates, mark the limits of the sterol band identified through being aligned with the stain of *Reference solution A* along the edges of the fluorescence, and additionally include the area of the zones 2–3 mm above and below the visible zones corresponding to *Reference solution A*. Remove the silica gel in the marked area into a filter funnel with a G3 porous septum. Add 10 mL of hot chloroform, mix carefully with the metal spatula, filter under vacuum, and collect the filtrate in

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the conical flask attached to the filter funnel. Wash the residue in the funnel three times with ether, 10 mL each time, and collect the filtrate in the same flask attached to the funnel. Evaporate the filtrate to a volume of 4–5 mL, transfer the residual solution to a previously weighed 10-mL test tube with a tapering bottom and a sealing stopper, and evaporate to dryness by mild heating in a gentle stream of nitrogen. Dissolve the residue in a few drops of acetone, and evaporate again to dryness. Place at 105° for approximately 10 min, allow to cool in a desiccator, and weigh.

Treat *Sample solution B* and *Sample solution C* the same way as prescribed for *Sample solution A*.

Determination of the sterols

Sample solution D: To the test tube containing the sterol fraction separated from Almond Oil by thin-layer chromatography add a freshly prepared mixture of anhydrous pyridine, hexamethyldisilazane, and chlorotrimethylsilane (9:3:1) in the ratio of 50 µL for every mg of sterols, avoiding any uptake of moisture. [NOTE—This reagent is also commercially available and used equivalently.]

Stopper the test tube, and shake carefully until the sterols are completely dissolved. Allow it to stand for at least 15 min at ambient temperature, and centrifuge for a few minutes if necessary. Use the supernatant. The slight opalescence that may form is normal and does not cause an anomaly. However, the formation of a white floc or the appearance of a pink color is indicative of the presence of moisture or deterioration of the reagent. If these occur, repeat the test.

Reference solution E: To 9 parts of the sterols separated from canola oil by thin-layer chromatography add 1 part of cholesterol. Treat the mixture in the same way as prescribed in *Sample solution D*.

Reference solution F: Treat the sterols separated from sunflower oil by thin-layer chromatography in the same way as prescribed in *Sample solution D*.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: Glass or fused-silica capillary; length 20–30 m, internal diameter 0.25–0.32 mm, coated with a 0.10–0.30-µm layer of stationary phase G27 or G36

Temperatures

Column: 260 ± 5°

Injection port: 280°

Detector: 290°

Carrier gas: Either helium with a linear velocity of 20–35 cm/s or hydrogen with a linear velocity of 30–50 cm/s

Injection volume: 1 µL

Injection type: Split, 50:1 to 100:1

System suitability

Samples: *Reference solution E* and *Reference solution F*

Suitability requirements

The retention time for β-sitosterol is 20 ± 5 min, and all of the sterols present must be separated. [NOTE—For peak identification purposes, the chromatogram *Reference solution E* shows four principal peaks corresponding to cholesterol, brassicasterol, campesterol, and β-sitosterol; the chromatogram obtained with *Reference solution F* shows four principal peaks corresponding to campesterol, stigmasterol, β-sitosterol, and Δ7-stigmastenol. The retention times of the sterols with reference to β-sitosterol are given in *Table 2*.]

Table 2. Relative Retention Times of Sterols for Two Different Columns

Identification	G36 Column	G27 Column
Cholesterol	0.67	0.63
Brassicasterol	0.73	0.71
24-Methylene-cholesterol	0.82	0.80
Campesterol	0.83	0.81
Campestanol	0.85	0.82
Stigmasterol	0.88	0.87
Δ7-Campesterol	0.93	0.92
Δ5,23-Stigmastadienol	0.95	0.95
Clerosterol	0.96	0.96
β-Sitosterol	1.00	1.00
Sitostanol	1.02	1.02
Δ5-Avenasterol	1.03	1.03
Δ5,24-Stigmastadienol	1.08	1.08
Δ7-Stigmastenol	1.12	1.12
Δ7-Avenasterol	1.16	1.16

Analysis

Samples: *Sample solution D*, *Reference solution E*, and *Reference solution F*

Calculate the percentage of each individual sterol in the sterol fraction of Almond Oil taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak area due to the sterol component to be determined

r_T = sum of the peak areas due to the components indicated in *Table 2*

Acceptance criteria: Almond Oil exhibits the composition profiles of sterols given in *Table 3*.

Table 3

Component	Percentage (%)
Cholesterol	≤0.7
Brassicasterol	≤0.3
Campesterol	≤5.0
Stigmasterol	≤4.0
β-Sitosterol	73.0–87.0
Δ5-Avenasterol	≥5.0
Δ7-Stigmastenol	≤3.0
Δ7-Avenasterol	≤3.0

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant, and well-filled containers. No storage requirements specified.
- **LABELING:** Label it to indicate the name and quantity of any added antioxidants.

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Aluminum Monostearate

Aluminum, dihydroxy(octadecanoato-*O*-)-;
Dihydroxy(stearato)aluminum [7047-84-9].

DEFINITION

Aluminum Monostearate is a compound of aluminum with a mixture of solid organic acids obtained from sources of vegetable or animal origin, and consists mainly of variable proportions of aluminum monostearate ($C_{18}H_{37}AlO_4$) and aluminum monopalmitate ($C_{16}H_{33}AlO_4$). The content of stearic acid in the fatty acid fraction is NLT 40.0% of the total content. The sum of stearic acid and palmitic acid in the fatty acid fraction is NLT 90.0% of the total content. Aluminum Monostearate contains the equivalent of NLT 14.7% and NMT 16.7% of aluminum oxide (Al_2O_3) on the dried basis. Additionally, it contains small amounts of the aluminum salts of other fatty acids.

IDENTIFICATION

• **A. Identification Tests—General** ⟨191⟩, *Chemical Identification Tests, Aluminum*
Sample: 1 g
Analysis: Heat the *Sample* with a mixture of 25 mL of water and 5 mL of hydrochloric acid for 1 h, replacing the water as it evaporates.
Acceptance criteria: Fatty acids are liberated, floating as an oily layer on the surface of the liquid, and the water layer meets the requirements.

• **B.** The retention times of the two major peaks of the *Sample solution* correspond to those of the *Standard solution*, as obtained in the *Content of Stearic Acid and Palmitic Acid* test in the *Assay*.

ASSAY

• **Content of Aluminum**
Sample: 5 g
Analysis
Sample: *Sample*
Weigh the *Sample* in a covered platinum crucible that previously has been ignited for 20 min, cooled over anhydrous magnesium perchlorate, and weighed. Heat the open crucible gently, without allowing the *Sample* to burst into flame, and gradually increase the heat until the ash is white. Ignite the ash for 20 min after the organic matter is removed, and cool. Add 15 mL of water, cover the crucible with a small watch glass, and boil gently for 5 min, using a small stirring rod to break up any large lumps of ash. Decant the solution through ashless filter paper, retaining most of the ash in the crucible. Repeat the extraction with water twice, passing the solutions through the same filter. Transfer the ash to the filter by means of a fine stream of water, and wash the crucible and the residue three times with warm water. Transfer the filter paper and the residue to the crucible, dry, and ignite for 20 min after the filter paper has burned away. Following the ignition period, cover the crucible, cool over anhydrous magnesium perchlorate for 15 min, and weigh the residue of aluminum oxide (Al_2O_3) rapidly. Repeat the ignition until constant weight is attained, using 20-min ignition periods and 15-min cooling periods. From the weight of the residue remaining in the crucible, calculate the content of aluminum oxide (Al_2O_3).
Acceptance criteria: 14.7%–16.7% on the dried basis

• **Content of Stearic Acid and Palmitic Acid**
Boron trifluoride–methanol solution: 140 g/L of boron trifluoride in methanol
Sample solution: Dissolve 100 mg of Aluminum Monostearate in a small conical flask fitted with a suitable reflux attachment with 5 mL of *Boron trifluoride–methanol solution*. Boil under reflux for 10 min. Add 4.0 mL of *n*-heptane through the condenser, and boil again under reflux for 10 min. Allow to cool. Add 20 mL of a saturated solution of sodium chloride. Shake, and allow the layers to separate. Dry the organic layer over 0.1 g of anhydrous sodium sulfate. Dilute 1.0 mL of this solution with *n*-heptane to 10.0 mL.
Standard solution: Prepare as directed in the *Sample solution* using 50 mg each of USP Stearic Acid RS and USP Palmitic Acid RS.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 30-m × 0.32-mm; coated with a 0.5-µm film of phase G16
Temperatures
Injection port: 220°
Detector: 260°
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	—	70	2
70	5	240	5

Carrier gas: Helium, passed through a bed of molecular sieve for drying, if necessary
Flow rate: 2.4 mL/min
Injection volume: 1 µL
System suitability
Sample: *Standard solution*
[Note—The relative retention times for methyl palmitate and methyl stearate are about 0.9 and 1.0, respectively.]
Suitability requirements
Resolution: NLT 5.0 between the methyl palmitate and methyl stearate peaks
Relative standard deviation: NMT 3.0% for the methyl stearate and methyl palmitate peaks (from six replicate injections of the *Standard solution*); NMT 1.0% for the ratio of the peak areas of methyl palmitate to the peak areas of methyl stearate (from six replicate injections of the *Standard solution*)
Analysis
Sample: *Sample solution*
Calculate the percentage of stearic acid ($C_{18}H_{36}O_2$) in the fatty acid fraction of the sample taken:

$$\text{Result} = (r_{U1}/r_T) \times 100$$

r_{U1} = peak area due to methyl stearate
r_T = sum of all the peak areas, excluding the solvent peak

Calculate the percentage of palmitic acid ($C_{16}H_{32}O_2$) in the fatty acid fraction of the sample taken:

$$\text{Result} = (r_{U2}/r_T) \times 100$$

r_{U2} = peak area due to methyl palmitate
r_T = sum of all the peak areas, excluding the solvent peak

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Acceptance criteria
Stearic acid: NLT 40.0%
Sum of stearic acid and palmitic acid: NLT 90.0%

IMPURITIES
• **ARSENIC** ⟨211⟩, *Procedures, Method I*
Test preparation: To 3.75 g of Aluminum Monostearate add 12.5 mL of hydrochloric acid and 0.5 mL of bromine TS, and heat on a steam bath until a transparent layer of melted fatty acid forms. Add 50 mL of water, heat on a hot plate until the volume is about 25 mL, and filter while hot. Cool, dilute the filtrate with water to 50 mL, and to a 10-mL aliquot of this solution add 2.5 mL of hydrochloric acid, then dilute with water to 55 mL.
Acceptance criteria: NMT 4 µg/g; the *Test preparation* meets the requirements, omitting the addition of 20 mL of 7 N sulfuric acid specified for *Procedure*.

SPECIFIC TESTS
• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry at 105° for 3 h.
Acceptance criteria: NMT 3.0%
• **ACIDITY AND ALKALINITY**
Sample solution: To 1.0 g of Aluminum Monostearate add 20 mL of carbon dioxide-free water and boil on a steam bath for 1 min with continuous shaking. Cool and filter. To 10 mL of the filtrate add 0.05 mL of bromothymol blue TS.
Analysis: Titrate with 0.01 N hydrochloric acid or 0.01 N sodium hydroxide.
Acceptance criteria: NMT 0.5 mL of 0.01 N hydrochloric acid or 0.01 N sodium hydroxide is required to change the color of the indicator.

Add the following:

▲• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Acid Value, Method I*
Sample: 1.0 g
Analysis: Dissolve the *Sample* in 50 mL of a mixture of equal volumes of alcohol and ether (which has been neutralized to phenolphthalein with 0.1 N potassium hydroxide or 0.1 N sodium hydroxide). Pass the suspension through dry filter paper, wash the vessel and the filter paper with a small amount of a mixture of neutralized ethanol and diethyl ether (1:1), and combine the filtrate and the washings. Proceed as directed in the chapter starting with "Add 1 mL of phenolphthalein TS".
Acceptance criteria: NMT 1 ▲(NF 1-Dec-2019)
• **BACTERIAL ENDOTOXINS TEST** ⟨85⟩: If labeled for use in preparing parenteral dosage forms, it also meets the following requirements. The level of bacterial endotoxins is such that the requirement in the relevant dosage form monograph(s) in which Aluminum Monostearate is used can be met. Where the label states that Aluminum Monostearate must be subjected to further processing during the preparation of injectable dosage forms, the level of bacterial endotoxins is such that the requirement in the relevant dosage form monograph(s) in which Aluminum Monostearate is used can be met.

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers.
• **LABELING:** Label it to indicate the content of stearic acid and the sum of stearic acid and palmitic acid content in the fatty acid fraction and to indicate the fatty acids used to produce Aluminum Monostearate are from sources of vegetable or animal origin. Where Aluminum Monostearate must be subjected to further processing during the preparation of injectable dosage forms to ensure acceptable levels of bacterial endotoxins, it is so labeled.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Palmitic Acid RS
USP Stearic Acid RS

Aluminum Oxide

Al_2O_3 101.96
[1344-28-1].

DEFINITION
Aluminum Oxide contains NLT 47.0% and NMT 60.0% of Al_2O_3.

IDENTIFICATION
• **IDENTIFICATION TESTS—GENERAL,** *Aluminum* ⟨191⟩
Sample: 2.5 g
Analysis: Dissolve the *Sample* in 15 mL of hydrochloric acid on a heated water bath. Dilute with water to 100 mL.
Acceptance criteria: Meets the requirements

ASSAY
• **PROCEDURE**
Sample: 800 mg
Analysis: Add the *Sample* to 10 mL of 2 M hydrochloric acid on a heated water bath. Cool, and dilute with water to 50.0 mL. To 10.0 mL of the solution, add dilute ammonia solution (460 mL of strong ammonia solution/L) until a precipitate begins to appear. Add the smallest quantity of 0.6 M hydrochloric acid needed to dissolve the precipitate, and dilute with water to 20 mL. Transfer 20 mL of the *Sample* to a 500-mL conical flask, add 25.0 mL of 0.1 M edetate disodium and 10 mL of a mixture of equal volumes of a 155 g/L solution of ammonium acetate and 2 N acetic acid. Boil for 2 min, then cool. Add 50 mL of ethanol and 3 mL of a freshly prepared 250 mg/L solution of dithizone in alcohol. Titrate (see *Titrimetry* ⟨541⟩) the excess of edetate sodium with 0.1 M zinc sulfate until the color changes from greenish-blue to reddish-violet. Each mL of 0.1 M edetate sodium is equivalent to 5.098 mg of Al_2O_3.
Acceptance criteria: 47.0%–60.0% of Al_2O_3

IMPURITIES
INORGANIC IMPURITIES
• **Arsenic,** *Method I* ⟨211⟩
Sample solution: Dissolve 2.5 g of Aluminum Oxide in 15 mL of hydrochloric acid on a heated water bath. Dilute with water to 100 mL. Use 30 mL of this solution for the test.
Acceptance criteria: NMT 4 ppm
• **CHLORIDE AND SULFATE,** *Chloride* ⟨221⟩
Chloride solution: 0.824 mg/mL of sodium chloride
Standard solution: On the day of use, dilute 1.0 mL of *Chloride solution* with water to 100 mL. Mix 10 mL of this solution (5 ppm Cl) with 5 mL of water.
Sample solution: Dissolve 0.1 g of Aluminum Oxide with heating in 10 mL of dilute nitric acid and dilute with water to 100 mL. Dilute 5 mL of the solution with water to 15 mL.
Analysis: Proceed as directed.
Acceptance criteria: The *Sample solution* shows no more chloride than the *Standard solution* (NMT 10,000 ppm).
• **CHLORIDE AND SULFATE,** *Sulfate* ⟨221⟩
Sulfate stock solution: 1.8 mg/mL of potassium sulfate
Sulfate solution: On the day of use, dilute 1.0 mL of *Sulfate stock solution* with water to 100 mL (10 ppm SO_4).
Standard solution: Add 3 mL of a 250 mg/mL solution of barium chloride to 4.5 mL of *Sulfate solution*. Shake and allow to stand for 1 min.

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Sample solution: Dissolve 2.5 g of Aluminum Oxide in 15 mL of hydrochloric acid on a heated water bath. Dilute with water to 100 mL. Dilute 4 mL of this solution with water to 100 mL.

Analysis: To two separate glass cylinders, add 2.5 mL of *Standard solution*. Then to cylinder 1, add 15 mL of the *Sample solution* and 0.5 mL of acetic acid. To cylinder 2 add 15 mL of the *Sulfate solution* and 0.5 mL of acetic acid.

Acceptance criteria: After 5 min, any opalescence in cylinder 1 containing the *Sample solution* is not more intense than that of cylinder 2 containing the *Sulfate solution* (NMT 10,000 ppm).

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩: The total aerobic bacterial count is NMT 1000 cfu/g and the total combined molds and yeasts count is NMT 100 cfu/g. It also meets the requirements of the tests for absence of *Escherichia coli* and bile-tolerant Gram-negative bacteria.
- **APPEARANCE OF SOLUTION**

Dilute hydrochloric acid: 10 mg/mL

Control solution: [NOTE—Prepare immediately before use.] Mix 9.6 mL of ferric chloride CS, 0.2 mL of cobaltous chloride CS, and 0.2 mL of cupric sulfate CS with *Dilute hydrochloric acid* to make 10 mL, and dilute 1.5 mL of this solution with the *Dilute hydrochloric acid* to 100 mL.

Sample solution: 2.5 g of Aluminum Oxide in 15 mL of hydrochloric acid on a heated water bath. Dilute with water to 100 mL.

Analysis: Make the comparison by viewing 2.0 mL of *Sample solution* and 2.0 mL of *Control solution* downward in matched color-comparison tubes against a white surface (see *Color and Achromicity* ⟨631⟩.

Acceptance criteria: The *Sample solution* is not more intensely colored than the *Control solution*.

Change to read:

- **CLARITY OF SOLUTION**

Hydrazine sulfate solution: 10 mg/mL of hydrazine sulfate. Allow to stand for 4–6 h before use. [**CAUTION**—Hydrazine sulfate is highly toxic. Avoid skin contact.]

Methenamine solution: 2.5 g of methenamine to a 100-mL glass-stoppered flask, add 25.0 mL of water, insert the glass stopper, and mix to dissolve.

Primary opalescent mixture: To the flask containing *Methenamine solution*, add 25.0 mL of *Hydrazine sulfate solution*, mix, and allow to stand for 24 h. [NOTE—This suspension is stable for 2 months. Mix before use, and do not use if it adheres to the container.]

Opalescent standard: Dilute 15.0 mL of the *Primary opalescent mixture* with water to 1000.0 mL. [NOTE—Use this suspension within 24 h after preparation.]

Reference suspension: Transfer 30.0 mL of *Opalescence standard* to a 100-mL volumetric flask, and dilute with water to volume.

Sample solution: Use the *Sample solution* from the test for *Appearance of Solution*.

Analysis: Transfer a sufficient portion of the *Sample solution* to a test tube of colorless, transparent, neutral glass with a flat base and an internal diameter of 15–25 mm to obtain a depth of 40 mm. Similarly transfer a portion of the *Reference suspension* to a separate matching test tube. Compare the *Sample solution* and the *Reference suspension* in diffused daylight, viewing vertically against a black background (see ▲*Visual Comparison* ⟨630⟩▲ (CN 1-May-2019)). [NOTE—The *Sample solution* is to be compared to the *Reference suspension* 5 min after preparation of the *Reference suspension*.]

Acceptance criteria: The *Sample solution* is not more opalescent than the *Reference suspension*.

- **ALKALINE IMPURITIES**

Sample: 1.0 g

Analysis: Shake the *Sample* with 20 mL of carbon dioxide-free water for 1 min and filter. To 10 mL of the filtrate add 0.1 mL of phenolphthalein TS.

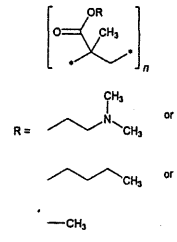
Acceptance criteria: Any pink color disappears with the addition of 0.3 mL of 0.1 M hydrochloric acid.

- **NEUTRALIZING CAPACITY:** Carry out the test at 37°. Disperse 0.5 g of aluminum oxide in 100 mL of water, heat, add 100.0 mL of 0.1 M hydrochloric acid, previously heated, and stir continuously: the pH of the solution after 10, 15, and 20 min is NLT 1.8, 2.3, and 3.0 respectively and is at no time greater than 4.5. Add 10.0 mL of 0.5 M hydrochloric acid, previously heated, stir continuously for 1 h, and titrate with 0.1 M sodium hydroxide to pH 3.5: NMT 35.0 mL of 0.1 M sodium hydroxide is required.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in a tight container at a temperature not exceeding 30°.

Amino Methacrylate Copolymer



Ratio: $CH_2CH_2N(CH_3)_2$:C_4H_9:CH_3 = 2:1:1

Poly((2-dimethylaminoethyl)methacrylate, butyl methacrylate, methyl methacrylate) (2:1:1);

N,N-Dimethylaminoethyl methacrylate–butyl methacrylate–methyl methacrylate copolymer (2:1:1) [24938-16-7].

DEFINITION

Amino Methacrylate Copolymer is a polymerized copolymer of (2-dimethylaminoethyl) methacrylate, butyl methacrylate, and methyl methacrylate. It contains NLT 20.8% and NMT 25.5% of dimethylaminoethyl groups ($C_4H_{10}N$), calculated on the dried basis.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
- **B.**

Sample solution: 1 mL of the *Sample solution* in the test for *Viscosity*

Analysis: Pour the *Sample solution* onto a glass plate, and allow the solvent to evaporate.

Acceptance criteria: A clear, colorless film results.

ASSAY

- **PROCEDURE**

Sample: 200 mg

Blank: 4 mL of water and 96 mL of glacial acetic acid

Supplement NF 38

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Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.1 N perchloric acid VS
Endpoint detection: Potentiometric
Analysis: Dissolve the *Sample* in a mixture of 4 mL of water and 96 mL of glacial acetic acid. Titrate with the *Titrant* to a potentiometric endpoint. Perform a blank determination. Calculate the percentage of dimethylaminoethyl groups ($C_4H_{10}N$) in the portion of the sample taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

V_S = *Titrant* volume consumed by the *Sample* (mL)
V_B = *Titrant* volume consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 72.1 mg/mEq
W = *Sample* weight (mg)

Acceptance criteria: 20.8%–25.5% on the dried basis

IMPURITIES

• **Residue on Ignition** ⟨281⟩**:** NMT 0.1%

• **Limit of Butyl Methacrylate and Methyl Methacrylate**
Buffer: 8.9 g/L of anhydrous dibasic sodium phosphate and 8.5 g/L of monobasic potassium phosphate. Adjust with phosphoric acid to a pH of 2.0. This is pH 2.0 phosphate buffer (0.0625 M).
Mobile phase: Methanol and *Buffer* (55:45)
Diluent: Acetonitrile and *Buffer* (40:60)
Standard solution: Dissolve 20 mg of butyl methacrylate and 10 mg of methyl methacrylate in 3 mL of *n*-butanol. Dilute with *Diluent* to 10 mL. Dilute 1.0 mL of this solution with *Diluent* to 250.0 mL, and mix. This solution contains about 8 µg/mL of butyl methylcrylate and 4 µg/mL of methyl methacrylate.
Sample solution: 20 mg/mL of Amino Methacrylate Copolymer in *Diluent*
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 205 nm
Column: 4.6-mm × 12-cm; packing L1
Flow rate: 2 mL/min
Injection volume: 50 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Resolution: NLT 10 between butyl methacrylate and methyl methacrylate
Relative standard deviation: NMT 3.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of each monomer in the portion of Amino Methacrylate Copolymer taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak response of each monomer (butyl methacrylate or methyl methacrylate) from the *Sample solution*
r_S = peak response of each monomer (butyl methacrylate or methyl methacrylate) from the *Standard solution*
C_S = concentration of each monomer (butyl methacrylate or methyl methacrylate) in the *Standard solution* (µg/mL)
C_U = concentration of Amino Methacrylate Copolymer in the *Sample solution* (mg/mL)
F = conversion factor, 10^{-3} mg/µg

Acceptance criteria: NMT 0.1% for each monomer

• **Limit of 2-Dimethylaminoethyl Methacrylate**
Buffer: 3.4 g/L of monobasic potassium phosphate. This is the monobasic potassium phosphate solution (0.025 M).
Mobile phase: Tetrahydrofuran and *Buffer* (75:25)
Standard solution: 8 µg/mL of 2-dimethylaminoethyl methacrylate in tetrahydrofuran
Sample solution: 20 mg/mL of Amino Methacrylate Copolymer in tetrahydrofuran
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 215 nm
Column: 4.6-mm × 12-cm; packing L8
Flow rate: 2 mL/min
Injection volume: 50 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 2.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of 2-dimethylaminoethyl methacrylate in the portion of Amino Methacrylate Copolymer taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak response of 2-dimethylaminoethyl methacrylate from the *Sample solution*
r_S = peak response of 2-dimethylaminoethyl methacrylate from the *Standard solution*
C_S = concentration of 2-dimethylaminoethyl methacrylate in the *Standard solution* (µg/mL)
C_U = concentration of Amino Methacrylate Copolymer in the *Sample solution* (mg/mL)
F = conversion factor, 10^{-3} mg/µg

Acceptance criteria: NMT 0.1%

SPECIFIC TESTS

• **Viscosity—Rotational Methods** ⟨912⟩
Sample solution: Dissolve 12.5 g in a mixture of 35.0 g of acetone and 52.5 g of isopropyl alcohol. [Note—Reserve a portion of this solution for the *Color of Solution* test.]
Analysis: Equip a suitable rotational viscometer with an adapter comprising a cylindrical spindle rotating within an accurately machined chamber (or tube).[1] Pipet the *Sample solution* in the specified volume, which is recommended by the instrument manufacturer, into the chamber (or tube), and ensure that the temperature of the test specimen is at 20 ± 0.1°. The cylindrical spindle rotates at the value of rpm, which corresponds to a rate of shear of approximately 37 s^{-1}.[2] Measure the apparent viscosity following the instrument manufacturer's directions.
Acceptance criteria: Viscosity, 3–6 mPa · s

• **Color of Solution**
Sample solution: Use the *Sample solution* prepared in the test for *Viscosity*.
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

[1] A commercial device is available from Brookfield as an ultra-low (UL) viscosity adapter. The adapter comprises a 0.4-cm diameter shaft, an accurately machined chamber (or tube) with an internal diameter of 2.8 cm and a depth of 13.5 cm, and a cylindrical spindle 2.5 cm in diameter and 9.1 cm in height.
[2] For the Brookfield UL adapter, the cylindrical spindle rotates at 30 rpm, which corresponds to a rate of shear of approximately 37 s^{-1}.

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Mode: Vis
Analytical wavelength: 420 nm
Cell: 1 cm
Analysis: Determine the absorbance of the *Sample*, using water as the blank.
Acceptance criteria: NMT 0.300

• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry a sample at 110° for 3 h.
Acceptance criteria: NMT 2.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers, and store at a temperature below 30°.

• **USP REFERENCE STANDARDS** ⟨11⟩
USP Amino Methacrylate Copolymer RS

Strong Ammonia Solution

NH_3 17.03
Ammonia [7664-41-7].

DEFINITION

Strong Ammonia Solution is a solution of NH_3, containing NLT 27.0% and NMT 31.0% (w/w) of NH_3. On exposure to air, it loses ammonia rapidly.
[**CAUTION**—Use care in handling Strong Ammonia Solution because of the caustic nature of the Solution and the irritating properties of its vapor. Cool the container well before opening, and cover the closure with a cloth or similar material while opening. Do not taste Strong Ammonia Solution, and avoid inhalation of its vapor.]

IDENTIFICATION

• **A.**
Analysis: Hold a glass rod moistened with hydrochloric acid near the surface of the Solution.
Acceptance criteria: Dense, white fumes are produced.

ASSAY

• **PROCEDURE**
Sample: Transfer quickly a portion of Strong Ammonia Solution to a stoppered, thick-walled container (a pressure bottle is suitable) to obtain a column height of about 20 cm, insert the stopper, and cool the container and contents to 10° or lower. Accurately weigh a glass-stoppered, 125-mL conical flask containing 35.0 mL of 1 N sulfuric acid VS. Insert a graduated 10-mL measuring pipet into the cooled solution, allow the liquid to rise in the pipet without vacuum, remove the pipet, wipe off adhering liquid, and discard the first mL of the solution permitted to run from the pipet. Hold the pipet just above the surface of the 1 N sulfuric acid VS in the conical flask, and transfer about 2 mL of the solution into the flask. Insert the stopper, and again weigh to obtain the weight of the portion taken of the *Sample*.
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Residual titration
Titrant: 1 N sulfuric acid VS
Back titrant: 1 N sodium hydroxide VS
Blank: Proceed as directed for the *Sample*, omitting the Strong Ammonia Solution
Endpoint detection: Colorimetric
Analysis
Calculate the percentage of NH_3 in the portion of the *Sample* taken:

$$\text{Result} = [(B - V) \times N \times F \times 100]/W$$

B = 1 N sodium hydroxide VS consumed by the *Blank* (mL)
V = 1 N sodium hydroxide VS consumed by the *Sample* (mL)
N = actual normality of the *Back titrant* (mEq/mL)
F = equivalency factor, 17.03 mg/mEq
W = weight of the *Sample* (mg)

Titrate the excess acid with 1 N sodium hydroxide VS, using methyl red TS as the indicator. Perform a blank determination. Each mL of 1 N sulfuric acid is equivalent to 17.03 mg of NH_3.
Acceptance criteria: 27.0%–31.0%

IMPURITIES

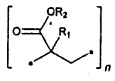
• **LIMIT OF NONVOLATILE RESIDUE**
Sample: 10 mL
Analysis: Evaporate the *Sample* in a tared platinum or porcelain dish to dryness, and dry at 105° for 1 h.
Acceptance criteria: NMT 5 mg of residue remains (0.05%).

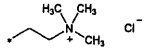
• **READILY OXIDIZABLE SUBSTANCES**
Sample solution: 4 mL of Strong Ammonia Solution
Analysis: Mix the *Sample solution* with 6 mL of water, and add a slight excess of 2 N sulfuric acid and 0.10 mL of 0.1 N potassium permanganate.
Acceptance criteria: The pink color does not completely disappear within 10 min.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers, at a temperature not above 25°.

Ammonio Methacrylate Copolymer



$R_1 = CH_3$; $R_2 = CH_3$ or
$R_1 = H$; $R_2 = C_2H_5$ or
$R_1 = CH_3$; $R_2 =$ 

Ratio of ethyl acrylate groups to methyl methacrylate groups to trimethylammonioethyl methacrylate groups is about 1:2:0.2 as being Type A or 1:2:0.1 as being Type B.
Poly(ethyl acrylate, methyl methacrylate, trimethylammonioethyl methacrylate chloride) (1: 2: 0.2);
Ethyl acrylate–methyl methacrylate–trimethylammonioethyl methacrylate chloride copolymer (1: 2: 0.2);
Poly(ethyl acrylate, methyl methacrylate, trimethylammonioethyl methacrylate chloride) (1: 2: 0.1);
Ethyl acrylate–methyl methacrylate–trimethylammonioethyl methacrylate chloride copolymer (1: 2: 0.1) [33434-24-1].

DEFINITION

Ammonio Methacrylate Copolymer is a polymerized copolymer of ethyl acrylate, methyl methacrylate, and a low content of methacrylic acid ester with quaternary ammonium groups (trimethylammonioethyl methacrylate chloride). The *Assay* requirements differ for the two types as set forth in the accompanying table.

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	Ammonio Methacrylate Units, Dried Basis (%)	
Type	Min.	Max.
A	8.85	11.96
B	4.48	6.77

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
- **B.** Meets the requirements of the *Assay*

ASSAY

- **PROCEDURE**

Sample: 1 g of Ammonio Methacrylate Copolymer Type A, or 2 g of Type B, previously dried

Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.1 N perchloric acid VS
Endpoint detection: Potentiometric

Analysis
Sample: *Sample*
Dissolve the *Sample* in 75 mL of glacial acetic acid at about 50° within 30 min. After the solution cools down to room temperature, add 25 mL of 0.6% cupric acetate in glacial acetic acid. Titrate this solution and a blank, and make any necessary corrections. Each mL of 0.1 N perchloric acid is equivalent to 20.772 mg of ammonio methacrylate ($C_9H_{18}ClNO_2$) units.

Acceptance criteria
Type A: 8.85%–11.96%
Type B: 4.48%–6.77%

IMPURITIES

INORGANIC IMPURITIES

- **Residue on Ignition** ⟨281⟩: NMT 0.1%

ORGANIC IMPURITIES

- **Procedure: Limit of Monomers**

Sodium perchlorate solution: 35 mg/mL of sodium perchlorate ($NaClO_4 \cdot H_2O$)

Mobile phase: Dilute phosphoric acid with water to obtain a solution with a pH of 2.0. Mix 4 volumes of this solution with 1 volume of methanol.

Standard stock solution: 1.6 mg/mL of ethyl acrylate and 0.2 mg/mL of methyl methacrylate in methanol

Standard solution: Dilute 1 mL of the *Standard stock solution* with methanol to 100 mL. Add 10 mL of this solution to 5 mL of the *Sodium perchlorate solution*. This solution contains about 10.67 µg/mL of ethyl acrylate and 1.33 µg/mL of methyl methacrylate.

Sample solution: Dissolve 5 g of Ammonio Methacrylate Copolymer in methanol, and dilute with the same solvent to 50 mL. Add 5 mL of the *Sodium perchlorate solution* dropwise to 10 mL of this solution while continuously stirring. Remove the precipitated polymer by centrifugation. Use the clear supernatant. Calculate the concentration of Ammonio Methacrylate Copolymer, in mg/mL, as C_U.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 202 nm
Column: 4.6-mm × 12-cm; packing L1
Flow rate: 2 mL/min
Injection size: 50 µL

System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for ethyl acrylate and methyl methacrylate are 1.00 and 1.14, respectively.]
Suitability requirements
Resolution: NLT 1.0 between the pair of analytes
Relative standard deviation: NMT 5.0% for each analyte

Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of ethyl acrylate (or methyl methacrylate) in the portion of Ammonio Methacrylate Copolymer taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak response of ethyl acrylate (or methyl methacrylate) from the *Sample solution*
r_S = peak response of ethyl acrylate (or methyl methacrylate) from the *Standard solution*
C_S = concentration of ethyl acrylate (or methyl methacrylate) in the *Standard solution* (µg/mL)
C_U = concentration of Ammonio Methacrylate Copolymer in the *Sample solution* (mg/mL)
F = conversion factor, 10^{-3} mg/µg

Acceptance criteria
Ethyl acrylate: NMT 100 ppm
Methyl methacrylate: NMT 50 ppm

SPECIFIC TESTS

- **LOSS ON DRYING** ⟨731⟩: Dry a sample in a vacuum at 80° for 5 h: it loses NMT 3.0% of its weight.
- **VISCOSITY—ROTATIONAL METHODS** ⟨912⟩

Sample solution: Place 52.5 g of isopropyl alcohol and 35.0 g of acetone in a conical flask with a ground-glass joint. Add a quantity of Ammonio Methacrylate Copolymer, equivalent to 12.5 g of solids on the dried basis, while stirring until the polymer has dissolved completely.

Analysis: Equip a suitable rotational viscometer with an adapter comprising a cylindrical spindle rotating within an accurately machined chamber (or tube).[1] Pipet the *Sample solution* in the specified volume, which is recommended by the instrument manufacturer, into the chamber (or tube), and ensure that the temperature of the test specimen is at 20 ± 0.1°. The cylindrical spindle rotates at the value of rpm, which corresponds to a rate of shear of approximately 37 s^{-1}.[2] Measure the apparent viscosity following the instrument manufacturer's directions.

Acceptance criteria: 1–15 mPa · s

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and store at a temperature not exceeding 30°.
- **LABELING:** Label it to state whether it is Type A or Type B.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Ammonio Methacrylate Copolymer, Type A RS
Poly(ethyl acrylate, methyl methacrylate, trimethylammonioethyl methacrylate chloride) (1: 2: 0.2);
Ethyl acrylate–methyl methacrylate–trimethylammonioethyl methacrylate chloride copolymer (1: 2: 0.2).

[1] A commercial device is available from Brookfield as an ultra-low (UL) viscosity adapter. The adapter comprises a 0.4-cm diameter shaft, an accurately machined chamber (or tube) with an internal diameter of 2.8 cm and a depth of 13.5 cm, and a cylindrical spindle 2.5 cm in diameter and 9.1 cm in height.
[2] For the Brookfield UL adapter, the cylindrical spindle rotates at 30 rpm, which corresponds to a rate of shear of approximately 37 s^{-1}.

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USP Ammonio Methacrylate Copolymer, Type B RS
Poly(ethyl acrylate, methyl methacrylate, trimethylammonioethyl methacrylate chloride) (1: 2: 0.1);
Ethyl acrylate–methyl methacrylate–trimethylammonioethyl methacrylate chloride copolymer (1: 2: 0.1).

Ammonio Methacrylate Copolymer Dispersion

DEFINITION

Ammonio Methacrylate Copolymer Dispersion is an aqueous dispersion of Ammonio Methacrylate Copolymer Type A or B in water. It may contain suitable antimicrobial preservatives and alkalizing agents. The *Assay* requirements differ for the two types, as set forth in the accompanying table.

	Ammonio Methacrylate Units, Dried Basis (%)	
Type	Min.	Max.
A	10.18	13.73
B	6.11	8.26

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
Sample: Residue obtained in the test for *Loss on Drying*
Acceptance criteria: Meets the requirements

ASSAY

• **PROCEDURE**
Sample: Dry under vacuum 2 g of Ammonio Methacrylate Copolymer Dispersion Type A, or 4 g of Ammonio Methacrylate Copolymer Dispersion Type B, at 90° for 30 min.
Blank: 75 mL of glacial acetic acid
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.1 N perchloric acid VS
Endpoint detection: Potentiometric
Analysis: Dissolve the *Sample* in 75 mL of glacial acetic acid at about 50°, within about 30 min. After the solution has cooled down, add 25 mL of 0.6% cupric acetate solution in glacial acetic acid. Titrate this solution with the *Titrant*. Perform a blank determination.
Calculate the percentage of ammonio methacrylate ($C_9H_{18}ClNO_2$) units in the portion of the *Sample* taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

V_S = *Titrant* volume consumed by the *Sample* (mL)
V_B = *Titrant* volume consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 207.72 mg/mEq
W = *Sample* weight (mg)

Acceptance criteria
Type A: 10.18%–13.73%
Type B: 6.11%–8.26%

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩
Analysis: Using mild heating conditions (e.g., steam bath, sand bath, etc.) to avoid loss of material, evaporate the dispersion to dryness before ignition.
Acceptance criteria: NMT 0.5% calculated on the undried dispersion basis

• **LIMIT OF MONOMERS**
Sodium perchlorate solution: 35 mg/mL of sodium perchlorate ($NaClO_4 \cdot H_2O$)
Mobile phase: Dilute phosphoric acid with water to obtain a solution having a pH of 2.0. Mix four volumes of this solution with one volume of methanol, filter, and degas.
Standard stock solution: 1.6 mg/mL of ethyl acrylate and 0.2 mg/mL of methyl methacrylate in methanol
Standard solution: Dilute 1 mL of the *Standard stock solution* with methanol to 100 mL. Add 10 mL of this solution to 5 mL of *Sodium perchlorate solution*.
Sample solution: Dissolve 5 g of Dispersion in methanol, and dilute with the same solvent to 50 mL. Add 5 mL of *Sodium perchlorate solution* dropwise to 10 mL of this solution while continuously stirring. Remove the precipitated polymer by centrifugation. Use the clear supernatant. Calculate the concentration of Dispersion, in mg/mL, as C_U.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 202 nm
Column: 4.6-mm × 12-cm; packing L1
Flow rate: 2 mL/min
Injection volume: 50 µL
System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for ethyl acrylate and methyl methacrylate are 1.00 and 1.14, respectively.]
Suitability requirements
Resolution: NLT 1.0 between the pair of analytes
Relative standard deviation: NMT 5.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of ethyl acrylate and methyl methacrylate in the portion of Dispersion taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak response of ethyl acrylate or methyl methacrylate from the *Sample solution*
r_S = peak response of ethyl acrylate or methyl methacrylate from the *Standard solution*
C_S = concentration of ethyl acrylate or methyl methacrylate in the *Standard solution* (µg/mL)
C_U = nominal concentration of Dispersion in the *Sample solution* (mg/mL)
F = conversion factor, 10^{-3} mg/µg

Acceptance criteria: NMT 0.002% of methyl methacrylate and NMT 0.008% of ethyl acrylate

SPECIFIC TESTS

• **VISCOSITY—ROTATIONAL METHODS** ⟨912⟩
Analysis: Use a viscometer equipped with a spindle having a cylinder 1.88 cm in diameter and 6.51 cm high attached to a shaft 0.32 cm in diameter. The distance from the top of the cylinder to the lower tip of the shaft is 0.75 cm, and the immersion depth is 8.15 cm. Adjust the temperature to 20 ± 0.10°. With the spindle rotating at 30 rpm, immediately record the scale reading. Multiply the scale reading by the constant for the viscometer spindle and speed used to obtain the viscosity in centipoises.

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Acceptance criteria: NMT 100 mPa · s

- **LOSS ON DRYING** ⟨731⟩
  Analysis: Dry a sample at 110° for 6 h.
  Acceptance criteria: 68.5%–71.5%
- **COAGULUM CONTENT**
  Sample: 100 g
  Analysis: Weigh a stainless steel sieve having 125-µm openings, and filter the *Sample* through it. Wash the sieve with distilled water until a clear filtrate is obtained, and dry the sieve to constant weight at 105°.
  Acceptance criteria: The weight of the residue does not exceed 1000 mg, corresponding to NMT 1%.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, at a temperature not exceeding 25° for Type A and not exceeding 30° for Type B. Protect from freezing.
- **LABELING:** Label it to state whether it is Type A or Type B. Label it to indicate the name and quantity of any added antimicrobial preservative or alkalizing agent.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Ammonio Methacrylate Copolymer, Type A RS
  USP Ammonio Methacrylate Copolymer, Type B RS

Ammonium Carbonate

Carbonic acid, monoammonium salt mixture with ammonium carbamate;
Monoammonium carbonate mixture with ammonium carbamate [8000-73-5].

DEFINITION

Ammonium Carbonate consists of ammonium bicarbonate (NH_4HCO_3) and ammonium carbamate (NH_2COONH_4) in varying proportions. It yields NLT 30.0% and NMT 34.0% of ammonia (NH_3).

IDENTIFICATION

- **A.** When heated, it is volatilized without charring, and the vapor is alkaline to moistened litmus paper. A solution (1 in 20) effervesces with acids.

ASSAY

- **PROCEDURE**
  Sample solution: Place 10 mL of water in a weighing bottle, tare the bottle and its contents, add 2 g of Ammonium Carbonate, and weigh.
  Titrimetric system
  (See *Titrimetry* ⟨541⟩.)
  Mode: Residual titration
  Titrant: 1 N sulfuric acid VS
  Back-titrant: 1 N sodium hydroxide VS
  Blank: 50.0 mL of 1 N sulfuric acid VS, accurately measured
  Endpoint detection: Colorimetric
  Analysis: Transfer the *Sample solution* to a 250-mL flask, add 50.0 mL of 1 N sulfuric acid VS, and when the solution has been effected, add methyl orange TS. Titrate the excess acid with 1 N sodium hydroxide VS. Perform a blank determination. Calculate the percentage of ammonia (NH_3) in the sample taken.

$$\text{Result} = [(B - V) \times N \times F \times 100]/W$$

B = 1 N sodium hydroxide VS consumed by the *Blank* (mL)
V = 1 N sodium hydroxide VS consumed by the sample (mL)
N = actual normality of the *Back-tritrant* (mEq/mL)
F = equivalency factor, 17.03 mg/mEq
W = weight of the sample (mg)

Acceptance criteria: 30.0%–34.0%

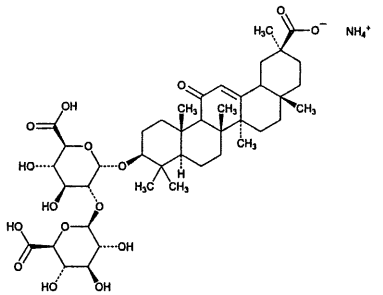
IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%
- **CHLORIDE AND SULFATE,** *Chloride* ⟨221⟩: A 2.0-g portion shows no more chloride than corresponds to 0.10 mL of 0.020 N hydrochloric acid (0.0035%).
- **CHLORIDE AND SULFATE,** *Sulfate* ⟨221⟩: A 2.0-g portion shows no more sulfate than corresponds to 0.10 mL of 0.020 N sulfuric acid (0.005%).

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at a temperature not above 30°.

Ammonium Glycyrrhizate



$C_{42}H_{62}O_{16} \cdot NH_3$ 840.08

Monoammonium glycyrrhizinate;
Glycyrrhizic acid ammonium salt;
α-D-Glucopyranosiduronic acid, (3β,20β)-20-carboxy-11-oxo-30-norolean-12-en-3-yl 2-*O*-β-D-glucopyranuronosyl-, ammonium salt (1:1);
α-D-Glucopyranosiduronic acid, (3β,20β)-20-carboxy-11-oxo-30-norolean-12-en-3-yl 2-*O*-β-D-glucopyranuronosyl-, monoammonium salt [53956-04-0].

DEFINITION

Ammonium Glycyrrhizate is a mixture of ammonium 18α- and 18β-glycyrrhizate (ammonium salt of (20β)-3β-[[2-*O*-(β-D-glucopyranosyluronic acid)-α-D-glucopyranosyluronic acid]oxy]-11-oxoolean-12-en-29-oic acid), and the 18β-isomer is the main component. It contains NLT 78.0% and NMT 102.0% of ammonium 18α- and 18β-glycyrrhizate, on the anhydrous basis.

IDENTIFICATION

- **A.** The retention times of the peaks of 18α- and 18β-glycyrrhizic acid from the *Sample solution* correspond to those from the *System suitability solution*, as obtained in the *Content of Ammonium 18α- and 18β-Glycyrrhizate*. [NOTE—The peak of 18α-glycyrrhizic acid could be absent in the *Sample solution*.]
- **B. IDENTIFICATION TESTS—GENERAL,** *Ammonium* ⟨191⟩
  Acceptance criteria: Meets the requirements

ASSAY

- **CONTENT OF AMMONIUM 18α- AND 18β-GLYCYRRHIZATE**
  Mobile phase: Acetonitrile, glacial acetic acid, and water (38:1:61)
  Standard solution: 0.5 mg/mL of USP Glycyrrhizic Acid RS in *Mobile phase*

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System suitability solution: 0.5 mg/mL of USP Ammonium Glycyrrhizate RS in *Mobile phase*
Sample solution: 0.5 mg/mL of Ammonium Glycyrrhizate in *Mobile phase*
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 3.9-mm × 30-cm analytical column; 5–10 µm packing L1
Flow rate: 2.0 mL/min
Injection volume: 10 µL
System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The relative retention times of 18β-glycyrrhizic acid and 18α-glycyrrhizic acid are about 1.0 and 1.2, respectively, *System suitability solution*.]
Suitability requirements
Resolution: NLT 2.0 between the peaks due to 18β-glycyrrhizic acid and 18α-glycyrrhizic acid, *System suitability solution*
Relative standard deviation: NMT 2.0%, *Standard solution*
Analysis
Samples: *Standard solution*, *System suitability solution*, and *Sample solution*
Determine the peak areas for each isomer (18α-glycyrrhizic acid or 18β-glycyrrhizic acid).
Calculate the percentage of ammonium 18α-glycyrrhizate (or ammonium 18β-glycyrrhizate) in the portion of Ammonium Glycyrrhizate taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{W(Salt)}/M_{W(Acid)}) \times 100$$

r_U = peak area of the 18α-glycyrrhizic acid (or 18β-glycyrrhizic acid) in the *Sample solution*
r_S = peak area of the 18β-glycyrrhizic acid in the *Standard solution*
C_S = concentration of the USP Glycyrrhizic Acid RS in the *Standard solution* (mg/mL)
C_U = concentration of the *Sample solution* (mg/mL)
$M_{W(Salt)}$ = molecular weight of ammonium glycyrrhizate, 840.08 g/mol
$M_{W(Acid)}$ = molecular weight of glycyrrhizic acid, 821.59 g/mol

Acceptance criteria: The total percentage of ammonium 18α-glycyrrhizate and ammonium 18β-glycyrrhizate is 78.0%–102.0%, and the percentage of ammonium 18α-glycyrrhizate is NMT 13.0%, on the anhydrous basis.

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩
Sample: 1.0 g
Acceptance criteria: NMT 0.5%
• **LIMIT OF ORGANIC IMPURITIES**
Mobile phase, System suitability solution, and Chromatographic system: Proceed as directed in *Content of Ammonium 18α- and 18β-Glycyrrhizate*.
Sample solution: 1.0 mg/mL of Ammonium Glycyrrhizate in the *Mobile phase*
Reference solution A: 0.05 mg/mL of Ammonium Glycyrrhizate in the *Mobile phase*, prepared from the *Sample solution*
Reference solution B: 0.057 mg/mL of Ammonium Glycyrrhizate in the *Mobile phase*, prepared from the *Sample solution*
System suitability
Sample: *System suitability solution*
[NOTE—The relative retention times for 24-hydroxyglycyrrhizinic acid, 18β-glycyrrhizic acid, and 18α-glycyrrhizic acid are about 0.7, 1.0 and 1.2, respectively.]
Suitability requirements
Resolution: NLT 2.0 between the peaks due to 18β-glycyrrhizic acid and 18α-glycyrrhizic acid
Analysis
Samples: *System suitability solution*, *Reference solution A*, *Reference solution B*, and *Sample solution*
Acceptance criteria: See *Table 1*.

Table 1

Name	Relative Retention Time	Acceptance Criteria
24-Hydroxyglycyrrhizinic acid[a]	0.7	NMT the sum of the areas of the peaks in the chromatogram from *Reference solution B*, corresponding to NMT 5.7%
Any other impurity	—	For each impurity, NMT 0.4 times the sum of the areas of the peaks in the chromatogram from *Reference solution A*, corresponding to NMT 2.0%
Sum of other impurities	—	NMT 1.6 times the sum of the areas of the peaks in the chromatogram from *Reference solution A*, corresponding to NMT 8.0%
Disregard limit	—	0.04 times the sum of the areas of the peaks in the chromatogram from *Reference solution A*, corresponding to 0.2%

[a] (4β,20β)-3β-[[2-*O*-(β-D-Glucopyranosyluronic acid)-α-D-glucopyranosyluronic acid]oxy]-23-hydroxy-11-oxoolean-12-en-29-oic acid.

SPECIFIC TESTS

• **OPTICAL ROTATION,** *Specific Rotation* ⟨781⟩
Sample solution: 10.0 mg/mL of Ammonium Glycyrrhizate in 50% ethanol
Acceptance criteria: +49.0 to +55.0 on the anhydrous basis
• **WATER DETERMINATION,** *Method Ia* ⟨921⟩
Sample: 0.25 g
Acceptance criteria: NMT 6.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers, and store in a cool, dry place.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Ammonium Glycyrrhizate RS
USP Glycyrrhizic Acid RS

Ammonium Phosphate

$(NH_4)_2HPO_4$ 132.06
Phosphoric acid, diammonium salt;
Diammonium phosphate [7783-28-0].

DEFINITION

Ammonium Phosphate contains NLT 96.0% and NMT 102.0% of $(NH_4)_2HPO_4$.

IDENTIFICATION

• **A. IDENTIFICATION TESTS—GENERAL,** *Ammonium* ⟨191⟩: A solution (1 in 20) meets the requirements.
• **B. IDENTIFICATION TESTS—GENERAL,** *Phosphate* ⟨191⟩: A solution (1 in 20) meets the requirements.

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ASSAY
• **PROCEDURE**
Sample: 600 mg of Ammonium Phosphate
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.1 N sulfuric acid VS
Endpoint detection: potentiometric
Analysis: Dissolve the *Sample* in 40 mL of water, and titrate with 0.1 N sulfuric acid VS to a pH of 4.6. Each mL of 0.1 N sulfuric acid is equivalent to 13.21 mg of $(NH_4)_2HPO_4$.
Acceptance criteria: 96.0%–102.0%

IMPURITIES
• **CHLORIDE AND SULFATE,** *Chloride* ⟨221⟩**:** A 1.0-g portion shows no more chloride than corresponds to 0.40 mL of 0.020 N hydrochloric acid (0.03%).
• **CHLORIDE AND SULFATE,** *Sulfate* ⟨221⟩**:** A 0.20-g portion shows no more sulfate than corresponds to 0.30 mL of 0.020 N sulfuric acid (0.15%).
• **ARSENIC,** *Method I* ⟨211⟩**:** NMT 3 ppm

SPECIFIC TESTS
• **PH** ⟨791⟩**:** 7.6–8.2, in a solution (1 in 100)

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight containers.

Ammonium Sulfate

$(NH_4)_2SO_4$ 132.14
Ammonium sulfate [7783-20-2].

DEFINITION
Ammonium Sulfate contains NLT 99.0% and NMT 100.5% of $(NH_4)_2SO_4$.

IDENTIFICATION
• **A. IDENTIFICATION TESTS—GENERAL,** *Ammonium* ⟨191⟩**:** A solution (1 in 20) meets the requirements.
• **B. IDENTIFICATION TESTS—GENERAL,** *Sulfate* ⟨191⟩**:** A solution (1 in 20) meets the requirements.

ASSAY
• **PROCEDURE**
Sample: 2.5 g of Ammonium Sulfate
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Residual titration
Titrant: 1 N sodium hydroxide VS
Back titrant: 1 N sulfuric acid VS
Endpoint detection: Colorimetric
Blank: 50.0 mL of 1 N sodium hydroxide VS, accurately measured
Analysis: Add the *Sample* to a 500-mL conical flask and dissolve in 50 mL of water. Add 50.0 mL of 1 N sodium hydroxide VS, place a filter funnel loosely in the neck of the flask, and boil until ammonia is expelled (about 10–15 min), as determined with litmus paper. Cool, add 0.15 mL of thymol blue TS, and titrate the excess sodium hydroxide with 1 N sulfuric acid VS. Perform a blank determination.
Calculate the percentage of ammonium sulfate $[(NH_4)_2SO_4]$ in the *Sample* taken:

$$\text{Result} = [(B - V) \times N \times F \times 100]/W$$

B = 1 N sulfuric acid VS consumed by the *Blank* (mL)
V = 1 N sulfuric acid VS consumed by the *Sample* (mL)
N = actual normality of the *Back titrant* (mEq/mL)
F = equivalency factor, 66.07 mg/mEq
W = weight of the *Sample* (mg)

Acceptance criteria: 99.0%–100.5%

IMPURITIES
• **RESIDUE ON IGNITION** ⟨281⟩
Sample: 20 g
Acceptance criteria: NMT 0.005%
• **LIMIT OF INSOLUBLE MATTER**
Sample: 20 g
Analysis: Transfer the *Sample* to a covered beaker, and dissolve in 200 mL of water. Heat to boiling, and warm on a steam bath for 1 h. Filter the hot solution through a tared sintered-glass crucible of medium porosity (10–15 µm). Wash the beaker and the filter with hot water, dry the crucible at 105°, cool in a desiccator, and weigh.
Acceptance criteria: NMT 1 mg of insoluble matter is found (0.005%).
• **LIMIT OF PHOSPHATE**
Standard phosphate solution, Phosphate reagent A, and **Phosphate reagent B:** Prepare as directed for *Phosphate in Reagents* under *Reagents, Indicators, and Solutions—General Tests for Reagents.*
Sample: 4.0 g
Control: 0.2 mL of *Standard phosphate solution*
Analysis
[NOTE—The tests for the *Sample* and the *Control* are made preferably in matched color-comparison tubes.]
Dissolve the *Sample* in 25 mL of 0.5 N sulfuric acid, add 1 mL each of *Phosphate reagent A* and *Phosphate reagent B,* and allow to stand at room temperature for 2 h. Proceed with the *Control* using the same quantities of the same reagents as in the test for the *Sample.*
Acceptance criteria: Any blue color obtained from the *Sample* should not exceed that produced from the *Control* (NMT 5 ppm).
• **CHLORIDE AND SULFATE,** *Chloride* ⟨221⟩
Standard chloride solution: Transfer 165 mg of sodium chloride to a 100-mL volumetric flask. Dissolve in and dilute with water to volume. Transfer 10.0 mL to a 1000-mL volumetric flask, and dilute with water to volume to obtain a solution having a concentration of 10 µg/mL of chloride.
Acceptance criteria: A 2-g portion shows no more chloride than corresponds to 1.0 mL of *Standard chloride solution* (NMT 5 ppm).
• **LIMIT OF NITRATE**
Standard nitrate solution and **Brucine sulfate solution:** Prepare as directed for *Nitrate in Reagents* under *Reagents, Indicators, and Solutions—General Tests for Reagents.*
Sample solution: Dissolve 1.0 g in 3 mL of water by heating in a boiling water bath, and add *Brucine sulfate solution* to make 50 mL.
Control solution: To 1.0 mL of *Standard nitrate solution* add 1.0 g ammonium sulfate, then add *Brucine sulfate solution* to make 50 mL.
Blank: 50 mL of *Brucine sulfate solution*
Analysis: Heat the *Sample solution, Control solution,* and *Blank* in a boiling water bath for 15 min with periodic gentle swirling, then cool rapidly in an ice bath to room temperature. Adjust a suitable spectrophotometer with the *Blank* to zero absorbance at 410 nm. Determine the absorbance of the *Sample solution,* note the result, and adjust the instrument with the *Sample solution* to zero

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absorbance. Determine the absorbance of the *Control solution*.
Acceptance criteria: The absorbance reading for the *Sample solution* does not exceed that for the *Control solution* (NMT 10 ppm).
• **IRON** ⟨241⟩
Sample solution: Dissolve 2.0 g in 40 mL of water, and add 2 mL of hydrochloric acid.
Acceptance criteria: NMT 5 ppm

SPECIFIC TESTS
• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic microbial count does not exceed 1000 cfu/g, and the total combined molds and yeasts count does not exceed 10 cfu/g.
• **pH** ⟨791⟩**:** 5.0–6.0 in a solution (1 in 20)

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements are specified.

Amylene Hydrate



$C_5H_{12}O$ 88.15
2-Butanol, 2-methyl-;
tert-Pentyl alcohol [75-85-4].

DEFINITION
Amylene Hydrate contains NLT 99.0% and NMT 100.0% of $C_5H_{12}O$.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F**▲ (CN 1-May-2020)
• **B. PROCEDURE**
Sample solution: 1 in 10
Analysis: To 10 mL of the *Sample solution* quickly add 5 mL of a 1-in-100 solution of vanillin in sulfuric acid.
Acceptance criteria: A violet-red color is produced.

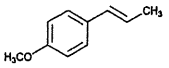
ASSAY
• **PROCEDURE**
Sample solution: Amylene Hydrate
Chromatographic system
Mode: GC
Detector: Thermal conductivity
Column: 4-mm × 2-cm glass column; packed with chromatographic support S2 (under typical conditions)
Temperature
Column: 190°
Injector: 200°
Detector block: 200°
Carrier gas: Helium
Flow rate: 50 mL/min
Injection size: 0.4 µL
Analysis
Sample: *Sample solution*
From the area under the curve, calculate the percentage (a/a) of $C_5H_{12}O$ in the Amylene Hydrate taken.
Acceptance criteria: 99.0%–100.0%

IMPURITIES
INORGANIC IMPURITIES
• **Limit of Nonvolatile Residue**
Sample: 10 mL
Analysis: Evaporate the *Sample* in a tared porcelain dish on a steam bath to a volume of about 1 mL, and allow it to evaporate spontaneously to dryness while protected from dust.
Acceptance criteria: The residue, if any, is colorless, and when dried at 105° for 1 h, weighs NMT 2 mg (0.02%).
ORGANIC IMPURITIES
• **Procedure**
Sample solution: 1 in 20
Analysis: To 10 mL of the *Sample solution* add 0.10 mL of 0.10 N potassium permanganate.
Acceptance criteria: The pink color does not completely disappear within 10 min.

SPECIFIC TESTS
• **SPECIFIC GRAVITY** ⟨841⟩**:** 0.803–0.807
• **DISTILLING RANGE,** *Method I* ⟨721⟩**:** It distills completely at between 97° and 103°, a correction factor of 0.037°/mm being applied as necessary.
• **WATER:** The relative retention time of water on the gas chromatographic column used in the *Assay* is approximately 0.2 times that of Amylene Hydrate. From the area under the curve obtained in the *Assay*, calculate the percentage (a/a) of water in the Amylene Hydrate taken: NMT 0.5% is found.
• **ALDEHYDE**
Sample solution: 1 in 20
Analysis: To 10 mL of the *Sample solution* add 1 mL of silver–ammonium nitrate TS, and heat the mixture on a water bath at 60° for 10 min.
Acceptance criteria: No darkening occurs.

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight containers.

Anethole



$C_{10}H_{12}O$ 148.20
Benzene, 1-methoxy-4-(1-propenyl)-, (*E*)-;
(*E*)-*p*-Propenylanisole [4180-23-8].
Synthetic [104-46-1].

DEFINITION
Anethole is obtained from Anise Oil and other sources, or it is prepared synthetically. It contains NLT 98.0% and NMT 102.0% of (*E*)-1-methyl-4-(1-propenyl) benzene (or *trans*-anethole) ($C_{10}H_{12}O$).

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F**▲ (CN 1-May-2020)
• **B. CHROMATOGRAPHIC IDENTITY**
Analysis: Proceed as directed in the *Assay for trans-Anethole*.
Acceptance criteria: The retention time of the major peak, excluding the internal standard peak, of the *Sample solution* corresponds to the anethole peak of the *Standard solution*.

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ASSAY

• **ASSAY FOR *trans*-ANETHOLE**

Internal standard solution: 2 mg/mL of USP Menthol RS (internal standard) in hexanes

Standard solution: 2 mg/mL of USP Anethole RS in *Internal standard solution* (use a freshly prepared solution)

Sample solution: 2 mg/mL of Anethole in *Internal standard solution* (use a freshly prepared solution)

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.53-mm × 30-m capillary; bonded with a 1.0-µm layer of phase G16

Temperatures

Injection port: 250°

Detector: 250°

Column: 125° (isothermally)

Carrier gas: Helium

Flow rate: 10 mL/min

Injection volume: 1.0 µL

Injection type: Split injection; split ratio of 10:1

Run time: 30 min

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for menthol and *trans*-anethole are about 0.5 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 15 between menthol and *trans*-anethole

Tailing factor: 0.8–2.0 for menthol and *trans*-anethole peaks

Relative standard deviation: NMT 2.0% for the peak response ratio of *trans*-anethole to the internal standard

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of *trans*-anethole ($C_{10}H_{12}O$) in the portion of Anethole taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of *trans*-anethole to the internal standard (peak response of *trans*-anethole/peak response of the internal standard) from the *Sample solution*

R_S = peak response ratio of *trans*-anethole to the internal standard (peak response of *trans*-anethole/peak response of the internal standard) from the *Standard solution*

C_S = concentration of USP Anethole RS in the *Standard solution* (mg/mL)

C_U = concentration of Anethole in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–102.0%

IMPURITIES

• **LIMIT OF *cis*-ANETHOLE, *p*-ANISALDEHYDE, AND OTHER VOLATILE IMPURITIES**

Internal standard solution: 0.01 mg/mL of USP Menthol RS (internal standard) in hexanes

System suitability solution: 4 mg/mL of USP Anethole RS in *Internal standard solution* after exposure to UV light for 1 h

Standard solution: 0.01 mg/mL of USP Anethole RS and 0.01 mg/mL of USP p-Anisaldehyde RS in *Internal standard solution* (use a freshly prepared solution)

Sample solution: 2 mg/mL of Anethole in *Internal standard solution* (use a freshly prepared solution)

Chromatographic system: Proceed as directed in the *Assay*, except use a *Run time* of 60 min.

System suitability

Samples: *System suitability solution* and *Standard solution*

[NOTE—See *Table 1* for the relative retention times.]

Table 1

Name	Relative Retention Time
Menthol (internal standard)	0.5
cis-Anethole	0.75
trans-Anethole	1.0
p-Anisaldehyde	2.2

Suitability requirements

Resolution: NLT 5 between *cis*-anethole and *trans*-anethole, *System suitability solution*

Relative standard deviation: NMT 5.0% for the peak response ratio of *trans*-anethole to the internal standard and NMT 5.0% for the peak response ratio of *p*-anisaldehyde to the internal standard, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of *p*-anisaldehyde in the portion of Anethole taken:

$$\text{Result} = (R_{U1}/R_{S1}) \times (C_{S1}/C_U) \times 100$$

R_{U1} = peak response ratio of *p*-anisaldehyde to the internal standard (peak response of *p*-anisaldehyde/peak response of the internal standard) from the *Sample solution*

R_{S1} = peak response ratio of *p*-anisaldehyde to the internal standard (peak response of *p*-anisaldehyde/peak response of the internal standard) from the *Standard solution*

C_{S1} = concentration of USP p-Anisaldehyde RS in the *Standard solution* (mg/mL)

C_U = concentration of Anethole in the *Sample solution* (mg/mL)

Calculate the percentage of *cis*-anethole or any other unspecified volatile impurity in the portion of Anethole taken:

$$\text{Result} = (R_{U2}/R_{S2}) \times (C_{S2}/C_U) \times 100$$

R_{U2} = peak response ratio of *cis*-anethole or any other unspecified impurity to the internal standard (peak response of *cis*-anethole or any other unspecified impurity/peak response of the internal standard) from the *Sample solution*

R_{S2} = peak response ratio of *trans*-anethole to the internal standard (peak response of *trans*-anethole/peak response of the internal standard) from the *Standard solution*

C_{S2} = concentration of USP Anethole RS in the *Standard solution* (mg/mL)

C_U = concentration of Anethole in the *Sample solution* (mg/mL)

Acceptance criteria: Disregard peaks that are less than 0.05% for any unspecified impurities and any peaks due to solvent.

Total impurities including *cis*-anethole, *p*-anisaldehyde, and all other unspecified impurities: NMT 2.0%

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• **LIMIT OF PHENOLS**
Sample: 1 mL
Analysis: Shake the *Sample* with 20 mL of water, and allow the liquids to separate. Pass the water layer through a filter paper previously moistened with water, and to 10 mL of the filtrate add 3 drops of ferric chloride TS.
Acceptance criteria: No purple or purplish color is produced.

SPECIFIC TESTS
• **SPECIFIC GRAVITY** ⟨841⟩: 0.983–0.988
• **DISTILLING RANGE,** *Method I* ⟨721⟩: 231°–237°, a correction factor of 0.063°/mm being applied as necessary
• **OPTICAL ROTATION,** *Angular Rotation* ⟨781A⟩: −0.15° to +0.15°
• **REFRACTIVE INDEX** ⟨831⟩: 1.557–1.561

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
• **LABELING:** Label to indicate whether it is of natural sources or is prepared synthetically.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Anethole RS
USP p-Anisaldehyde RS
USP Menthol RS

Anise Oil

DEFINITION
Anise Oil is the volatile oil distilled with steam from the dried, ripe fruit of *Pimpinella anisum* L. (Fam. Apiaceae) or from the dried ripe fruit of *Illicium verum* Hook. f. (Fam. Illiciaceae). [NOTE—If solid material has separated, carefully warm the Anise Oil until it is completely liquefied, and mix before using.]

IMPURITIES
• **LIMIT OF PHENOLS**
Sample solution: Recently distilled Anise Oil in 90% alcohol (1 in 3)
Acceptance criteria: The *Sample solution* is neutral to moistened litmus paper, and develops no blue or brownish color upon the addition of 1 drop of ferric chloride TS to 5 mL of the solution.

SPECIFIC TESTS
• **SOLUBILITY IN 90% ALCOHOL:** One volume dissolves in 3 volumes of 90% alcohol.
• **SPECIFIC GRAVITY** ⟨841⟩: 0.978–0.988
• **CONGEALING TEMPERATURE** ⟨651⟩: NLT 15°
• **OPTICAL ROTATION,** *Angular Rotation* ⟨781A⟩: −2° to +1°
• **REFRACTIVE INDEX** ⟨831⟩: 1.553–1.560 at 20°

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-filled, tight containers, and avoid exposure to excessive heat.
• **LABELING:** The label states the Latin binomial name and, following the official name, the part of the plant source from which the article was derived. The label also states that if solid material has separated, carefully warm the oil until it is completely liquefied, and mix before using.

Arginine
—see Arginine General Monographs

Aromatic Elixir

DEFINITION
Prepare Aromatic Elixir as follows.

Suitable essential oil(s)	
Syrup	375 mL
Talc	30 g
Alcohol, Purified Water, each a sufficient quantity to make	1000 mL

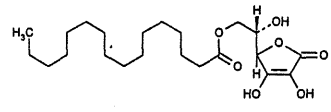
Dissolve the oil(s) in *Alcohol* to make 250 mL. To this solution add the *Syrup* in several portions, agitating vigorously after each addition. Then add the required quantity of *Purified Water* in the same manner. Mix *Talc* with the liquid, and pass through a filter, dampened with *Diluted Alcohol*, and return the filtrate until a clear liquid is obtained.

OTHER COMPONENTS
• **ALCOHOL DETERMINATION,** *Method II* ⟨611⟩: 21.0%–23.0% of C_2H_5OH, with acetone being used as the internal standard

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Package in tight containers.

Ascorbic Acid
—see Ascorbic Acid General Monographs

Ascorbyl Palmitate



$C_{22}H_{38}O_7$ 414.53
L-Ascorbic acid, 6-hexadecanoate;
L-Ascorbic acid 6-palmitate [137-66-6].

DEFINITION
Ascorbyl Palmitate contains NLT 95.0% and NMT 100.5% of ascorbyl palmitate ($C_{22}H_{38}O_7$), calculated on the dried basis.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
• **B. CHROMATOGRAPHIC IDENTITY**
Analysis: Proceed as directed in the *Assay*.
Acceptance criteria: The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*.

ASSAY
• **PROCEDURE**
Mobile phase: Acetonitrile, methanol, 0.01 M perchloric acid (10:65:25)
Standard solution: 0.5 mg/mL of USP Ascorbyl Palmitate RS in methanol
Sample solution: 0.5 mg/mL of Ascorbyl Palmitate in methanol

Supplement 4

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Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 245 nm
Column: 4.6-mm × 15-cm; 5-µm packing L7
Temperatures
Column: 25°
Autosampler: 5°
[NOTE—If an autosampler is not used, keep the *Standard solution* and *Sample solution* at 5° before injection.]
Flow rate: 1.0 mL/min
Injection volume: 10 µL
Run time: 15 min
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 1.0%
Tailing factor: 0.8–2.0
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate, on the dried basis, the percentage of Ascorbyl Palmitate taken:

$$\text{Result} = [(r_U/r_S) \times (C_S/C_U)] \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP Ascorbyl Palmitate RS in the *Standard solution* (mg/mL)
C_U = concentration of Ascorbyl Palmitate in the *Sample solution* (mg/mL)

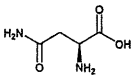
Acceptance criteria: 95.0%–100.5% on the dried basis

IMPURITIES
• **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 0.1%

SPECIFIC TESTS
• **OPTICAL ROTATION,** *Specific Rotation* ⟨781S⟩**:** +21° to +24°
Sample solution: 100 mg/mL in methanol
• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry a sample in vacuum at 60° for 1 h.
Acceptance criteria: NMT 2.0%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight containers, and store in a cool, dry place.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Ascorbyl Palmitate RS

Asparagine



$C_4H_8N_2O_3 \cdot H_2O$ 150.13
$C_4H_8N_2O_3$ 132.12
L-Asparagine;
L-α-Aminosuccinamic acid, monohydrate [5794-13-8].
Anhydrous [70-47-3].

DEFINITION
Asparagine is anhydrous, or contains one molecule of water of hydration. It contains NLT 98.0% and NMT 101.5% of $C_4H_8N_2O_3$, as L-asparagine, calculated on the dried basis.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
[NOTE—Use USP Asparagine Anhydrous RS and USP Asparagine Monohydrate RS for the evaluation of the anhydrous and monohydrate forms of Asparagine, respectively.]

ASSAY
• **PROCEDURE**
Sample: 130 mg
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.1 N perchloric acid VS
Blank: 3 mL of formic acid in 50 mL of glacial acetic acid
Endpoint detection: Potentiometrically
Analysis: Dissolve the *Sample* with 3 mL of formic acid in 50 mL of glacial acetic acid. Perform a blank determination. Calculate the percentage of asparagine ($C_4H_8N_2O_3$) in the *Sample* taken:

$$[(V - B) \times N \times F \times 100]/W$$

V = volume of *Titrant* consumed by the *Sample* (mL)
B = volume of *Titrant* consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor for asparagine, 132.1 mg/mEq
W = weight of the *Sample* (mg)

Acceptance criteria: 98.0%–101.5% on the dried basis

IMPURITIES
• **RESIDUE ON IGNITION** ⟨281⟩
Sample: 1.0 g
Acceptance criteria: NMT 0.1%
• **LEAD** ⟨251⟩
Sample: 1 g
Control: 5 mL of *Diluted Standard Lead Solution* (5 µg of Pb)
Acceptance criteria: NMT 5 ppm
• **CHROMATOGRAPHIC PURITY**
Standard solution: 0.05 mg/mL of USP Asparagine Anhydrous RS or USP Asparagine Monohydrate RS
[NOTE—Use USP Asparagine Anhydrous RS and USP Asparagine Monohydrate RS for the evaluation of the anhydrous and monohydrate forms of Asparagine, respectively.]
Sample solution: 10 mg/mL
Chromatographic system
(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
Adsorbent: 0.25-mm layer of chromatographic silica gel mixture
Application volume: 5 µL
Developing solvent system: Butyl alcohol, glacial acetic acid, and water (3:1:1)
Spray reagent: 2 mg/mL of ninhydrin in butyl alcohol and glacial acetic acid (19:1)
Analysis
Samples: *Standard solution* and *Sample solution*
Proceed as directed in the general test chapter, and then dry the plate at 80° for 30 min. Spray the plate with the *Spray reagent*, heat at 80° for 10 min, and examine under white light.
Acceptance criteria: No secondary spot from the *Sample solution* is larger or more intense than the principal spot

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from the *Standard solution* (0.5%), and NMT 1.0% of total impurities is found.

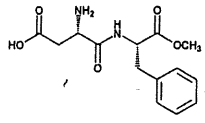
SPECIFIC TESTS

- **OPTICAL ROTATION,** *Specific Rotation* ⟨781S⟩
  Sample solution: 10 mg/mL, in 6 N hydrochloric acid
  Acceptance criteria: +33.0° to +36.5°, measured at 20°
- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic microbial count does not exceed 1000 cfu/g, and the total combined molds and yeasts count does not exceed 100 cfu/g.
- **LOSS ON DRYING** ⟨731⟩**:** Dry a sample at 130° for 3 h: the anhydrous form loses NMT 1.0% of its weight, and the monohydrate loses between 11.5% and 12.5% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed, light-resistant containers. Store at room temperature.
- **LABELING:** Label it to indicate whether it is anhydrous or the monohydrate.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Asparagine Anhydrous RS
  USP Asparagine Monohydrate RS

Aspartame



$C_{14}H_{18}N_2O_5$ 294.30

L-Phenylalanine, *N*-L-α-aspartyl-, 1-methyl ester; 3-Amino-*N*-(α-carboxyphenethyl)succinamic acid *N*-methyl ester [22839-47-0].

DEFINITION

Aspartame contains NLT 98.0% and NMT 102.0% of aspartame ($C_{14}H_{18}N_2O_5$), calculated on the dried basis.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197M**▲ (CN 1-May-2020)
  [NOTE—Do not dry specimens.]

ASSAY

- **PROCEDURE**

Buffer: 0.05 M monobasic potassium phosphate, adjusted with phosphoric acid to a pH of 4.3
Mobile phase: Methanol and *Buffer* (18:82)
Diluent: Methanol and water (1:9)
System suitability solution: 0.1 mg/mL each of USP Aspartame Related Compound A RS and USP L-Phenylalanine RS in *Diluent*
Standard solution: 0.5 mg/mL of USP Aspartame RS in *Diluent*
Sample solution: 0.5 mg/mL of Aspartame in *Diluent*. [NOTE—Avoid heat and excessive holding times.]
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 210 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 40°
Flow rate: 2 mL/min
Injection volume: 20 µL
Run time: 30 min
System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The relative retention times for the L-phenylalanine and aspartame related compound A peaks are 0.6 and 1.0, respectively.]
Suitability requirements
Resolution: NLT 8 between the L-phenylalanine and aspartame related compound A peaks, *System suitability solution*
Tailing factor: NMT 1.5, *Standard solution*
Relative standard deviation: NMT 1.0%, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of aspartame ($C_{14}H_{18}N_2O_5$) in the portion of Aspartame taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of aspartame from the *Sample solution*
r_S = peak area of aspartame from the *Standard solution*
C_S = concentration of USP Aspartame RS in the *Standard solution* (mg/mL)
C_U = concentration of Aspartame in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–102.0% on the dried basis

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 0.2%
- **LIMIT OF 5-BENZYL-3,6-DIOXO-2-PIPERAZINEACETIC ACID**

Mobile phase, Diluent, System suitability solution, and **Chromatographic system:** Proceed as directed in the *Assay*.
Standard solution: 75 µg/mL of USP Aspartame Related Compound A RS in *Diluent*
Sample solution: 5 mg/mL of Aspartame in *Diluent*. [NOTE—Avoid heat and excessive holding times.]
System suitability
Samples: *System suitability solution* and *Standard solution*
Suitability requirements
Resolution: NLT 8 between the L-phenylalanine and aspartame related compound A peaks, *System suitability solution*
Tailing factor: NMT 2.0, *Standard solution*
Relative standard deviation: NMT 4.0%, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
[NOTE—Continue the elution of the *Sample solution* for 45 min.]
Calculate the percentage of aspartame related compound A in the portion of Aspartame taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of aspartame related compound A from the *Sample solution*
r_S = peak response of aspartame related compound A from the *Standard solution*
C_S = concentration of 5-benzyl-3,6-dioxo-2-piperazineacetic acid in the *Standard solution* (mg/mL)
C_U = concentration of Aspartame in the *Sample solution* (mg/mL)

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Acceptance criteria: NMT 1.5%

• **CHROMATOGRAPHIC PURITY**

Mobile phase, Diluent, System suitability solution, and **Chromatographic system:** Proceed as directed in the *Assay*.

Sample stock solution: Proceed as directed in the *Sample solution* in the test for *Limit of 5-Benzyl-3,6-dioxo-2-piperazineacetic Acid*.

Sample solution: 0.1 mg/mL of Aspartame from the *Sample stock solution* in *Diluent*

System suitability

Sample: *System suitability solution*

Suitability requirements

Resolution: NLT 8 between the L-phenylalanine and aspartame related compound A peaks, *System suitability solution*

Analysis

Samples: *Sample stock solution* and *Sample solution*

[NOTE—Continue the elution of the *Sample stock solution* for 45 min.]

Acceptance criteria: The sum of all of the peak responses of the *Sample stock solution*, excluding the 5-benzyl-3,6-dioxo-2-piperazineacetic acid and aspartame peak responses, is NMT the aspartame peak response of the *Sample solution*, corresponding to NMT 2.0% of chromatographic impurities.

SPECIFIC TESTS

• **TRANSMITTANCE**

Sample solution: 10 mg/mL of Aspartame in 2 N hydrochloric acid, prepared by means of sonication

Analysis: Determine the transmittance in a 1-cm cell at 430 nm with a suitable spectrophotometer.

Acceptance criteria: Transmittance of NLT 0.95, corresponding to an absorbance of NMT about 0.022

• **OPTICAL ROTATION,** *Specific Rotation* ⟨781S⟩

Sample solution: 40 mg/mL in 15 N formic acid

Acceptance criteria: +14.5° to +16.5°, determined at 20° within 30 min after preparation of the *Sample solution*

• **LOSS ON DRYING** ⟨731⟩

Analysis: Dry at 105° for 4 h.

Acceptance criteria: NMT 4.5%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Aspartame RS

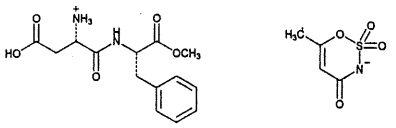
USP Aspartame Related Compound A RS

5-Benzyl-3,6-dioxo-2-piperazineacetic acid.

$C_{13}H_{14}N_2O_4$ 262.27

USP L-Phenylalanine RS

Aspartame Acesulfame



$C_{18}H_{23}O_9N_3S$ 457.45

Aspartame acesulfame salt;

[2-Carboxy-β-(*N*-b-methoxycarbonyl-2-phenyl) ethylcarbamoyl)]ethanaminium 6-methyl-4-oxo-1,2,3-oxathiazin-3-ide-2,2-dioxide;

L-Phenylalanine, L-α-aspartyl-2-methyl ester compound with 6-methyl-1,2,3-oxathiazin-4(3*H*)-one 2,2-dioxide (1:1) [106372-55-8].

DEFINITION

Aspartame Acesulfame contains NLT 63.0% and NMT 66.0% of aspartame, calculated on the dried basis. It contains NLT 34.0% and NMT 37.0% of acesulfame, calculated as the acid form on the dried basis.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

[NOTE—Do not dry specimens.]

ASSAY

• **PROCEDURE**

Sample: 0.150 g

Analysis: Dissolve the *Sample* in 50 mL of dehydrated alcohol. Titrate with 0.1 N tetrabutylammonium hydroxide in methanol/isopropyl alcohol VS. Potentiometrically determine the volumes of titrant, in mL, at the first equivalence point (V_1) and at the second equivalence point (V_2). Perform a blank titration with 50 mL of dehydrated alcohol, and designate the volume of titrant, in mL, as V_B.

Calculate the percentage of acesulfame (*Result 1*) and aspartame (*Result 2*) in the portion of Aspartame Acesulfame taken:

$$\text{Result 1} = 163N(V_1 - V_B)/10W$$

$$\text{Result 2} = 294N(V_2 - V_1)/10W$$

N = normality of the titrant

W = weight of Aspartame Acesulfame taken (g)

Acceptance criteria: Aspartame, 63.0%–66.0% on the dried basis; acesulfame, 34.0%–37.0% as the acid form on the dried basis

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 1.1%

• **LIMIT OF POTASSIUM**

Potassium stock solution: 19.0 µg/mL of potassium chloride, previously dried at 105° for 2 h, in water. This solution contains 10 µg of potassium/mL.

Standard solution A: Transfer 10.0 mL of *Potassium stock solution* to a 100-mL volumetric flask. Add 2.0 mL of sodium chloride solution (1 in 5) and 1.0 mL of hydrochloric acid, and dilute with water to volume (1.0 µg/mL of potassium).

Standard solution B: Transfer 15.0 mL of *Potassium stock solution* to a 100-mL volumetric flask. Add 2.0 mL of sodium chloride solution (1 in 5) and 1.0 mL of hydrochloric acid, and dilute with water to volume (1.5 µg/mL of potassium).

Standard solution C: Transfer 20.0 mL of *Potassium stock solution* to a 100-mL volumetric flask. Add 2.0 mL of sodium chloride solution (1 in 5) and 1.0 mL of hydrochloric acid, and dilute with water to volume (2.0 µg/mL of potassium).

Sample stock solution: 3.0 mg/mL of Aspartame Acesulfame

Sample solution: Transfer 10 mL of *Sample stock solution* to a 100-mL volumetric flask. Add 2.0 mL of sodium chloride solution (1 in 5) and 1.0 mL of hydrochloric acid, and dilute with water to volume. Filter the solution.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

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Analytical wavelength: 766.5 nm (potassium emission line)
Lamp: Potassium hollow-cathode
Flame: Air–acetylene
Blank: Water
Standard curve
Samples: *Standard solution A*, *Standard solution B*, and *Standard solution C*
Plot: Absorbance values versus their corresponding concentration (µg/mL) of potassium
Analysis
Sample: *Sample solution*
From the *Standard curve*, determine the concentration of potassium in the *Sample solution*.
Calculate the percentage of potassium in the portion of Aspartame Acesulfame taken:

$$\text{Result} = (C_S/C_U) \times 100$$

C_S = concentration of potassium in the *Sample solution* from the *Standard curve* (µg/mL)
C_U = concentration of aspartame acesulfame in the *Sample solution* (g/mL)

Acceptance criteria: NMT 0.5%

• **RELATED COMPOUNDS**
Diluent: Methanol and water (1:9)
Mobile phase: Prepare a solution by dissolving 5.6 g of monobasic potassium phosphate in 820 mL of water in a 1-L volumetric flask, adjusting with phosphoric acid to a pH of 4.3, diluting with methanol to volume, and mixing.
Standard solution: 25 µg/mL of USP Aspartame Related Compound A RS in *Diluent*
Sample solution: 5 mg/mL in *Diluent*
[NOTE—Avoid heat and excessive holding times.]
Instrumental conditions
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 210 nm
Column: 4.6-mm × 25-cm; packing L1
Column temperature: 40°
Flow rate: 1.5 mL/min
Injection size: 20 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Tailing factor: NMT 2.0
Relative standard deviation: NMT 4.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of aspartame related compound A in the portion of Aspartame Acesulfame taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of aspartame related compound A from the *Sample solution*
r_S = peak response of aspartame related compound A from the *Standard solution*
C_S = concentration of USP Aspartame Related Compound A RS in the *Standard solution* (mg/mL)
C_U = concentration of Aspartame Acesulfame in the *Sample solution* (mg/mL)

Acceptance criteria: NMT 0.5%

• **CHROMATOGRAPHIC PURITY**
Diluent, Mobile phase, and Instrumental conditions: Proceed as directed in *Related Compounds*.
Acesulfame solution: 2 mg/mL of USP Acesulfame Potassium RS in *Diluent*
Sample stock solution: Proceed as directed for *Sample solution* under *Related Compounds*.
Sample solution: 75 µg/mL of Aspartame Acesulfame from *Sample stock solution* in *Diluent*
Analysis
Samples: *Acesulfame solution*, *Sample stock solution*, and *Sample solution*
[NOTE—Continue the elution of the *Sample stock solution* for twice the retention time of the aspartame peak.]
Acceptance criteria: The sum of the responses of all the peaks of the *Sample stock solution*, excluding the aspartame related compound A, acesulfame, and aspartame peak responses, is not greater than the aspartame peak response from the *Sample solution* (1.0%).

SPECIFIC TESTS

• **OPTICAL ROTATION,** *Specific Rotation* ⟨781S⟩
15 N formic acid: To a 1-L volumetric flask add approximately 300 mL of water, then weigh 719 g of 96% formic acid into this flask, and mix. Cool the solution to 20°. Dilute with water to volume.
Sample solution: 62 mg/mL in *15 N formic acid*
Acceptance criteria: +14.5° to +16.5°, determined at 20° within 30 min after preparation of the *Sample solution*.
[NOTE—Divide the calculated specific rotation by 0.646 to correct for the aspartame content in Aspartame Acesulfame.]

• **LOSS ON DRYING** ⟨731⟩**:** Dry a sample at 105° for 4 h: it loses NMT 0.5% of its weight.

• **TRANSMITTANCE:** The transmittance of a 1-in-100 solution of Aspartame Acesulfame in distilled water, determined in a 1-cm cell at 430 nm with a suitable spectrophotometer, is NLT 0.95.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store in a cool, dry place.

• **USP REFERENCE STANDARDS** ⟨11⟩
USP Acesulfame Potassium RS
USP Aspartame Acesulfame RS
USP Aspartame Related Compound A RS
5-Benzyl-3,6-dioxo-2-piperazineacetic acid.
$C_{13}H_{14}N_2O_4$ 262.27

Barium Hydroxide Lime—*see Barium Hydroxide Lime General Monographs*

Behenoyl Polyoxylglycerides

DEFINITION
Behenoyl Polyoxylglycerides is a mixture of monoesters, diesters, and triesters of glycerol and monoesters and diesters of polyethylene glycols. The polyethylene glycols used have a mean molecular weight of 400. It is produced by esterification of glycerol and polyethylene glycols with behenic acid.

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IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
- **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩

Standard solution: 50 mg/mL of USP Behenoyl Polyoxylglycerides RS in methylene chloride
Sample solution: 50 mg/mL of Behenoyl Polyoxylglycerides in methylene chloride
Chromatographic system
Application volume: 10 µL
Developing solvent system: Ether and hexanes (70:30)
Spray reagent: 0.1-mg/mL solution of rhodamine B in alcohol
Analysis: Proceed as directed in the chapter. Then spray the plate with *Spray reagent*, and locate the spots on the plate by examination under UV light at a wavelength of 365 nm.
Acceptance criteria: The R_F values of the principal spots of the *Sample solution* correspond to those of the *Standard solution*.

- **C.** It meets the requirements in *Specific Tests* (see *Table 1*) for *Fats and Fixed Oils, Fatty Acid Composition* ⟨401⟩.

IMPURITIES

- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩

Sample solution: 2.0 g
Acceptance criteria: NMT 0.1%

- **ALKALINE IMPURITIES**

Sample: 5.0 g
Analysis: Heat the *Sample* slightly until it melts. Add 10 mL of alcohol and 0.05 mL of bromophenol blue TS, and mix well. While the solution is still warm, titrate with 0.01 N hydrochloric acid VS to change the color to yellow.
Acceptance criteria: NMT 1.0 mL of 0.01 N hydrochloric acid

- **LIMIT OF FREE ETHYLENE OXIDE AND DIOXANE**

Analysis: Proceed as directed in *Ethylene Oxide and Dioxane* ⟨228⟩, *Method I*.
Acceptance criteria
Ethylene oxide: NMT 1 µg/g
Dioxane: NMT 10 µg/g

- **LIMIT OF FREE GLYCEROL**

Sample: 1.2 g
Periodic acetic acid solution: Dissolve 0.446 g of sodium periodate in 2.5 mL of a 25% (v/v) solution of sulfuric acid, and dilute with glacial acetic acid to 100.0 mL.
Potassium iodide solution: 75 mg/mL of potassium iodide
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Residual titration
Titrant: 0.1 M sodium thiosulfate VS
Endpoint detection: Visual
Analysis: Dissolve the *Sample* in 25 mL of methylene chloride, heating if necessary. Cool, and add 100 mL of water and 25.0 mL of *Periodic acetic acid solution*. Shake, and allow to stand for 30 min. Add 40 mL of *Potassium iodide solution*, and allow to stand for 1 min. Add 1 mL of starch TS, and titrate the liberated iodine with *Titrant*. Perform a blank determination, and make any necessary correction. Each mL of *Titrant* is equivalent to 2.3 mg of glycerin.
Acceptance criteria: NMT 3.0%

SPECIFIC TESTS

- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩

Sample: 2.0 g
Acceptance criteria: NMT 4.0

- **FATS AND FIXED OILS,** *Fatty Acid Composition* ⟨401⟩: Behenoyl Polyoxylglycerides exhibits the composition profile of fatty acids in *Table 1*.

Table 1

Carbon-Chain Length	No. of Double Bonds	Percentage (%)
12	0	≤1.0
14	0	≤1.0
16	0	≤3.0
18	0	≤5.0
20	0	≤10.0
22	0	≥83.0
22	1	≤3.0
24	0	≤3.0

- **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩

Sample: 1.0 g
Analysis: Proceed as directed in the chapter. If the volume of 0.5 N sodium hydroxide VS required for the titration is less than 2 mL, a more dilute titrant may be used.
Acceptance criteria: 30–50

- **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩

Sample: 2.0 g
Acceptance criteria: NMT 6.0

- **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩

Sample: 2.0 g
Acceptance criteria: 120–140

- **WATER DETERMINATION,** *Method I* ⟨921⟩

Sample: 1.0 g
Analysis: Use anhydrous pyridine as the solvent.
Acceptance criteria: NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, protected from heat and moisture. Store at NMT 35°.
- **LABELING:** Label it to indicate the average nominal molecular weight of polyethylene glycols used as part of the official title.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Behenoyl Polyoxylglycerides RS

Bentonite

Bentonite [1302-78-9].

DEFINITION
Bentonite is a native, colloidal, hydrated aluminum silicate.

IDENTIFICATION

- **A. X-RAY DIFFRACTION** ⟨941⟩

Sample A: Add 2 g in small portions to 100 mL of water, with intense agitation. Allow to stand for 12 h to ensure complete hydration. Place 2 mL of the mixture so obtained on a suitable glass slide, and allow to air-dry at room temperature to produce an oriented film. Place the slide in a vacuum desiccator over a free surface of ethylene glycol. Evacuate the desiccator, and close the stopcock so that the ethylene glycol saturates the desiccator chamber. Allow to stand for 12 h.
Sample B: Prepare a random powder specimen of Bentonite.

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Analysis
Samples: *Sample A* and *Sample B*
Record the X-ray diffraction pattern of the samples, and determine the *d* values.
Acceptance criteria: The largest peak in the pattern of *Sample A* corresponds to a *d* value between 15.0 and 17.2 Å. The major peak in the region between 1.48 and 1.54 Å from the pattern of *Sample B* is between 1.492 and 1.504 Å.

IMPURITIES

- **ARSENIC,** *Method I* ⟨211⟩

Test preparation: Transfer 8.0 g to a 250-mL beaker containing 100 mL of dilute hydrochloric acid (1 in 25), mix, and cover with a watch glass. Boil gently, with occasional stirring, for 15 min without allowing excessive foaming. Pass the hot supernatant through a rapid-flow filter paper into a 200-mL volumetric flask, and wash with four 25-mL portions of hot dilute hydrochloric acid (1 in 25), collecting the washings in the volumetric flask. Cool the combined filtrates to room temperature, and add dilute hydrochloric acid (1 in 25) to volume. Transfer 25 mL of this solution to a generator flask, and dilute with water to 35 mL.
Standard preparation: Transfer 5 mL of *Standard Arsenic Solution* (5 µg) to a generator flask, and dilute with water to 35 mL.
Analysis: Proceed as directed in the chapter, determining the absorbances.
Acceptance criteria: NMT 5 ppm; the absorbance due to any red color from the *Test preparation* does not exceed that produced by the *Standard preparation.*

- **LEAD**

[NOTE—The *Standard solution* and the *Sample solution* may be modified, if necessary, to obtain solutions of suitable concentrations, adaptable to the linear or working range of the instrument. It may be necessary to correct the value obtained for the *Sample solution* for interference from the sample specimen matrix.]
Standard solution: On the day of use, dilute 3.0 mL of lead nitrate stock solution TS with water to 100 mL. Each mL of the *Standard solution* contains the equivalent of 3 µg of lead.
Sample solution: Transfer 3.75 g to a 250-mL beaker containing 100 mL of dilute hydrochloric acid (1 in 25), stir, and cover with a watch glass. Boil for 15 min, then cool to room temperature, and pass through a rapid-flow filter paper into a 400-mL beaker. Wash the filter with four 25-mL portions of hot water, collecting the washings in the 400-mL beaker. Concentrate the combined extracts by gentle boiling to approximately 20 mL. If a precipitate appears, add 2–3 drops of nitric acid, heat to boiling, and cool to room temperature. Pass the concentrated extracts through a rapid-flow filter paper into a 50-mL volumetric flask. Transfer the remaining contents of the 400-mL beaker through the filter paper and into the flask with water. Dilute with water to volume.
Instrumental conditions
Mode: Atomic absorption spectrophotometry
Analytical wavelength: 283.3 nm
Lamp: Lead hollow cathode with deuterium arc background correction
Flame: Air and acetylene
Analysis: Determine the absorbances of the *Standard solution* and the *Sample solution.*
Acceptance criteria: The absorbance of the *Sample solution* is NMT that of the *Standard solution* (40 µg/g).

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** Meets the requirements of the test for absence of *Escherichia coli*
- **pH** ⟨791⟩

Analysis: Disperse 4.0 g in 200 mL of water, mixing vigorously to facilitate wetting.
Acceptance criteria: 9.5–10.5

- **LOSS ON DRYING** ⟨731⟩

Analysis: Dry at 105° for 2 h.
Acceptance criteria: 5.0%–8.0%

- **GEL FORMATION:** Mix 6 g with 300 mg of magnesium oxide. Add the mixture, in several divided portions, to 200 mL of water contained in a blender of approximately 500-mL capacity. Blend thoroughly for 5 min at high speed, transfer 100 mL of the mixture to a 100-mL graduated cylinder, and allow to remain undisturbed for 24 h. NMT 2 mL of supernatant appears on the surface.
- **SWELLING POWER:** To 100 mL of water contained in a glass-stoppered cylinder of 100-mL capacity add 2 g in portions, dropping it upon the surface of the water, and allow each portion to settle before adding the next. The mass at the bottom gradually swells until it occupies an apparent volume of NLT 24 mL at the end of a 2-h period.
- **FINENESS OF POWDER:** Sprinkle 2 g on 20 mL of water contained in a mortar. Allow to swell, disperse evenly with a pestle, and dilute with water to 100 mL. Pour the suspension through a No. 200 standard sieve, and wash the sieve thoroughly with water. No grit is felt when the fingers are rubbed over the wire mesh of the sieve.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **LABELING:** Label it to indicate that absorption of atmospheric moisture should be avoided following the opening of the original package, preferably by storage of the remainder of the contents in a tight container.

Purified Bentonite

DEFINITION
Purified Bentonite is a colloidal montmorillonite that has been processed to remove grit and nonswellable ore components.

IDENTIFICATION

Change to read:

- **A. X-RAY DIFFRACTION** ⟨941⟩

▲Sample: 2 g
Analysis 1: Add the *Sample* in small portions to 100 mL of water with intense agitation. Allow to stand for 12 h to ensure complete hydration. Place 2 mL of the resulting mixture on a suitable glass slide, and allow to air-dry at room temperature to produce an oriented film. Place the slide in a vacuum desiccator over a free surface of ethylene glycol. Evacuate the desiccator, and close the stopcock so that the ethylene glycol saturates the desiccator chamber. Allow to stand for 12 h. Record the X-ray diffraction pattern, and calculate the *d* values.
Acceptance criteria 1: The largest peak corresponds to a *d* value between 15.0 and 17.2 Å.
Analysis 2: Prepare a random powder specimen of Purified Bentonite, record the X-ray diffraction pattern, and determine the *d* values in the region between 1.48 and 1.54 Å.

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Acceptance criteria 2: A peak is found at 1.492–1.504 Å. ▲ (NF 1-Dec-2019)

ASSAY

Delete the following:

▲• **ALUMINUM CONTENT AND MAGNESIUM CONTENT**

[NOTE—The *Standard solutions* and the *Sample solution* may be diluted quantitatively with water, if necessary, to obtain solutions of suitable concentrations adaptable to the linear or working range of the instrument.]

Aluminum content

Aluminum standard stock solution: Dissolve 1.000 g of aluminum in a mixture of 10 mL of hydrochloric acid and 10 mL of water by gentle heating. Transfer the solution to a 1000-mL volumetric flask, and dilute with water to volume. This solution contains the equivalent of 1 mg/mL of aluminum.

Aluminum standard solutions: Transfer 2-, 5-, and 10-mL aliquots of the *Aluminum standard stock solution* to separate 100-mL volumetric flasks containing 200 mg of sodium chloride, and dilute each with water to volume.

Sample stock solution: Transfer 0.200 g of Purified Bentonite to a 25-mL platinum crucible containing 1.0 g of lithium metaborate, and mix. Using a muffle furnace or a suitable burner, heat slowly at first, and ignite at 1000°–1200° for 15 min. Cool, place the crucible in a 100-mL beaker containing 25 mL of dilute nitric acid (50 mg/mL), and add an additional 50 mL of the dilute acid, filling and submerging the upright crucible. Place a polyfluorocarbon-coated magnetic stirring bar into the crucible, and stir gently to dissolve. Pour the contents into a 250-mL beaker, and remove the crucible. Warm the solution, transfer through a rapid-flow filter paper with the aid of water into a 200-mL volumetric flask, and dilute with water to volume.

Sample solution: Pipet 20 mL of the *Sample stock solution* into a 100-mL volumetric flask. Add 20 mL of sodium chloride solution (10 mg/mL), and dilute with water to volume.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometer equipped with a single-slot burner

Analytical wavelength: 309 nm

Lamp: Aluminum hollow-cathode

Flame: Oxidizing acetylene–air–nitrous oxide

Analysis

Samples: *Aluminum standard solutions* and *Sample solution*

Determine the aluminum content of the Purified Bentonite in the *Sample solution* from the linear regression equation calculated from the absorbances and concentrations of the *Aluminum standard solutions*.

Magnesium content

Lanthanum solution: Stir 88.30 g of lanthanum chloride ($LaCl_3$) with 500 mL of 6 N hydrochloric acid to dissolve, transfer with the aid of water to a 1000-mL volumetric flask, and dilute with water to volume.

Magnesium standard stock solution: Place 1.000 g of magnesium in a 250-mL beaker containing 20 mL of water, and carefully add 20 mL of hydrochloric acid, warming, if necessary, to complete the reaction. Transfer the solution to a 1000-mL volumetric flask, and dilute with water to volume. This solution contains the equivalent of 1 mg/mL of magnesium. Transfer 10.0 mL of this solution to a 1000-mL volumetric flask, and dilute with water to volume.

Magnesium standard solutions: Transfer 5-, 10-, 15-, and 20-mL aliquots of the *Magnesium standard stock solution* to separate 100-mL volumetric flasks. To each flask add 20.0 mL of *Lanthanum solution*, and dilute with water to volume.

Sample stock solution: Use the *Sample stock solution* prepared as directed for *Aluminum content*.

Sample solution: Transfer 25 mL of the *Sample stock solution* to a 50-mL volumetric flask, and dilute with water to volume. Transfer 5.0 mL of this solution to a 100-mL volumetric flask, add 20.0 mL of *Lanthanum solution*, and dilute with water to volume.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometer equipped with a single-slot burner

Analytical wavelength: 285 nm

Lamp: Magnesium hollow-cathode

Flame: Reducing flame of acetylene–air

Analysis

Samples: *Magnesium standard solutions* and *Sample solution*

Determine the magnesium content of the Purified Bentonite in the *Sample solution* from the linear regression equation calculated from the absorbances and concentrations of the *Magnesium standard solutions*.

Acceptance criteria: The ratio of the aluminum content to the magnesium content is 3.5–5.5. ▲ (NF 1-Dec-2019)

IMPURITIES

• **ARSENIC** ⟨211⟩, *Procedures, Method I*

Standard solution: 5 mL of *Standard Arsenic Solution* (5 µg of As)

Sample solution: Transfer 13.3 g to a 250-mL beaker containing 100 mL of dilute hydrochloric acid (1 in 25), mix, cover with a watch glass, and boil gently, with occasional stirring, for 15 min without allowing excessive foaming. Allow the insoluble material to settle, and decant the hot supernatant through a rapid-flow filter paper into a 200-mL volumetric flask, retaining as much sediment as possible in the beaker. Add 25 mL of hot dilute hydrochloric acid (1 in 25) to the residue in the beaker, stir, heat to boiling, allow the insoluble material to settle, and decant the supernatant through the filter into the 200-mL volumetric flask. Repeat the extraction with four additional 25-mL portions of hot dilute hydrochloric acid (1 in 25), decanting each hot supernatant through the filter into the volumetric flask. At the last extraction, transfer as much of the insoluble material as possible onto the filter. Cool the combined filtrates to room temperature, and add dilute hydrochloric acid (1 in 25) to volume.

Analysis: Using a 25-mL aliquot of *Sample solution* and the *Standard solution*, treat them as directed in the *Procedure*.

Acceptance criteria: NMT 3 µg/g; the absorbance due to any red color from the *Sample solution* does not exceed that produced by the *Standard solution*.

• **LEAD**

[NOTE—The *Standard solution* and the *Sample solution* may be modified, if necessary, to obtain solutions of suitable concentrations, adaptable to the linear or working range of the instrument.]

Standard solution: On the day of use, dilute 3.0 mL of lead nitrate stock solution TS to 100 mL. Each milliliter of the *Standard solution* contains the equivalent of 3 µg of lead.

Sample solution: Transfer 10.0 g of Purified Bentonite to a 250-mL beaker containing 100 mL of dilute hydrochloric acid (1 in 25), stir, cover with a watch glass, and boil for

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15 min. Cool to room temperature, and allow the insoluble matter to settle. Decant the supernatant through a rapid-flow filter paper into a 400-mL beaker. Add 25 mL of hot water to the insoluble matter in the 250-mL beaker, stir, allow the insoluble matter to settle, and decant the supernatant through the filter into the 400-mL beaker. Repeat the extraction with two additional 25-mL portions of water, decanting each supernatant portion through the filter into the 400-mL beaker. Wash the filter with 25 mL of hot water, collecting this filtrate in the 400-mL beaker. Concentrate the combined extracts by gentle boiling to approximately 20 mL. If a precipitate appears, add 2–3 drops of nitric acid, heat to boiling, and cool to room temperature. Pass the concentrated extracts through a rapid-flow filter paper into a 50-mL volumetric flask. Transfer the remaining contents of the 400-mL beaker through the filter paper and into the flask with water. Dilute with water to volume.

Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometer equipped with a deuterium arc background correction and a single-slot burner
Analytical wavelength: 284 nm
Lamp: Lead hollow-cathode
Flame: Air–acetylene
Analysis: Determine the absorbances of the *Standard solution* and the *Sample solution.*
Acceptance criteria: The absorbance of the *Sample solution* is NMT that of the *Standard solution* (15 µg/g).

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic microbial count does not exceed 1×10^3 cfu/g. It meets the requirements of the test for absence of *Escherichia coli.*
- **pH** ⟨791⟩
  Sample suspension: 50 mg/mL
  Acceptance criteria: 9.0–10.0
- **LOSS ON DRYING** ⟨731⟩
  Analysis: Dry at 110° to constant weight.
  Acceptance criteria: NMT 8.0%

Change to read:

- **VISCOSITY**
  Sample: After determining *Loss on Drying*, weigh a quantity of Purified Bentonite equivalent to 25.0 g on the dried basis. Over a period of a few seconds, transfer the undried sample specimen to a suitable 1-L blender jar containing an amount of water, maintained at a temperature of 25 ± 2°, that is sufficient to produce a mixture weighing 500 g. Blend for 3 min, accurately timed, at 14,000–15,000 rpm (high speed). [1] ▲(NF 1-Dec-2019)
  [NOTE—Heat generated during blending causes a temperature rise to ▲33 ± 3°.▲(NF 1-Dec-2019)]
  Analysis: Transfer the contents of the blender to a 600-mL beaker and allow to stand for 5 min.▲(NF 1-Dec-2019) Using a suitable rotational viscometer[2] ▲(NF 1-Dec-2019) equipped with a spindle having a cylinder 1.87 cm in diameter and 0.69 cm high attached to a shaft 0.32 cm in diameter, the distance from the top of the cylinder to the lower tip of the shaft being 2.54 cm, and the immersion depth being 5.00 cm (No. 2 spindle), operate the viscometer at 60 rpm for 6 min, accurately timed, and record the scale reading.
  Acceptance criteria: 40–200 mPa · s

Delete the following:

- ▲• **ACID DEMAND**
  Sample: After determining *Loss on Drying* ⟨731⟩, weigh a quantity of Purified Bentonite equivalent to 5.00 g.
  Analysis: Disperse the *Sample* in 500 mL of water with the aid of a suitable blender fitted with a 1-L jar. Using a stopwatch, designate zero time. With constant mixing, add 3.0-mL portions of 0.100 N hydrochloric acid at 5, 65, 125, 185, 245, 305, 365, 425, 485, 545, 605, 665, and 725 s, and add a 1.0-mL portion at 785 s. Determine the pH potentiometrically at 840 s.
  Acceptance criteria: NMT 4.0▲(NF 1-Dec-2019)

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.

Bentonite Magma

DEFINITION

Prepare Bentonite Magma as follows.

Bentonite	50 g
Purified Water, a sufficient quantity to make	1000 g

Sprinkle the *Bentonite,* in portions, upon 800 g of hot *Purified Water,* allowing each portion to become thoroughly wetted without stirring. Allow it to stand with occasional stirring for 24 h. Stir until a uniform magma is obtained, add *Purified Water* to make 1000 g, and mix.
The Magma may also be prepared by mechanical means such as by use of a blender, as follows. Place 500 g of *Purified Water* in the blender, and while the machine is running, add the *Bentonite.* Add *Purified Water* to make up to 1000 g or up to the operating capacity of the blender. Blend the mixture for 5–10 min, add *Purified Water* to make 1000 g, and mix.

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** It meets the requirements of the tests for absence of *Escherichia coli.*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Package in tight containers.

Benzaldehyde



C_7H_6O 106.12
Benzoic aldehyde;
Phenyl formaldehyde [100-52-7].

DEFINITION

Benzaldehyde contains NLT 98.0% and NMT 102.0% of benzaldehyde (C_7H_6O).

[1] A suitable blender is available from Waring as Waring Commercial Blender Model 7009G or equivalent with 1-L glass jar and tachometer adapter, Model CAC24 or equivalent.
[2] A suitable viscometer is available from Brookfield as Viscometer Model LVF or LVT, or equivalent.

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IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F**▲ (CN 1-May-2020)

ASSAY

• **PROCEDURE**

Solution A: Acetonitrile and glacial acetic acid (1000:1, v/v)

Solution B: Glacial acetic acid and water (1:1000, v/v)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	18	82
20	60	40
30	60	40

System suitability solution: 0.1 mg/mL of USP Benzoic Acid RS and 0.06 mg/mL of USP Methylparaben RS in acetonitrile

Standard solution: 0.15 mg/mL of USP Benzaldehyde RS in acetonitrile

Sample solution: 0.15 mg/mL of Benzaldehyde in acetonitrile

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 235 nm

Detector temperature: 25°

Column: 4.6-mm × 25-cm; 5-µm packing L1

Flow rate: 1.2 mL/min

Injection volume: 10 µL

System suitability

Samples: *System suitability solution* and *Standard solution*

Suitability requirements

Resolution: NLT 2.0 between benzoic acid and methylparaben, *System suitability solution*

Tailing factor: NMT 2.0 for benzoic acid and methylparaben, *System suitability solution*

Relative standard deviation: NMT 2.0% for benzaldehyde, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of benzaldehyde (C_7H_6O) in the portion of Benzaldehyde taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of benzaldehyde from the *Sample solution*

r_S = peak response of benzaldehyde from the *Standard solution*

C_S = concentration of USP Benzaldehyde RS in the *Standard solution* (mg/mL)

C_U = concentration of Benzaldehyde in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–102.0%

IMPURITIES

• **LIMIT OF HYDROCYANIC ACID**

Sample solution: Shake 0.5 mL of Benzaldehyde with 5 mL of water, add 0.5 mL of 1 N sodium hydroxide and 0.1 mL of ferrous sulfate TS, and warm the mixture gently.

Acceptance criteria: Upon the addition of a slight excess of hydrochloric acid, no greenish-blue color or blue precipitate is produced within 15 min.

• **LIMIT OF NITROBENZENE**

Sample solution: Dissolve 1 mL of Benzaldehyde in 20 mL of alcohol, and mix with 10 mL of water.

Analysis: Add 1-g portions of zinc and 1-mL portions of 2 N sulfuric acid, as needed, to maintain a brisk evolution of hydrogen for 1 h. Filter, evaporate the liquid to 20 mL, and boil 10 mL of the concentrated liquid with 1 drop of potassium dichromate TS.

Acceptance criteria: No purplish color is produced.

• **CHLORINATED COMPOUNDS**

Analysis: Wind a strip of 20-mesh copper gauze 1.5 cm wide and 5 cm long around the end of a copper wire. Heat the gauze in the nonluminous flame of a Bunsen burner until it glows without coloring the flame green. Permit the gauze to cool, and heat several times until a thick coat of oxide has formed. With a medicine dropper, apply 2 drops of Benzaldehyde to the cooled gauze, ignite, and permit it to burn freely in the air. Again cool the gauze, add 2 more drops of Benzaldehyde, and burn as before. Repeat this process until a total of 6 drops have been added and ignited. Then hold the gauze in the outer edge of the Bunsen flame, adjusted to a height of 4 cm.

Acceptance criteria: Not even a transient green color is imparted to the flame.

• **LIMIT OF ETHYLBENZENE, CYCLOHEXYLMETHANOL, BENZYL ALCOHOL, AND BENZOIC ACID**

Sample solution: Neat Benzaldehyde

Standard solution: 0.1% of USP Ethylbenzene RS, 0.1% of USP Cyclohexylmethanol RS, 0.2% of USP Benzoic Acid RS, and 0.2% of USP Benzaldehyde RS in USP Benzyl Alcohol RS

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.32-mm × 30-m fused silica capillary, coated with a 0.5-µm layer of phase G16

Temperatures

Detector: 310°

Injection port: 200°

Column: See *Table 2*.

Table 2

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
50	5	220	35

Carrier gas: Helium

Flow rate: 1.2 mL/min

Injection volume: 0.1 µL

Injection type: Splitless injection

System suitability

Sample: *Standard solution*

[NOTE—For relative retention times, see *Table 3*.]

Table 3

Component	Relative Retention Time
Ethylbenzene	0.45
Benzaldehyde	1.00
Cyclohexylmethanol	1.03

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Table 3 *(continued)*

Component	Relative Retention Time
Benzyl alcohol	1.45
Benzoic acid	2.04

System suitability requirements
Resolution: NLT 3.0 between benzaldehyde and cyclohexylmethanol
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of each impurity in the portion of Benzaldehyde taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of each impurity from the *Sample solution*
r_T = sum of all the peak responses from the *Sample solution*

Acceptance criteria
Each individual impurity: NMT 1.0%
Total impurities: NMT 2.0%

SPECIFIC TESTS
• **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 1.5%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-filled, tight, light-resistant containers. Store at room temperature, and avoid exposure to excessive heat.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Benzaldehyde RS
USP Benzoic Acid RS
USP Benzyl Alcohol RS
USP Cyclohexylmethanol RS
USP Ethylbenzene RS
USP Methylparaben RS

Compound Benzaldehyde Elixir

DEFINITION
Compound Benzaldehyde Elixir contains 0.05% Benzaldehyde in a suitably flavored and sweetened hydroalcoholic vehicle.

OTHER COMPONENTS
• **ALCOHOL DETERMINATION,** *Method I* ⟨611⟩**:** 3.0%–5.0%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Package in tight, light-resistant containers.

Benzalkonium Chloride

Ammonium, alkyldimethyl(phenylmethyl)-, chloride; Alkylbenzyldimethylammonium chloride [8001-54-5].

DEFINITION
Benzalkonium Chloride is a mixture of alkylbenzyldimethylammonium chlorides of the general formula:

$$[C_6H_5CH_2N(CH_3)_2R]Cl$$

in which R represents a mixture of alkyls, including all or some of the group beginning with n-C_8H_{17} and extending through higher homologs, with n-$C_{12}H_{25}$, n-$C_{14}H_{29}$, and n-$C_{16}H_{33}$ composing the major portion. On the anhydrous basis, the content of the n-$C_{12}H_{25}$ homolog is NLT 40.0%, and the content of the n-$C_{14}H_{29}$ homolog is NLT 20.0% of the total alkylbenzyldimethylammonium chloride content. The amount of the n-$C_{12}H_{25}$ and n-$C_{14}H_{29}$ homolog components together is NLT 70.0% of the total alkylbenzyldimethylammonium chloride content. The total alkylbenzyldimethylammonium chloride content, calculated on the anhydrous basis, with allowance made for the amount of residue on ignition, is NLT 97.0% and NMT 103.0% of $[C_6H_5CH_2N(CH_3)_2R]Cl$.

IDENTIFICATION
• **A.**
Analysis: To 2 mL of a solution (1 in 100) add 1 mL of 2 N nitric acid.
Acceptance criteria: A white precipitate is formed and is dissolved after adding 5 mL of alcohol.
• **B.**
Analysis: Dissolve 200 mg in 1 mL of sulfuric acid, add 100 mg of sodium nitrate, and heat on a steam bath for 5 min. Cool, dilute with water to 10 mL, add 500 mg of zinc dust, and warm for 5 min on a steam bath. To 2 mL of the clear supernatant, add 1 mL of sodium nitrite solution (1 in 20), cool in ice water, and then add 3 mL of a solution of 500 mg of 2-naphthol in 10 mL of 6 N ammonium hydroxide.
Acceptance criteria: An orange-red color is produced.
• **C. IDENTIFICATION TESTS—GENERAL,** *Chloride* ⟨191⟩**:** The solution in a mixture of equal volumes of water and alcohol meets the requirements.
• **D.** The retention times of the major peaks for benzalkonium chloride of the *Sample solution* correspond to those of the *Standard solution*, as obtained in the test for *Ratio of Alkyl Components*.

ASSAY
• **RATIO OF ALKYL COMPONENTS**
Solution A: Adjust a 0.1 M solution of sodium acetate with glacial acetic acid to a pH of 5.0.
Mobile phase: Acetonitrile and *Solution A* (9:11). Acetonitrile and *Solution A* may be adjusted from (2:3) to (3:2) to meet system suitability requirements.
Standard solution: 4 mg/mL of benzalkonium chloride from USP Benzalkonium Chloride RS and water
Sample solution: 4 mg/mL of Benzalkonium Chloride
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 3.9-mm × 30-cm; packing L10, or 4.6-mm × 25-cm; 10-µm packing L10
Flow rate: 2 mL/min
Injection volume: 20 µL
System suitability
Sample: *Standard solution*
[NOTE—See *Table 1*. Relative retention times are provided for information only, and the Standard should be used to ensure appropriate peak identification.]

Table 1

Name	Relative Retention Time
C_{10} homolog	0.9

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Table 1 *(continued)*

Name	Relative Retention Time
C_{12} homolog	1.0
C_{14} homolog	1.3
C_{16} homolog	1.7

Suitability requirements
Resolution: NLT 1.5 between the C_{12} and C_{14} homologs
Relative standard deviation: NMT 2.0% for the C_{12} homolog
Analysis
Samples: *Standard solution* and *Sample solution*
Identify the homolog peaks by comparison of the retention times of the *Sample solution* with those of the *Standard solution*.
Calculate the percentage of each quaternary ammonium homolog in the portion of Benzalkonium Chloride taken:

$$\text{Result} = \frac{r_U \times M_r}{\sum_i (r_U \times M_r)} \times 100$$

r_U = peak area of each homolog from the *Sample solution*
M_r = molecular weight of each homolog. The molecular weights of C_{10}, C_{12}, C_{14}, and C_{16} homologs are 312, 340, 368, and 396, respectively.

Acceptance criteria: On the anhydrous basis, the content of the *n*-$C_{12}H_{25}$ homolog is NLT 40.0% and the content of the *n*-$C_{14}H_{29}$ homolog is NLT 20.0% of the total alkylbenzyldimethylammonium chloride content. The amount of the *n*-$C_{12}H_{25}$ and *n*-$C_{14}H_{29}$ homolog components together is NLT 70.0% of the total alkylbenzyldimethylammonium chloride content.

• **TOTAL ALKYLBENZYLDIMETHYLAMMONIUM CHLORIDES**
Sample: Weigh a quantity of Benzalkonium Chloride equivalent to 500 mg of anhydrous benzalkonium chloride.
Analysis: Transfer the *Sample*, with the aid of 35 mL of water, to a glass-stoppered, 250-mL conical separator containing 25 mL of methylene chloride. Add 10 mL of 0.1 N sodium hydroxide, and 10.0 mL of freshly prepared potassium iodide solution (1 in 20). Insert the stopper into the separator, shake, allow the layers to separate, and discard the methylene chloride layer. Wash the aqueous layer with three 10-mL portions of methylene chloride, and discard the washings. Transfer the aqueous layer to a glass-stoppered, 250-mL conical flask, and rinse the separator with three 5-mL portions of water, adding the washings to the flask. Add 40 mL of cold hydrochloric acid to the flask, mix, and titrate with 0.05 M potassium iodate VS until the solution becomes light brown in color. Add 5 mL of methylene chloride, insert the stopper into the flask, and shake vigorously. Continue the titration, dropwise, with shaking after each addition, until the methylene chloride layer no longer changes color and the aqueous layer is clear yellow. Record the titrant volume, V_t, in mL. Perform a blank determination, using 20 mL of water as the sample, and record the titrant volume, V_b, in mL. [NOTE—$V_b > V_t$.] The difference between the two titrations represents the amount of potassium iodate equivalent to the weight of benzalkonium chloride in the sample. Each mL of 0.05 M potassium iodate is equivalent to $x/10$ mg of benzalkonium chloride, where x represents the average molecular weight of the sample, derived by summing, for all homologs, the products:

$$\text{Result } (x) = \sum_i [(r_U/r_T) \times M_r]$$

r_U = peak area of each homolog from the *Ratio of Alkyl Components* test
r_T = sum of all the peak areas of the homologs from the *Ratio of Alkyl Components* test
M_r = molecular weight of each homolog. The molecular weights of the C_{10}, C_{12}, C_{14}, and C_{16} homologs are 312, 340, 368, and 396, respectively.

Acceptance criteria: 97.0%–103.0% on the anhydrous basis

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩: NMT 2.0%

• **LIMIT OF AMINES AND AMINE SALTS**
Sample: 5.0 g of Benzalkonium Chloride
Analysis: Dissolve the *Sample* by heating carefully (e.g., on top of a steam bath with water as the steam source) in 20 mL of a mixture of methanol and 1 N hydrochloric acid VS (97:3). [NOTE—The mixed solution, however, must not reach the boiling point.] Add 100 mL of isopropyl alcohol, and pass a stream of nitrogen slowly through the solution. Gradually add 12.0 mL of 0.1 N tetrabutylammonium hydroxide VS while recording the potentiometric titration curve.
Acceptance criteria: If the curve shows two inflection points, the volume of titrant added between the two points is NMT 5.0 mL, corresponding to NMT 0.1 mmol/g of amines and amine salts. If the curve shows no point of inflection, the substance being examined does not comply with the test. If the curve shows one point of inflection, repeat the test, but add 3.0 mL of a 25.0 mg/mL solution of dimethyldecylamine in isopropyl alcohol before the titration. If after addition of 12.0 mL of the titrant, the titration curve shows only one point of inflection, the substance being examined does not comply with the test.

• **LIMIT OF BENZYL ALCOHOL, BENZALDEHYDE, AND (CHLOROMETHYL)BENZENE**
[NOTE—Prepare the solutions immediately before use.]
Solution A: Dissolve 1.09 g of sodium 1-hexanesulfonate and 6.9 g of monobasic sodium phosphate in water in a 1000-mL volumetric flask, adjust with phosphoric acid to a pH of 3.5, and dilute with water to volume.
Solution B: Methanol
Mobile phase: See *Table 2*.

Table 2

Time (min)	Solution A (%)	Solution B (%)
0	80	20
10	80	20
14	50	50
35	50	50
36	20	80
55	20	80
56	80	20
65	80	20

Standard solution A: 0.25 mg/mL of USP Benzyl Alcohol RS in methanol

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Standard solution B: 0.075 mg/mL of USP Benzaldehyde RS in methanol
Standard solution C: 0.025 mg/mL of USP Benzyl Alcohol RS in methanol, prepared from *Standard solution A* and methanol
Sample solution: 50 mg/mL of Benzalkonium Chloride in methanol
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 210 nm for benzyl alcohol and (chloromethyl)benzene; UV 257 nm for benzaldehyde
Column: 4.6-mm × 15-cm; 5-µm packing L1
Column temperature: 30°
Flow rate: 1.0 mL/min
Injection volume: 20 µL
System suitability
Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*
[NOTE—See *Table 3* for relative retention times.]

Table 3

Name	Relative Retention Time
Benzyl alcohol	1.0
Benzaldehyde	1.3
(Chloromethyl)benzene	2.4

Suitability requirements
Relative standard deviation: NMT 5.0% for benzyl alcohol, *Standard solution A*
Signal-to-noise ratio: NLT 10 for the principal peak, *Standard solution C*
Analysis
Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*
Calculate the content of (chloromethyl)benzene by multiplying the peak area of (chloromethyl)benzene by 1.3. [NOTE—The correction factor is used to adjust for baseline shift.]
Acceptance criteria
Benzyl alcohol: The response of the benzyl alcohol peak from the *Sample solution* is NMT that of the benzyl alcohol peak from *Standard solution A*, corresponding to NMT 0.5%.
Benzaldehyde: The response of the benzaldehyde peak from the *Sample solution* is NMT that of the benzaldehyde peak from *Standard solution B*, corresponding to NMT 0.15%.
(Chloromethyl)benzene: The response of the (chloromethyl)benzene peak from the *Sample solution* is NMT 0.1 times that of the principal peak from *Standard solution A*, corresponding to NMT 0.05%.

SPECIFIC TESTS
• **ACIDITY OR ALKALINITY**
Sample: 0.5 g of Benzalkonium Chloride
Analysis: Dissolve the *Sample* in water, dilute with water to 50 mL, and mix. Add 0.1 mL of bromocresol purple TS.
Acceptance criteria: NMT 0.5 mL of 0.1 N hydrochloric acid or 0.1 N sodium hydroxide is required to change the color of the indicator.
• **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 15.0%
• **WATER-INSOLUBLE MATTER:** A solution (1 in 10) is free from turbidity and insoluble matter.

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Benzaldehyde RS
USP Benzalkonium Chloride RS
USP Benzyl Alcohol RS

Benzalkonium Chloride Solution

DEFINITION
Benzalkonium Chloride Solution contains NLT 95.0% and NMT 105.0% of the labeled amount of benzalkonium chloride in a solution having a concentration of 1.0% or more; and NLT 93.0% and NMT 107.0% of the labeled amount of benzalkonium chloride in a solution having a concentration of less than 1.0%. It may contain a suitable coloring agent and may contain NMT 10% of alcohol.
[**CAUTION**—Mixing Benzalkonium Chloride Solution with ordinary soaps and anionic detergents may decrease or destroy the bacteriostatic activity of the Solution.]

IDENTIFICATION
• **A.**
Analysis: To 2 mL of a solution having an equivalent of 10 mg/mL of benzalkonium chloride add 1 mL of 2 N nitric acid.
Acceptance criteria: A white precipitate is formed and is dissolved after adding 5 mL of alcohol.
• **B. IDENTIFICATION TESTS—GENERAL** ⟨191⟩, *Chloride*: A solution of it in a mixture of equal volumes of water and alcohol meets the requirements.
• **C.**
Analysis: Dissolve the residue, obtained by evaporating on a steam bath a volume of Solution equivalent to 200 mg of benzalkonium chloride, in 1 mL of sulfuric acid. Add 100 mg of sodium nitrate, and heat on a steam bath for 5 min. Cool, dilute with water to 10 mL, add 500 mg of zinc dust, and warm for 5 min on a steam bath. To 2 mL of the clear supernatant add 1 mL of sodium nitrite solution (1 in 20), cool in ice water, then add 3 mL of a solution of 500 mg of 2-naphthol in 10 mL of 6 N ammonium hydroxide.
Acceptance criteria: An orange-red color is produced.
• **D. CHROMATOGRAPHIC IDENTITY**
Analysis: Proceed as directed in the test for *Ratio of Alkyl Components*.
Acceptance criteria: The retention times of the major peaks for benzalkonium chloride of the *Sample solution* correspond to those of the *Standard solution*.

ASSAY
• **RATIO OF ALKYL COMPONENTS**
Solution A: Adjust a 0.1 M solution of sodium acetate with glacial acetic acid to a pH of 5.0.
Mobile phase: Acetonitrile and *Solution A* (9:11). Acetonitrile and *Solution A* may be adjusted from (2:3) to (3:2) to meet system suitability requirements.
Standard solution: 4 mg/mL of benzalkonium chloride prepared from USP Benzalkonium Chloride RS and water
Sample solution: Transfer a volume of Solution, equivalent to 400 mg of benzalkonium chloride, to a 100-mL volumetric flask, and dilute with water to volume.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm

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Column: 3.9-mm × 30-cm; packing L10 or 4.6-mm × 25-cm; 10-µm packing L10
Flow rate: 2 mL/min
Injection volume: 20 µL
System suitability
Sample: *Standard solution*
[NOTE—See *Table 1*. Relative retention times are provided for information only, and the Standard should be used to ensure appropriate peak identification.]

Table 1

Name	Relative Retention Time
C_{10} homolog	0.9
C_{12} homolog	1.0
C_{14} homolog	1.3
C_{16} homolog	1.7

Suitability requirements
Resolution: NLT 1.5 between the C_{12} and C_{14} homologs
Relative standard deviation: NMT 2.0% for the C_{12} homolog
Analysis
Samples: *Standard solution* and *Sample solution*
Identify the homolog peaks by comparison of the retention times from the *Sample solution* with those of the *Standard solution*.
Calculate the percentage of each quaternary ammonium homolog in the portion of Solution taken:

$$\text{Result} = \frac{r_U \times M_r}{\sum_i (r_U \times M_r)} \times 100$$

r_U = peak area of each homolog from the *Sample solution*
M_r = molecular weight of each homolog. The molecular weights of the C_{10}, C_{12}, C_{14}, and C_{16} homologs are 312, 340, 368, and 396, respectively.

Acceptance criteria: On the solid basis, the content of the *n*-$C_{12}H_{25}$ homolog is NLT 40.0%, and the content of the *n*-$C_{14}H_{29}$ homolog is NLT 20.0% of the total alkylbenzyldimethylammonium chloride content. The amount of the *n*-$C_{12}H_{25}$ and *n*-$C_{14}H_{29}$ homolog components together is NLT 70.0% of the total alkylbenzyldimethylammonium chloride content.

• **TOTAL ALKYLBENZYLDIMETHYLAMMONIUM CHLORIDES**
Sample solution: Evaporate or dilute with water to 30 mL a volume of Solution equivalent to 500 mg of benzalkonium chloride.
Analysis: Transfer the *Sample solution*, with the aid of a minimum quantity of water, to a glass-stoppered, 250-mL conical separator. Transfer 25 mL of methylene chloride. Add 10 mL of 0.1 N sodium hydroxide, and 10.0 mL of freshly prepared potassium iodide solution (1 in 20), insert the stopper in the separator, shake, allow the layers to separate, and discard the methylene chloride layer. Wash the aqueous layer with three 10-mL portions of methylene chloride, and discard the washings. Transfer the aqueous layer to a glass-stoppered, 250-mL conical flask, and rinse the separator with three 5-mL portions of water, adding the washings to the flask. Add 40 mL of cold hydrochloric acid to the flask, mix, and titrate with 0.05 M potassium iodate VS until the solution becomes light brown in color. Add 5 mL of methylene chloride, insert the stopper into the flask, and shake vigorously. Continue the titration, dropwise, with shaking after each addition, until the methylene chloride layer no longer changes color and the aqueous layer is clear yellow. Record the titrant volume, V_T, in mL. Perform a blank determination, using 20 mL of water as the sample, and record the titrant volume, V_B, in mL. [NOTE—$V_B > V_T$.] The difference between the two titrations represents the amount of potassium iodate equivalent to the weight of benzalkonium chloride in the sample. Each mL of 0.05 M potassium iodate is equivalent to $x/10$ mg of benzalkonium chloride, where x represents the average molecular weight of the sample, derived by summing, for all homologs, the products:

$$\text{Result } (x) = \sum_i [(r_U/r_T) \times M_r]$$

r_U = peak area of each homolog from the test for *Ratio of Alkyl Components*
r_T = sum of all the peak areas of the homologs from the test for *Ratio of Alkyl Components*
M_r = molecular weight of each homolog. The molecular weights of the C_{10}, C_{12}, C_{14}, and C_{16} homologs are 312, 340, 368, and 396, respectively.

Acceptance criteria
For labeled concentrations of NLT 1.0%: 95.0%–105.0%
For labeled concentrations less than 1.0%: 93.0%–107.0%

OTHER COMPONENTS

• **ALCOHOL CONTENT** (if added)
Diluent: 2-Propanol and water (8:2)
Internal standard solution: 0.005 g/mL of tertiary butyl alcohol in water
Alcohol stock solution: 0.015 g/mL of alcohol (C_2H_5OH) in water
Standard solutions: Introduce 1, 2, and 4 mL, respectively, of *Alcohol stock solution* into three separate and identical 25-mL volumetric flasks. To each flask add a 5-mL portion of the *Internal standard solution*. Dilute with *Diluent* to volume, and mix thoroughly. The *Standard solutions* contain 0.0006, 0.0012, and 0.0024 g/mL of alcohol (C_2H_5OH), respectively.
Sample solution: Weigh an appropriate amount of Solution into a 25-mL volumetric flask, and pipet 5 mL of the *Internal standard solution* into the flask. Dilute with *Diluent* to volume, and mix thoroughly to obtain a solution containing 0.0006–0.0024 g/mL of alcohol (C_2H_5OH).
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.32-mm × 15-m glass or quartz capillary; 1.8-µm layer of phase G43
Temperatures
Injection port: 250°
Detector: 320°
Column: See *Table 2*.

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Table 2

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
50	—	50	4
50	10	70	0
70	50	300	4

Run time: 14.6 min
Carrier gas: Helium
Flow rate: See *Table 3*.

Table 3

Initial Flow (mL/min)	Flow Ramp (mL/min²)	Final Flow (mL/min)	Hold Time at Final Flow (min)
1.4	—	1.4	6
1.4	5	3	8.6

Injection volume: 0.5 µL
Injection type: Split, 75:1
Inlet liner: 4-mm liner with deactivated glass wool
System suitability
Sample: *Standard solution* containing 0.0012 g/mL of alcohol (C_2H_5OH)
[NOTE—The relative retention times for alcohol, 2-propanol, and tertiary butyl alcohol are 0.75, 0.90, and 1.0, respectively.]
Suitability requirements
Resolution: NLT 2.0 between 2-propanol and tertiary butyl alcohol
Relative standard deviation: NMT 2% for the peak response ratio of alcohol to the internal standard
Analysis
Samples: *Standard solutions* and *Sample solution*
Plot the peak response ratios of alcohol to tertiary butyl alcohol in the *Standard solutions* versus the content, in g/mL, of alcohol, and draw the straight line best fitting the plotted points. From the graph obtained, determine the content, *C*, in g/mL, of alcohol (C_2H_5OH) in the *Sample solution*.
Calculate the percentage of alcohol (C_2H_5OH) in the portion of Solution (v/v) taken:

$$\text{Result} = (V \times C \times D_S)/(W \times D_A) \times 100$$

V = volume of the *Sample solution*, 25 mL
D_S = density of the Solution (g/mL)
W = weight of Solution taken to prepare the *Sample solution* (g)
D_A = density of alcohol (g/mL)

Acceptance criteria: If present, 95.0%–105.0% of the labeled amount of alcohol (C_2H_5OH)

IMPURITIES

• **LIMIT OF AMINES AND AMINE SALTS**
Sample: A quantity of Solution equivalent to 5.0 g of benzalkonium chloride
Analysis: Dissolve the *Sample* by heating carefully (e.g., on top of a steam bath with water as the steam source) in 20 mL of a mixture of methanol and 1 N hydrochloric acid VS (97:3). [NOTE—The mixed solution, however, must not reach the boiling point.] Add 100 mL of isopropyl alcohol, and pass a stream of nitrogen slowly through the solution. Gradually add 12.0 mL of 0.1 N tetrabutylammonium hydroxide VS while recording the potentiometric titration curve.
Acceptance criteria: If the curve shows two inflection points, the volume of titrant added between the two points is NMT 5.0 mL, corresponding to NMT 0.1 mmol/g of amines and amine salts. If the curve shows no point of inflection, the substance being examined does not comply with the test. If the curve shows one point of inflection, repeat the test, but add 3.0 mL of a 25.0 mg/mL solution of dimethyldecylamine in isopropyl alcohol before the titration. If after the addition of 12.0 mL of the titrant, the titration curve shows only one point of inflection, the substance being examined does not comply with the test.

• **LIMIT OF BENZYL ALCOHOL, BENZALDEHYDE, AND (CHLOROMETHYL)BENZENE**
[NOTE—Prepare the solutions immediately before use.]
Solution A: Dissolve 1.09 g of sodium 1-hexanesulfonate and 6.9 g of monobasic sodium phosphate in water in a 1000-mL volumetric flask, adjust with phosphoric acid to a pH of 3.5, and dilute with water to volume.
Solution B: Methanol
Mobile phase: See *Table 4*.

Table 4

Time (min)	Solution A (%)	Solution B (%)
0	80	20
10	80	20
14	50	50
35	50	50
36	20	80
55	20	80
56	80	20
65	80	20

Standard solution A: 0.25 mg/mL of USP Benzyl Alcohol RS in methanol
Standard solution B: 0.075 mg/mL of USP Benzaldehyde RS in methanol
Standard solution C: 0.025 mg/mL of USP Benzyl Alcohol RS in methanol, prepared from *Standard solution A* and methanol
Sample solution: Determine the density of the Solution. Dilute a quantity of the Solution equivalent to 2.5 g of benzalkonium chloride with methanol to 50.0 mL. This solution contains 50 mg/mL of benzalkonium chloride.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 210 nm for benzyl alcohol and (chloromethyl)benzene; UV 257 nm for benzaldehyde
Column: 4.6-mm × 15-cm; 5-µm packing L1
Column temperature: 30°
Flow rate: 1.0 mL/min
Injection volume: 20 µL
System suitability
Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*
[NOTE—See *Table 5* for relative retention times.]

NF Monographs

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Table 5

Name	Relative Retention Time
Benzyl alcohol	1.0
Benzaldehyde	1.3
(Chloromethyl)benzene	2.4

Suitability requirements
Relative standard deviation: NMT 5.0% for benzyl alcohol, *Standard solution A*
Signal-to-noise ratio: NLT 10 for the principal peak, *Standard solution C*
Analysis
Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*
Calculate the content of (chloromethyl)benzene by multiplying the peak area of (chloromethyl)benzene by 1.3. [NOTE—The correction factor is used to adjust for baseline shift.]
Acceptance criteria
Benzyl alcohol: The response of the benzyl alcohol peak from the *Sample solution* is NMT that of the benzyl alcohol peak from *Standard solution A*, corresponding to NMT 0.5%.
Benzaldehyde: The response of the benzaldehyde peak from the *Sample solution* is NMT that of the benzaldehyde peak from *Standard solution B*, corresponding to NMT 0.15%.
(Chloromethyl)benzene: The response of the (chloromethyl)benzene peak from the *Sample solution* is NMT 0.1 times that of the principal peak from *Standard solution A*, corresponding to NMT 0.05%.

SPECIFIC TESTS
- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩: A solution containing less than 5.0% of benzalkonium chloride meets the requirements of the test for absence of *Pseudomonas aeruginosa*.
- **ACIDITY OR ALKALINITY**
Sample solution: Evaporate or dilute with carbon dioxide-free water to prepare a 50-mL solution of 10 mg/mL of benzalkonium chloride in water.
Analysis: To the *Sample solution* add 0.1 mL of bromocresol purple TS.
Acceptance criteria: NMT 0.5 mL of 0.1 N hydrochloric acid or 0.1 N sodium hydroxide is required to change the color of the indicator.

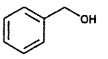
ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers, and prevent contact with metals.
- **LABELING:** Label it to indicate the concentration of benzalkonium chloride, and to indicate the name and quantity of the coloring agent added. The labeling also indicates the concentration of alcohol added.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Benzaldehyde RS
USP Benzalkonium Chloride RS
USP Benzyl Alcohol RS

Benzethonium Chloride—*see Benzethonium Chloride General Monographs*

Benzoic Acid—*see Benzoic Acid General Monographs*

Benzyl Alcohol

Portions of the monograph text that are national *USP* text, and are not part of the harmonized text, are marked with symbols (•,) to specify this fact.



C_7H_8O 108.14
Phenylmethanol [100-51-6].

DEFINITION
Benzyl Alcohol contains NLT 98.0% and NMT the equivalent of 100.5% of phenylmethanol (C_7H_8O).

IDENTIFICATION

Change to read:

- **•A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F.▲** (CN 1-May-2020) On undried specimen,

ASSAY
- **PROCEDURE**
Phenolphthalein solution: Dissolve 0.1 g of phenolphthalein in 80 mL of ethanol (96%), and dilute with water to 100.0 mL. To test for sensitivity, add 100 mL of carbon dioxide-free water to 0.1 mL of the *Phenolphthalein solution*. The solution is colorless. NMT 0.2 mL of 0.02 M sodium hydroxide is required to change the color to pink.
Sample: 0.900 g
Analysis: To the *Sample* add 15 mL of a freshly prepared mixture of dried pyridine and acetic anhydride (7:1), and heat under a reflux condenser on a boiling water bath for 30 min. Cool, and add 25 mL of water. Using 0.25 mL of *Phenolphthalein solution* as the indicator, titrate with 1 M sodium hydroxide VS. Carry out a blank titration.
Calculate the percentage content of phenylmethanol (C_7H_8O):

$$\text{Result} = 10.81 \times (n_1 - n_2)/m$$

n_1 = amount of 1 M sodium hydroxide used for the blank (mL)
n_2 = amount of 1 M sodium hydroxide used for the sample (mL)
m = amount of sample taken (g)

Acceptance criteria: 98.0%–100.5%

IMPURITIES
- **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩: NMT 5
- **RESIDUE ON EVAPORATION**
Analysis: After ensuring that the Benzyl Alcohol complies with the test for *Fats and Fixed Oils, Peroxide Value*, evaporate 10.0 g to dryness in a tared quartz or porcelain crucible or platinum dish on a hot plate at a temperature not exceeding 200°. Ensure that the Benzyl Alcohol does not boil during evaporation. Dry the residue on the hot plate for 1 h, and allow to cool in a desiccator.
Acceptance criteria: The residue weighs NMT 5 mg, corresponding to NMT 0.05%.

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• **ORGANIC IMPURITIES, BENZALDEHYDE, AND OTHER RELATED SUBSTANCES**

Sample solution: Use the Benzyl Alcohol sample under examination.

Standard solution A: Dissolve 0.100 g of ethylbenzene in 10.0 mL of the *Sample solution*. Dilute 2.0 mL of this solution with the *Sample solution* to 20.0 mL.

Standard solution B: Dissolve 2.000 g of dicyclohexyl in 10.0 mL of the *Sample solution*. Dilute 2.0 mL of this solution with the *Sample solution* to 20.0 mL.

Reference solution A (for use in nonparenteral applications): Dissolve 0.750 g of benzaldehyde and 0.500 g of cyclohexylmethanol in the *Sample solution*, and dilute with the *Sample solution* to 25.0 mL. Add 1.0 mL of this solution to a mixture of 2.0 mL of *Standard solution A* and 3.0 mL of *Standard solution B*, and dilute with the *Sample solution* to 20.0 mL.

Reference solution B (for use in parenteral applications): Dissolve 0.250 g of benzaldehyde and 0.500 g of cyclohexylmethanol in the *Sample solution*, and dilute with the *Sample solution* to 25.0 mL. Add 1.0 mL of this solution to a mixture of 2.0 mL of *Standard solution A* and 2.0 mL of *Standard solution B*, and dilute with the *Sample solution* to 20.0 mL.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Carrier: Helium, chromatography grade

Carrier linear velocity: 25 cm/s, at 50°

Detector temperature: 310°

Column: 30-m × 0.32-mm; 0.5-µm film thickness, G16

Temperature

Injector: 200°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
50	5	220	35

System suitability

Sample: For nonparenteral applications, use *Reference solution A*. For parenteral applications, use *Reference solution B*.

[NOTE—The retention time of benzyl alcohol is about 26 min. The relative retention times for ethylbenzene, dicyclohexyl, benzaldehyde, cyclohexylmethanol, and benzyl alcohol are about 0.28, 0.59, 0.68, 0.71, and 1.0, respectively.]

Injection volume: 0.1 µL without air plug

Suitability requirements

Sensitivity: Adjust the sensitivity of the detector so that the height of the peak due to ethylbenzene is NLT 30% of the full scale of the recorder.

Resolution: NLT 3.0 between the peaks corresponding to benzaldehyde and cyclohexylmethanol

Analysis

Samples: *Sample solution* and *Reference solution A* for nonparenteral applications and *Reference solution B* for parenteral applications

Acceptance criteria (nonparenteral applications): If any peaks are present in the chromatogram obtained with the *Sample solution* that have the same retention times as the peaks due to ethyl benzene and dicyclohexyl, subtract the areas of any such peaks from the peak areas at these retention times in the chromatograms of *Reference solution A* or *Reference solution B* (corrected peak areas of ethylbenzene and dicyclohexyl). Any such peaks in the *Sample solution* should be included in the assessments for the total of other peaks.

In the chromatogram obtained with the *Sample solution*, the area of any peak corresponding to benzaldehyde is NMT the difference between the area of the peak due to benzaldehyde in the chromatogram obtained with *Reference solution A* (0.15%) and the area of the peak due to benzaldehyde in the chromatogram obtained with the *Sample solution*.

In the chromatogram obtained with the *Sample solution*, the area of any peak corresponding to cyclohexylmethanol is NMT the difference between the area of the peak due to cyclohexylmethanol in the chromatogram obtained with *Reference solution A* (0.10%) and the area of the peak due to cyclohexylmethanol in the chromatogram obtained with the *Sample solution*.

In the chromatogram obtained with the *Sample solution*, the sum of the areas of any peak with a relative retention time less than that of benzyl alcohol and apart from the peaks due to benzaldehyde and cyclohexylmethanol is NMT four times the area of ethylbenzene in *Reference solution A*, corrected if necessary as described above (0.04%).

In the chromatogram obtained with the *Sample solution*, the sum of the areas of any peak with a relative retention time greater than that of benzyl alcohol is NMT the area of dicyclohexyl in *Reference solution A*, corrected if necessary as described above (0.3%).

Disregard any peak with an area less than 0.01 times that of the peak due to ethylbenzene in the chromatogram of *Reference solution A*, corrected if necessary as described above.

Acceptance criteria (parenteral applications): If any peaks are present in the chromatogram obtained with the *Sample solution* that have the same retention times as the peaks due to ethyl benzene and dicyclohexyl, subtract the areas of any such peaks from the peak areas at these retention times in the chromatograms of *Reference solution A* or *Reference solution B* (corrected peak areas of ethylbenzene and dicyclohexyl). Any such peaks in the *Sample solution* should be included in the assessments for the total of other peaks.

In the chromatogram obtained with the *Sample solution*, the area of any peak corresponding to benzaldehyde is NMT the difference between the area of the peak due to benzaldehyde in the chromatogram obtained with *Reference solution B* (0.05%) and the area of the peak due to benzaldehyde in the chromatogram obtained with the *Sample solution*.

In the chromatogram obtained with the *Sample solution*, the area of any peak corresponding to cyclohexylmethanol is NMT the difference between the area of the peak due to cyclohexylmethanol in the chromatogram obtained with *Reference solution B* (0.10%) and the area of the peak due to cyclohexylmethanol in the chromatogram obtained with the *Sample solution*.

In the chromatogram obtained with the *Sample solution*, the sum of the areas of any peak with a relative retention time less than that of benzyl alcohol and apart from the peaks due to benzaldehyde and cyclohexylmethanol is NMT two times the area of ethylbenzene in *Reference solution B*, corrected if necessary as described above (0.02%).

In the chromatogram obtained with the *Sample solution*, the sum of the areas of any peak with a relative retention time greater than that of benzyl alcohol is NMT the area of dicyclohexyl in *Reference solution B*, corrected if necessary as described above (0.2%).

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Disregard any peak with an area less than 0.01 times that of the peak due to ethylbenzene in the chromatogram of *Reference solution B*, corrected if necessary as described above.

SPECIFIC TESTS

• **ACIDITY**

Phenolphthalein solution: Prepare as directed in the *Assay*.

Analysis: To 10 mL of Benzyl Alcohol add 10 mL of ethanol (96%) and 1 mL of *Phenolphthalein solution*.

Acceptance criteria: NMT 1 mL of 0.1 M sodium hydroxide is required to change the color of the indicator to pink.

Change to read:

• **•CLARITY OF SOLUTION**

[NOTE—The *Sample solution* is to be compared to *Reference suspension 1* in diffused daylight 5 min after preparation of *Reference suspension 1*.]

Hydrazine solution: Transfer 1.0 g of hydrazine sulfate to a 100-mL volumetric flask, dissolve in and dilute with water to volume, and mix. Allow to stand 4–6 h before use.

Methenamine solution: Transfer 2.5 g of methenamine to a 100-mL glass-stoppered flask, add 25.0 mL of water, insert the glass stopper, and mix to dissolve.

Primary opalescent suspension: [NOTE—This suspension is stable for 2 months, provided it is stored in a glass container free from surface defects. The suspension must not adhere to the glass and must be well mixed before use.] Transfer 25.0 mL of *Hydrazine solution* to the *Methenamine solution* in the 100-mL glass-stoppered flask. Mix, and allow to stand for 24 h.

Opalescence standard: [NOTE—This suspension should not be used beyond 24 h after preparation.] Transfer 15.0 mL of the *Primary opalescent suspension* to a 1000-mL volumetric flask, dilute with water to volume, and mix.

Reference suspension 1: Transfer 5.0 mL of the *Opalescence standard* to a 100-mL volumetric flask, and dilute with water to volume.

Reference suspension 2: Transfer 10.0 mL of the *Opalescence standard* to a second 100-mL volumetric flask, and dilute with water to volume.

Sample solution: Dissolve 2.0 g of Benzyl Alcohol in 60 mL of water.

Analysis: Transfer a sufficient portion of the *Sample solution* to a test tube of colorless, transparent, neutral glass with a flat base and an internal diameter of 15–25 mm, to obtain a depth of 40 mm. Similarly transfer portions of *Reference suspension 1*, *Reference suspension 2*, and water to separate matching test tubes. Compare the *Sample solution*, *Reference suspension 1*, *Reference suspension 2*, and water in diffused daylight, viewing vertically against a black background (see ▲*Visual Comparison* ⟨630⟩▲ (CN 1-May-2019)). [NOTE—The diffusion of light must be such that *Reference suspension 1* can readily be distinguished from water, and that *Reference suspension 2* can readily be distinguished from *Reference suspension 1*.]

Acceptance criteria: The *Sample solution* shows the same clarity as that of water, or its opalescence is not more pronounced than that of *Reference suspension 1*.♦

Change to read:

• **•COLOR OF SOLUTION**

Sample solution: Use the *Sample solution* prepared in the test for *Clarity of Solution*.

Analysis: Transfer a sufficient portion of the *Sample solution* to a test tube of colorless, transparent, neutral glass with a flat base and an internal diameter of 15–25 mm, to obtain a depth of 40 mm. Similarly transfer a portion of water to a separate matching test tube. Compare the color of the *Sample solution* with that of water in diffused daylight, viewing vertically against a white background (see ▲*Visual Comparison* ⟨630⟩▲ (CN 1-May-2019)).

Acceptance criteria: The *Sample solution* has the color of water.♦

• **REFRACTIVE INDEX** ⟨831⟩: 1.538–1.541 at 20°

ADDITIONAL REQUIREMENTS

• **•LABELING:** Where Benzyl Alcohol is intended for use in the manufacture of parenteral applications, it is so labeled.♦

• **•PACKAGING AND STORAGE:** Preserve in tight containers, protected from light.♦

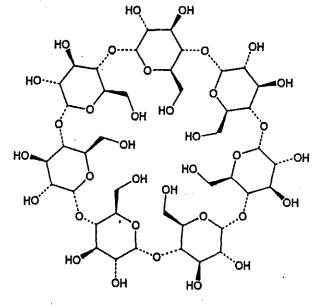
• **• USP REFERENCE STANDARDS**

USP Benzyl Alcohol RS

♦

Benzyl Benzoate—*see Benzyl Benzoate General Monographs*

Betadex



$(C_6H_{10}O_5)_7$ 1134.98

Beta Cyclodextrin [7585-39-9].

DEFINITION

Betadex is a nonreducing cyclic compound composed of seven alpha-(1–4) linked D-glucopyranosyl units. It contains NLT 98.0% and NMT 102.0% of betadex $(C_6H_{10}O_5)_7$, calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

• **A.** **▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K.**▲ (CN 1-May-2020) On undried specimen

• **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

• **C.** It meets the requirements of the test for *Optical Rotation*.

• **D.**

Sample: 0.2 g

Analysis: Mix the *Sample* with 2 mL of iodine TS, warm in a water bath to dissolve, and allow to stand at room temperature.

Acceptance criteria: A yellow-brown precipitate is formed.

ASSAY

• **PROCEDURE**

Mobile phase: Methanol and water (7:93)

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System suitability solution: 0.5 mg/mL of USP Alpha Cyclodextrin RS, 0.5 mg/mL of USP Beta Cyclodextrin RS, and 0.5 mg/mL of USP Gamma Cyclodextrin RS
Standard solution: 1.0 mg/mL of USP Beta Cyclodextrin RS
Sample stock solution: Transfer 250 mg of Betadex to a 25-mL volumetric flask, and dissolve in water with the aid of heat if necessary. Cool, and dilute with water to volume.
Sample solution: 1 mg/mL of Betadex, prepared from the *Sample stock solution*, diluted with water
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Refractive index
Column: 4.6-mm × 15-cm; 5-µm packing L1
Temperatures
Column: 30°
Detector: 40°
Flow rate: 1.5 mL/min
Injection volume: 50 µL
System suitability
Sample: *System suitability solution*
[NOTE—The relative retention times for gamma cyclodextrin, alpha cyclodextrin, and beta cyclodextrin are 0.4, 0.5, and 1.0, respectively.]
Suitability requirements
Resolution: NLT 1.5 between the gamma cyclodextrin and alpha cyclodextrin peaks
Tailing factor: 0.8–2.0 for the three cyclodextrins
Relative standard deviation: NMT 2.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of betadex $(C_6H_{10}O_5)_7$ in the portion of sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP Beta Cyclodextrin RS in the *Standard solution* (mg/mL)
C_U = concentration of Beta Cyclodextrin in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–102.0% on the anhydrous basis

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 0.1%
• **LIMIT OF REDUCING SUGARS**
Cupric solution: 150 mg/mL of cupric sulfate
Tartrate solution: 25 mg/mL of anhydrous sodium carbonate, 25 mg/mL of potassium sodium tartrate, 20 mg/mL of sodium bicarbonate, and 200 mg/mL of anhydrous sodium sulfate
Cupric–tartaric solution: Immediately before use, mix 1 part of *Cupric solution* with 25 parts of *Tartrate solution*.
Ammonium molybdate solution: 100 mg/mL of ammonium molybdate
Disodium arsenate solution: 60 mg/mL of disodium arsenate
Ammonium molybdate reagent: Mix 10 mL of *Disodium arsenate solution*, 50 mL of *Ammonium molybdate solution*, and 90 mL of diluted sulfuric acid, and dilute with water to 200 mL.
Sample solution: Transfer 1.0 g of Betadex (as calculated on the anhydrous basis) to a 100-mL volumetric flask. Dissolve in and dilute with water that has been previously boiled and cooled to room temperature to volume, and mix. To 1 mL of this solution add 1 mL of *Cupric–tartaric solution*. Heat on a water bath for 10 min, then cool to room temperature. Add 10 mL of *Ammonium molybdate reagent*, and allow to stand for 15 min.
Standard stock solution: 20 mg/L of USP Dextrose RS
Standard solution: Prepare at the same time as the *Sample solution*. To 1 mL of the *Standard stock solution* add 1 mL of *Cupric–tartaric solution*. Heat on a water bath for 10 min, then cool to room temperature. Add 10 mL of *Ammonium molybdate reagent*, and allow to stand for 15 min.
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: Vis
Analytical wavelength: 740 nm
Blank: Water
Analysis
Samples: *Sample solution* and *Standard solution*
Acceptance criteria: 0.2%; the absorbance of the *Sample solution* is NMT that of the *Standard solution*.
• **LIMIT OF LIGHT-ABSORBING IMPURITIES**
Sample solution: Transfer 1.0 g of Betadex (as calculated on the anhydrous basis) to a 100-mL volumetric flask. Dissolve in and dilute with water that has been previously boiled and cooled to room temperature to volume. Mix, and pass through a filter of 0.2-µm pore size.
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: UV/Vis
Analytical wavelength: 230–750 nm
Cell: 1 cm
Blank: Water
Analysis
Sample: *Sample solution*
Acceptance criteria
Absorbance:
NMT 0.10, 230–350 nm
NMT 0.05, 350–750 nm
• **RELATED COMPOUNDS**
Mobile phase, System suitability solution, Chromatographic system, and System suitability: Proceed as directed in the *Assay*.
Standard solution: 0.05 mg/mL each of USP Alpha Cyclodextrin RS, USP Beta Cyclodextrin RS, and USP Gamma Cyclodextrin RS from the *System suitability solution*, diluted with water
Sample solution: Use the *Sample stock solution* from the *Assay*.
Analysis
Samples: *Standard solution* and *Sample solution*
Acceptance criteria
Alpha cyclodextrin: 0.25%; the area of any peak corresponding to alpha cyclodextrin is NMT 0.5 times the area of the corresponding peak from the *Standard solution*.
Gamma cyclodextrin: 0.25%; the area of any peak corresponding to gamma cyclodextrin is NMT 0.5 times the area of the corresponding peak from the *Standard solution*.
Other related substances: 0.5%; the sum of all of the peak areas, excluding the alpha cyclodextrin, beta cyclodextrin, gamma cyclodextrin, and artifact peaks is NMT the area of the peak due to betadex (beta cyclodextrin) from the *Standard solution*.

SPECIFIC TESTS

• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g. It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

NF Monographs

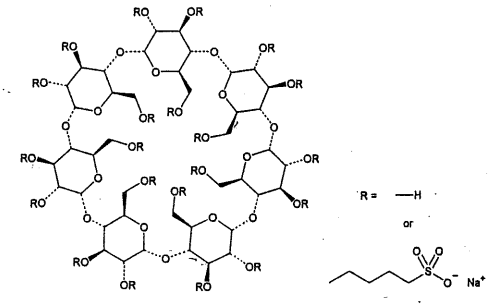
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- **OPTICAL ROTATION,** *Specific Rotation* ⟨781S⟩
  Sample solution: 10 mg/mL in water
  Acceptance criteria: +160° to +164°, determined at 20°
- **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 14.0%
- **COLOR AND CLARITY OF SOLUTION**
  Sample solution: Dissolve 0.2 g in 20.0 mL of freshly boiled and cooled water.
  Acceptance criteria: The resulting solution is clear and colorless.
- **pH** ⟨791⟩
  Sample solution: Add 0.1 mL of a saturated solution of potassium chloride to 10 mL of a 10 mg/mL aqueous solution of Betadex.
  Acceptance criteria: 5.0–8.0

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Alpha Cyclodextrin RS
  USP Beta Cyclodextrin RS
  USP Gamma Cyclodextrin RS
  USP Dextrose RS

Betadex Sulfobutyl Ether Sodium



$C_{42}H_{70-n}O_{35} \cdot (C_4H_8SO_3Na)_n$ 2163 when $n = 6.5$
Beta cyclodextrin sulfobutyl ethers, sodium salts;
Beta cyclodextrin sulfobutyl ether sodium [182410-00-0].

DEFINITION

Betadex Sulfobutyl Ether Sodium is prepared by alkylation of betadex using 1,4-butane sultone under basic conditions. The average degree of substitution in betadex is NLT 6.2 and NMT 6.9. It contains NLT 95.0% and NMT 105.0% of $C_{42}H_{70-n}O_{35} \cdot (C_4H_8SO_3Na)_n$ (n = 6.2–6.9), calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy:* **197K**▲ (CN 1-May-2020)
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.
- **C.** It meets the requirements of the test for *Average Degree of Substitution*.
- **D. IDENTIFICATION TESTS—GENERAL,** *Sodium* ⟨191⟩

ASSAY

- **PROCEDURE**
  Mobile phase: 0.1 M potassium nitrate in a mixture of acetonitrile and water (1:4)
  Standard solution: 10 mg/mL of USP Betadex Sulfobutyl Ether Sodium RS in *Mobile phase*
  Sample solution: 10 mg/mL of Betadex Sulfobutyl Ether Sodium in *Mobile phase*
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: LC
  Detector: Refractive index
  Detector temperature: 35 ± 2°
  Column: 7.8-mm × 30-cm analytical column; packing L37.
  [NOTE—Rinse the column with a solution of acetonitrile and water (1:9) at the completion of the run series.]
  Flow rate: 1.0 mL/min
  Injection size: 20 µL
  System suitability
  Sample: *Standard solution*
  Suitability requirements
  Relative standard deviation: NMT 2.0%
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Calculate the percentage of betadex sulfobutyl ether sodium $[C_{42}H_{70-n}O_{35} \cdot (C_4H_8SO_3Na)_n]$ in the portion of Betadex Sulfobutyl Ether Sodium taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response for betadex sulfobutyl ether sodium from the *Sample solution*
r_S = peak response for betadex sulfobutyl ether sodium from the *Standard solution*
C_S = concentration of USP Betadex Sulfobutyl Ether Sodium RS in the *Standard solution* (mg/mL)
C_U = concentration of Betadex Sulfobutyl Ether Sodium in the *Sample solution* (mg/mL)

Acceptance criteria: 95.0%–105.0% on the anhydrous basis

IMPURITIES

- **LIMIT OF BETA CYCLODEXTRIN (BETADEX)**
  Solution A: 25 mM sodium hydroxide
  Solution B: 250 mM sodium hydroxide and 1 M potassium nitrate
  Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
4	100	0
5	0	100
10	0	100
11	100	0
20	100	0

Standard solution: 2 µg/mL of USP Beta Cyclodextrin RS
Sample solution: 2 mg/mL of Betadex Sulfobutyl Ether Sodium
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability* and *Ion Chromatography* ⟨1065⟩.)
Mode: IC
Detector: Pulsed amperometry (amperometric cell with gold working electrode and silver reference electrode)
Column
Guard: 4.0-mm × 5-cm anion-exchange; packing L61

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Analytical: 4.0-mm × 25-cm anion-exchange; packing L61
Column temperature: 50 ± 2°
Flow rate: 1.0 mL/min
Injection size: 20 µL
Waveform for pulsed amperometric detector: See *Table 2*.

Table 2

Time (s)	Voltage (V)
0.00	0.10
0.30	Start integration
0.50	0.10
0.50	Stop integration
0.51	0.60
0.59	0.60
0.60	−0.60
0.65	−0.60

System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 5.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of beta cyclodextrin (betadex) in the portion of Betadex Sulfobutyl Ether Sodium taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak response for beta cyclodextrin from the *Sample solution*
r_S = peak response for beta cyclodextrin from the *Standard solution*
C_S = concentration of USP Beta Cyclodextrin RS in the *Standard solution* (µg/mL)
C_U = concentration of Betadex Sulfobutyl Ether Sodium in the *Sample solution* (mg/mL)
F = conversion factor (10^{-3} mg/µg)

Acceptance criteria: NMT 0.1%

• **LIMIT OF 1,4-BUTANE SULTONE**
Internal standard solution: 0.25 µg/mL of diethyl sulfone
Standard stock solution A: 0.5 µg/mL of 1,4-butane sultone
Standard stock solution B: 1.0 µg/mL of 1,4-butane sultone
Standard stock solution C: 2.0 µg/mL of 1,4-butane sultone
Sample stock solution: 250 mg/mL of Betadex Sulfobutyl Ether Sodium in the *Internal standard solution*
Blank solution, and Sample solutions A, B, C, and D: Follow *Table 3* to place the quantities of *Internal standard solution*, each *Standard stock solution*, *Sample stock solution*, water, or methylene chloride in each glass test tube with a stopper. [NOTE—A screw-capped, 10-mL test tube is suitable.] Mix on a vortex mixer each test tube for 30 s, and allow it stand for at least 5 min or until complete separation of the phase. Extract the organic phase into a GC vial and seal. [NOTE—With great care take the minimum possible amount of aqueous phase.] Added quantities of 1,4-butane sultone in *Sample solutions A, B, C,* and *D* are 0.5, 1.0, 2.0, and 0 µg, respectively.

Table 3

Sample Name	Solution 1 Added (mL)	Solution 2 Added (mL)	Methylene Chloride Added (mL)
Blank solution	Internal standard solution, 4.0	Water, 1.0	1.0
Sample solution A	Sample stock solution, 4.0	Standard stock solution A, 1.0	1.0
Sample solution B	Sample stock solution, 4.0	Standard stock solution B, 1.0	1.0
Sample solution C	Sample stock solution, 4.0	Standard stock solution C, 1.0	1.0
Sample solution D	Sample stock solution, 4.0	Water, 1.0	1.0

[NOTE—Prepare immediately before use.]
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.32-mm × 25-m fused-silica capillary column; 0.5-µm layer of phase G46
Temperature
Detector: 270°
Injection port: 200°
Column: See the temperature program in *Table 4*.

Table 4

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
100	10	200	—
200	35	270	5

Carrier gas: Helium, typically at 12 psi inlet pressure
Injection size: 1.0 µL
Injection type: Splitless injection for 0.5 min, then split at 50 mL/min. [NOTE—The use of an appropriate splitless injection liner is recommended.]
System suitability
Sample: *Sample solution B*
[NOTE—The relative retention times for diethyl sulfone and 1,4-butane sultone are 0.7 and 1.0, respectively.]
Suitability requirements
Relative standard deviation: NMT 10.0%
Analysis
Samples: *Blank solution, Sample solutions A, B, C,* and *D*
Correct the ratio of peak responses of the 1,4-butane sultone to diethyl sulfone in *Sample solution A, B, C,* or *D* by subtracting the ratio of peak responses of the 1,4-butane sultone to ethyl sulfone in the *Blank solution*. Plot the corrected ratio of peak response of 1,4-butane sultone to peak response of diethyl sulfone in *Sample solution A, B, C* or *D*, versus the added quantity, in µg, of 1,4-butane sultone. Extrapolate the line joining the points on the graph until it meets the quantity axis. The distance between this point and the intersection of the axes represents the quantity of 1,4-butane sultone, *A*, in µg, in the 4-mL portion of *Sample stock solution*. Calculate the content of 1,4-butane sultone in the portion of Betadex Sulfobutyl Ether Sodium taken:

$$\text{Result} = A/(V_{Ext} \times C_U \times F)$$

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A = determined above
V_{Ext} = volume of the *Sample stock solution* used in the extraction step, 4.0 mL
C_U = concentration of Betadex Sulfobutyl Ether Sodium in the *Sample stock solution* (mg/mL)
F = conversion factor (10^{-3} g/mg)

Acceptance criteria: NMT 0.5 ppm

• **LIMIT OF SODIUM CHLORIDE, 4-HYDROXYBUTANE-1-SULFONIC ACID, AND BIS(4-SULFOBUTYL) ETHER DISODIUM**

Solution A: 5 mM sodium hydroxide, degas in a closed vessel for 15 min
Solution B: 25 mM sodium hydroxide, degas in a closed vessel for 15 min
Mobile phase: See *Table 5*.

Table 5

Time (min)	Solution A (%)	Solution B (%)
0	100	0
4	100	0
10	70	30
24	70	30
25	100	0
40	100	0

Column wash solution A: 50 mM sodium citrate
Column wash solution B: 150 mM sodium hydroxide
Standard solution: Prepare a solution having known concentrations of 8 µg/mL of USP Sodium Chloride RS, 4 µg/mL of 4-hydroxybutane-1-sulfonic acid, and 4 µg/mL of bis(4-sulfobutyl) ether disodium.
Sample solution: 4 mg/mL of Betadex Sulfobutyl Ether Sodium
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability* and *Ion Chromatography* ⟨1065⟩.)
Mode: IC
Detector: Conductivity
Range: 30 µS
Current: 100 mA
Column: [NOTE—At the end of each run, clean the column using *Column wash solution A* at a flow rate of 1 mL/min for 35 min then using *Column wash solution B* at the same flow rate for 35 min.]
Guard: 4.0-mm × 5.0-cm anion-exchange; packing L61
Analytical: 4.0-mm × 25-cm anion-exchange; packing L61
Column temperature: 30°
Suppressor: Micromembrane anion autosuppressor[1] or a suitable chemical suppression system
Suppressant: Autosuppression
Flow rate: 1.0 mL/min
Injection size: 20 µL
System suitability
Sample: *Standard solution*
[NOTE—Relative retention times are provided for information only. The relative retention times for 4-hydroxybutane-1-sulfonate ion, chloride ion, and bis(sulfobutyl) ether ion are 1.0, 1.4, and 8.6, respectively.]

[1] Available as Anion Self-Regenerating Suppressor (ASRS) from Dionex Inc., or equivalent.

Suitability requirements
Resolution: NLT 2.0
Relative standard deviation: NMT 10.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of sodium chloride, 4-hydroxybutane-1-sulfonic acid, or bis(sulfobutyl) ether disodium in the portion of Betadex Sulfobutyl Ether Sodium taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak response for sodium chloride, 4-hydroxybutane-1-sulfonic acid, or bis(sulfobutyl) ether disodium from the *Sample solution*
r_S = peak response for sodium chloride, 4-hydroxybutane-1-sulfonic acid, or bis(sulfobutyl) ether disodium from the *Standard solution*
C_S = concentration of sodium chloride, 4-hydroxybutane-1-sulfonic acid, or bis(sulfobutyl) ether disodium in the *Standard solution* (µg/mL)
C_U = concentration of Betadex Sulfobutyl Ether Sodium in the *Sample solution* (mg/mL)
F = conversion factor (10^{-3} mg/µg)

Acceptance criteria
Sodium chloride: NMT 0.2%
4-Hydroxybutane-1-sulfonic acid: NMT 0.09%
Bis(sulfobutyl) ether disodium: NMT 0.05%

SPECIFIC TESTS

• **BACTERIAL ENDOTOXINS TEST** ⟨85⟩**:** The level of bacterial endotoxins is such that the requirement under the relevant dosage form monograph(s) in which Betadex Sulfobutyl Ether Sodium is used can be met. Where the label states that Betadex Sulfobutyl Ether Sodium must be subjected to further processing during the preparation of injectable dosage forms, the level of bacterial endotoxins is such that the requirement under the relevant dosage form monograph(s) in which Betadex Sulfobutyl Ether Sodium is used can be met.

• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic microbial count does not exceed 100 cfu/g, and the total combined molds and yeasts count does not exceed 50 cfu/g. It meets the requirements of the test for absence of *Escherichia coli*.

• **CLARITY OF SOLUTION**
Sample solution: 30% (w/v) solution
Analysis: Examine the *Sample solution* using a light box against white and black backgrounds, and record the presence of any haze, fluorescence, fibers, specks, or other foreign matter.
Acceptance criteria: The solution is clear, and essentially free from particles of foreign matter.

• **AVERAGE DEGREE OF SUBSTITUTION**
Run electrolyte: 30 mM benzoic acid and adjusted to a pH that is suitable for the instrument used by addition of 100 mM tris(hydroxymethyl) aminomethane buffer. [NOTE—Due to variation between capillaries, a single universally applicable electrolyte pH is not specified. Instead, the optimal pH associated with each individual capillary should be determined according to the instrumental manual.]
Standard solution: 10 mg/mL of USP Betadex Sulfobutyl Ether Sodium RS

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Sample solution: 10 mg/mL of Betadex Sulfobutyl Ether Sodium

Capillary rinsing procedure: Use separate run electrolyte vials for capillary rinse and sample analysis. Perform pre-analysis rinses on a daily basis before each analysis: rinse the capillary with 0.1 N sodium hydroxide for 30 min, with water for NLT 2 h, and with *Run electrolyte* for NLT 1 h. Perform pre-injection rinses prior to each injection as follows. Rinse the capillary with 0.1 N sodium hydroxide for NLT 1 min, and with *Run electrolyte* for NLT 3 min. If a new capillary is being used, in addition to the regular rinses described above, a new capillary requires rinsing before its first use. Rinse the new capillary with 1 M sodium hydroxide for 1 h, followed by a 2-h water rinse.

Electrophoretic system

(See *Capillary Electrophoresis* ⟨1053⟩.)

Mode: High-performance CE

Detector: Inverse UV 200 nm, with a bandwidth of 20 nm. [NOTE—A detection wavelength of 205 nm with a bandwidth of 10 nm may be used as an alternative.]

Column: 50-µm × 50-cm fused silica column

Column temperature: 25°

Applied voltage: 0.00 to +30.00 kV linear ramp over 10 min, then at 30 kV for a further 20 min

Injection size: Equal volumes at 0.5 psi for 10 s

System suitability

Sample: *Standard solution*

[NOTE—See *Table 6* for the approximate relative migration times for betadex sulfobutyl ether sodium peaks I–X (betadex sulfobutyl ether sodium peaks I, II, III, ..., X, contains beta cyclodextrin molecule with 1, 2, 3, ..., 10 sulfobutyl substituent(s), respectively). The relative migration times are for informational purposes only to aid in peak identification.]

Table 6

Betadex Sulfobutyl Ether Sodium Peaks I–X	Relative Migration Time
I	0.58
II	0.63
III	0.69
IV	0.77
V	0.83
VI	0.91
VII	1.00
VIII	1.10
IX	1.20
X	1.30

Suitability requirements

Resolution: NLT 0.9, between betadex sulfobutyl ether sodium peak IX and betadex sulfobutyl ether sodium peak X

Analysis

Samples: *Run electrolyte*, water, *Standard solution*, and *Sample solution*

Inject the *Standard solution* and *Sample solution* by applying differential pressure of 0.5 psi, equivalent to 34 mbar, for 10 s, followed by injection of *Run electrolyte* at 0.5 psi for 2 s. [NOTE—Pressure injections should be made with a vial of water or *Run electrolyte* at the outlet end of the capillary.]

Record the electropherograms, and measure the peak responses for the individual betadex sulfobutyl ether sodium peaks (I to X). Calculate the corrected peak area, A_i, for each peak in the eletropherogram:

$$\text{Corrected Peak Area } A_i = \frac{\text{Peak Area} \times \text{Effective Capillary Length (cm)}}{\text{Migration Time}}$$

Normalize the corrected peak areas by presenting each as a percentage of the total corrected substitution envelope area:

$$\text{Normalized Area, } NA_i = \frac{A_i}{\sum_{i=1}^{n} A_i} \times 100$$

n = highest level of substitution

Determine the average degree of substitution:

$$\text{Average Degree of Substitution} = \frac{\sum_{i=1}^{n} (\text{Level of Substitution for Peak} \times NA_i)}{100}$$

Acceptance criteria: 6.2–6.9 for average degree of substitution

For each of betadex sulfobutyl ether sodium peaks I–X, see limit range (% peak area) in *Table 7*.

Table 7

Betadex Sulfobutyl Ether Sodium Peaks I–X	Limit Range (% Peak Area)
I	0–0.3
II	0–0.9
III	0.5–5.0
IV	2.0–10.0
V	10.0–20.0
VI	15.0–25.0
VII	20.0–30.0
VIII	10.0–25.0
IX	2.0–12.0
X	0–4.0

- **pH** ⟨791⟩: 4.0–6.8, in a 30% (w/v) solution in carbon dioxide-free water
- **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 10.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at room temperature. Protect from moisture.
- **LABELING:** Label it to indicate its use in the manufacture of injectable dosage forms.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Beta Cyclodextrin RS
  USP Betadex Sulfobutyl Ether Sodium RS
  USP Sodium Chloride RS

Boric Acid

H_3BO_3 61.83

Boric acid (H_3BO_3) [10043-35-3].

NF Monographs

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DEFINITION
Boric Acid contains NLT 99.5% and NMT 100.5% of boric acid (H_3BO_3), calculated on the dried basis.

IDENTIFICATION
• **A. IDENTIFICATION TESTS—GENERAL,** *Borate* ⟨191⟩**:** Meets the requirements

ASSAY
• **PROCEDURE**
Sample solution: Dissolve 2 g of Boric Acid in 100 mL of a mixture of glycerin and water (1:1), previously neutralized to phenolphthalein TS.
Analysis: Titrate the *Sample solution* with 1 N sodium hydroxide VS. Discharge the pink color by the addition of 50 mL of glycerin, neutralized to phenolphthalein TS, and continue the titration until the pink color reappears. Each mL of 1 N sodium hydroxide is equivalent to 61.83 mg of H_3BO_3.
Acceptance criteria: 99.5%–100.5% on the dried basis

SPECIFIC TESTS
• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry over silica gel for 5 h.
Acceptance criteria: NMT 0.5%
• **SOLUBILITY IN ALCOHOL**
Sample solution: 1 g in 10 mL of boiling alcohol
Acceptance criteria: It dissolves completely.
• **COMPLETENESS OF SOLUTION**
Sample solution: 1 g in 25 mL of water
Acceptance criteria: It produces a clear solution.

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers.
• **LABELING:** Label the container with a warning that it is not for internal use.

Butane

C_4H_{10} 58.12
n-Butane [106-97-8].

DEFINITION
Butane contains NLT 97.0% of butane (C_4H_{10}).
[**CAUTION**—Butane is highly flammable and explosive.]

IDENTIFICATION
• **A. INFRARED ABSORPTION:** Exhibits maxima, among others, at the following wavelengths, in µm: 3.4 (vs), 6.8 (s), 7.2 (m), and 10.4 (m)
• **B.**
Sample: Use an empty stainless steel cylinder equipped with a stainless steel valve, having a capacity of NLT 200 mL, and a pressure rating of 240 psi or more. Dry the cylinder with the valve open at 110° for 2 h, and evacuate the hot cylinder to less than 1 mm of mercury. Close the valve, cool, and weigh. Connect one end of a charging line tightly to the butane container and the other end loosely to the empty cylinder. Carefully open the butane container, and allow the butane to flush out the charging line through the loose connection. Avoid excessive flushing, which causes moisture to freeze in the charging line and connections. Tighten the fitting on the empty cylinder, and open the empty cylinder valve, allowing the butane to flow into the evacuated cylinder. Continue sampling until the desired amount of butane is obtained, then close the butane container valve, and finally close the sample cylinder valve. [**CAUTION**—Do not overload the sample cylinder; hydraulic expansion due to temperature change can cause overloaded cylinders to explode.] Weigh the charged sample cylinder, and determine the weight.
Analysis: Determine the vapor pressure of the *Sample* at 21° by means of a suitable pressure gauge.
Acceptance criteria: 205–235 kPa absolute (30–34 psia)

ASSAY
• **PROCEDURE**
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Thermal conductivity
Column: 3-mm × 6-m aluminum; packed with 10 weight percent of liquid phase G30 on support S1D
Column temperature: 33°
Carrier gas: Helium
Flow rate: 50 mL/min
Injection volume: 2 µL
System suitability
Sample: *n*-Butane
Suitability requirements: The peak responses of *n*-butane in the chromatograms from duplicate determinations agree within 1%.
Analysis
Samples: Connect one Butane cylinder to the chromatograph through a suitable sampling valve and a flow control valve downstream from the sampling valve. Flush the liquid specimen through the sampling valve, taking care to avoid entrapment of gas or air in the sampling valve.
Calculate the purity by dividing 100 times the *n*-butane response by the sum of all of the responses.
Acceptance criteria: NLT 97.0%

SPECIFIC TESTS
• **HIGH-BOILING RESIDUES**
Sample: Use the *Sample* from *Identification* test *B*.
Analysis: Prepare a cooling coil from copper tubing (about 6-mm outside diameter × about 6.1-m long) to fit into a vacuum-jacketed flask. Immerse the cooling coil in a mixture of dry ice and acetone in a vacuum-jacketed flask, and connect one end of the tubing to the *Sample*. Carefully open the sample cylinder valve, flush the cooling coil with about 50 mL of the *Sample*, and discard this portion of liquefied sample. Continue delivering liquefied sample from the cooling coil, and collect it in a previously chilled 1000-mL sedimentation cone until the cone is filled to the 1000-mL mark. Allow the sample to evaporate, using a warm water bath maintained at about 40° to reduce evaporating time. When all of the liquid has evaporated, rinse the sedimentation cone with two 50-mL portions of pentane, and combine the rinsings in a tared 150-mL evaporating dish. Transfer 100 mL of the pentane solvent to a second tared 150-mL evaporating dish, place both evaporating dishes on a water bath, evaporate to dryness, and heat the dishes in an oven at 100° for 60 min. Cool the dishes in a desiccator, and weigh. Repeat the heating for 15-min periods until successive weighings are within 0.1 mg, and calculate the weight of the residue obtained from the *Sample* as the difference between the weights of the residues in the two evaporating dishes.
Acceptance criteria: NMT 5 µg/mL
• **ACIDITY OF RESIDUE**
Sample solution: Add 10 mL of water to the residue obtained in the test for *High-Boiling Residues*.
Analysis: Mix the *Sample solution* by swirling for 30 s, add 2 drops of methyl orange TS, insert the stopper in the tube, and shake vigorously.

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Acceptance criteria: No pink or red color appears in the aqueous layer.

• **LIMIT OF SULFUR COMPOUNDS**

Analysis: Carefully open the container valve to produce a moderate flow of gas. Do not direct the gas stream toward the face, but deflect a portion of the stream toward the nose.

Acceptance criteria: The odor is free from the characteristic odor of sulfur compounds.

• **WATER DETERMINATION** ⟨921⟩

Sample: 100 g of the *Sample* from *Identification* test *B*

Analysis: Proceed as directed in the chapter with the following modifications. (a) Provide the closed-system titrating vessel with an opening through which passes a coarse-porosity gas dispersion tube connected to a sampling cylinder. (b) Dilute the *Reagent* with anhydrous methanol to give a water equivalence factor of 0.2–1.0 mg/mL; age this diluted solution for NLT 16 h before standardization. (c) Introduce the *Sample* into the titration vessel through the gas dispersion tube at a rate of about 100 mL/min; if necessary, heat the sample cylinder gently to maintain this flow rate.

Acceptance criteria: NMT 0.001%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight cylinders, and prevent exposure to excessive heat.

Butyl Alcohol



$CH_3CH_2CH_2CH_2OH$ 74.12

1-Butanol;
1-Butyl alcohol;
n-Butanol;
n-Butyl alcohol [71-36-3].

DEFINITION

Butyl Alcohol is *n*-butyl alcohol. It contains NLT 99.5% of *n*-butyl alcohol ($C_4H_{10}O$).

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F**▲ (CN 1-May-2020)

• **B.** The retention time of the major peak from the *Sample solution* corresponds to the 1-butanol peak from the *System suitability solution*, as obtained in the *Assay*.

ASSAY

• **PROCEDURE**

System suitability solution: USP 1-Butanol RS and USP 2-Methyl-1-Propanol RS (1:1)

Reference solution: 0.1% of Butyl Alcohol in water

Sample solution: Butyl Alcohol (neat)

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.53-mm × 30-m; coated with a 3.0-µm thickness of phase G43

Temperatures

Detector: 250°

Injection port: 140°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
40	—	40	20
40	10	240	20

Carrier gas: Helium

Flow rate: 4.8–4.9 mL/min

Injection volume: 1 µL

Injection type: Split injection, split ratio is 30:1

[NOTE—A needle wash with the *Sample solution* is recommended to minimize the carry-over.]

System suitability

Sample: *System suitability solution*

[NOTE—The 2-methyl-1-propanol peak typically shows at about 11 min, and the 1-butanol peak at about 15 min. See *Table 2*.]

Table 2

Component	Relative Retention Time (RRT)
2-Methyl-1-propanol	0.7
1-Butanol	1.0

System suitability requirements

Resolution: NLT 2.0 between 2-methyl-1-propanol and 1-butanol

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Reference solution* and *Sample solution*

Calculate the percentage of butyl alcohol in the portion of sample taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of butyl alcohol

r_T = sum of all the peaks except the peaks each of which with an area less than 0.1 times the area of the major peak from the *Reference solution*

Acceptance criteria: NLT 99.5%

IMPURITIES

• **LIMIT OF BUTYRALDEHYDE, 2-BUTANOL, ISOBUTYL ALCOHOL (2-METHYL-1-PROPANOL), AND BUTYL ETHER**

Reference solution: 0.1% of Butyl Alcohol in water

Standard solution: 0.2% of USP Butyraldehyde RS, 0.2% of butyl ether, 0.1% of USP 2-Methyl-1-Propanol RS, and 0.1% of USP 2-Butanol RS in Butyl Alcohol

Sample solution and **Chromatographic system:** Proceed as directed in the *Assay*.

System suitability

Sample: *Standard solution*

[NOTE—See *Table 3*.]

Table 3

Component	Relative Retention Time (RRT)
Butyraldehyde	0.45
2-Butanol	0.5
2-Methyl-1-propanol	0.7

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Table 3 *(continued)*

Component	Relative Retention Time (RRT)
1-Butanol	1.0
Butyl ether	1.8

System suitability requirements
Resolution: NLT 1.5 between all adjacent peaks
Analysis
Samples: *Reference solution*, *Standard solution*, and *Sample solution*
If any peaks are present in the chromatogram from the *Sample solution* that have the same retention times as the peaks due to butyraldehyde, 2-butanol, isobutyl alcohol (2-methyl-1-propanol), and butyl ether, subtract the areas of any such peaks from the peak areas at these retention times in the chromatogram from the *Standard solution*.

$$\text{Result } (\Delta r) = r_S - r_U$$

r_S = peak response of each individual impurity (butyraldehyde, 2-butanol, isobutyl alcohol, or butyl ether) in the *Standard solution*
r_U = peak response of each individual impurity (butyraldehyde, 2-butanol, isobutyl alcohol, or butyl ether), if present, in the *Sample solution*

Acceptance criteria: See *Table 4*. Disregard any peak with an area less than 0.1 times the area of the major peak from the *Reference solution*, corresponding to 0.01%.

Table 4

Impurity	Percentage (%)
Butyraldehyde	In the chromatogram from the *Sample solution*, the area of any peak corresponding to butyraldehyde, r_U, is NMT half of the difference (Δr) between the area of the peak due to butyraldehyde in the chromatogram from the *Standard solution* and the area of the peak due to butyraldehyde in the chromatogram from the *Sample solution*, corresponding to NMT 0.1%.
2-Butanol	In the chromatogram from the *Sample solution*, the area of any peak corresponding to 2-butanol, r_U, is NMT the difference (Δr) between the area of the peak due to 2-butanol in the chromatogram from the *Standard solution* and the area of the peak due to 2-butanol in the chromatogram from the *Sample solution*, corresponding to NMT 0.1%.
Isobutyl alcohol (2-Methyl-1-propanol)	In the chromatogram from the *Sample solution*, the area of any peak corresponding to 2-methyl-1-propanol, r_U, is NMT the difference (Δr) between the area of the peak due to 2-methyl-1-propanol in the chromatogram from the *Standard solution* and the area of the peak due to 2-methyl-1-propanol in the chromatogram from the *Sample solution*, corresponding to NMT 0.1%.
Butyl ether	In the chromatogram from the *Sample solution*, the area of any peak corresponding to butyl ether, r_U, is NMT the difference (Δr) between the area of the peak due to butyl ether in the chromatogram from the *Standard solution* and the area of the peak due to butyl ether in the chromatogram from the *Sample solution*, corresponding to NMT 0.2%.

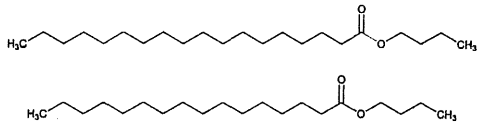
SPECIFIC TESTS

- **ACIDITY**
  Sample: 74 mL (60 g)
  Analysis: Titrate the *Sample* with 0.020 N alcoholic potassium hydroxide, using phenolphthalein TS as the indicator, until a pink color persists for NLT 15 s.
  Acceptance criteria: NMT 2.5 mL is consumed.
- **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 0.1%
- **LIMIT OF NONVOLATILE RESIDUE**
  Sample: 100 mL
  Analysis: Evaporate the *Sample* in a tared porcelain dish on a steam bath, and dry at 105° for 30 min.
  Acceptance criteria: The weight of the residue does not exceed 4 mg (0.004%).

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and prevent exposure to excessive heat.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Butyraldehyde RS
  USP 1-Butanol RS
  USP 2-Butanol RS
  USP 2-Methyl-1-Propanol RS

Butyl Palmitostearate



DEFINITION

Butyl Palmitostearate is a mixture of the butyl ester of stearic acid ($C_{18}H_{36}O_2$) and the butyl ester of palmitic acid ($C_{16}H_{32}O_2$). It contains 40.0%–80.0% of butyl stearate ($C_{22}H_{44}O_2$). The total percentage of butyl stearate ($C_{22}H_{44}O_2$) and butyl palmitate ($C_{20}H_{40}O_2$) is NLT 90.0%.

IDENTIFICATION

- **A.** It meets the requirements of the test for *Content of Butyl Palmitate and Butyl Stearate*.

ASSAY

- **CONTENT OF BUTYL PALMITATE AND BUTYL STEARATE**
  Standard solution A: 2.0 mg/mL of USP Butyl Stearate RS in chloroform
  Standard solution B: 1.0 mg/mL of USP Butyl Palmitate RS in chloroform
  System suitability solution: 1.4 µg/mL of USP Butyl Stearate RS and 1.0 µg/mL of USP Butyl Palmitate RS in chloroform, prepared from *Standard solution A*, *Standard solution B*, and chloroform
  Sample solution: 2.0 mg/mL of Butyl Palmitostearate in chloroform
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: GC
  Detector: Flame ionization
  Column: 0.32-mm × 30-m capillary; bonded with a 0.25-µm layer of thickness of phase G27
  Temperatures
  Detector: 280°
  Injection port: 280°
  Column: See *Table 1*.

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Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
60	—	60	2
60	4	250	20.5

Carrier gas: Helium
Flow rate: 1.4 mL/min
Injection volume: 1 µL
Injection type: Split injection, split ratio is 5:1
System suitability
Samples: *Standard solution A* and *System suitability solution*
[NOTE—The relative retention times for butyl palmitate and butyl stearate are 0.91 and 1.00, respectively.]
System suitability requirements
Resolution: NLT 20 between butyl palmitate and butyl stearate, *System suitability solution*
Tailing factor: NMT 2.0 for the butyl stearate peak, *System suitability solution*
Relative standard deviation: NMT 3.0% for the butyl stearate peak, *Standard solution A*
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Identify the butyl palmitate and butyl stearate peaks in the *Sample solution* based on those in *Standard solution A* and *Standard solution B*.
Calculate the percentage of butyl palmitate or butyl stearate in the portion of Butyl Palmitostearate taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of butyl palmitate or butyl stearate from the *Sample solution*
r_S = peak response of butyl palmitate from *Standard solution B* or peak response of butyl stearate from *Standard solution A*
C_S = concentration of USP Butyl Palmitate RS in *Standard solution B* or concentration of USP Butyl Stearate RS in *Standard solution A*
C_U = concentration of Butyl Palmitostearate in the *Sample solution*

Acceptance criteria
Content of butyl stearate: 40.0%–80.0%
Sum of butyl stearate and butyl palmitate: NLT 90.0%

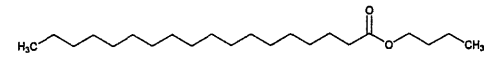
SPECIFIC TESTS

- **MELTING RANGE OR TEMPERATURE,** *Class II* ⟨741⟩**:** 17°–24°
- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩**:** NMT 0.5
- **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩**:** NMT 1.0
- **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩**:** 165–180
- **WATER DETERMINATION,** *Method Ia* ⟨921⟩**:** NMT 0.5%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. Store at room temperature. Keep away from heat and sources of ignition.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Butyl Palmitate RS
USP Butyl Stearate RS

Butyl Stearate



$C_{22}H_{44}O_2$ 340.59
Octadecanoic acid, butyl ester;
Butyl octadecanoate [123-95-5].

DEFINITION

Butyl Stearate consists of the esters of butyl alcohol and fatty acids composed chiefly of stearic acid ($C_{18}H_{36}O_2$) and a minor amount of palmitic acid ($C_{16}H_{32}O_2$). It contains NLT 90.0% of butyl stearate [$C_{22}H_{44}O_2$ (M_W 340.59)], and the total of butyl stearate ($C_{22}H_{44}O_2$) and butyl palmitate [$C_{20}H_{40}O_2$ (M_W 312.53)] is NLT 96.0%.

IDENTIFICATION

- **A.** It meets the requirements of the test for *Content of Butyl Palmitate and Butyl Stearate*.

ASSAY

- **CONTENT OF BUTYL PALMITATE AND BUTYL STEARATE**
Standard solution A: 2.0 mg/mL of USP Butyl Stearate RS in chloroform
Standard solution B: 0.2 mg/mL of USP Butyl Palmitate RS in chloroform
System suitability solution: 1.4 µg/mL of USP Butyl Stearate RS and 1.0 µg/mL of USP Butyl Palmitate RS in chloroform, prepared from *Standard solution A*, *Standard solution B*, and chloroform
Sample solution: 2.0 mg/mL of Butyl Stearate in chloroform
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.32-mm × 30-m capillary; bonded with a 0.25-µm layer of thickness of phase G27
Temperatures
Detector: 280°
Injection port: 280°
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
60	—	60	2
60	4	250	20.5

Carrier gas: Helium
Flow rate: 1.4 mL/min
Injection volume: 1 µL
Injection type: Split injection, split ratio is 5:1.
System suitability
Samples: *System suitability solution* and *Standard solution A*
[NOTE—The relative retention times for butyl palmitate and butyl stearate are 0.91 and 1.00, respectively.]
System suitability requirements
Resolution: NLT 20 between butyl palmitate and butyl stearate, *System suitability solution*
Tailing factor: NMT 2.0 for the butyl stearate peak, *System suitability solution*
Relative standard deviation: NMT 3.0% for the butyl stearate peak, *Standard solution A*
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

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Identify the butyl palmitate and butyl stearate peaks in the *Sample solution* based on those in *Standard solution A* and *Standard solution B*.
Calculate the percentage of butyl palmitate or butyl stearate in the portion of Butyl Stearate taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

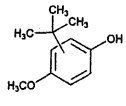
r_U = peak response of butyl palmitate or butyl stearate from the *Sample solution*
r_S = peak response of butyl palmitate from *Standard solution B* or peak response of butyl stearate from *Standard solution A*
C_S = concentration of USP Butyl Palmitate RS in *Standard solution B* or concentration of USP Butyl Stearate RS in *Standard solution A*
C_U = concentration of Butyl Stearate in the *Sample solution*

Acceptance criteria: NLT 90.0% of butyl stearate and NLT 96.0% for the sum of butyl stearate and butyl palmitate

SPECIFIC TESTS
- **MELTING RANGE OR TEMPERATURE,** *Class II* ⟨741⟩**:** 19°–24°
- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩**:** NMT 0.5
- **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩**:** NMT 1.0
- **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩**:** 165–180
- **WATER DETERMINATION,** *Method Ia* ⟨921⟩**:** NMT 0.5%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers. Store at room temperature. Keep away from heat and sources of ignition.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Butyl Palmitate RS
  USP Butyl Stearate RS

Butylated Hydroxyanisole



$C_{11}H_{16}O_2$ 180.24
Phenol, (1,1-dimethylethyl)-4-methoxy-;
tert-Butyl-4-methoxyphenol [25013-16-5].

DEFINITION
Butylated Hydroxyanisole is predominantly 3-*tert*-butyl-4-hydroxyanisole, with varying amounts of 2-*tert*-butyl-4-hydroxyanisole. It contains NLT 98.5% of butylated hydroxyanisole ($C_{11}H_{16}O_2$) as a sum of the two isomers.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197A**▲ (CN 1-May-2020)
  Analysis: Determine the position of the most intense 3-*tert*-butyl-4-hydroxyanisole peaks within 10 cm^{-1} of 682, 815, 855, 914, 1031, 1196, 1413, and 1504 cm^{-1} in a spectrum of USP 3-*tert*-Butyl-4-hydroxyanisole RS. Compare the peak positions of Butylated Hydroxyanisole to those of USP 3-*tert*-Butyl-4-hydroxyanisole RS.
  Acceptance criteria: All peak positions determined from Butylated Hydroxyanisole are within 5 cm^{-1} of those determined from USP 3-*tert*-Butyl-4-hydroxyanisole RS.
- **B.**
  Solution A: 5% acetic acid, prepared by diluting 50 mL of glacial acetic acid in a 1-L flask with water to volume
  Mobile phase: Acetonitrile and *Solution A* (65:35)
  Standard solution: 0.4 mg/mL of USP 3-*tert*-Butyl-4-hydroxyanisole RS and 0.1 mg/mL of USP 2-*tert*-Butyl-4-hydroxyanisole RS in *Mobile phase*
  Sample solution: 0.5 mg/mL of Butylated Hydroxyanisole in *Mobile phase*
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: LC
  Detector: UV 275 nm
  Column: 3.0-mm × 15-cm; 3-µm packing L1
  Column temperature: 40°
  Flow rate: 0.75 mL/min
  Injection volume: 10 µL
  Run time: NLT 15 min
  Analysis
  Samples: *Standard solution* and *Sample solution*
  [NOTE—2-*tert*-Butyl-4-hydroxyanisole and 3-*tert*-butyl-4-hydroxyanisole coelute under these chromatographic conditions. However, a small shoulder of 2-*tert*-butyl-4-hydroxyanisole may be seen on the left-hand side of the 3-*tert*-butyl-4-hydroxyanisole peak. The retention time of the 3-*tert*-butyl-4-hydroxyanisole peak is about 2.1 min.]
  Acceptance criteria: The retention time of the main peak of the *Sample solution* corresponds to that of the *Standard solution*. The chromatographic profile of the *Sample solution* should be similar to that of the *Standard solution* and exhibit only 1 major peak corresponding to butylated hydroxyanisole.

ASSAY

Change to read:

- **PROCEDURE**
  Solution A: Prepare as directed in *Identification B*.
  Mobile phase: Acetonitrile and *Solution A* (45:55)
  ▲System suitability solution:▲ (NF 1-May-2019) 90 µg/mL of USP 3-*tert*-Butyl-4-hydroxyanisole RS and 10 µg/mL of USP 2-*tert*-Butyl-4-hydroxyanisole RS*Mobile phase*
  ▲Standard solution A: 90 µg/mL of USP 3-*tert*-Butyl-4-hydroxyanisole RS in *Mobile phase*
  Standard solution B: 10 µg/mL of USP 2-*tert*-Butyl-4-hydroxyanisole RS in *Mobile phase*▲ (NF 1-May-2019)
  Sample solution: 100 µg/mL of Butylated Hydroxyanisole in *Mobile phase*
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: LC
  Detector: UV 290 nm
  Column: 4.6-mm × 75-mm; 3.5-µm packing L1
  Column temperature: 30°
  Flow rate: 1.2 mL/min
  Injection volume: 20 µL
  System suitability
  Samples: *▲System suitability solution, Standard solution A,* and *Standard solution B*▲ (NF 1-May-2019)
  [NOTE—The retention times of 2-*tert*-butyl-4-hydroxyanisole and 3-*tert*-butyl-4-hydroxyanisole are about 4.2 and 4.6 min, respectively.]
  Suitability requirements
  Resolution: NLT 1.5 between the 3-*tert*-butyl-4-hydroxyanisole isomer and 2-*tert*-butyl-4-hydroxyanisole isomer peaks, *▲System suitability solution*▲ (NF 1-May-2019)

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Tailing factor: NMT 1.5, ▲*Standard solution A* and *Standard solution B*▲ (NF 1-May-2019)
Relative standard deviation: NMT 2.0% for the 3-*tert*-butyl-4-hydroxyanisole isomer and 2-*tert*-butyl-4-hydroxyanisole isomer peaks, ▲*Standard solution A* and *Standard solution B*▲ (NF 1-May-2019)

Analysis
Samples: ▲*Standard solution A, Standard solution B,*▲ (NF 1-May-2019) and *Sample solution*
Measure the peak areas for each isomer.
Calculate the percentage of each isomer in the portion of Butylated Hydroxyanisole taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

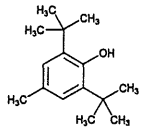
r_U = peak area of the corresponding isomer from the *Sample solution*
r_S = peak area of the corresponding isomer from ▲*Standard solution A* or *Standard solution B*▲ (NF 1-May-2019)
C_S = concentration of the appropriate Reference Standard in ▲*Standard solution A* or *Standard solution B*▲ (NF 1-May-2019) (µg/mL)
C_U = concentration of Butylated Hydroxyanisole in the *Sample solution* (µg/mL)

[NOTE—Calculate the percentage of butylated hydroxyanisole ($C_{11}H_{16}O_2$) in the portion of Butylated Hydroxyanisole taken by adding the quantities of the two isomers.]
Acceptance criteria: NLT 98.5%

IMPURITIES
• **RESIDUE ON IGNITION** ⟨281⟩
Sample: 10 g
Acceptance criteria: NMT 0.01%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP 2-*tert*-Butyl-4-hydroxyanisole RS
$C_{11}H_{16}O_2$ 180.25
USP 3-*tert*-Butyl-4-hydroxyanisole RS
$C_{11}H_{16}O_2$ 180.25

Butylated Hydroxytoluene



$C_{15}H_{24}O$ 220.35
Phenol, 2,6-bis(1,1-dimethylethyl)-4-methyl-; 2,6-Di-*tert*-butyl-*p*-cresol [128-37-0].

DEFINITION
Butylated Hydroxytoluene contains NLT 99.0% and NMT 101.5% of butylated hydroxytoluene ($C_{15}H_{24}O$).

IDENTIFICATION

Change to read:

• **A.** ▲**SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K.**▲ (CN 1-May-2020) Undried sample
• **B.** The retention time of the butylated hydroxytoluene peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*. The chromatographic profile of the *Sample solution* should exhibit only one major peak corresponding to the main compound.

ASSAY
• **PROCEDURE**
Solution A: 5% Acetic acid, prepared by diluting 50 mL of glacial acetic acid in a 1-L flask with water to volume
Mobile phase: Acetonitrile and *Solution A* (65:35)
Standard solution: 0.5 mg/mL of USP Butylated Hydroxytoluene RS in *Mobile phase*
Sample solution: 0.5 mg/mL of Butylated Hydroxytoluene in *Mobile phase*
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 275 nm
Column: 3.0-mm × 15-cm; 3-µm packing L1
Column temperature: 40°
Flow rate: 0.75 mL/min
Injection volume: 10 µL
Run time: 15 min
System suitability
Sample: *Standard solution*
[NOTE—The retention time of butylated hydroxytoluene is about 10.8 min.]
Suitability requirements
Tailing factor: NMT 1.5
Relative standard deviation: NMT 0.5%
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of butylated hydroxytoluene in the portion of Butylated Hydroxytoluene taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of butylated hydroxytoluene from the *Sample solution*
r_S = peak area of butylated hydroxytoluene from the *Standard solution*
C_S = concentration of USP Butylated Hydroxytoluene RS in the *Standard solution*
C_U = concentration of Butylated Hydroxytoluene in the *Sample solution*

Acceptance criteria: 99.0%–101.5%

IMPURITIES
• **RESIDUE ON IGNITION** ⟨281⟩
Sample: 50 g
Analysis: Transfer the *Sample* to a tared crucible, ignite until thoroughly charred, and cool. Moisten the ash with 1 mL of sulfuric acid, and complete the ignition by heating at 800 ± 25° for 15-min periods to constant weight.
Acceptance criteria: NMT 0.002%
• **ORGANIC IMPURITIES**
Mobile phase and **Chromatographic system:** Proceed as directed in the *Assay*, except extend the *Run time* to 20 min.
Standard solution: 2 µg/mL of USP Butylated Hydroxytoluene RS in *Mobile phase*
Sample solution: 2.0 mg/mL of Butylated Hydroxytoluene in *Mobile phase*

Supplement 1

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System suitability
Sample: *Standard solution*
[NOTE—The retention time of butylated hydroxytoluene is about 10.8 min.]
Suitability requirements
Signal-to-noise ratio: NLT 40
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the corrected peak area of each impurity in the *Sample solution*:

$$\text{Result} = r_U/F$$

r_U = peak area of the individual impurity from the *Sample solution*
F = relative response factor (see *Table 1*)

Acceptance criteria
Individual impurity: NMT 0.1%; the corrected peak area of each impurity in the *Sample solution* is NMT the area of the principal peak in the *Standard solution*.
Total impurities: NMT 0.7%; the sum of all the corrected peak areas in the chromatogram of the *Sample solution*, excluding the butylated hydroxytoluene peak area is NMT 7 times the butylated hydroxytoluene peak area of the *Standard solution*.

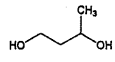
Table 1

Name	Relative Retention Time	Relative Response Factor
p-Cresol or *m*-cresol[a]	0.12	1.9
3-*tert*-Butyl-4-hydroxyanisole (BHA)	0.19	1.1
3,5-Di-*tert*-butyl-4-hydroxybenzoic acid	0.20	3.6
2-*tert*-Butyl-4-methylphenol or 2-*tert*-butyl-5-methylphenol[b]	0.27	1.7
3,5-Di-*tert*-butyl-4-hydroxy benzaldehyde	0.37	6.6
4,6-Di-*tert*-butyl-*m*-cresol	0.66	1.1
2,6-Di-*tert*-butyl-phenol	0.77	0.9
Any unspecified impurity	—	1.0

[a] The *p*-cresol and *m*-cresol peaks are not separated under the method conditions.
[b] The 2-*tert*-butyl-4-methylphenol and 2-*tert*-butyl-5-methylphenol peaks are not separated under the method conditions.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Butylated Hydroxytoluene RS

Butylene Glycol



$C_4H_{10}O_2$ ($HOCH_2CH_2CH(OH)CH_3$) 90.12
Butane-1,3-diol;
1,3-Butylene glycol [107-88-0].

DEFINITION
Butylene Glycol contains NLT 98.0% and NMT 102.0% of butane-1,3-diol ($C_4H_{10}O_2$), calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F▲** (CN 1-May-2020)
- **B. CHROMATOGRAPHIC IDENTITY**
  Analysis: Proceed as directed in the *Assay*.
  Acceptance criteria: The retention time of butane-1,3-diol in the *Sample solution* corresponds to that in the *Standard solution*.

ASSAY
- **PROCEDURE**
  Standard solution: 1.0 mg/mL of USP Butane-1,3-diol RS and 1.0 mg/mL of USP Propylene Glycol RS (internal standard) in methanol
  Sample solution: 1.0 mg/mL of Butylene Glycol and 1.0 mg/mL of USP Propylene Glycol RS (internal standard) in methanol
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: GC
  Detector: Flame ionization
  Column: 0.53-mm × 30-m capillary; bonded with a 1.0-µm layer of phase G16 or G47
  Temperatures
  Detector: 240°
  Injection port: 230°
  Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
40	0	40	3
40	10	80	0
80	5	130	2
130	10	170	0
170	25	240	0

Carrier gas: Hydrogen
Flow rate: 10 mL/min
Injection volume: 1.0 µL
Injection type: Split injection; split ratio is 2:1.
Liner: Single gooseneck liner with wool
System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for propylene glycol and butane-1,3-diol are 1.00 and 1.22, respectively.]
Suitability requirements
Resolution: NLT 15 between propylene glycol and butane-1,3-diol
Tailing factor: 0.8–2.0
Relative standard deviation: NMT 2% for the peak response ratio of butane-1,3-diol to the internal standard
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of butane-1,3-diol in the portion of Butylene Glycol taken:

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$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of butane-1,3-diol to the internal standard (peak response of butane-1,3-diol/peak response of the internal standard) from the *Sample solution*

R_S = peak response ratio of butane-1,3,-diol to the internal standard (peak response of butane-1,3-diol/peak response of the internal standard) from the *Standard solution*

C_S = concentration of USP Butane-1,3-diol RS in the *Standard solution* (mg/mL)

C_U = concentration of Butylene Glycol in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–102.0% on the anhydrous basis

IMPURITIES

- **Residue on Ignition** ⟨281⟩: NMT 0.05% determined on 2 g
- **Limit of Lead**

[Note—For the preparation of all aqueous solutions and for the rinsing of glassware before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin. For digestion, use acid-cleaned, high-density polyethylene, polypropylene, polytef, or quartz tubes. Select all reagents to have as low a content of lead as practicable, and store all reagent solutions in borosilicate glass containers. Cleanse glassware before use by soaking in warm 8 N nitric acid for 30 min and rinsing with deionized water. Store final diluted solutions in acid-cleaned plastic or polytef tubes or bottles.]

Matrix modifier solution: 200 mg/mL of magnesium nitrate. Just before use, transfer 1.0 mL of this solution to a 10-mL volumetric flask, and dilute with 5% nitric acid to volume.

Alternative matrix modifier solution: Just before use, add 0.3 mL of commercially available 10,000 µg/mL palladium standard solution and 5 mL of commercially available 10,000 µg/mL magnesium nitrate standard solution to 9.7 mL of 5% nitric acid, and mix well. [Note—*Alternative matrix modifier solution* can be used to replace the *Matrix modifier solution*. If the alternative solution is used, then the air-ashing step in the furnace program (see *Table 2*) can be omitted.]

Lead nitrate stock solution: Dissolve 159.8 mg of lead nitrate in 100 mL of water to which has been added 1 mL of nitric acid, and then dilute with water to 1000 mL. Prepare and store this solution in glass containers free from soluble lead salts.

Standard stock solution: Transfer 10.0 mL of *Lead nitrate stock solution* to a 100-mL volumetric flask, add 40 mL of water and 5 mL of nitric acid, and dilute with water to volume. Transfer 1.0 mL of this solution to a second 100-mL volumetric flask, dilute with 5% nitric acid to volume, and mix. This solution contains 0.1 µg/mL of lead.

Standard solutions: Transfer portions of *Standard stock solution* to four suitable containers, and dilute with 5% nitric acid to obtain *Standard solutions* having lead concentrations of 100, 50, 25, and 10 ng/mL, respectively.

Sample solution: [Note—Perform this procedure in a fume hood.] Transfer 1.5 g of Butylene Glycol to two digestion tubes, labeled "Sample solution" and "Temperature monitor solution", and add 0.75 mL of nitric acid to each tube. Place a thermometer in the tube labeled "Temperature monitor solution", and use the *Temperature monitor solution* solely to monitor temperature to be within the ranges specified by the method. Warm both solutions slowly to 90°–95° to avoid spattering. Heat until all brown vapors have dissipated and the samples no longer have a rust-colored tint. This typically takes 20–30 min. Allow the samples to cool. Add 0.5 mL of 50% hydrogen peroxide dropwise to both solutions, heat to 90°–95° for 5 min, and cool. Add a second 0.5-mL portion of 50% hydrogen peroxide dropwise to both solutions, and heat to 90°–100° for 5–10 min or until the solutions are clear. Cool, and transfer the *Sample solution* to a 10-mL volumetric flask. Rinse the tube labeled "Sample solution" with 5% nitric acid, add the rinsing to the volumetric flask, dilute with 5% nitric acid to volume, and mix.

Standard blank: 5% nitric acid

Sample blank: Transfer 1.5 g of water to a digestion tube, and proceed as directed for the *Sample solution*, beginning with "add 0.75 mL of nitric acid".

Instrumental conditions

Mode: Graphite furnace atomic absorption with pyrolytically-coated graphite tubes and adequate means of background correction

Analytical wavelength: Lead emission line at 283.3 nm

Lamp: Lead hollow-cathode

Furnace program: See *Table 2*. [Note—The temperature program may be modified to obtain optimum furnace temperatures.]

If the *Matrix modifier solution* is used, the furnace controller must be able to handle two gas flows to facilitate air ashing. Argon is used as the purge gas for the furnace for all steps but the char. Oxygen ashing is used to avoid build up of residue during the char step. Breathing-quality air is used as the alternative gas for the air ashing. The long (60 s) "Cool down" step prior to atomization ensures that the air used for the oxygen ashing (char) is cleared from the furnace.

Autosampler

Sample volume: 20 µL

Table 2 (Butylene Glycol)

Step	Temperature (°)	Ramp (s)	Hold Time (s)	Gas	Gas Flow Rate (mL/min)	Read (s)
Dry	200	20	30	Argon	300	—
Char (ash)	750	40	40	Air[a]	300	—
Cool down	20	1	60	Argon	300	—
Atomize	1800	0	10	Argon	Stop flow	10
Clean	2600	1	7	Argon	300	—
Cool down	20	1	5	Argon	300	—

[a] If *Matrix modifier solution* is used, air ashing must be used in the experiment. If *Alternative matrix modifier solution* is used, air can be substituted with argon.

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Alternative volume: 5 µL of *Matrix modifier solution* (or *Alternative matrix modifier solution*)

Analysis

Samples: 5 µL of the *Matrix modifier solution* (or *Alternative matrix modifier solution*) added into each 20-µL aliquot of the four *Standard solutions*; a mixture of 5 µL of the *Matrix modifier solution* (or *Alternative matrix modifier solution*) and 20 µL of the *Sample solution*; a mixture of 5 µL of the *Matrix modifier solution* (or *Alternative matrix modifier solution*) and 20 µL of the *Standard blank*; and a mixture of 5 µL of the *Matrix modifier solution* (or *Alternative matrix modifier solution*) and 20 µL of the *Sample blank*. Use peak area measurements for all quantitations.

Using the *Standard blank* to set the instrument to zero, determine the integrated absorbances of the *Standard solutions*. Plot the integrated absorbances of the *Standard solutions* versus their contents of lead, in ng/mL, and draw the line best fitting the four points to determine the calibration curve. Similarly determine the integrated absorbances of the *Sample solution* and the *Sample blank*. Correct the absorbance value of the *Sample solution* by subtracting from it the absorbance value obtained from the *Sample blank*.

Calculate the concentration of lead, in µg/g, in the portion of Butylene Glycol taken:

$$\text{Result} = [(V \times C_L)/W] \times F$$

V = volume of the *Sample solution*, 10 mL
C_L = concentration of lead in the *Sample solution*, as determined from the calibration curve (ng/mL)
W = weight of Butylene Glycol taken to prepare the *Sample solution* (g)
F = conversion factor, 10^{-3} µg/ng

Acceptance criteria: NMT 2 µg/g

• **LIMIT OF 4-HYDROXY-2-BUTANONE, BUTANE-2,3-DIOL, ETHYLENE GLYCOL, BUTANE-1,2-DIOL, BUTANE-1,4-DIOL, DIETHYLENE GLYCOL, AND OTHER ORGANIC IMPURITIES**

System suitability solution: 1.0 mg/mL of USP Butane-1,3-diol RS, 1.0 mg/mL of USP Propylene Glycol RS, 0.01 mg/mL of 4-hydroxy-2-butanone, 0.01 mg/mL of butane-2,3-diol, 0.01 mg/mL of butane-1,2-diol, 0.01 mg/mL of USP Ethylene Glycol RS, 0.01 mg/mL of butane-1,4-diol, and 0.01 mg/mL of USP Diethylene Glycol RS in methanol

Sensitivity solution: 1.0 mg/mL of USP Butane-1,3-diol RS, 1.0 mg/mL of USP Propylene Glycol RS, 0.001 mg/mL of 4-hydroxy-2-butanone, 0.001 mg/mL of butane-2,3-diol, 0.001 mg/mL of butane-1,2-diol, 0.001 mg/mL of USP Ethylene Glycol RS, 0.001 mg/mL of butane-1,4-diol, and 0.001 mg/mL of USP Diethylene Glycol RS in methanol

Sample solution: 5 mg/mL of Butylene Glycol in methanol

Chromatographic system: Proceed as directed in the *Assay*.

System suitability

Samples: *System suitability solution* and *Sensitivity solution* [NOTE—See *Table 3*.]

Table 3

Name	Relative Retention Time
4-Hydroxy-2-butanone	0.91
Butane-2,3-diol	0.98
Propylene glycol	1.00
Ethylene glycol	1.05
Butane-1,2-diol	1.13
Butane-1,3-diol	1.22
Butane-1,4-diol	1.49
Diethylene glycol	1.54

Suitability requirements

Resolution: NLT 1.5 between butane-2,3-diol and propylene glycol, *System suitability solution*

Relative standard deviation: NMT 2% for the peak response ratio of butane-1,3-diol to propylene glycol, *System suitability solution*

Signal-to-noise ratio: NLT 20 for any of the following peaks: 4-hydroxy-2-butanone; butane-2,3-diol; ethylene glycol; butane-1,2-diol; butane-1,4-diol; and diethylene glycol, *Sensitivity solution*

Analysis

Samples: *System suitability solution* and *Sample solution*

Identify each individual impurity peak in the *Sample solution* based on that in the *System suitability solution*.

Calculate the percentage of each individual impurity in the portion of Butylene Glycol taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of each individual impurity in the *Sample solution*
r_T = sum of all the peaks in the *Sample solution* excluding those due to solvent or reagents

Acceptance criteria

Each individual impurity: NMT 0.1%

Total impurities: NMT 2.0%

SPECIFIC TESTS

• **ACIDITY AND ALKALINITY**

Sample solution: A solution of Butylene Glycol (1 in 5)

Analysis: Perform a pH measurement.

Acceptance criteria: pH value is 5.5–7.0.

• **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 0.5%

ADDITIONAL REQUIREMENTS

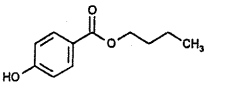
• **PACKAGING AND STORAGE:** Preserve in tight containers. Do not store above 50°. Protect from moisture.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Butane-1,3-diol RS
USP Diethylene Glycol RS
USP Ethylene Glycol RS
USP Propylene Glycol RS

Butylparaben

Portions of the monograph text that are national *USP* text, and are not part of the harmonized text, are marked with symbols (•,•) to specify this fact.



$C_{11}H_{14}O_3$ 194.23

Benzoic acid, 4-hydroxy-, butyl ester;
Butyl *p*-hydroxybenzoate [94-26-8].

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DEFINITION
Butylparaben contains NLT 98.0% and NMT 102.0% of $C_{11}H_{14}O_3$.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197M**▲ (CN 1-May-2020)
- **B. MELTING RANGE OR TEMPERATURE** ⟨741⟩: 68°–71°

ASSAY
- **PROCEDURE**

Mobile phase, Sample solution, Standard solution B, and **Chromatographic system:** Proceed as directed in the procedure for *Related Substances.*

System suitability

Sample: *Standard solution B*

Suitability requirements

Relative standard deviation: NMT 0.85% for six injections

Analysis

Samples: *Sample solution* and *Standard solution B*

Calculate the percentage of Butylparaben in the *Sample solution*:

$$\text{Result} = P \times (r_U \times C_S)/(r_S \times C_U)$$

P = labeled purity of USP Butylparaben RS expressed as a percentage

r_U = peak area of butylparaben from the *Sample solution*

C_S = concentration of butylparaben in *Standard solution B* (mg/mL)

r_S = peak area of butylparaben from *Standard solution B*

C_U = concentration of Butylparaben in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–102.0%

IMPURITIES
- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%, determined on a 1.0-g sample
- **RELATED SUBSTANCES**

Mobile phase: Methanol and a 6.8 g/L solution of potassium dihydrogen phosphate (1:1 v/v)

Sample solution: Dissolve 50.0 mg of Butylparaben in 2.5 mL of methanol, and dilute with *Mobile phase* to 50.0 mL. Dilute 10.0 mL of this solution with *Mobile phase* to 100.0 mL.

Standard solution A: 5.0 µg/mL each of *p*-hydroxybenzoic acid, USP Propylparaben RS, and USP Butylparaben RS in *Mobile phase*

Standard solution B: Dissolve 50.0 mg of USP Butylparaben RS in 2.5 mL of methanol, and dilute with *Mobile phase* to 50.0 mL. Dilute 10.0 mL of this solution with *Mobile phase* to 100.0 mL.

Standard solution C: Dilute 1.0 mL of the *Sample solution* with *Mobile phase* to 20.0 mL. Dilute 1.0 mL of this solution with *Mobile phase* to 10.0 mL.

Standard solution D: 50 µg/mL of iso-butylparaben in *Mobile phase*

Standard solution E: *Standard solution D* in *Standard solution B* (1 in 100)

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability.*)

Mode: LC

Detector: UV 272 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Column temperature: 35°

Flow rate: 1.3 mL/min

Injection volume: 10 µL

Run time: About 1.5 times the retention time of butylparaben

System suitability

Sample: *Standard solutions A* and *E*

[NOTE—The retention time of butylparaben is about 22 min; the relative retention times for *p*-hydroxybenzoic acid, propylparaben, and iso-butylparaben with a reference to butylparaben are about 0.1, 0.5, and 0.9 min, respectively.]

Suitability requirements

Resolution: NLT 5.0 between the propylparaben and butylparaben peaks from *Standard solution A* and NLT 1.5 between the iso-butylparaben and butylparaben peaks from *Standard solution E*

Analysis

Samples: *Sample solution* and *Standard solution C*

[NOTE—Disregard any limit that is 0.2 times the area of the principal peak from *Standard solution C* (0.1%).]

Acceptance criteria

***p*-Hydroxybenzoic acid:** The peak area from the *Sample solution*, multiplied by 1.4 to correct for the calculation of content, is NMT the area of the principal peak from *Standard solution C* (0.5%).

Unspecified impurities: The peak area of each impurity from the *Sample solution* is NMT the area of the principal peak from *Standard solution C* (0.5%).

Total impurities: The total peak area for all impurities from the *Sample solution* is NMT twice the area of the principal peak from *Standard solution C* (1.0%).

SPECIFIC TESTS
- **ACIDITY:** To 2 mL of *Butylparaben solution* prepared in the *Color of Solution* test add 3 mL of alcohol, 5 mL of carbon dioxide-free water, and 0.1 mL of bromocresol green TS. Titrate with 0.10 N sodium hydroxide.

Acceptance criteria: NMT 0.1 mL is required to produce a blue color.
- **COLOR OF SOLUTION**

Butylparaben solution: Dissolve 1 g in alcohol, and dilute with alcohol to 10 mL.

Acceptance criteria: This solution is clear and not more intensely colored than alcohol or a solution prepared immediately before use by mixing 2.4 mL of ferric chloride CS, 1.0 mL of cobaltous chloride CS, and 0.4 mL of cupric sulfate CS with 0.3 N hydrochloric acid to make 10 mL, and diluting 5 mL of this solution with 0.3 N hydrochloric acid to make 100 mL. Make the comparison by viewing the solutions downward in matched color-comparison tubes against a white surface (see *Color and Achromicity* ⟨631⟩).

ADDITIONAL REQUIREMENTS
- **▲PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Butylparaben RS

USP Propylparaben RS

▲

Calcium Carbonate

—see Calcium Carbonate General Monographs

NF Monographs

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Calcium Chloride—*see Calcium Chloride General Monographs*

Tribasic Calcium Phosphate

$Ca_5(OH)(PO_4)_3$ 502.31
Calcium hydroxide phosphate [12167-74-7].

DEFINITION
Tribasic Calcium Phosphate consists of a variable mixture of calcium phosphates with the approximate composition ($10CaO \cdot 3P_2O_5 \cdot H_2O$). It contains NLT 34.0% and NMT 40.0% of calcium (Ca).

IDENTIFICATION
• **A.**
Sample solution: Dissolve 100 mg in 5 mL of diluted nitric acid.
Analysis: Warm the *Sample solution*, and add 2 mL of ammonium molybdate TS.
Acceptance criteria: A yellow precipitate is formed.
• **B.**
Sample: 100 mg of Tribasic Calcium Phosphate
Analysis: Dissolve the *Sample* by warming in 10 mL of 2 N hydrochloric acid. Add 2.5 mL of ammonia TS dropwise, with shaking, and then add 5 mL of ammonium oxalate TS.
Acceptance criteria: A white precipitate is formed.

ASSAY
• **PROCEDURE**
Sample: 150 mg of Tribasic Calcium Phosphate
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.05 M edetate disodium VS
Endpoint detection: Visual
Analysis: Dissolve the *Sample*, with the aid of gentle heat if necessary, in a mixture of hydrochloric acid and water (5:3) contained in a 250-mL beaker equipped with a magnetic stirrer, and cautiously add 125 mL of water. With constant stirring, add in the following order: 0.5 mL of triethanolamine, 300 mg of hydroxy naphthol blue, and from the titration buret, about 23 mL of *Titrant*. Add sodium hydroxide solution (45 in 100) until the initial red color changes to clear blue. Continue to add it dropwise until the color changes to violet, and add an additional 0.5 mL. The pH is 12.3–12.5. Continue the titration dropwise with *Titrant* to the appearance of a clear blue endpoint that persists for NLT 60 s.
Calculate the percentage of calcium (Ca) in the portion of the *Sample* taken:

$$\text{Result} = [(V_S \times M \times F)/W] \times 100$$

V_S = *Titrant* volume consumed by the *Sample* (mL)
M = actual molarity of the *Titrant* (mmol/mL)
F = equivalency factor, 40.08 mg/mmol
W = *Sample* weight (mg)

Acceptance criteria: 34.0%–40.0%

IMPURITIES
• **CHLORIDE AND SULFATE** ⟨221⟩, *Chloride*
Standard: 1.0 mL of 0.020 N hydrochloric acid
Sample: 0.500 g of Tribasic Calcium Phosphate
Analysis: Dissolve the *Sample* in 25 mL of 2 N nitric acid, and add 1 mL of silver nitrate TS.
Acceptance criteria: NMT 0.14%
• **CHLORIDE AND SULFATE** ⟨221⟩, *Sulfate*
Standard: 1.0 mL of 0.020 N sulfuric acid
Sample: 0.500 g of Tribasic Calcium Phosphate
Analysis: Dissolve the *Sample* in the smallest possible amount of 3 N hydrochloric acid. Dilute with water to 100 mL, and filter, if necessary. To 25 mL of the filtrate add 1 mL of barium chloride TS.
Acceptance criteria: NMT 0.8%
• **ARSENIC** ⟨211⟩, *Method I*
Test preparation: Dissolve 1.0 g in just sufficient 3 N hydrochloric acid.
Acceptance criteria: NMT 3 ppm
• **BARIUM**
Sample: 500 mg of Tribasic Calcium Phosphate
Analysis: Mix the *Sample* with 10 mL of water, heat, add hydrochloric acid, dropwise, until solution is effected, and then add 2 drops of the acid in excess. Filter, and add to the filtrate 1 mL of potassium sulfate TS.
Acceptance criteria: No turbidity appears within 15 min.
• **CARBONATE**
Sample: 2 g of Tribasic Calcium Phosphate
Analysis: Mix the *Sample* with 20 mL of water, and add 3 N hydrochloric acid, dropwise, to effect solution.
Acceptance criteria: No effervescence is produced.
• **ACID-INSOLUBLE SUBSTANCES**
Analysis: If an insoluble residue remains in the test for *Carbonate*, boil the solution, filter, wash the residue well with hot water until the last washing is free from chloride, and ignite the residue to constant weight.
Acceptance criteria: NMT 0.2%; the weight of the residue is NMT 4 mg.
• **DIBASIC SALT AND CALCIUM OXIDE**
Sample: 1.5 g of Tribasic Calcium Phosphate
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Residual titration
Titrant: 1 N hydrochloric acid VS
Back-titrant: 0.1 N sodium hydroxide VS
Endpoint detection: Potentiometric
Analysis: Dissolve the *Sample* by warming with 25.0 mL of *Titrant*. Cool, and slowly titrate the excess of *Titrant*, while agitating constantly, with the *Back-titrant* to a pH of 4.0.
Acceptance criteria: 13.0–14.3 mL of 1 N hydrochloric acid is consumed for each g of salt, calculated on the ignited basis.
• **LIMIT OF FLUORIDE**
[NOTE—Prepare and store all solutions in plastic containers.]
Buffer solution: 294 mg/mL of sodium citrate dihydrate in water
Standard stock solution: 1.1052 mg/mL of USP Sodium Fluoride RS in water
Standard solution: Transfer 20.0 mL of *Standard stock solution* to a 100-mL volumetric flask containing 50.0 mL of *Buffer solution*, dilute with water to volume, and mix. Each mL of this solution contains 100 µg of fluoride ion.
Sample solution: Transfer 2.0 g of Tribasic Calcium Phosphate to a beaker containing a plastic-coated stirring bar. Add 20 mL of water and 3.0 mL of hydrochloric acid, and stir until dissolved. Add 50.0 mL of *Buffer solution* and sufficient water to make 100 mL.
Electrode system: Use a fluoride-specific ion-indicating electrode and a silver–silver chloride reference electrode connected to a pH meter capable of measuring potentials with a minimum reproducibility of ±0.2 mV (see *pH* ⟨791⟩).
Analysis
Samples: *Standard solution* and *Sample solution*
Standard response line: Transfer 50.0 mL of *Buffer solution* and 3.0 mL of hydrochloric acid to a beaker, and

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add water to make 100 mL. Add a plastic-coated stirring bar, insert the electrodes into the solution, stir for 15 min, and read the potential, in mV. Continue stirring, and at 5-min intervals add 100, 100, 300, 500, and 500 µL of *Standard solution*, reading the potential 5 min after each addition. Plot the logarithms of the cumulative fluoride ion concentrations (0.1, 0.2, 0.5, 1.0, and 1.5 µg/mL) versus potential, in mV.

Rinse and dry the electrodes, insert them into the *Sample solution*, stir for 5 min, and read the potential, in mV. From the measured potential and the *Standard response line* determine the concentration, *C* (in µg/mL), of fluoride ion in the *Sample solution*.

Calculate the content of fluoride in the portion of Tribasic Calcium Phosphate taken:

$$\text{Result} = (V \times C)/W$$

V = volume of the *Sample solution* (mL)
C = concentration of fluoride ion in the *Sample solution* determined from the *Standard response line* (µg/mL)
W = weight of Tribasic Calcium Phosphate taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 75 ppm

• **LIMIT OF NITRATE**

Sample: 200 mg of Tribasic Calcium Phosphate

Analysis: Mix the *Sample* with 5 mL of water, and add just sufficient hydrochloric acid to effect solution. Dilute with water to 10 mL, add 0.20 mL of indigo carmine TS, then add, with stirring, 10 mL of sulfuric acid.

Acceptance criteria: The blue color persists for NLT 5 min.

• **WATER-SOLUBLE SUBSTANCES**

Sample: 2 g of Tribasic Calcium Phosphate

Analysis: Digest the *Sample* with 100 mL of water on a steam bath for 30 min. Cool, add sufficient water to restore the original volume, stir well, and filter. Evaporate 50 mL of the filtrate in a tared porcelain dish on a steam bath to dryness, and dry the residue at 120° to constant weight.

Acceptance criteria: NMT 0.5%; the weight of the residue is NMT 5 mg.

SPECIFIC TESTS

• **LOSS ON IGNITION** ⟨733⟩

Analysis: Ignite at 800° for 30 min.

Acceptance criteria: NMT 8.0%

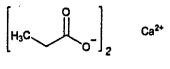
ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Sodium Fluoride RS

Calcium Propionate



$C_6H_{10}CaO_4$ 186.22

[4075-81-4].

DEFINITION

Calcium Propionate contains NLT 98.0% and NMT 100.5% of calcium propionate ($C_6H_{10}CaO_4$), calculated on the anhydrous basis.

IDENTIFICATION

• **A.** A solution of Calcium Propionate (1 in 20) imparts a transient yellowish red color to a nonluminous flame.

ASSAY

• **PROCEDURE**

Sample solution: 4 mg/mL of Calcium Propionate

Analysis: While stirring, preferably with a magnetic stirrer, add 30 mL of 0.05 M edetate disodium VS from a 50-mL buret to 100 mL of the *Sample solution*. Add 15 mL of 1 N sodium hydroxide and 300 mg of hydroxy naphthol blue indicator, and continue the titration to a blue endpoint. Each mL of 0.05 M edetate disodium VS is equivalent to 9.311 mg of calcium propionate ($C_6H_{10}CaO_4$).

Acceptance criteria: 98.0%–100.5% on the anhydrous basis

IMPURITIES

• **LIMIT OF FLUORIDE**

Buffer: 294.1 mg/mL of sodium citrate dihydrate

Standard solution: 2.21 mg/mL of USP Sodium Fluoride RS. [NOTE—Store solution in a plastic bottle.] On the day of use, transfer 5.0 mL of the resulting solution to a 1000-mL volumetric flask, dilute with water to volume, and mix. Each mL of this solution contains 5 µg of fluoride ion.

Electrode system: Use a fluoride-specific, ion-indicating electrode and a silver–silver chloride reference electrode connected to a pH meter capable of measuring potentials with a minimum reproducibility of ±0.2 mV (see *pH* ⟨791⟩).

Standard response line: Transfer 1.0, 2.0, 3.0, 5.0, 10.0, and 15.0 mL of the *Standard solution* to separate 250-mL plastic beakers. Add 50 mL of water, 5 mL of 1 N hydrochloric acid, 10 mL of *Buffer*, and 10 mL of 0.2 M edetate disodium to each beaker, and mix. Transfer each solution to separate 100-mL volumetric flasks, dilute with water to volume, and mix. Transfer a 50-mL portion of each solution to separate 125-mL plastic beakers, and read the potential, in mV, of each solution using the electrode system. Plot the calibration curve versus potential, in mV, on two-cycle semilogarithmic paper with µg of fluoride per 100 mL of solution on the logarithmic scale.[1]

Sample: 1.0 g

Analysis: Transfer the *Sample* to a 150-mL glass beaker, add 10 mL of water, and, while stirring continuously, slowly add 20 mL of 1 N hydrochloric acid to dissolve the *Sample*. Boil rapidly for 1 min. Transfer to a 250-mL plastic beaker, and cool rapidly in ice water. Add 15 mL of *Buffer* and 10 mL of 0.2 M edetate disodium, and mix. Adjust the pH to 5.5 ± 0.1 with 1 N hydrochloric acid or 1 N sodium hydroxide, if necessary. Transfer to a 100-mL volumetric flask, dilute with water to volume, and mix. Transfer a 50-mL portion of this solution to a 125-mL plastic beaker, and record the potential using the electrode system. Determine the concentration of fluoride, in µg/mL, in the *Sample*.

Acceptance criteria: NMT 30 µg/g

• **LIMIT OF LEAD**

25% Sulfuric acid solution: Cautiously add 100 mL of sulfuric acid to 300 mL of water with constant stirring while cooling in an ice bath.

Standard solutions: On the day of use, transfer 5.0, 10.0, and 20.0 mL of standard lead solution TS to three separate 100-mL volumetric flasks, add 10 mL of 3 N hydrochloric acid to each, and dilute with water to volume corresponding to 0.5-, 1.0-, and 2.0-µg/mL standards.

Sample blank: Add 5 mL of *25% Sulfuric acid solution* into an evaporating dish. Within a hood, place the dish on a

[1] A suitable source for semilogarithmic paper can be found at http://statland.org/GraphPaper/gpaper.html.

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steam bath to evaporate most of the water. Place the dish on a burner, and slowly pre-ash the sample by expelling most of the sulfuric acid. Place the dish in a muffle furnace that has been set at 525°, and ash the sample until the residue appears free from carbon. Cool, and cautiously wash down the inside of the evaporation dish with water. Add 5 mL of 1 N hydrochloric acid. Place the dish on a steam bath, and evaporate to dryness. Add 1.0 mL of 3 N hydrochloric acid and approximately 5 mL of water, and heat briefly on a steam bath to dissolve any residue. Transfer the solution quantitatively to a 10-mL volumetric flask, dilute to volume, and mix.

Sample solution: Place 10 g of Calcium Propionate, to the nearest 0.1 mg, into an evaporating dish. Add a sufficient amount of *25% Sulfuric acid solution*, and distribute the sulfuric acid uniformly through the sample. Proceed as directed under *Sample blank* beginning with "Within a hood..."

Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption
Analytical wavelength: 283.3 nm
Lamp: Lead electrodeless discharge
Flame: Air–acetylene
Slit width: 0.7 nm
Instrument blank: Water

Standard curve
Samples: *Standard solutions* and *Sample blank*
Plot: Corrected absorbance values versus their corresponding concentration (µg/mL). [NOTE—Determine corrected absorbance values by subtracting the absorbance of the *Sample blank* from the absorbance of the *Standard solutions*.]

Analysis
Samples: *Sample blank* and *Sample solution*
[NOTE—Determine corrected absorbance values by subtracting the absorbance of the *Sample blank* from the absorbance of the *Sample solution*.]
From the *Standard curve*, determine the lead concentration in the *Sample solution*.
Calculate the lead content in the portion of Calcium Propionate taken:

$$\text{Result} = (C_S \times V)/W$$

C_S = concentration of lead from the *Standard curve* (µg/mL)
V = final volume of the sample (mL)
W = weight of the sample taken (g)

Acceptance criteria: NMT 2 µg/g

• **MAGNESIUM** (as MgO)
Magnesium standard solution: Dissolve 50.0 mg of magnesium metal in 1 mL of hydrochloric acid in a 1000-mL volumetric flask, dilute with water to volume, and mix.
Sample solution: Place 400.0 mg of Calcium Propionate, 5 mL of 2.7 N hydrochloric acid, and about 10 mL of water in a small beaker, and dissolve the Calcium Propionate by heating on a hot plate.
Analysis: Evaporate the *Sample solution* to a volume of about 2 mL, and cool. Transfer the residual liquid to a 100-mL volumetric flask, dilute with water to volume, and mix. Dilute 7.5 mL of this solution with water to 20 mL, add 2 mL of 1 N sodium hydroxide and 0.05 mL of a 1:1000 solution of thiazole yellow, and mix. Allow to stand for 10 min, and shake. Any color produced does not exceed that produced by 1.0 mL of *Magnesium standard solution* in the same volume as that of a control containing 2.5 mL of the *Sample solution* (corresponding to 10 mg of Calcium Propionate) and the quantities of the reagents used in the test.
Acceptance criteria: The solution passes the test (about 0.4%).

SPECIFIC TESTS
• **WATER-INSOLUBLE SUBSTANCES**
Sample: 10 g
Analysis: Dissolve the *Sample* in 100 mL of hot water, pass through a tared filtering crucible, wash the insoluble residue with hot water, and dry at 105° to constant weight.
Acceptance criteria: NMT 0.2%
• **pH** ⟨791⟩**:** 7.5–10.5, in a solution (1 in 10)
• **WATER DETERMINATION** ⟨921⟩, *Method I*: NMT 5.0%
• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry at 105° for 2 h.
Acceptance criteria: NMT 4%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Store in tightly closed containers. No storage requirements specified.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Sodium Fluoride RS

Calcium Saccharate—*see Calcium Saccharate General Monographs*

Calcium Silicate

DEFINITION
Calcium Silicate, crystalline or amorphous, is a compound of calcium oxide and silicon dioxide. It contains NLT 4.0% of calcium oxide and NLT 35.0% of silicon dioxide.

IDENTIFICATION
• **A. IDENTIFICATION TESTS—GENERAL,** *Calcium* ⟨191⟩
Sample solution: Mix 0.5 g with 10 mL of 3 N hydrochloric acid. Filter, and neutralize the filtrate to litmus paper with 6 N ammonium hydroxide.
Acceptance criteria: The neutralized filtrate meets the requirements.
• **B.**
Analysis: Prepare a bead by fusing a few crystals of sodium ammonium phosphate on a platinum loop in the flame of a gas burner. Place the hot, transparent bead in contact with the specimen of Calcium Silicate, and again fuse.
Acceptance criteria: Silica floats about in the bead, producing, upon cooling, an opaque bead having a web-like structure.

ASSAY
• **SILICON DIOXIDE**
Sample: Calcium Silicate in the appropriate amount (see *Table 1*)

Table 1

Sample Weight (mg)	Calcium Oxide Content (%)
400	>25
600	11–25
1000	4–10

Analysis: Transfer the *Sample* to a beaker, add 5 mL of water and 10 mL of perchloric acid, and heat until dense white

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fumes of perchloric acid are evolved. Cover the beaker with a watch glass, and continue to heat for 2 h. Allow to cool, add 30 mL of water, filter, and wash the precipitate with 200 mL of hot water. [NOTE—Retain the combined filtrate and washings for use in the assay for *Calcium Oxide*.] Transfer the filter paper and its contents to a platinum crucible, heat slowly to dryness, then heat sufficiently to char the filter paper. Ignite at about 900°–1000° to constant weight. Moisten the residue with 5 drops of perchloric acid, add 15 mL of hydrofluoric acid, heat cautiously on a hot plate until all of the acid is driven off, and ignite at a temperature NLT 1000° to constant weight. Cool in a desiccator, and weigh: the loss in weight represents the weight of SiO_2.

Acceptance criteria: NLT 35.0% of silicon dioxide (SiO_2) and 90.0%–110.0% of the content stated in the labeling, or within the range of percentages stated in the labeling

• **CALCIUM OXIDE**

Sample solution: Use the combined filtrate and washings retained from the assay for *Silicon Dioxide*.

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Direct titration

Titrant: 0.05 M edetate disodium VS

Endpoint detection: Visual

Analysis: Neutralize the *Sample solution* to litmus with 1 N sodium hydroxide. Add, while stirring, 10 mL of 0.05 M edetate disodium VS from a 50-mL buret. Add 15 mL of 1 N sodium hydroxide and 300 mg of hydroxy naphthol blue, and continue the titration to a blue endpoint.

Calculate Z, the percentage of calcium oxide (CaO) in the sample taken:

$$Z = [(V \times M \times F)/W] \times 100$$

V = volume of *Titrant* consumed by the *Sample solution* (mL)
M = actual molarity of the *Titrant* (mmol/mL)
F = equivalency factor, 56.08 mg/mmol
W = weight of the sample (mg)

Calculate the percentage of the labeled amount of calcium oxide (CaO):

$$\text{Result} = (Z/L) \times 100$$

Z = percentage of CaO in the sample taken as calculated above
L = labeled amount of CaO

Acceptance criteria: NLT 4.0% of calcium oxide (CaO) and 90.0%–110.0% of the content stated in the labeling, or within the range of percentages stated in the labeling

IMPURITIES

• **LOSS ON IGNITION** ⟨733⟩**:** Transfer 1 g to a suitable tared crucible, dry at 105° for 2 h, and ignite at 900° to constant weight: it loses NMT 20.0% of its weight.

Change to read:

• **LIMIT OF LEAD**

Palladium matrix modifier: Palladium nitrate (1% Pd)[1]

Magnesium matrix modifier: Magnesium nitrate (2% Mg)[2]

Nitric acid, 65% [7697-37-2]: Use a suitable grade with a content of NLT 65.0%.[3]

Nitric acid diluent: Transfer 42 mL of *Nitric acid, 65%* to a 1000-mL volumetric flask. Dilute with water to volume.

Matrix modifier solution: Transfer 1.0 mL of *Palladium matrix modifier* and 100 µL of *Magnesium matrix modifier* to a 20-mL volumetric flask. Dilute with water to volume, and mix.

Lead standard solution: A solution containing $Pb(NO_3)_2$ in 0.5 M nitric acid corresponding to 1000 mg of ▲lead/L▲ (ERR 1-Mar-2019)[4]

Standard solution: Transfer 100 µL of *Lead standard solution* to a 100-mL volumetric flask. Dilute with water to volume, and mix. This solution contains the equivalent of 1.0 µg/mL of lead.

Calibration solutions

Calibration solution A: Transfer 50 mL of *Nitric acid diluent* to a 100-mL volumetric flask, and dilute with water to volume (0 µg/mL of lead).

Calibration solution B: Transfer 1 mL of *Standard solution* and 50 mL of *Nitric acid diluent* to a 100-mL volumetric flask, and dilute with water to volume (0.01 µg/mL of lead).

Calibration solution C: Transfer 5 mL of *Standard solution* and 50 mL of *Nitric acid diluent* to a 100-mL volumetric flask, and dilute with water to volume (0.05 µg/mL of lead).

Sample solution: Weigh 2.0 g of Calcium Silicate into a 150-mL beaker. Mix with 50 mL of *Nitric acid diluent*. Cover with a watch glass, and boil for 20 min. Allow to cool. With the aid of a vacuum, pass through a glass filter,[5] and wash the filter several times with water. Transfer the filtrate into a 100-mL volumetric flask. Dilute with water to volume, and mix.

System suitability solution: Transfer 5 mL of *Standard solution* and 50 mL of *Nitric acid diluent* into a 100-mL volumetric flask. Dilute with water to volume, and mix. This solution contains the equivalent of 0.05 µg of lead/mL.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Graphite furnace atomic absorption spectrophotometer

Analytical wavelength: 283.3 nm (lead emission line)

Lamp: Lead hollow-cathode lamp and an adequate means of Zeeman background correction

Carrier gas: Argon

Under typical conditions, the *Sample solution* and *Calibration solutions* volumes are 20 µL, the volume of the *Matrix modifier solution* is 10 µL, the injection temperature is 20°, and the oven conditions are as follows (see *Table 2*). [NOTE—These conditions may be optimized for each instrument.]

Table 2

Step	Temperature (°)
Drying 1	110
Drying 2	130
Pyrrolysis	950
Read	1800

[1] A suitable grade is available as catalog number RCMMPD10KN-50 from VWR, www.vwr.com.

[2] Available as catalog number RCMMMG20KN-50, from VWR, www.vwr.com.

[3] A suitable grade is available as catalog number 441-2 from EMD Chemicals, www.emdchemicals.com.

[4] A suitable grade is available as catalog number 1.19776 from EMD Chemicals, www.emdchemicals.com.

[5] Glass filter: Whatman GF/B glass microfiber filters, 1.0 µm, Whatman no. 1821-090, is suitable. Available as catalog number 28497-492, from VWR, www.vwr.com.

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Table 2 *(continued)*

Step	Temperature (°)
Clean out	2450

Analysis

Samples: *Calibration solutions, Sample solution,* and *System suitability solution*

Plot the absorbance of each *Calibration solution* versus its content of lead, in µg/mL, and draw the best straight line fitting the three points. From this plot, determine the concentration, in µg/mL, of lead in the *Sample solution*: the correlation coefficient is NLT 0.99, and the recovery for the *System suitability solution* is 85%–115%.

Calculate the quantity, in ppm, of lead in the *Sample solution.*

$$\text{Result} = (C \times V)/W$$

C = concentration of the *Sample solution* (µg/mL)
V = volume of the *Sample solution* (mL)
W = weight of sample taken (g)

Acceptance criteria: NMT 10 ppm

• **LIMIT OF FLUORIDE**

[NOTE—Store all solutions in polytef containers.]

Buffer solution: 294 mg/mL of sodium citrate

Ionic strength adjustment buffer: Transfer 42 mL of hydrochloric acid, 121 g of tris(hydroxymethyl) aminomethane, and 115 g of sodium tartrate to a 500-mL volumetric flask containing 250 mL of water. Stir to dissolve, and dilute with water to volume.

Standard stock solution: 221 µg/mL of USP Sodium Fluoride RS. This stock solution contains 100 µg/mL of fluoride ion.

Sample solution: Transfer 2.0 g of Calcium Silicate to a 100-mL polytef beaker containing a magnetic stir bar. Add 20 mL of water and 2.0 mL of hydrochloric acid. Cover with a watch glass, and heat to a vigorous boil for 1 min, stirring continuously. Remove from heat, and cool. Transfer the cooled suspension to a 100-mL polytef beaker. Add 25 mL of *Buffer solution,* and adjust with ammonium hydroxide or hydrochloric acid to a pH of 5–6. Add 50 mL of *Ionic strength adjustment buffer* and water to make 100 mL of solution.

Electrode system: Use a fluoride-specific ion-indicating electrode and a suitable reference electrode connected to a pH meter capable of measuring potentials with a reproducibility of ±0.2 mV (see *pH* ⟨791⟩).

Standard response line: Obtain a standard response line with four standard solutions containing 0, 0.10, 0.20, and 0.40 µg/mL of fluoride as follows. Add 23 mL of water, 2 mL of hydrochloric acid, and 25 mL of *Buffer solution* to a 100-mL plastic beaker. Adjust with ammonium hydroxide to a pH of 5–6, and add *Ionic strength adjustment buffer* to obtain 100 mL of solution. Insert the electrode into the solution, stir for at least 15 min, and record the potential for the standard solution containing 0 µg/mL of fluoride. When the electrode has stabilized, add 100 µL of the *Standard stock solution* to the beaker, and stir. Allow the electrode to stabilize for 5 min, and measure the potential for the standard solution containing 0.10 µg/mL of fluoride. Similarly add another 100 and 200 µL of the *Standard stock solution* and record the potential for the standard solutions, containing 0.20 µg/mL of fluoride and 0.40 µg/mL of fluoride, respectively. After each addition, continue to stir for 5 min before recording the reading.

Analysis: Insert the calibrated electrode into the *Sample solution,* stir for 5 min, and record the measurement. From the measured potential of the *Sample solution* and the *Standard response line,* determine the concentration, C, in µg/mL, of fluoride ion in the *Sample solution.*

Calculate the quantity, in ppm, of fluoride in Calcium Silicate:

$$\text{Result} = (C \times V)/W$$

C = concentration of fluoride ion in the *Sample solution* (µg/mL)
V = volume of the *Sample solution* (mL)
W = weight of Calcium Silicate in the *Sample solution* (g)

Acceptance criteria: NMT 50 ppm

SPECIFIC TESTS

• **PH** ⟨791⟩**:** 8.4–11.2, determined in a well-mixed aqueous suspension (1 in 20)

• **RATIO OF SILICON DIOXIDE TO CALCIUM OXIDE:** Divide the percentage of silicon dioxide obtained in the test for *Silicon Dioxide* by the percentage of calcium oxide obtained in the test for *Calcium Oxide.*

Acceptance criteria: 0.5–20

• **SUM OF CaO, SiO_2, AND LOSS ON IGNITION:** The sum of the percentages obtained in the three tests is NLT 90.0%.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

• **LABELING:** The labeling states the claimed percentage or range of percentages for the content of calcium oxide and for the content of silicon dioxide.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Sodium Fluoride RS

Calcium Stearate

Octadecanoic acid, calcium salt;
Calcium stearate [1592-23-0].

DEFINITION

Calcium Stearate is a compound of calcium with a mixture of solid organic acids obtained from sources of vegetable or animal origin and consists mainly of variable proportions of calcium stearate ($C_{36}H_{70}CaO_4$) and calcium palmitate ($C_{32}H_{62}CaO_4$). It contains NLT 6.4% and NMT 7.4% of calcium (Ca), calculated on the dried basis. The content of stearic acid in the fatty acid fraction is NLT 40.0% of the total content. The sum of stearic acid and palmitic acid in the fatty acid fraction is NLT 90.0% of the total content.

IDENTIFICATION

• **A. IDENTIFICATION TESTS—GENERAL,** *Calcium* ⟨191⟩

Sample: 1 g

Analysis: Heat the *Sample* with a mixture of 25 mL of water and 5 mL of hydrochloric acid.

Acceptance criteria: Fatty acids are liberated and appear as an oily layer floating on the surface of the liquid. The water layer meets the requirements.

• **B.** The retention times of the major peaks of the *Sample solution* correspond to those of the *Standard solution,* as obtained in the *Assay* for *Content of Stearic Acid and Palmitic Acid.*

ASSAY

• **CONTENT OF CALCIUM**

Sample: 1.2 g

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

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Mode: Direct titration
Titrant: 0.05 M edetate disodium VS
Endpoint detection: Colorimetric
Analysis: Boil the *Sample* with 50 mL of 1 N sulfuric acid for about 3 h, using a watch glass cover to avoid splattering, or until the separated fatty acid layer is clear. Add water, if necessary, to maintain the original volume. [NOTE—Stirring may be helpful in obtaining a clear layer and decreasing extraction time.] Cool, filter, and wash the filter and the flask thoroughly with water until the last washing is not acid to litmus. Neutralize the filtrate with 1 N sodium hydroxide to litmus. While stirring, preferably with a magnetic stirrer, titrate with 0.05 M edetate disodium VS as follows. Add about 30 mL from a 50-mL buret, then add 15 mL of 1 N sodium hydroxide and 300 mg of hydroxy naphthol blue. Continue the titration to a blue endpoint. Each mL of 0.05 M edetate disodium is equivalent to 2.003 mg of calcium.
Acceptance criteria: 6.4%–7.4% on the dried basis

• **CONTENT OF STEARIC ACID AND PALMITIC ACID**
Boron trifluoride–methanol solution: 140 g/L of boron trifluoride in methanol
Sample solution: Dissolve 100 mg of Calcium Stearate in a small conical flask fitted with a suitable reflux attachment with 5 mL of *Boron trifluoride–methanol solution*. Boil under reflux for 10 min. Add 4.0 mL of *n*-heptane through the condenser, and boil again under reflux for 10 min. Allow to cool. Add 20 mL of a saturated solution of sodium chloride. Shake, and allow the layers to separate. Remove about 2 mL of the organic layer, and dry it over 0.2 g of anhydrous sodium sulfate. Dilute 1.0 mL of this solution with *n*-heptane to 10.0 mL.
Standard solution: Prepare as directed in the *Sample solution*, using 50 mg of USP Stearic Acid RS and 50 mg of USP Palmitic Acid RS.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 30-m × 0.32-mm fused silica; 0.5-µm layer of phase G16
Temperatures
Injection port: 220°
Detector: 260°
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	—	70	2
70	5	240	5

Carrier gas: Helium, passed through a bed of molecular sieve for drying, if necessary
Flow rate: 2.4 mL/min
Injection volume: 1 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Resolution: NLT 5.0 between the methyl palmitate and methyl stearate peaks. [NOTE—The relative retention times for methyl palmitate and methyl stearate are about 0.9 and 1.0, respectively.]
Relative standard deviation: NMT 3.0% for the methyl stearate and methyl palmitate peaks; NMT 1.0% for the ratio of the peak areas of methyl palmitate to the peak areas of methyl stearate, from 6 replicate injections
Analysis: Calculate the percentage of stearic acid ($C_{18}H_{36}O_2$) in the fatty acid fraction of the sample taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak area due to methyl stearate
r_T = sum of all the peak areas, excluding the solvent peak

Calculate the percentage of palmitic acid ($C_{16}H_{32}O_2$) in the fatty acid fraction of the sample taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak area due to methyl palmitate
r_T = sum of all the peak areas, excluding the solvent peak

Acceptance criteria
Stearic acid: NLT 40.0%
Sum of stearic acid and palmitic acid: NLT 90.0%

SPECIFIC TESTS
• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry a sample at 105° to constant weight.
Acceptance criteria: NMT 4.0%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers.
• **LABELING:** Label it to indicate the content of stearic acid in the fatty acid fraction and to indicate the fatty acids used to produce calcium stearate are from sources of vegetable or animal origin.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Palmitic Acid RS
USP Stearic Acid RS

Calcium Sulfate

$CaSO_4$ 136.14
$CaSO_4 \cdot 2H_2O$ 172.17
Sulfuric acid, calcium salt (1:1);
Calcium sulfate (1:1) [7778-18-9].
Dihydrate [10101-41-4].

DEFINITION
Calcium Sulfate is anhydrous or contains two molecules of water of hydration. It contains NLT 98.0% and NMT 101.0% of $CaSO_4$, calculated on the dried basis.

IDENTIFICATION
• **A. IDENTIFICATION TESTS—GENERAL,** *Calcium* ⟨191⟩
Sample solution: Dissolve 200 mg by warming in a mixture of 4 mL of 3 N hydrochloric acid and 16 mL of water.
Acceptance criteria: Meets the requirements
• **B. IDENTIFICATION TESTS—GENERAL,** *Sulfate* ⟨191⟩
Sample solution: Dissolve 200 mg by warming in a mixture of 4 mL of 3 N hydrochloric acid and 16 mL of water.
Acceptance criteria: Meets the requirements

ASSAY
• **PROCEDURE**
Sample: Dissolve 300 mg of Calcium Sulfate in 100 mL of water and 4 mL of 3 N hydrochloric acid. Boil, if necessary, to dissolve, and cool before titrating.

Supplement 1

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Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.05 M edetate disodium VS
Endpoint detection: Visual
Analysis: While stirring, preferably with a magnetic stirrer, add to the *Sample solution*, in the order named, 0.5 mL of triethanolamine, 300 mg of hydroxy naphthol blue, and, from a 50-mL buret, 30 mL of 0.05 M edetate disodium VS. Add sodium hydroxide solution (45 in 100) until the initial red color changes to clear blue, continue to add dropwise until the color changes to violet, and add an additional 0.5 mL. The pH is 12.3–12.5. Continue the titration dropwise with 0.05 M edetate disodium VS to the appearance of a clear-blue endpoint that persists for NLT 60 s.
Calculate the percentage of Calcium Sulfate ($CaSO_4$) in the Sample taken:

$$\text{Result} = [(V \times M \times F)/W] \times 100$$

V = volume of *Titrant* consumed by the *Sample* (mL)
M = actual molarity of the *Titrant* (mM/mL)
F = equivalency factor, 136.14 mg/mM
W = weight of the *Sample* (mg)

Acceptance criteria: 98.0%–101.0% on the dried basis

IMPURITIES
• **Iron** ⟨241⟩
Sample solution: Dissolve 100 mg in 8 mL of 3 N hydrochloric acid, and dilute with water to 47 mL.
Acceptance criteria: NMT 100 ppm

SPECIFIC TESTS
• **Loss on Drying** ⟨731⟩
Samples: Neat
Analysis: Dry a sample at a temperature NLT 250° to constant weight.
Acceptance criteria: NMT 1.5% for the anhydrous form and 19.0%–23.0% for the dihydrate

ADDITIONAL REQUIREMENTS
• **Packaging and Storage:** Preserve in well-closed containers.
• **Labeling:** Label it to indicate whether it is anhydrous or the dihydrate.

Anhydrous Dibasic Calcium Phosphate

—see Dibasic Calcium Phosphate Dihydrate General Monographs

Dibasic Calcium Phosphate Dihydrate

—see Dibasic Calcium Phosphate Dihydrate General Monographs

Candelilla Wax

[8006-44-8].

DEFINITION
Candelilla Wax is the purified wax obtained from the leaves of the candelilla plant, *Euphorbia antisyphilitica* Zucc.

IDENTIFICATION

Change to read:

• **A. ▲Spectroscopic Identification Tests** ⟨197⟩, *Infrared Spectroscopy*: **197F**▲ (CN 1-May-2020)
Sample: Use a thin film of melted *Candelilla Wax*.
Acceptance criteria: Meets the requirements
• **B.** It meets the requirements of the test in *Specific Tests* for *Melting Range or Temperature, Class II* ⟨741⟩.

IMPURITIES
• **Limit of Lead** ⟨251⟩
Sample: 3.3 g
Control: 10 µg of lead from a *Diluted Standard Lead Solution* (1 µg/mL of lead)
Analysis: Weigh the *Sample* into a porcelain dish, heat on a hot plate until completely charred, then heat in a muffle furnace at 480° for 8 h or overnight, and cool. Cautiously add 5 mL of nitric acid, evaporate to dryness on a hot plate, then heat again in the muffle furnace for exactly 15 min, and cool. Extract the ash with two 10-mL portions of water, filtering each extract into a separator. Leach any insoluble material on the filter with 6 mL of *Ammonium Citrate Solution*, 2 mL of *Hydroxylamine Hydrochloride Solution*, and 5 mL of water, adding the filtered washings to the separator. Continue as directed in *Lead* ⟨251⟩, *Procedure*, beginning with "Add two drops of phenol red TS", and use 10 mL of *Diluted Standard Lead Solution* for a control.
Acceptance criteria: The color of the *Sample solution* does not exceed that in the control, corresponding to NMT 3 µg/g.

SPECIFIC TESTS
• **Melting Range or Temperature,** *Class II* ⟨741⟩**:** 68.5°–72.5°
• **Fats and Fixed Oils,** *Acid Value* ⟨401⟩
Sample: 3 g
Analysis: Weigh the *Sample* in a 250-mL flask, add 50 mL of a mixture of isopropyl alcohol and toluene (5:4), attach the flask to a reflux condenser, and boil gently until the wax is completely dissolved. Examine the solution: no insoluble particles are present. Remove the flask from the condenser, add 1 mL of phenolphthalein TS, and titrate immediately with 0.5 N alcoholic potassium hydroxide VS to a faint, reddish-yellow color. [Note—Do not allow the solution to cool; titrate at a warm temperature after refluxing. After the titration, reserve the solution for use in the test for *Saponification Value*.]
Calculate the acid value as the number of mg of potassium hydroxide required to neutralize the free acids in 1 g of Candelilla Wax.
Acceptance criteria: 12–22
• **Fats and Fixed Oils,** *Saponification Value* ⟨401⟩
Sample solution: Use the solution from the test for *Acid Value*.
Analysis: To the *Sample solution* add 15.0 mL of 0.5 N alcoholic potassium hydroxide VS, reflux for 3–4 h, and titrate the excess alkali with 0.5 N hydrochloric acid VS to a yellow-amber color. Perform a blank determination under the same conditions (see *Titrimetry* ⟨541⟩, *Residual Titrations*). The saponification value is the summation of the ester value so obtained and the acid value obtained above.
Acceptance criteria: 43–65

ADDITIONAL REQUIREMENTS
• **Packaging and Storage:** Preserve in well-closed containers. No storage requirements specified.
• **USP Reference Standards** ⟨11⟩
USP Candelilla Wax RS

NF Monographs

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Canola Oil

Low erucic acid rapeseed oil;
LEAR oil.

DEFINITION
Canola Oil is the refined fixed oil obtained from the seeds of *Brassica napus* or *Brassica campestris* (Fam. Cruciferae). A suitable antioxidant may be added.

IDENTIFICATION
- **A. Identity by Fatty Acid Composition**
  Analysis: Proceed as directed in the test for *Fats and Fixed Oils* ⟨401⟩, *Procedures, Fatty Acid Composition.*
  Acceptance criteria: Meets the composition profile of fatty acids in *Table 1*
- **B. Identity by Triglyceride Profile**
  Analysis: Proceed as directed in *Identification of Fixed Oils by Thin-Layer Chromatography* ⟨202⟩, *Identification, Method I* or *Method II.*
  Acceptance criteria: Meets the requirements in the chapter

SPECIFIC TESTS
- **Fats and Fixed Oils** ⟨401⟩, *Procedures, Fatty Acid Composition*: Canola Oil exhibits the composition profile of fatty acids in *Table 1*.

Table 1

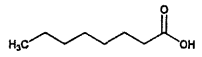
Carbon-Chain Length	Number of Double Bonds	Percentage (%)
<14	—	<0.1
14	0	<0.2
16	0	<6.0
16	1	<1.0
18	0	<2.5
18	1	>50
18	2	<40
18	3	<14
20	0	<1.0
20	1	<2.0
22	0	<0.5
22[a]	1	≤2.0
24	0	<0.2
24	1	<0.2

[a] Erucic acid.

- **Limit of Erucic Acid:** NMT 2.0%, as determined in the test for *Fats and Fixed Oils* ⟨401⟩, *Procedures, Fatty Acid Composition*
- **Specific Gravity** ⟨841⟩: 0.906–0.920
- **Fats and Fixed Oils** ⟨401⟩, *Procedures, Acid Value*: NMT 6.0
- **Fats and Fixed Oils** ⟨401⟩, *Procedures, Iodine Value*: 110–126
- **Fats and Fixed Oils** ⟨401⟩, *Procedures, Peroxide Value*: NMT 10.0
- **Fats and Fixed Oils** ⟨401⟩, *Procedures, Saponification Value*: 178–193
- **Fats and Fixed Oils** ⟨401⟩, *Procedures, Unsaponifiable Matter*: NMT 1.5%
- **Refractive Index** ⟨831⟩: 1.465–1.467 at 40°

ADDITIONAL REQUIREMENTS
- **Packaging and Storage:** Preserve in tight containers, and avoid contact with metals. Fill to the top or flush partially filled containers with nitrogen. No storage requirements specified.
- **Labeling:** Label it to indicate the name and concentration of any added antioxidant.

Caprylic Acid



$C_8H_{16}O_2$ 144.21
Octanoic acid;
1-Heptanecarboxylic acid [124-07-2].

DEFINITION
Caprylic Acid contains NLT 99.0% of caprylic acid ($C_8H_{16}O_2$), calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

- **A. ▲Spectroscopic Identification Tests** ⟨197⟩, *Infrared Spectroscopy*: **197F** ▲ (CN 1-May-2020)
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay.*

ASSAY
- **Procedure**
  Standard solution: 10 mg/mL of USP Caprylic Acid RS in ethyl acetate
  Sample solution: 10 mg/mL of Caprylic Acid in ethyl acetate
  System suitability solution: 10 µg/mL of Caprylic Acid in ethyl acetate, prepared from the *Sample solution*
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability.*)
  Mode: GC
  Detector: Flame ionization
  Column: 0.25-mm × 30-cm fused silica capillary column bonded with a 0.25-µm layer of phase G25
  Temperatures
  Detector: 250°
  Injection port: 250°
  Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
100	—	100	1
100	5	220	10

Carrier gas: Helium
Flow rate: 1.5 mL/min
Injection volume: 1 µL
Injection type: Split injection. The split ratio is about 1:100.
System suitability
Samples: *Standard solution* and *System suitability solution*

NF Monographs

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Suitability requirements
Relative standard deviation: NMT 2.0%, *Standard solution*
Signal-to-noise ratio: NLT 5 for the major peak, *System suitability solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of caprylic acid ($C_8H_{16}O_2$) in the portion of Caprylic Acid taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of caprylic acid from the *Sample solution*
r_S = peak response of caprylic acid from the *Standard solution*
C_S = concentration of USP Caprylic Acid RS in the *Standard solution* (mg/mL)
C_U = concentration of Caprylic Acid in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 99.0% on the anhydrous basis

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%
- **LIMIT OF RELATED LINEAR AND BRANCHED ALKYL CARBOXYLIC ACIDS, RELATED ESTERS, CYCLIC ESTERS AND KETONE**

Standard solution, System suitability solution, Sample solution, Chromatographic system, and System suitability: Proceed as directed in the *Assay*.
Analysis
Sample: *Sample solution*
Calculate the percentage of each impurity in the portion of Caprylic Acid taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of each individual impurity in the *Sample solution*
r_T = sum of all the peaks except the peak due to solvent and peaks below the *Disregard limit* (see *Table 2*) in the *Sample solution*

Acceptance criteria: See *Table 2*.
Disregard any peak with an area less than 0.5 times the area of the major peak from the *System suitability solution*.

Table 2

Impurity	Percentage (%)
Each individual	NMT 0.3
Total	NMT 0.5

SPECIFIC TESTS

- **BACTERIAL ENDOTOXINS TEST** ⟨85⟩: The level of bacterial endotoxins is such that the requirement in the relevant dosage form monograph(s) in which Caprylic Acid is used can be met. Where the label states that Caprylic Acid must be subjected to further processing during the preparation of injectable dosage forms, the level of bacterial endotoxins is such that the requirement in the relevant dosage form monograph(s) in which Caprylic Acid is used can be met.
- **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩: NMT 10.0
- **WATER DETERMINATION,** *Method Ia* ⟨921⟩: NMT 0.7%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and store at room temperature.
- **LABELING:** Where Caprylic Acid must be subjected to further processing during the preparation of injectable dosage forms to ensure acceptable levels of bacterial endotoxins, it is so labeled.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Caprylic Acid RS

Caprylocaproyl Polyoxylglycerides

Former Title: Caprylocaproyl Macrogolglycerides

DEFINITION

Caprylocaproyl Polyoxylglycerides is a mixture of monoesters, diesters, and triesters of glycerol and monoesters and diesters of polyethylene glycols. The polyethylene glycols used have a mean molecular weight between 200 and 400. It is produced by partial alcoholysis of medium-chain triglycerides with polyethylene glycol, by esterification of glycerol and polyethylene glycol with caprylic acid and capric acid, or as a mixture of glycerol esters and ethylene oxide condensate with caprylic acid and capric acid. It may contain free polyethylene glycols.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F**▲ (CN 1-May-2020)
- **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩
Standard solution: 50 mg/mL of USP Caprylocaproyl Polyoxylglycerides RS in methylene chloride
Sample solution: 50 mg/mL of Caprylocaproyl Polyoxylglycerides in methylene chloride
Application volume: 50 µL
Developing solvent system: Ether and hexanes (7:3)
Spray reagent: 0.1 mg/mL of rhodamine B in alcohol
Analysis
Samples: *Standard solution* and *Sample solution*
Proceed as directed in the chapter. Then spray the plate with *Spray reagent*, and locate the spots on the plate by examination under UV light at a wavelength of 365 nm.
Acceptance criteria: The R_F values of the principal spots of the *Sample solution* correspond to those of the *Standard solution*.
- **C.** It meets the requirements of the test for *Fats and Fixed Oils* ⟨401⟩, *Fatty Acid Composition*.

IMPURITIES

- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩: NMT 0.1%
- **ALKALINE IMPURITIES**
Sample: 5.0 g of Caprylocaproyl Polyoxylglycerides
Analysis: To the *Sample* add 10 mL of alcohol and 0.05 mL of bromophenol blue TS, and mix well. Titrate with 0.01 N hydrochloric acid VS to change the color to yellow.
Acceptance criteria: NMT 1.0 mL of 0.01 N hydrochloric acid is required.
- **LIMIT OF FREE ETHYLENE OXIDE AND DIOXANE**
Analysis: Proceed as directed in *Ethylene Oxide and Dioxane* ⟨228⟩, *Method I*.
Acceptance criteria
Ethylene oxide: NMT 1 µg/g
Dioxane: NMT 10 µg/g
- **LIMIT OF FREE GLYCEROL**
Sample: 1.20 g
Blank: 25 mL of methylene chloride

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Periodic acetic acid solution: Dissolve 0.446 g of sodium periodate in 2.5 mL of a 25% (v/v) solution of sulfuric acid, and dilute with glacial acetic acid to 100.0 mL.
Potassium iodide solution: 75 mg/mL of potassium iodide
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Residual titration
Titrant: 0.1 M sodium thiosulfate
Analysis: Dissolve the *Sample* in 25 mL of methylene chloride, heating if necessary. Cool, and add 100 mL of water and 25.0 mL of *Periodic acetic acid solution*. Shake, and allow to stand for 30 min. Add 40 mL of *Potassium iodide solution*, and allow to stand for 1 min. Add 1 mL of starch TS, and titrate the liberated iodine with *Titrant*. Perform a blank determination.
Calculate the percentage of free glycerol in the portion of Caprylocaproyl Polyoxylglycerides taken:

$$\text{Result} = [(V_B - V_S) \times N \times E \times F/W] \times 100$$

V_B = *Titrant* volume consumed by the *Blank* (mL)
V_S = *Titrant* volume consumed by the *Sample* (mL)
N = actual normality of the *Titrant* (mEq/mL)
E = equivalency factor for glycerol, 23 mg/mEq
F = unit conversion factor, 10^{-3} g/mg
W = *Sample* weight taken for the titration (g)

Acceptance criteria: NMT 5.0%

SPECIFIC TESTS
- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩**:** NMT 2.0, determined on a 2.0-g specimen
- **FATS AND FIXED OILS,** *Fatty Acid Composition* ⟨401⟩**:** Caprylocaproyl Polyoxylglycerides exhibit the composition profile of fatty acids shown in *Table 1*, as determined in the section *Fatty Acid Composition* under *Fats and Fixed Oils* ⟨401⟩.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
6	0	≤2.0
8	0	50.0–80.0
10	0	20.0–50.0
12	0	≤3.0
14	0	≤1.0

- **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩**:** The hydroxyl value is within the range specified in *Table 2* for the labeled type, when determined on a 1.0-g specimen.

Table 2

Ethylene Oxide Units per Molecule (Nominal Value)	Type of Polyethylene Glycol	Hydroxyl Value
4	200	80–120
6	300	140–180
8	400	170–205

- **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩**:** NMT 2.0
- **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩**:** NMT 6.0, determined on a 2.0-g specimen
- **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩**:** The saponification value is within the range specified in *Table 3* for the labeled type, determined on a 2.0-g specimen.

Table 3

Ethylene Oxide Units per Molecule (Nominal Value)	Type of Polyethylene Glycol	Saponification Value
4	200	265–285
6	300	170–190
8	400	85–105

- **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 1.0%, determined on a 1.0-g specimen. Use as the solvent anhydrous pyridine or a mixture of methylene chloride and anhydrous methanol (7:3).

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light and moisture. Store at controlled room temperature.
- **LABELING:** Label it to indicate the type and the average nominal molecular weight of polyethylene glycol used, or to indicate the number of ethylene oxide units per molecule (nominal value), as part of the official title.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Caprylocaproyl Polyoxylglycerides RS

Caramel

DEFINITION
Caramel is a concentrated solution of the product obtained by heating sugar or glucose until the sweet taste is destroyed and a uniform dark brown mass results, a small amount of alkali or of alkaline carbonate or a trace of mineral acid being added while heating.
[NOTE—Where included in articles for coloring purposes, Caramel complies with the regulations of the FDA concerning color additives (21 CFR 73.85, caramel).]

IMPURITIES
- **ARSENIC,** *Method II* ⟨211⟩**:** NMT 3 ppm
- **LEAD** ⟨251⟩**:** NMT 10 ppm
- **ASH:** It swells when incinerated, and forms a coke-like charcoal that burns off only after prolonged heating at a high temperature. It yields NMT 8.0% of ash.

SPECIFIC TESTS
- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*
- **SPECIFIC GRAVITY** ⟨841⟩**:** NLT 1.30
- **PURITY:** The addition of 0.5 mL of phosphoric acid to 20 mL of a solution (1 in 20) produces no precipitate.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers.

Caraway

DEFINITION
Caraway is the dried, ripe fruit of *Carum carvi* L. (Fam. Apiaceae).

CONTAMINANTS
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Pesticide Residue Analysis***:** Meets the requirements

Supplement 1

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SPECIFIC TESTS

• **BOTANICAL CHARACTERISTICS**

Macroscopic

Unground Caraway: Separate mericarps about 4–7 mm in length and 1–2 mm in diameter. The mericarp is oblong, curved, and tapers toward the base and apex, to which half of the stylopodium is attached. Externally, it is dark brown to weak brown and shows five lighter-colored, filiform, primary ribs, between each pair of which a secondary rib occurs on the dorsal surface.

Powdered Caraway: Moderate yellowish brown to light olive brown

Microscopic

Unground Caraway: The mericarp is nearly equilaterally pentagonal in transverse section and shows a fibrovascular bundle in each primary rib. The epicarp consists of tabular polygonal epidermal cells with thick outer walls possessing a striated cuticle and with occasional stomata. The mesocarp, located between primary rib regions, is composed of collapsed, thin-walled parenchyma normally bearing four dorsal vittae in the intervals and two commissural vittae and sometimes one or more additional vittae. The endocarp consists of broad, slightly undulate, inner epidermal cross cells that are coherent with the collapsed cells of the spermoderm. The endosperm consists of thick-walled reserve parenchyma containing fixed oil and aleurone grains up to 10 µm in diameter, the latter with embedded rosette aggregates of calcium oxalate up to 4 µm in diameter. An embryo is embedded in the upper end of the endosperm.

Powdered Caraway: It shows fragments of the epicarp with striped cuticle, numerous polyhedral endosperm cells containing aleurone grains with embedded rosette aggregates of calcium oxalate, few fragments of slightly lignified fibers and spiral vessels, fragments of cross cells of endocarp, orange to yellow fragments of vittae, and no reticulate parenchyma.

• **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Foreign Organic Matter*: NMT 3.0%

• **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Acid-Insoluble Ash*: NMT 1.5%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers. Preserve against attack by insects.

• **LABELING:** The label states the Latin binomial and, following the official name, the part(s) of the plant contained in the article.

Caraway Oil

DEFINITION

Caraway Oil is the volatile oil distilled from the dried, ripe fruit of *Carum carvi* L. (Fam. Apiaceae). It contains NLT 50.0% of *d*-carvone ($C_{10}H_{14}O$).

ASSAY

• **PROCEDURE**

Sample: 10 mL of Caraway Oil

Analysis: Transfer the *Sample* to a cassia flask, and add 50 mL of a saturated solution of sodium sulfite that has been made neutral to 2 drops of phenolphthalein TS by means of a saturated sodium bisulfite solution. Heat the flask in boiling water, and shake it repeatedly, neutralizing the mixture from time to time by adding a few drops of the saturated sodium bisulfite solution. When no coloration appears upon the addition of a few more drops of phenolphthalein TS and heating for 15 min, cool the mixture to room temperature. When the liquids have separated more completely, add a sufficient volume of the saturated sodium bisulfite solution to raise the lower level of the oily layer to the graduated portion of the neck of the flask.

Acceptance criteria: The volume of the residual oily liquid does not exceed 5 mL, indicating the presence of NLT 50.0% of *d*-carvone ($C_{10}H_{14}O$) in the portion of Oil taken.

SPECIFIC TESTS

• **SOLUBILITY IN 80% ALCOHOL:** One volume dissolves in 8 volumes of 80% alcohol.

• **SPECIFIC GRAVITY** ⟨841⟩: 0.900–0.910

• **OPTICAL ROTATION,** *Angular Rotation* ⟨781A⟩: +70° to +80°

• **REFRACTIVE INDEX** ⟨831⟩: 1.484–1.488 at 20°

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

• **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant source from which the article was derived.

Carbomer 934

DEFINITION

Carbomer 934 is a high molecular weight polymer of acrylic acid cross-linked with allyl ethers of sucrose. Carbomer 934, previously dried under vacuum at 80° for 1 h, contains NLT 56.0% and NMT 68.0% of carboxylic acid (–COOH) groups. The viscosity of a neutralized 0.5% aqueous dispersion of Carbomer 934 is between 30,500 and 39,400 mPa · s.

IDENTIFICATION

• **A.**

Sample dispersion: 10 mg/mL

Analysis 1: To one portion of the *Sample dispersion* add thymol blue TS.

Acceptance criteria 1: An orange color is produced.

Analysis 2: To another portion of the *Sample dispersion* add cresol red TS.

Acceptance criteria 2: A yellow color is produced.

• **B.**

Sample dispersion: 10 mg/mL

Analysis: Adjust the *Sample dispersion* with 1 N sodium hydroxide to a pH of 7.5.

Acceptance criteria: A very viscous gel is produced.

ASSAY

• **CARBOXYLIC ACID CONTENT**

Sample: 400 mg, previously dried

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Direct titration

Electrode: Calomel–glass

Titrant: 0.25 N sodium hydroxide VS

Endpoint detection: Potentiometric

Analysis: Slowly add the *Sample* to 400 mL of water in a 1000-mL beaker, while stirring continuously at about 1000 rpm, with the stirrer shaft set at the side of the beaker at an angle of 60° and with the propeller positioned near the bottom of the beaker. Continue stirring for 15 min. Reduce the stirring speed, and titrate potentiometrically with *Titrant*. After each addition of *Titrant* allow 1 min for mixing before recording the pH.

Calculate the carboxylic acid content as a percentage of carboxylic acid groups:

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$$\text{Result} = [(V \times N/W) \times F] \times 100$$

V = *Titrant* volume consumed (mL)
N = actual normality of the *Titrant* (mEq/mL)
W = *Sample* weight (mg)
F = equivalency factor for the carboxylic acid (–COOH) group, 45.02 mEq/mg

Acceptance criteria: 56.0%–68.0% on the dried basis

IMPURITIES

• **LIMIT OF BENZENE**

Standard solution: 0.2 mg/mL of benzene in methanol. Quantitatively dilute this solution with organic-free water (see *Residual Solvents* ⟨467⟩) to obtain a solution having a known concentration of about 1.0 µg/mL.

Sample solution: Transfer 20 mg of Carbomer 934 to a 100-mL volumetric flask. Add 75 mL of sodium chloride solution (20 mg/mL), and mix by mechanical means until homogeneous (usually about 30 min). Dilute with sodium chloride solution (20 mg/mL) to volume, and mix until homogeneous (usually less than 1 min). This solution must be analyzed within 3 h of preparation.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Columns
Guard: 0.53-mm × 5-m silica, deactivated with phenylmethyl siloxane
Analytical: 0.53-mm × 30-m fused silica, coated with 3.0-µm G43 stationary phase
Temperatures
Injection port: 140°
Detector: 260°
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
40	—	40	20
40	Rapidly	240	20

Carrier gas: Helium
Linear velocity: 35 cm/s
Injection volume: 1 µL
Injection type: Splitless
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 15%
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of benzene in the portion of Carbomer 934 taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of benzene from the *Sample solution*
r_S = peak response of benzene from the *Standard solution*
C_S = concentration of benzene in the *Standard solution* (µg/mL)
C_U = concentration of Carbomer 934 in the *Sample solution* (µg/mL)

Acceptance criteria: NMT 0.5%

SPECIFIC TESTS

• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry a sample under vacuum at 80° for 1 h.
Acceptance criteria: NMT 2.0%

• **VISCOSITY—ROTATIONAL METHODS** ⟨912⟩
Sample: 2.50 g, dried under vacuum at 80° for 1 h
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Electrode: Calomel–glass
Titrant: 180 mg/mL of sodium hydroxide
Endpoint detection: pH
Analysis: Carefully add the *Sample* to 500 mL of water in a 1000-mL beaker, while stirring continuously at 1000 ± 10 rpm, with the stirrer shaft set to the side of the beaker at an angle of 60° and with the propeller positioned near the bottom of the beaker. Allow 45–90 s for addition of the *Sample* at a uniform rate, being sure that loose aggregates of powder are broken up, and continue stirring at 1000 ± 10 rpm for 15 min. Remove the stirrer, and place the beaker containing the dispersion in a 25 ± 0.1° water bath for 30 min. Insert the stirrer to a depth necessary to ensure that air is not drawn into the dispersion, and, while stirring at 300 ± 10 rpm, titrate with *Titrant* to a pH of of 7.3–7.8 by adding the *Titrant* below the surface. Stir for 2–3 min until neutralization is complete. Then determine the final pH. If the pH is less than 7.3, raise it with additional sodium hydroxide. If it is more than 7.8, discard the mucilage, and prepare another, using a smaller amount of sodium hydroxide for titration.
Return the neutralized mucilage to the 25° water bath for 1 h. Measure the pH again, and make certain that the mucilage pH is 7.3–7.8. Perform the viscosity determination without delay to avoid slight viscosity changes that occur 75 min after neutralization.
Equip a suitable rotational viscometer with a spindle having a cylinder 1.5 cm in diameter and 0.2 cm high attached to a shaft 0.3 cm in diameter, the distance from the top of the cylinder to the lower tip of the shaft being 3.02 cm.[1] The spindle rotates at 20 rpm at an immersion depth of 4.9 cm. Follow the instrument manufacturer's directions to measure the apparent viscosity.
Acceptance criteria: 30,500–39,400 mPa · s

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.

• **LABELING:** Label it to indicate that it is not intended for internal use. A carbomer homopolymer manufactured using benzene and complying with the unique requirements of this monograph will be officially titled Carbomer 934 and will not be referred to as Carbomer Homopolymer.

Carbomer 934P

DEFINITION

Carbomer 934P is a high molecular weight polymer of acrylic acid, cross-linked with allyl ethers of sucrose or pentaerythritol. Carbomer 934P, previously dried under vacuum at 80° for 1 h, contains NLT 56.0% and NMT 68.0% of carboxylic acid (–COOH) groups. The viscosity of a

[1] Available as an RV6 spindle from Brookfield, or the equivalent.

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neutralized 0.5% aqueous dispersion of Carbomer 934P is between 29,400 and 39,400 mPa · s.

IDENTIFICATION

• **A.**
Sample dispersion: 10 mg/mL
Analysis 1: To one portion of the *Sample dispersion* add thymol blue TS.
Acceptance criteria 1: An orange color is produced.
Analysis 2: To another portion of the *Sample dispersion* add cresol red TS.
Acceptance criteria 2: A yellow color is produced.

• **B.**
Sample dispersion: 10 mg/mL
Analysis: Adjust the *Sample dispersion* with 1 N sodium hydroxide to a pH of 7.5.
Acceptance criteria: A very viscous gel is produced.

ASSAY

• **CARBOXYLIC ACID CONTENT**
Sample: 400 mg, previously dried
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Electrode: Calomel–glass
Titrant: 0.25 N sodium hydroxide VS.
Endpoint detection: Potentiometric
Analysis: Slowly add the *Sample* to 400 mL of water in a 1000-mL beaker, while stirring continuously at about 1000 rpm, with the stirrer shaft set at the side of the beaker at an angle of 60° and with the propeller positioned near the bottom of the beaker. Continue stirring for 15 min. Reduce the stirring speed, and titrate potentiometrically with *Titrant*. After each addition of *Titrant* allow 1 min for mixing before recording the pH.
Calculate the carboxylic acid content as a percentage of carboxylic acid groups:

$$\text{Result} = [(V \times N/W) \times F] \times 100$$

V = *Titrant* volume consumed (mL)
N = actual normality of the *Titrant* (mEq/mL)
W = *Sample* weight (mg)
F = equivalency factor for the carboxylic acid (–COOH) group, 45.02 mEq/mg

Acceptance criteria: 56.0%–68.0% on the dried basis

IMPURITIES

• **LIMIT OF BENZENE**
Standard solution: 0.2 mg/mL of benzene in methanol. Dilute this solution with organic-free water (see *Residual Solvents* ⟨467⟩) to obtain a solution having a known concentration of about 1.0 µg/mL.
Sample solution: Transfer 1 g of Carbomer 934P to a 100-mL volumetric flask. Add 75 mL of sodium chloride solution (20 mg/mL), and mix by mechanical means until homogeneous (usually about 30 min). Dilute with sodium chloride solution (20 mg/mL) to volume, and mix until homogeneous (usually less than 1 min). This solution must be analyzed within 3 h of preparation.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Columns
Guard: 0.53-mm × 5-m silica, deactivated with phenylmethyl siloxane
Analytical: 0.53-mm × 30-m fused silica, coated with 3.0-µm G43 stationary phase
Temperatures
Injection port: 140°
Detector: 260°
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
40	—	40	20
40	Rapidly	240	20

Carrier gas: Helium
Linear velocity: 35 cm/s
Injection volume: 1 µL
Injection type: Splitless
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 15%
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of benzene in the portion of Carbomer 934P taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of benzene from the *Sample solution*
r_S = peak response of benzene from the *Standard solution*
C_S = concentration of benzene in the *Standard solution* (µg/mL)
C_U = concentration of Carbomer 934P in the *Sample solution* (µg/mL)

Acceptance criteria: NMT 0.01%

SPECIFIC TESTS

• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry a sample under vacuum at 80° for 1 h.
Acceptance criteria: NMT 2.0%

• **VISCOSITY—ROTATIONAL METHODS** ⟨912⟩
Sample: 2.50 g, dried under vacuum at 80° for 1 h
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Electrode: Calomel–glass
Titrant: 180 mg/mL of sodium hydroxide
Endpoint detection: pH
Analysis: Carefully add the *Sample* to 500 mL of water in a 1000-mL beaker, while stirring continuously at 1000 ± 10 rpm, with the stirrer shaft set to the side of the beaker at an angle of 60° and with the propeller positioned near the bottom of the beaker. Allow 45–90 s for addition of the *Sample* at a uniform rate, being sure that loose aggregates of powder are broken up, and continue stirring at 1000 ± 10 rpm for 15 min. Remove the stirrer, and place the beaker containing the dispersion in a 25 ± 0.1° water bath for 30 min. Insert the stirrer to a depth necessary to ensure that air is not drawn into the dispersion, and while stirring at 300 ± 10 rpm, titrate with *Titrant* to a pH of 7.3–7.8 by adding the *Titrant* below the surface. Stir for 2–3 min until neutralization is complete. Then determine the final pH. If the pH is less than 7.3, raise it with additional sodium hydroxide. If it is more than 7.8, discard the mucilage, and prepare another, using a smaller amount of sodium hydroxide for titration.

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Return the neutralized mucilage to the 25° water bath for 1 h. Measure the pH again, and make certain that the mucilage pH is 7.3–7.8. Perform the viscosity determination without delay to avoid slight viscosity changes that occur 75 min after neutralization.

Equip a suitable rotational viscometer with a spindle having a cylinder 1.5 cm in diameter and 0.2 cm high attached to a shaft 0.3 cm in diameter, the distance from the top of the cylinder to the lower tip of the shaft being 3.02 cm.[1] The spindle rotates at 20 rpm at an immersion depth of 4.9 cm. Follow the instrument manufacturer's directions to measure the apparent viscosity.

Acceptance criteria: 29,400–39,400 mPa · s

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.
- **LABELING:** A carbomer homopolymer manufactured using benzene and complying with the unique requirements of this monograph will be officially titled Carbomer 934P and will not be referred to as Carbomer Homopolymer.

Carbomer 940

DEFINITION

Carbomer 940 is a high molecular weight polymer of acrylic acid cross-linked with allyl ethers of pentaerythritol. Carbomer 940, previously dried under vacuum at 80° for 1 h, contains NLT 56.0% and NMT 68.0% of carboxylic acid (–COOH) groups. The viscosity of a neutralized 0.5% aqueous dispersion of Carbomer 940 is between 40,000 and 60,000 mPa · s.

IDENTIFICATION

- **A.**

Sample dispersion: 10 mg/mL

Analysis 1: To one portion of the *Sample dispersion* add thymol blue TS.

Acceptance criteria 1: An orange color is produced.

Analysis 2: To another portion of the *Sample dispersion* add cresol red TS.

Acceptance criteria 2: A yellow color is produced.

- **B.**

Sample dispersion: 10 mg/mL

Analysis: Adjust the *Sample dispersion* with 1 N sodium hydroxide to a pH of 7.5.

Acceptance criteria: A very viscous gel is produced.

ASSAY

- **CARBOXYLIC ACID CONTENT**

Sample: 400 mg, previously dried

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Direct titration

Electrode: Calomel–glass

Titrant: 0.25 N sodium hydroxide VS

Endpoint detection: Potentiometric

Analysis: Slowly add the *Sample* to 400 mL of water in a 1000-mL beaker, while stirring continuously at about 1000 rpm, with the stirrer shaft set at the side of the beaker at an angle of 60° and with the propeller positioned near the bottom of the beaker. Continue stirring for 15 min. Reduce the stirring speed, and titrate potentiometrically with *Titrant*. After each addition of *Titrant* allow 1 min for mixing before recording the pH.

Calculate the carboxylic acid content as a percentage of carboxylic acid groups:

$$\text{Result} = [(V \times N/W) \times F] \times 100$$

V = *Titrant* volume consumed (mL)
N = actual normality of the *Titrant* (mEq/mL)
W = *Sample* weight (mg)
F = equivalency factor for the carboxylic acid (–COOH) group, 45.02 mEq/mg

Acceptance criteria: 56.0%–68.0% on the dried basis

IMPURITIES

- **LIMIT OF BENZENE**

Standard solution: 0.2 mg/mL of benzene in methanol. Dilute this solution with organic-free water (see *Residual Solvents* ⟨467⟩) to obtain a solution having a known concentration of about 1.0 µg/mL.

Sample solution: Transfer 20 mg of Carbomer 940 to a 100-mL volumetric flask. Add 75 mL of sodium chloride solution (20 mg/mL), and mix by mechanical means until homogeneous (usually about 30 min). Dilute with sodium chloride solution (20 mg/mL) to volume, and mix until homogeneous (usually less than 1 min). This solution must be analyzed within 3 h of preparation.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Columns

Guard: 0.53-mm × 5-m silica, deactivated with phenylmethyl siloxane

Analytical: 0.53-mm × 30-m fused silica, coated with 3.0-µm G43 stationary phase

Temperatures

Injection port: 140°

Detector: 260°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
40	—	40	20
40	Rapidly	240	20

Carrier gas: Helium

Linear velocity: 35 cm/s

Injection volume: 1 µL

Injection type: Splitless

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 15%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of benzene in the portion of Carbomer 940 taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of benzene from the *Sample solution*
r_S = peak response of benzene from the *Standard solution*
C_S = concentration of benzene in the *Standard solution* (µg/mL)

[1] Available as an RV6 spindle from Brookfield, or the equivalent.

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C_U = concentration of Carbomer 940 in the *Sample solution* (µg/mL)

Acceptance criteria: NMT 0.5%

SPECIFIC TESTS

• **Loss on Drying** ⟨731⟩
Analysis: Dry a sample under vacuum at 80° for 1 h.
Acceptance criteria: NMT 2.0%

• **Viscosity—Rotational Methods** ⟨912⟩
Sample: 2.50 g, dried under vacuum at 80° for 1 h
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Electrode: Calomel–glass
Titrant: 180 mg/mL of sodium hydroxide
Endpoint detection: pH
Analysis: Carefully add the *Sample* to 500 mL of water in a 1000-mL beaker, while stirring continuously at 1000 ± 10 rpm, with the stirrer shaft set to the side of the beaker at an angle of 60° and with the propeller positioned near the bottom of the beaker. Allow 45–90 s for addition of the *Sample* at a uniform rate, being sure that loose aggregates of powder are broken up, and continue stirring at 1000 ± 10 rpm for 15 min. Remove the stirrer, and place the beaker containing the dispersion in a 25 ± 0.1° water bath for 30 min. Insert the stirrer to a depth necessary to ensure that air is not drawn into the dispersion, and, while stirring at 300 ± 10 rpm, titrate with *Titrant* to a pH of 7.3–7.8 by adding the *Titrant* below the surface. Stir for 2–3 min until neutralization is complete. Then determine the final pH. If the pH is less than 7.3, raise it with additional sodium hydroxide. If it is more than 7.8, discard the mucilage, and prepare another, using a smaller amount of sodium hydroxide for titration.
Return the neutralized mucilage to the 25° water bath for 1 h. Measure the pH again, and make certain that the mucilage pH is 7.3–7.8. Perform the viscosity determination without delay to avoid slight viscosity changes that occur 75 min after neutralization.
Equip a suitable rotational viscometer with a spindle having a shaft about 0.3 cm in diameter, the distance from the top of the shaft to the lower tip of the shaft being 5.0 cm.[1] The spindle rotates at 20 rpm at an immersion depth of 5.5 cm. Follow the instrument manufacturer's directions to measure the apparent viscosity.
Acceptance criteria: 40,000–60,000 mPa · s

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Preserve in tight containers. No storage requirements specified.

• **Labeling:** Label to indicate that it is not intended for internal use. A carbomer homopolymer manufactured using benzene and complying with the unique requirements of this monograph will be officially titled Carbomer 940 and will not be referred to as Carbomer Homopolymer.

Carbomer 941

DEFINITION

Carbomer 941 is a high molecular weight polymer of acrylic acid cross-linked with allyl ethers of pentaerythritol. Carbomer 941, previously dried under vacuum at 80° for 1 h, contains NLT 56.0% and NMT 68.0% of carboxylic acid (–COOH) groups. The viscosity of a neutralized 0.5% aqueous dispersion of Carbomer 941 is between 4000 and 11,000 mPa · s.

IDENTIFICATION

• **A.**
Sample dispersion: 10 mg/mL
Analysis 1: To one portion of the *Sample dispersion* add thymol blue TS.
Acceptance criteria 1: An orange color is produced.
Analysis 2: To another portion of the *Sample dispersion* add cresol red TS.
Acceptance criteria 2: A yellow color is produced.

• **B.**
Sample dispersion: 10 mg/mL
Analysis: Adjust the *Sample dispersion* with 1 N sodium hydroxide to a pH of 7.5.
Acceptance criteria: A very viscous gel is produced.

ASSAY

• **Carboxylic Acid Content**
Sample: 400 mg, previously dried
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Electrode: Calomel–glass
Titrant: 0.25 N sodium hydroxide VS
Endpoint detection: Potentiometric
Analysis: Slowly add the *Sample* to 400 mL of water in a 1000-mL beaker, while stirring continuously at about 1000 rpm, with the stirrer shaft set at the side of the beaker at an angle of 60° and with the propeller positioned near the bottom of the beaker. Continue stirring for 15 min. Reduce the stirring speed, and titrate potentiometrically with *Titrant*. After each addition of *Titrant* allow 1 min for mixing before recording the pH.
Calculate the carboxylic acid content as a percentage of carboxylic acid groups:

$$\text{Result} = [(V \times N/W) \times F] \times 100$$

V = *Titrant* volume consumed (mL)
N = actual normality of the *Titrant* (mEq/mL)
W = *Sample* weight (mg)
F = equivalency factor for the carboxylic acid (–COOH) group, 45.02 mEq/mg

Acceptance criteria: 56.0%–68.0% on the dried basis

IMPURITIES

• **Limit of Benzene**
Standard solution: 0.2 mg/mL of benzene in methanol. Dilute this solution with organic-free water (see *Residual Solvents* ⟨467⟩) to obtain a solution having a known concentration of about 1.0 µg/mL.
Sample solution: Transfer 20 mg of Carbomer 941 to a 100-mL volumetric flask. Add about 75 mL of sodium chloride solution (20 mg/mL), and mix by mechanical means until homogeneous (usually about 30 min). Dilute with sodium chloride solution (20 mg/mL) to volume, and mix until homogenous (usually less than 1 min). This solution must be analyzed within 3 h of preparation.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Columns
Guard: 0.53-mm × 5-m silica, deactivated with phenylmethyl siloxane
Analytical: 0.53-mm × 30-m fused silica, coated with 3.0-µm G43 stationary phase

[1] Available as an RV7 spindle from Brookfield, or the equivalent.

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Temperatures
Injection port: 140°
Detector: 260°
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
40	—	40	20
40	Rapidly	240	20

Carrier gas: Helium
Linear velocity: 35 cm/s
Injection volume: 1 µL
Injection type: Splitless
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 15%
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of benzene in the portion of Carbomer 941 taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of benzene from the *Sample solution*
r_S = peak response of benzene from the *Standard solution*
C_S = concentration of benzene in the *Standard solution* (µg/mL)
C_U = concentration of Carbomer 941 in the *Sample solution* (µg/mL)

Acceptance criteria: NMT 0.5%

SPECIFIC TESTS
• **Loss on Drying** ⟨731⟩
Analysis: Dry a sample under vacuum at 80° for 1 h.
Acceptance criteria: NMT 2.0%
• **Viscosity—Rotational Methods** ⟨912⟩
Sample: 2.50 g, dried under vacuum at 80° for 1 h
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Electrode: Calomel–glass
Titrant: 180 mg/mL of sodium hydroxide
Endpoint detection: pH
Analysis: Carefully add the *Sample* to 500 mL of water in a 1000-mL beaker, while stirring continuously at 1000 ± 10 rpm, with the stirrer shaft set to the side of the beaker at an angle of 60° and with the propeller positioned near the bottom of the beaker. Allow 45–90 s for addition of the *Sample* at a uniform rate, being sure that loose aggregates of powder are broken up, and continue stirring at 1000 ± 10 rpm for 15 min. Remove the stirrer, and place the beaker containing the dispersion in a 25 ± 0.1° water bath for 30 min. Insert the stirrer to a depth necessary to ensure that air is not drawn into the dispersion, and while stirring at 300 ± 10 rpm, titrate with *Titrant* to a pH of 7.3–7.8 by adding the *Titrant* below the surface. Stir for 2–3 min until neutralization is complete. Then determine the final pH. If the pH is less than 7.3, raise it with additional sodium hydroxide. If it is more than 7.8, discard the mucilage, and prepare another, using a smaller amount of sodium hydroxide for titration.
Return the neutralized mucilage to the 25° water bath for 1 h. Measure the pH again, and make certain that the mucilage pH is 7.3–7.8. Perform the viscosity determination without delay to avoid slight viscosity changes that occur 75 min after neutralization.
Equip a suitable rotational viscometer with a spindle having a disk about 2.1 cm in diameter and 0.2 cm high, attached to a shaft 0.3 cm in diameter, the distance from the top of the disk to the lower tip of the shaft being 2.7 cm.[1] The spindle rotates at 20 rpm at an immersion depth of 4.9 cm. Follow the instrument manufacturer's directions to measure the apparent viscosity.
Acceptance criteria: 4,000–11,000 mPa · s

ADDITIONAL REQUIREMENTS
• **Packaging and Storage:** Preserve in tight containers. No storage requirements specified.
• **Labeling:** Label it to indicate that it is not intended for internal use. A carbomer homopolymer manufactured using benzene and complying with the unique requirements of this monograph will be officially titled Carbomer 941 and will not be referred to as Carbomer Homopolymer.

Carbomer 1342

DEFINITION
Carbomer 1342 is a high molecular weight copolymer of acrylic acid and a long-chain alkyl methacrylate cross-linked with allyl ethers of pentaerythritol. Carbomer 1342, previously dried under vacuum at 80° for 1 h, contains NLT 52.0% and NMT 62.0% of carboxylic acid (–COOH) groups. The viscosity of a neutralized 1.0% aqueous dispersion of Carbomer 1342 is between 9500 and 26,500 centipoises.

IDENTIFICATION
• **A.**
Sample dispersion: 10 mg/mL
Analysis 1: To one portion of the *Sample dispersion* add thymol blue TS.
Acceptance criteria 1: An orange color is produced.
Analysis 2: To another portion of the *Sample dispersion* add cresol red TS.
Acceptance criteria 2: A yellow color is produced.
• **B.**
Sample dispersion: 10 mg/mL
Analysis: Adjust the *Sample dispersion* with 1 N sodium hydroxide to a pH of 7.5.
Acceptance criteria: A very viscous gel is produced.

ASSAY
• **Carboxylic Acid Content**
Sample: 400 mg, previously dried
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Electrode: Calomel–glass
Titrant: 0.25 N sodium hydroxide VS
Endpoint detection: Potentiometric
Analysis: Slowly add the *Sample* to 400 mL of water in a 1000-mL beaker, while stirring continuously at about 1000 rpm, with the stirrer shaft set at the side of the beaker at an angle of 60° and with the propeller positioned near the bottom of the beaker. Continue stirring for 15 min. Reduce the stirring speed, and titrate potentiometrically

[1] Available as an RV5 spindle from Brookfield, or the equivalent.

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with *Titrant*. After each addition of *Titrant*, allow 1 min for mixing before recording the pH.
Calculate the carboxylic acid content as a percentage of carboxylic acid groups:

$$\text{Result} = [(V \times N/W) \times F] \times 100$$

V = *Titrant* volume consumed (mL)
N = actual normality of the *Titrant* (mEq/mL)
W = *Sample* weight (mg)
F = equivalency factor for the carboxylic acid (–COOH) group, 45.02 mEq/mg

Acceptance criteria: 52.0%–62.0% on the dried basis

IMPURITIES

• **LIMIT OF BENZENE**
Standard solution: 0.2 mg/mL of benzene in methanol. Dilute this solution with organic-free water (see *Residual Solvents* ⟨467⟩) to obtain a solution having a known concentration of about 1.0 µg/mL.
Sample solution: Transfer 50 mg of Carbomer 1342 to a 100-mL volumetric flask. Add 75 mL of sodium chloride solution (20 mg/mL), and mix by mechanical means until homogeneous (usually about 30 min). Dilute with sodium chloride solution (20 mg/mL) to volume, and mix until homogeneous (usually less than 1 min). This solution must be analyzed within 3 h of preparation.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Columns
Guard: 0.53-mm × 5-m silica, deactivated with phenylmethyl siloxane
Analytical: 0.53-mm × 30-m fused silica, coated with 3.0-µm G43 stationary phase
Temperatures
Injection port: 140°
Detector: 260°
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
40	—	40	20
40	Rapidly	240	20

Carrier gas: Helium
Linear velocity: 35 cm/s
Injection volume: 1 µL
Injection type: Splitless
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 15%
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of benzene in the portion of Carbomer 1342 taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of benzene from the *Sample solution*
r_S = peak response of benzene from the *Standard solution*
C_S = concentration of benzene in the *Standard solution* (µg/mL)
C_U = concentration of Carbomer 1342 in the *Sample solution* (µg/mL)

Acceptance criteria: NMT 0.2%

SPECIFIC TESTS

• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry a sample under vacuum at 80° for 1 h.
Acceptance criteria: NMT 2.0%
• **VISCOSITY—ROTATIONAL METHODS** ⟨912⟩
Sample: 5.00 g, dried under vacuum at 80° for 1 h
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Electrode: Calomel–glass
Titrant: 180 mg/mL of sodium hydroxide
Endpoint detection: pH
Analysis: Carefully add the *Sample* to 500 mL of water in a 1000-mL beaker, while stirring continuously at 1000 ± 10 rpm, with the stirrer shaft set to the side of the beaker at an angle of 60° and with the propeller positioned near the bottom of the beaker. Allow 45–90 s for addition of the sample at a uniform rate, being sure that loose aggregates of powder are broken up, and continue stirring at 1000 ± 10 rpm for 15 min. Insert the stirrer to a depth necessary to ensure that air is not drawn into the dispersion, and while stirring at 300 ± 10 rpm, titrate with *Titrant* to a pH of 7.3–7.8 by adding the *Titrant* below the surface. Stir for 2–3 min until neutralization is complete. Then determine the final pH. If the pH is less than 7.3, raise it with additional sodium hydroxide. If it is more than 7.8, discard the mucilage, and prepare another, using a smaller amount of sodium hydroxide for titration.
Return the neutralized mucilage to the 25° water bath for 1 h. Measure the pH again, and make certain that the mucilage pH is 7.3–7.8. Perform the viscosity determination without delay to avoid slight viscosity changes that occur 75 min after neutralization. Equip a suitable rotational viscometer with a spindle having a cylinder 1.5 cm in diameter and 0.2 cm high attached to a shaft 0.3 cm in diameter, the distance from the top of the cylinder to the lower tip of the shaft being 3.02 cm.[1] The spindle rotates at 20 rpm at an immersion depth of 4.9 cm. Follow the instrument manufacturer's directions to measure the apparent viscosity.
Acceptance criteria: 9500–26,500 centipoises

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers.
• **LABELING:** Label to indicate that it is not intended for internal use. A carbomer copolymer manufactured using benzene and complying with the unique requirements of this monograph will be officially titled Carbomer 1342 and will not be referred to as Carbomer Copolymer.

Carbomer Copolymer

DEFINITION

Carbomer Copolymer is a high molecular weight copolymer of acrylic acid and a long-chain alkyl methacrylate cross-linked with allyl ethers of polyalcohols.
[NOTE—The heading of this monograph does not constitute the official title for a Carbomer Copolymer

[1] Available as an RV6 spindle from Brookfield, or the equivalent.

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manufactured with the use of benzene. When benzene is used in the manufacturing process, the name will be Carbomer 1342, provided it complies with the existing requirements in the Carbomer 1342 monograph.]

IDENTIFICATION

Change to read:

- **A. ▲Spectroscopic Identification Tests** ⟨197⟩, *Infrared Spectroscopy*: **197K.**▲ (CN 1-May-2020) Exhibits main bands at or near (±5) wavenumbers (cm^{-1}) 1710, 1454, 1414, 1245, 1172, 1115, and 801, with the strongest band at 1710.
- **B.**

Sample: 5 g
Analysis: Add the *Sample* to 500 mL of water, and stir.
Acceptance criteria: A dispersion is formed, with a foam layer that persists after the dispersion is allowed to stand at room temperature for 1 h.

ASSAY

- **Content of Carboxylic Acid**

Sample: 400 mg, previously dried under vacuum at 80° for 1 h
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Electrode: Calomel–glass
Titrant: 0.25 N sodium hydroxide VS
Endpoint detection: Potentiometric
Analysis: Slowly add the *Sample* to 400 mL of water in a 1000-mL beaker, while stirring continually at 1000 rpm. The stirrer shaft is set at an angle of about 60° and to one side of the beaker, and the propeller is positioned near the bottom of the beaker. Continue stirring for 15 min. Reduce the stirring speed, and titrate with *Titrant*. After each addition of *Titrant*, allow 1 min for mixing before recording the pH.
Calculate the carboxylic acid content as a percentage of carboxylic acid groups in the portion of Carbomer Copolymer taken:

$$\text{Result} = [(V \times N \times M_r)/W] \times 100$$

V = *Titrant* volume consumed by the *Sample* (mL)
N = actual normality of the *Titrant* (mEq/mL)
M_r = molecular weight of the carboxylic acid (–COOH) group, 45.02
W = *Sample* weight (mg)

Acceptance criteria: 52.0%–62.0%

IMPURITIES

- **Limit of Ethyl Acetate and Cyclohexane**

[Note—This test is required only for those Carbomer Copolymers where the labeling indicates that ethyl acetate or a mixture of ethyl acetate and cyclohexane was used in the polymerization process.]
Standard stock solution: Transfer 5.0 mL of methanol to a 10-mL serum vial, insert a rubber septum, and seal with a metal cap. Add 25.0 µL of ethyl acetate and 20.0 µL of cyclohexane through the septum into the vial, and mix.
Standard solution: Transfer 20.0 mL of methanol to a 30-mL serum vial, insert a rubber septum, and seal with a metal cap. Through the rubber septum add 10 µL of methyl ethyl ketone (internal standard) and 50.0 µL of the *Standard stock solution*, and mix to obtain a solution containing 0.225 mg of ethyl acetate and 0.156 mg of cyclohexane.
Sample solution: Transfer 50 mg of Carbomer Copolymer to a 30-mL serum vial, add 20.0 mL of methanol, insert a rubber septum, and seal with a metal cap. Through the rubber septum add 10 µL of methyl ethyl ketone, and mix.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 2-mm × 3-m; packed with 1% liquid phase G25 on 60- to 80-mesh support S12
Temperatures
Detector: 250°
Injection port: 250°
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
115	—	115	4
115	6	175	5

Carrier gas: Helium
Flow rate: 33 mL/min
Injection volume: 2 µL
System suitability
Sample: *Standard solution*
[Note—The relative retention times for methyl ethyl ketone, ethyl acetate, and cyclohexane are about 0.7, 0.9, and 1.0, respectively.]
Suitability requirements
Resolution: NLT 1.0 between ethyl acetate and cyclohexane
Relative standard deviation: NMT 2.5%
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentages of ethyl acetate and cyclohexane in the portion of Carbomer Copolymer taken:

$$\text{Result} = (R_U/R_S) \times (W_S/W_U) \times 100$$

R_U = peak area ratio of the relevant analyte peak to the methyl ethyl ketone peak from the *Sample solution*
R_S = peak area ratio of the relevant analyte peak to the methyl ethyl ketone peak from the *Standard solution*
W_S = weight of ethyl acetate or cyclohexane in the *Standard solution* (mg)
W_U = weight of Carbomer Copolymer taken to prepare the *Sample solution* (mg)

Acceptance criteria
Ethyl acetate: NMT 0.5%
Cyclohexane: NMT 0.3%

- **Limit of Benzene**

Benzene solution: 1.0 mg/mL of benzene in dimethyl sulfoxide
Solvent solution: 0.1 µg/mL of *Benzene solution* in organic-free water (see *Residual Solvents* ⟨467⟩, *Identification, Control, and Quantification of Residual Solvents*)
Sodium chloride solution: 20 mg/mL of sodium chloride
Sample solution: Transfer 50 mg of Carbomer Copolymer to a 10-mL volumetric flask. Add 7.5 mL of *Sodium chloride solution*, and mix by mechanical means until homogeneous (usually about 30 min). Dilute with *Sodium chloride solution* to volume, and mix until homogeneous (usually less than 1 min). This solution must be analyzed within 3 h of preparation.

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Standard solution: Transfer 50 mg of Carbomer Copolymer to a 10-mL volumetric flask. Add 7.5 mL of *Sodium chloride solution*, and mix by mechanical means until homogeneous (usually about 30 min). Add 1.0 mL of the *Solvent solution*, dilute with *Sodium chloride solution* to volume, and mix until homogeneous (usually less than 1 min). This solution contains 0.01 µg/mL of benzene.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC, equipped with a headspace injector
Detector: Flame ionization
Column: 0.53-mm × 30-m fused silica; coated with 3.0-µm G43 stationary phase
Temperatures
Detector: 250°
Injection port: 140°
Column: See *Table 2*.

Table 2

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
40	—	40	20
40	10	240	20

Carrier gas: Helium
Flow rate: Linear velocity of 35 cm/s
Split ratio: 5:1
Injection volume: 1 mL (gaseous phase)
[NOTE—The following headspace conditions may be used: a pressurization time of 30 s and a transfer line temperature of 90°.]
Vial temperature: The vials are maintained at 80° for 60 min before headspace injection.

System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 15% for three replicate injections

Analysis
Samples: *Standard solution* and *Sample solution*
Transfer 10.0 mL each of the *Standard solution* and the *Sample solution* to separate headspace vials. Close the vials with a tight rubber membrane stopper coated with polytetrafluoroethylene, and secure with an aluminum crimped cap. Shake to obtain a homogeneous dispersion. Separately inject the gaseous phase of the *Standard solution* and the *Sample solution*.

Acceptance criteria: The benzene peak response from the *Sample solution* is NMT half of that from the *Standard solution*, corresponding to NMT 2 µg/g.

• **LIMIT OF ACRYLIC ACID**

Mobile phase: Dissolve 6.80 g of monobasic potassium phosphate in 300 mL of water, dilute with water to 500 mL, and mix. Dilute 100 mL of this solution with water to 1 L, adjust with phosphoric acid to a pH of 3.0 ± 0.1, and mix. Filter and degas.

Standard solution: 25 µg/mL of acrylic acid

Sample solution: Transfer 100 mg of Carbomer Copolymer to a tared serum vial. Add water to obtain 10.0 mL of solution. Cap the vial, and shake by mechanical means for 2 h. Add 2 drops of sodium hydroxide solution (500 mg/mL), and shake by hand for 15 s. Add 1.0 mL of calcium chloride solution (100 mg/mL), and shake until the gel collapses. Centrifuge for 15 min, and use the clear supernatant.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 200 nm
Column: 8-mm × 10-cm; packing L1
Flow rate: 1 mL/min
Injection volume: 10 µL

System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 5%

Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of free acrylic acid in the portion of Carbomer Copolymer taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak response of acrylic acid from the *Sample solution*
r_S = peak response of acrylic acid from the *Standard solution*
C_S = concentration of acrylic acid in the *Standard solution* (µg/mL)
C_U = concentration of Carbomer Copolymer in the *Sample solution* (mg/mL)
F = unit conversion, 10^{-3} mg/µg

Acceptance criteria: NMT 0.25%

SPECIFIC TESTS

• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry under vacuum at 80° for 1 h.
Acceptance criteria: NMT 2.0%

• **VISCOSITY—ROTATIONAL METHODS** ⟨912⟩
Sample: 5.00 g of Carbomer Copolymer, previously dried under vacuum at 80° for 1 h

Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Electrode: Calomel–glass
Titrant: 180 mg/mL of sodium hydroxide
Endpoint detection: Potentiometric

Analysis: Carefully add the *Sample* to 500 mL of water in a 1000-mL beaker, while stirring continuously at 1000 ± 50 rpm, with the stirrer shaft set to one side of the beaker at an angle of 60° and the propeller positioned near the bottom of the beaker. The stirrer used is a three-blade, 2-inch–diameter marine impeller. Add the *Sample* at a uniform rate for 45–90 s, being sure that loose aggregates of powder are broken up, and continue stirring at 1000 ± 50 rpm for 15 min. Remove the stirrer, and place the beaker containing the dispersion in a 25 ± 0.1° water bath for 30 min. Insert a paddle stirrer to a depth necessary to ensure that air is not drawn into the dispersion; and while stirring at 300 ± 25 rpm, titrate to a pH of 7.3–7.8 by adding *Titrant* below the surface. Stir 2–3 min until neutralization is complete. Then determine the final pH. [NOTE—If the pH is below 7.3, raise it with additional sodium hydroxide. If it is above 7.8, discard the mucilage, and prepare another batch, using a smaller amount of sodium hydroxide for titration.]

Return the beaker containing the neutralized mucilage to the 25 ± 0.1° water bath for 1 h. Measure the pH again, making certain that it is between 7.3 and 7.8. Using a rotational viscometer equipped with a suitable spindle at a spindle immersion depth as defined in *Table 3*, perform the viscosity determination without delay to avoid the slight viscosity changes that occur 75 min after neutralization.

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Table 3 (Carbomer Copolymer)

Viscosity Ranges (mPa · s)	Spindle No.	A[a] (cm)	B[b] (cm)	C[c] (cm)	D[d] (cm)	E[e] (cm)	Multiplier
100–400	1	5.6	2.2	0.3	2.7	6.1	5
400–1600	2	4.7	0.2	0.3	2.7	4.9	20
1000–4000	3	3.5	0.2	0.3	2.7	4.9	50
2000–8000	4	2.7	0.2	0.3	2.7	4.9	100
4000–16,000	5	2.1	0.2	0.3	2.7	4.9	200
10,000–40,000	6	1.5	0.2	0.3	3.0	4.9	500
40,000–160,000	7	—	—	0.3	—	5.5	2,000

[a] Cylinder diameter.
[b] Cylinder height.
[c] Shaft diameter.
[d] Distance from the top of the cylinder to the lower tip of the shaft.
[e] Spindle immersion depth.

The spindle rotates at 20 rpm. Follow the instrument manufacturer's directions to measure the apparent viscosity.
Acceptance criteria: See *Table 4*.

Table 4

Carbomer Copolymer	1% Viscosity Specification (mPa · s)
A	4,500–13,500
B	10,000–29,000
C	25,000–45,000

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, at a temperature not exceeding 45°.
- **LABELING:** If benzene has been used in the manufacturing process, the name of the article will be Carbomer 1342, provided it complies with and is labeled in accordance with the requirements set forth in that monograph. If benzene is not used in the manufacturing process, label it to indicate whether it is Type A, B, or C; and label it to state the measured viscosity, giving the viscosity measurement parameters, the concentration of the solution, and the type of equipment used; the solvent or solvents used in the polymerization process; and the nominal and residual solvent levels for each solvent.

Carbomer Homopolymer

DEFINITION

Carbomer Homopolymer is a high molecular weight polymer of acrylic acid cross-linked with allyl ethers of polyalcohols. Carbomer Homopolymer, previously dried, contains NLT 56.0% and NMT 68.0% of carboxylic acid (–COOH) groups. [NOTE—This monograph applies to, but is not limited to, Carbomer 934, Carbomer 934P, Carbomer 940, and Carbomer 941 manufactured without benzene. The heading of this monograph does not constitute the official title for a Carbomer Homopolymer manufactured with the use of benzene. When benzene is used in the manufacturing process, the name of the article will be Carbomer 934, Carbomer 934P, Carbomer 940, or Carbomer 941, whichever is appropriate.]

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K.▲** (CN 1-May-2020)**:** Exhibits main bands at or near (±5) wavenumbers (cm^{-1}) 1710, 1454, 1414, 1245, 1172, 1115, and 801, with the strongest band at 1710.
- **B.**
  Sample dispersion: 10 mg/mL
  Analysis: Adjust the *Sample dispersion* with 1 N sodium hydroxide to a pH of 7.5.
  Acceptance criteria: A viscous gel is produced.
- **C.**
  Analysis: Add 2 mL of calcium chloride solution (100 mg/mL), while stirring, to 10 mL of the gel obtained from *Identification* test *B*.
  Acceptance criteria: A white precipitate is produced immediately.
- **D.**
  Sample dispersion: 10 mg/mL
  Analysis 1: Add 0.5 mL of thymol blue TS to 10 mL of the *Sample dispersion*.
  Acceptance criteria 1: An orange color is produced.
  Analysis 2: To another 10 mL of the *Sample dispersion* add 0.5 mL of cresol red TS.
  Acceptance criteria 2: A yellow color is produced.

ASSAY

- **CONTENT OF CARBOXYLIC ACID**
  Sample: 400 mg, previously dried under vacuum at 80° for 1 h
  Titrimetric system
  (See *Titrimetry* ⟨541⟩.)
  Mode: Direct titration
  Electrode: Calomel–glass
  Titrant: 0.25 N sodium hydroxide VS
  Endpoint detection: Potentiometric
  Analysis: Slowly add the *Sample* to 400 mL of water in a 1000-mL beaker, while stirring continually at 1000 rpm. The stirrer shaft is set at an angle of about 60° and to one side of the beaker, and the propeller is positioned near the bottom of the beaker. Continue stirring for 15 min. Reduce the stirring speed, and titrate with *Titrant*. After each addition of *Titrant*, allow 1 min for mixing before recording the pH.
  Calculate the carboxylic acid content as a percentage of carboxylic acid groups in the portion of Carbomer Homopolymer taken:

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$$\text{Result} = [(V \times N \times M_r)/W] \times 100$$

V = *Titrant* volume consumed by the *Sample* (mL)
N = actual normality of the *Titrant* (mEq/mL)
M_r = molecular weight of the carboxylic acid (–COOH) group, 45.02
W = *Sample* weight (mg)

Acceptance criteria: 56.0%–68.0%

IMPURITIES

• **Residue on Ignition** ⟨281⟩
Sample: 1.0 g
Acceptance criteria: NMT 4.0%

• **Limit of Ethyl Acetate and Cyclohexane**
[Note—This test is required only for those Carbomer Homopolymers where ethyl acetate or a mixture of ethyl acetate and cyclohexane was used in the polymerization process.]
Standard stock solution: Transfer 5.0 mL of methanol to a 10-mL serum vial, insert a rubber septum, and seal with a metal cap. Add 25.0 µL of ethyl acetate and 20.0 µL of cyclohexane through the septum into the vial, and mix.
Standard solution: Transfer 20.0 mL of methanol to a 30-mL serum vial, insert a rubber septum, and seal with a metal cap. Through the rubber septum add 10 µL of methyl ethyl ketone (internal standard) and 50.0 µL of the *Standard stock solution*, and mix.
Sample solution: Transfer 50 mg of Carbomer Homopolymer to a 30-mL serum vial, add 20.0 mL of methanol, insert a rubber septum, and seal with a metal cap. Through the rubber septum add 10 µL of methyl ethyl ketone, and mix.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 2-mm × 3-m; packed with 1% liquid phase G25 on 60- to 80-mesh support S12
Temperatures
Detector: 250°
Injection port: 250°
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
115	—	115	4
115	6	175	5

Carrier gas: Helium
Flow rate: 33 mL/min
Injection volume: 2 µL
System suitability
Sample: *Standard solution*
[Note—The relative retention times for methyl ethyl ketone, ethyl acetate, and cyclohexane are about 0.7, 0.9, and 1.0, respectively.]
Suitability requirements
Resolution: NLT 1.0 between ethyl acetate and cyclohexane
Relative standard deviation: NMT 2.5%
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentages of ethyl acetate and cyclohexane in the portion of Carbomer Homopolymer taken:

$$\text{Result} = (R_U/R_S) \times (W_S/W_U) \times 100$$

R_U = peak area ratio of the relevant analyte peak to the methyl ethyl ketone peak from the *Sample solution*
R_S = peak area ratio of the relevant analyte peak to the methyl ethyl ketone peak from the *Standard solution*
W_S = weight of ethyl acetate or cyclohexane in the *Standard solution* (mg)
W_U = weight of Carbomer Homopolymer taken to prepare the *Sample solution* (mg)

Acceptance criteria
Ethyl acetate: NMT 0.5%
Cyclohexane: NMT 0.3%

• **Limit of Benzene**
Benzene solution: 1.0 mg/mL of benzene in dimethyl sulfoxide
Solvent solution: 0.1 µg/mL of *Benzene solution* in organic-free water (see *Residual Solvents* ⟨467⟩, *Identification, Control, and Quantification of Residual Solvents*).
Sodium chloride solution: 20 mg/mL of sodium chloride
Standard solution: Transfer 50 mg of Carbomer Homopolymer to a 10-mL volumetric flask. Add 7.5 mL of *Sodium chloride solution*, and mix by mechanical means until homogeneous (usually about 30 min). Add 1.0 mL of the *Solvent solution*, dilute with *Sodium chloride solution* to volume, and mix until homogeneous (usually less than 1 min). This solution contains 0.01 µg/mL of benzene.
Sample solution: Transfer 50 mg of Carbomer Homopolymer to a 10-mL volumetric flask. Add 7.5 mL of *Sodium chloride solution*, and mix by mechanical means until homogeneous (usually about 30 min). Dilute with *Sodium chloride solution* to volume, and mix until homogeneous (usually less than 1 min). This preparation must be analyzed within 3 h of preparation.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC, equipped with a headspace injector
Detector: Flame ionization
Column: 0.53-mm × 30-m fused silica; coated with a 3.0-µm G43 stationary phase
Temperatures
Detector: 250°
Injection port: 140°
Column: See *Table 2*.

Table 2

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
40	—	40	20
40	10	240	20

Carrier gas: Helium
Flow rate: At a linear velocity of 35 cm/s
Injection type: Split ratio, 5:1
Injection volume: 1 mL (gaseous phase)
[Note—The following headspace conditions may be used: a pressurization time of 30 s and a transfer line temperature of 90°.]
The vials are maintained at a temperature of 80° for 60 min before headspace injection.
System suitability
Sample: *Standard solution*

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Suitability requirements
Relative standard deviation: NMT 15% for three replicate injections
Analysis
Samples: *Standard solution* and *Sample solution*
Transfer 10.0 mL each of the *Standard solution* and *Sample solution* to separate headspace vials. Close the vials with a tight rubber membrane stopper coated with polytetrafluoroethylene, and secure with an aluminum crimped cap. Shake to obtain a homogeneous dispersion. Separately inject the gaseous phase of the *Standard solution* and the *Sample solution*.
Acceptance criteria: NMT 2 µg/g; the benzene peak response from the *Sample solution* is NMT half of that from the *Standard solution*.

• **LIMIT OF ACRYLIC ACID**
Solution A: 1.361 mg/mL of monobasic potassium phosphate
Solution B: Acetonitrile and *Solution A* (1:1)
Mobile phase: See *Table 3*.

Table 3

Time (min)	Solution A (%)	Solution B (%)
0	100	0
8	100	0
9	0	100
20	0	100
21	100	0
30	100	0

Diluent: 25 mg/mL of potassium alum
Standard solution: 12.5 µg/mL of acrylic acid in *Diluent*
Sample solution: Mix 100 mg of Carbomer Homopolymer with *Diluent*, and add *Diluent* to obtain 20.0 mL of suspension. Heat the suspension at 50° for 20 min with occasional shaking. Then shake the suspension continuously at room temperature for 60 min. Centrifuge, and use the clear supernatant.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 205 nm
Column: 4.6-mm × 12-cm; packing L1
Flow rate: 1 mL/min
Injection volume: 20 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 5%
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of free acrylic acid in the portion of Carbomer Homopolymer taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak response of acrylic acid from the *Sample solution*
r_S = peak response of acrylic acid from the *Standard solution*
C_S = concentration of acrylic acid in the *Standard solution* (µg/mL)
C_U = concentration of Carbomer Homopolymer in the *Sample solution* (mg/mL)
F = unit conversion, 10^{-3} mg/µg

Acceptance criteria: NMT 0.25%

SPECIFIC TESTS

• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry under vacuum at 80° for 1 h.
Acceptance criteria: NMT 2.0%

• **VISCOSITY—ROTATIONAL METHODS** ⟨912⟩
Sample: 2.50 g of Carbomer Homopolymer, previously dried
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Electrode: Calomel–glass
Titrant: 180 mg/mL of sodium hydroxide
Endpoint detection: Potentiometric
Analysis: Carefully add the *Sample* to 500 mL of water in a 800-mL beaker, while stirring continuously at 1000 ± 50 rpm. The stirrer shaft is set at an angle of 60° and positioned at one side of the beaker, and the propeller is positioned near the bottom of the beaker. The stirrer used should be a three-blade, 2-inch marine impeller. Add the *Sample* at a uniform rate over a period of 45–60 s, being sure that loose aggregates of powder are broken up, and continue stirring at 1000 ± 50 rpm for 15 min. [NOTE—Proper dispersion of the Carbomer Homopolymer resin is imperative for accurate viscosity readings.] Remove the stirrer, and allow the beaker containing the dispersion to stand at controlled room temperature for 30 min. Insert a paddle stirrer to a depth necessary to ensure that the air is not drawn into the dispersion, and while stirring at 300 ± 25 rpm, titrate with *Titrant* to a pH of 7.3–7.8. After adding the *Titrant*, stir with a paddle mixer at 300 ± 25 rpm for 2–3 min. [NOTE—After neutralization, care must be taken to avoid excessively high shearing, because aggressive mixing will break the polymer chains and reduce the viscosity reading.] Take the final pH reading with a pH meter to make sure it is between 7.3 and 7.8. [NOTE—If the pH is below 7.3, raise it with additional sodium hydroxide. If the pH is above 7.8, discard the mucilage, and prepare another using a smaller amount of sodium hydroxide for titration.]
Place the neutralized mucilage into a water bath maintained at 25 ± 0.1° for 1 h. Measure the pH again, and make certain it is between 7.3 and 7.8. Perform the viscosity determination without delay to avoid the slight viscosity changes that occur 75 min after neutralization.
Equip a suitable rotational viscometer[1] with a suitable spindle, as defined in *Table 4*.
The spindle rotates at 20 rpm. Follow the instrument manufacturer's directions to measure the apparent viscosity.
Acceptance criteria: See *Table 5*.

Table 5

Carbomer Homopolymer	Viscosity Specified (mPa · s)
A	4,000–11,000
B	25,000–45,000
C	40,000–60,000

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers, at a temperature not exceeding 45°.

• **LABELING:** Label it to indicate whether it is Type A, B, or C; and label it to state the measured viscosity, giving the viscosity measurement parameters, the concentration of

[1] Available as a Brookfield RV viscometer, or equivalent.

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Table 4 (Carbomer Homopolymer)

Viscosity Ranges (mPa · s)	Spindle No.	A[a] (cm)	B[b] (cm)	C[c] (cm)	D[d] (cm)	E[e] (cm)	Multiplier
100–400	1	5.6	2.2	0.3	2.7	6.1	5
400–1600	2	4.7	0.2	0.3	2.7	4.9	20
1000–4000	3	3.5	0.2	0.3	2.7	4.9	50
2000–8000	4	2.7	0.2	0.3	2.7	4.9	100
4000–16,000	5	2.1	0.2	0.3	2.7	4.9	200
10,000–40,000	6	1.5	0.2	0.3	3.0	4.9	500
40,000–160,000	7	—	—	0.3	—	5.5	2,000

[a] Cylinder diameter.
[b] Cylinder height.
[c] Shaft diameter.
[d] Distance from the top of the cylinder to the lower tip of the shaft.
[e] Spindle immersion depth.

the solution, and the type of equipment used; the solvent or solvents used in the polymerization process; and the nominal and residual solvent levels for each solvent.

Carbomer Interpolymer

DEFINITION

Carbomer Interpolymer is a carbomer homopolymer or copolymer that contains a block copolymer of polyethylene glycol and a long-chain alkyl acid ester.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS ⟨197⟩, *Infrared Spectroscopy*: 197K.▲** (CN 1-May-2020) Exhibits bands at or near (±5) wavenumbers (cm^{-1}) 1710, 1454, 1414, 1245, 1172, 1115, and 801, with the strongest band at 1710.
- **B.**
  Sample: 2.5 g
  Analysis: Without mixing, add the *Sample* to 500 mL of water in an 800-mL beaker, and allow to stand.
  Acceptance criteria: The powders wet out in NMT 60 min.
- **C.**
  Sample: 5 g
  Analysis: Add the *Sample* to 500 mL of water, stir, and adjust with 1 N sodium hydroxide to a pH of 7.5.
  Acceptance criteria: A viscous gel is formed.

ASSAY

- **CONTENT OF CARBOXYLIC ACID**
  Sample: 400 mg, previously dried under vacuum at 80° for 1 h
  Titrimetric system
  (See *Titrimetry* ⟨541⟩.)
  Mode: Direct titration
  Electrode: Calomel–glass
  Titrant: 0.25 N sodium hydroxide VS
  Endpoint detection: Potentiometric
  Analysis: Slowly add the *Sample* to 400 mL of water in a 1000-mL beaker, while stirring continually at 1000 rpm. The stirrer shaft is set at an angle of about 60° and to one side of the beaker, and the propeller is positioned near the bottom of the beaker. Continue stirring for 15 min. Reduce the stirring speed, and titrate with *Titrant*. After each addition of *Titrant*, allow 1 min for mixing before recording the pH.
  Calculate the carboxylic acid content as a percentage of carboxylic acid groups in the portion of Carbomer Interpolymer taken:

$$\text{Result} = [(V \times N \times M_r)/W] \times 100$$

V = *Titrant* volume consumed by the *Sample* (mL)
N = actual normality of the *Titrant* (mEq/mL)
M_r = molecular weight of the carboxylic acid (–COOH) group, 45.02
W = *Sample* weight (mg)

Acceptance criteria: 52.0%–62.0%

IMPURITIES

- **LIMIT OF ETHYL ACETATE AND CYCLOHEXANE**
  [NOTE—This test is required only for those Carbomer Interpolymers where the labeling indicates that ethyl acetate or a mixture of ethyl acetate and cyclohexane was used in the polymerization process.]
  Standard stock solution: Transfer 5.0 mL of methanol to a 10-mL serum vial, insert a rubber septum, and seal with a metal cap. Add 20.0 µL of ethyl acetate and 10.0 µL of cyclohexane through the septum into the vial, and mix.
  Standard solution: Transfer 20.0 mL of methanol to a 30-mL serum vial, insert a rubber septum, and seal with a metal cap. Through the rubber septum add 10 µL of methyl ethyl ketone (internal standard) and 50.0 µL of the *Standard stock solution*, and mix to obtain a solution containing 0.18 mg of ethyl acetate and 0.078 mg of cyclohexane.
  Sample solution: Transfer 50 mg of Carbomer Interpolymer to a 30-mL serum vial, add 20.0 mL of methanol, insert a rubber septum, and seal with a metal cap. Through the rubber septum add 10 µL of methyl ethyl ketone, and mix.
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: GC
  Detector: Flame ionization
  Column: 2-mm × 3-m; packed with 1% liquid phase G25 on 60- to 80-mesh support S12
  Temperatures
  Detector: 250°
  Injection port: 250°
  Column: See *Table 1*.

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Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
115	—	115	4
115	6	175	5

Carrier gas: Helium
Flow rate: 33 mL/min
Injection volume: 2 µL
System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for methyl ethyl ketone, ethyl acetate, and cyclohexane are about 0.7, 0.9, and 1.0, respectively.]
Suitability requirements
Resolution: NLT 1.0 between ethyl acetate and cyclohexane
Relative standard deviation: NMT 2.5%
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentages of ethyl acetate and cyclohexane in the portion of Carbomer Interpolymer taken:

$$\text{Result} = (R_U/R_S) \times (W_S/W_U) \times 100$$

R_U = peak area ratio of the relevant analyte peak to the methyl ethyl ketone peak from the *Sample solution*
R_S = peak area ratio of the relevant analyte peak to the methyl ethyl ketone peak from the *Standard solution*
W_S = weight of ethyl acetate or cyclohexane in the *Standard solution* (mg)
W_U = weight of Carbomer Interpolymer taken to prepare the *Sample solution* (mg)

Acceptance criteria
Ethyl acetate: NMT 0.35%
Cyclohexane: NMT 0.15%

• **LIMIT OF BENZENE**
Benzene solution: 1.0 mg/mL of benzene in dimethyl sulfoxide
Solvent solution: 0.1 µg/mL of *Benzene solution* in organic-free water (see *Residual Solvents* ⟨467⟩, *Identification, Control, and Quantification of Residual Solvents*)
Sodium chloride solution: 20 mg/mL of sodium chloride
Sample solution: Transfer 50 mg of Carbomer Interpolymer to a 10-mL volumetric flask. Add 7.5 mL of *Sodium chloride solution*, and mix by mechanical means until homogeneous (usually about 30 min). Dilute with *Sodium chloride solution* to volume, and mix until homogeneous (usually less than 1 min). This solution must be analyzed within 3 h of preparation.
Standard solution: Transfer 50 mg of Carbomer Interpolymer to a 10-mL volumetric flask. Add 7.5 mL of *Sodium chloride solution*, and mix by mechanical means until homogeneous (usually about 30 min). Add 1.0 mL of the *Solvent solution*, dilute with *Sodium chloride solution* to volume, and mix until homogeneous (usually less than 1 min). This solution contains 0.01 µg/mL of benzene.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC, equipped with a headspace injector
Detector: Flame ionization
Column: 0.53-mm × 30-m fused silica; coated with 3.0-µm G43 stationary phase
Temperatures
Detector: 250°
Injection port: 140°
Column: See *Table 2*.

Table 2

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
40	—	40	20
40	10	240	20

Carrier gas: Helium
Flow rate: Linear velocity of 35 cm/s
Split ratio: 5:1
Injection volume: 1 mL (gaseous phase)
[NOTE—The following headspace conditions may be used: a pressurization time of 30 s and a transfer line temperature of 90°.]
The vials are maintained at a temperature of 80° for 60 min before headspace injection.
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 15% for three replicate injections
Analysis
Samples: *Standard solution* and *Sample solution*
Transfer 10.0 mL each of the *Standard solution* and *Sample solution* to separate headspace vials. Close the vials with a tight rubber membrane stopper coated with polytetrafluoroethylene, and secure with an aluminum crimped cap. Shake to obtain a homogeneous dispersion. Separately inject the gaseous phase of the *Standard solution* and *Sample solution*.
Acceptance criteria: The benzene peak response from the *Sample solution* is NMT half of that from the *Standard solution*, corresponding to NMT 2 µg/g.

• **LIMIT OF ACRYLIC ACID**
Mobile phase: Dissolve 6.80 g of monobasic potassium phosphate in 300 mL of water, dilute with water to 500 mL, and mix. Dilute 100 mL of this solution with water to 1 L, adjust with phosphoric acid to a pH of 3.0 ± 0.1, and mix. Filter and degas.
Standard solution: 25 µg/mL of acrylic acid
Sample solution: Transfer 100 mg of Carbomer Interpolymer to a tared serum vial. Add water to obtain 10.0 mL of solution. Cap the vial, and shake by mechanical means for 2 h. Add 2 drops of sodium hydroxide solution (500 mg/mL), and shake by hand for 15 s. Add 1.0 mL of calcium chloride solution (100 mg/mL), and shake until the gel collapses. Centrifuge for 15 min, and use the clear supernatant.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 200 nm
Column: 8-mm × 10-cm; packing L1
Flow rate: 1 mL/min
Injection volume: 10 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 5%
Analysis
Samples: *Standard solution* and *Sample solution*

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Calculate the percentage of free acrylic acid in the portion of Carbomer Interpolymer taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak response of acrylic acid from the *Sample solution*
r_S = peak response of acrylic acid from the *Standard solution*
C_S = concentration of acrylic acid in the *Standard solution* (µg/mL)
C_U = concentration of Carbomer Interpolymer in the *Sample solution* (mg/mL)
F = unit conversion, 10^{-3} mg/µg

Acceptance criteria: NMT 0.25%

SPECIFIC TESTS

• **Loss on Drying** ⟨731⟩
Analysis: Dry under vacuum at 80° for 1 h.
Acceptance criteria: NMT 2.0%

• **Viscosity—Rotational Methods** ⟨912⟩
Carbomer Interpolymer A
Sample: 2.50 g of Carbomer Interpolymer, previously dried under vacuum at 80° for 1 h (0.5% aqueous dispersion)
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Electrode: Calomel–glass
Titrant: 180 mg/mL of sodium hydroxide
Endpoint detection: Potentiometric
Analysis: Carefully add the *Sample* to 500 mL of water in a 1000-mL beaker, while stirring continuously at 1000 ± 50 rpm, with the stirrer shaft set to one side of the beaker at an angle of 60° and the propeller positioned near the bottom of the beaker. The stirrer used is a three-blade, 2-inch–diameter marine impeller. Add the *Sample* at a uniform rate for 45–90 s, being sure that loose aggregates of powder are broken up, and continue stirring at 1000 ± 50 rpm for 15 min. Remove the stirrer, and place the beaker containing the dispersion in a 25 ± 0.1° water bath for 30 min. Insert a paddle stirrer to a depth necessary to ensure that air is not drawn into the dispersion; and while stirring at 300 ± 25 rpm, titrate to a pH of 7.3–7.8 by adding *Titrant* below the surface. Stir 2–3 min until neutralization is complete. Then determine the final pH. [NOTE—If the pH is below 7.3, raise it with additional sodium hydroxide. If it is above 7.8, discard the mucilage, and prepare another batch, using a smaller amount of sodium hydroxide for titration.]
Return the beaker containing the neutralized mucilage to the 25 ± 0.1° water bath for 1 h. Measure the mucilage pH again, making certain that it is between 7.3 and 7.8. Using a rotational viscometer equipped with a suitable spindle at a spindle immersion depth as defined in *Table 3*, perform the viscosity determination without delay to avoid the slight viscosity changes that occur 75 min after neutralization.
The spindle rotates at 20 rpm. Follow the instrument manufacturer's directions to measure the apparent viscosity.

Carbomer Interpolymer B
Sample: 5.00 g of Carbomer Interpolymer, previously dried under vacuum at 80° for 1 h (1.0% aqueous dispersion)
Analysis: Proceed as directed for *Viscosity—Rotational Methods* ⟨912⟩, *Carbomer Interpolymer A*, except adjust the pH of the dispersion to 5.8–6.3 instead of 7.3–7.8.

Carbomer Interpolymer C
Sample: 2.50 g of Carbomer Interpolymer, previously dried under vacuum at 80° for 1 h (0.5% aqueous dispersion)
Analysis: Proceed as directed for *Viscosity—Rotational Methods* ⟨912⟩, *Carbomer Interpolymer A*, except adjust the pH of the dispersion to 5.8–6.3 instead of 7.3–7.8.
Acceptance criteria: See *Table 4*.

Table 4

Carbomer Interpolymer	Viscosity Specifications (mPa · s)
A	45,000–65,000
B	47,000–77,000
C	8,500–16,500

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Preserve in tight containers, at a temperature not exceeding 45°.

• **Labeling:** Label it to indicate whether it is Type A, B, or C. Also label it to state the measured viscosity, giving the viscosity measurement parameters, the concentration of the solution, and the type of equipment used; the solvent or solvents used in the polymerization process; and the nominal and measured residual solvent levels for each solvent.

Table 3 (Carbomer Interpolymer)

Viscosity Ranges (mPa · s)	Spindle No.	A[a] (cm)	B[b] (cm)	C[c] (cm)	D[d] (cm)	E[e] (cm)	Multiplier
100–400	1	5.6	2.2	0.3	2.7	6.1	5
400–1600	2	4.7	0.2	0.3	2.7	4.9	20
1000–4000	3	3.5	0.2	0.3	2.7	4.9	50
2000–8000	4	2.7	0.2	0.3	2.7	4.9	100
4000–16,000	5	2.1	0.2	0.3	2.7	4.9	200
10,000–40,000	6	1.5	0.2	0.3	3.0	4.9	500
40,000–160,000	7	—	—	0.3	—	5.5	2,000

[a] Cylinder diameter.
[b] Cylinder height.
[c] Shaft diameter.
[d] Distance from the top of the cylinder to the lower tip of the shaft.
[e] Spindle immersion depth.

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Carbon Dioxide—*see Carbon Dioxide General Monographs*

Carboxymethylcellulose Calcium

Cellulose, carboxymethyl ether, calcium salt;
Cellulose carboxymethyl ether calcium salt [9050-04-8].

DEFINITION
Carboxymethylcellulose Calcium is the calcium salt of a polycarboxymethyl ether of cellulose.

IDENTIFICATION
• **A. Procedure**
Sample solution: Shake thoroughly 0.1 g of Carboxymethylcellulose Calcium with 10 mL of water, followed by 2 mL of 1 N sodium hydroxide, and allow to stand for 10 min. [Note—Save the unused portion of the *Sample solution* for use in *Identification* tests *B* and *C*.]
Analysis: To 1 mL of the *Sample solution* add water to make 5 mL. To 1 drop of the resulting solution, add 0.5 mL of chromotropic acid TS, and heat in a water bath for 10 min.
Acceptance criteria: A red-purple color develops.
• **B. Procedure**
Analysis: Shake 5 mL of the *Sample solution* prepared in *Identification* test *A* with 10 mL of acetone.
Acceptance criteria: A white, flocculent precipitate is formed.
• **C. Procedure**
Analysis: Shake 5 mL of the *Sample solution* prepared in *Identification* test *A* with 1 mL of ferric chloride TS.
Acceptance criteria: A brown, flocculent precipitate is formed.
• **D. Identification Tests—General,** *Calcium* ⟨191⟩
Analysis: Ignite 1 g to ash, dissolve the residue in 10 mL of water and 5 mL of 6 N acetic acid, and filter, if necessary. Boil the filtrate, cool, and neutralize with 6 N ammonium hydroxide.
Acceptance criteria: Meets the requirements

IMPURITIES
Inorganic Impurities
• **Residue on Ignition** ⟨281⟩: 10.0%–20.0%; use 1.0 g, previously dried
• **Chloride and Sulfate,** *Chloride* ⟨221⟩
Sample stock solution: Shake thoroughly 0.80 g with 50 mL of water, dissolve in 10 mL of 1 N sodium hydroxide, and add water to make 100 mL. [Note—Retain a portion of the *Sample stock solution* for use in the test for *Sulfate*.]
Sample solution: Heat 20 mL of the *Sample stock solution* with 10 mL of 2 N nitric acid in a water bath until a flocculent precipitate is formed, cool, centrifuge, and remove the supernatant. Wash the precipitate with three 10-mL portions of water by centrifuging each time, combine the supernatant and the washings, add water to make 100 mL, and mix.
Acceptance criteria: A 25-mL portion of the *Sample solution* shows no more chloride than is contained in 0.20 mL of 0.020 N hydrochloric acid (0.36%).
• **Chloride and Sulfate,** *Sulfate* ⟨221⟩
Sample solution: Heat 10 mL of the *Sample stock solution* prepared in the test for *Chloride* with 1 mL of hydrochloric acid in a water bath until a flocculent precipitate is formed. Cool, centrifuge, and remove the supernatant. Wash the precipitate with three 10-mL portions of water by centrifuging each time, combine the supernatant and the washings, add water to make 100 mL, and mix.
Acceptance criteria: A 25-mL portion of the *Sample solution* shows no more sulfate than is contained in 0.21 mL of 0.020 N sulfuric acid (1.0%).

SPECIFIC TESTS
• **Alkalinity:** Shake thoroughly 1.0 g with 50 mL of freshly boiled and cooled water, and add 2 drops of phenolphthalein TS: no red color develops.
• **Loss on Drying** ⟨731⟩**:** Dry a sample at 105° for 4 h: it loses NMT 10.0% of its weight.

ADDITIONAL REQUIREMENTS
• **Packaging and Storage:** Preserve in tight containers.

Low-Substituted Carboxymethylcellulose Sodium

Cellulose, carboxymethyl ether, sodium salt, low-substituted;
Carmellose sodium, low-substituted [9004-32-4].

DEFINITION
Low-Substituted Carboxymethylcellulose Sodium is the sodium salt of a partly *O*-(carboxymethylated) cellulose. It contains NLT 2.0% and NMT 4.5% of sodium (Na), calculated on the dried basis.

IDENTIFICATION

Add the following:

▲• **A. Spectroscopic Identification Tests** ⟨197⟩, *Infrared Spectroscopy*: **197A** or **197K**▲ (NF 1-May-2020)

Change to read:

• ▲**B.**▲ (NF 1-May-2020)
Solution A: 100 mg/mL of sodium hydroxide
Sample: 1 g
Analysis: Shake the *Sample* with 100 mL of *Solution A*.
Acceptance criteria: A suspension is produced.

Change to read:

• ▲**C.**▲ (NF 1-May-2020)
Sample: 1 g
Analysis: Shake the *Sample* with 50 mL of water. Transfer 1 mL to a test tube, and add 1 mL of water and 1 mL of 1-naphthol TS. Incline the test tube, and add carefully 2 mL of sulfuric acid down the side so that it forms a lower layer.
Acceptance criteria: A reddish-purple color develops at the interface.

Delete the following:

▲• **C.** It meets the requirements in *Impurities* for *Residue on Ignition*.▲ (NF 1-May-2020)
• **D. Identification Tests—General** ⟨191⟩, *Chemical Identification Tests, Sodium, A*
Sample solution: To the residue obtained in the test for *Residue on Ignition* add 1 mL of hydrochloric acid, evaporate on a water bath, and dissolve in 20 mL of water.
Acceptance criteria: Meets the requirements

ASSAY
• **Content of Sodium**
Analysis: Calculate the percentage of sodium in the portion of Low-Substituted Carboxymethylcellulose Sodium taken:

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$$\text{Result} = a \times (M \times A_r/M_r)$$

a = percentage obtained from the test for *Residue on Ignition*, determined separately
M = number of moles of sodium per mole of sodium sulfate, 2
A_r = atomic weight of sodium, 22.99
M_r = molecular weight of sodium sulfate, 142.04

Acceptance criteria: 2.0%–4.5% on the dried basis

IMPURITIES

• **Residue on Ignition** ⟨281⟩
Sample: 1.0 g
Analysis: Determine using a mixture of sulfuric acid and water (1:1) and an ignition temperature of 600 ± 50°.
Acceptance criteria: 6.5%–13.5%

• **Limit of Sodium Chloride and Sodium Glycolate**
Sodium chloride
Sample: 5 g
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.05 N silver nitrate VS
Electrode system: Silver electrode and mercurous sulfate electrode with a potassium sulfate bridge
Endpoint detection: Potentiometric
Analysis: Transfer the *Sample* to a 250-mL conical flask. Add 50 mL of water and 5 mL of 30% hydrogen peroxide, and heat on a water bath for 20 min, stirring occasionally to ensure hydration. Cool, and add 100 mL of water and 10 mL of nitric acid. Titrate with *Titrant*. Calculate the percentage of sodium chloride in the *Sample*:

$$\text{Result} = \{[(V \times N \times F)/W] \times [100/(100 - b)]\} \times 100$$

V = volume of *Titrant* (mL)
N = normality of the *Titrant* (mEq/mL)
F = equivalency factor for sodium chloride, 0.05844 g/mEq
W = weight of the *Sample* (g)
b = percentage obtained from the test for *Loss on Drying*, determined separately

Sodium glycolate
Standard stock solution: Transfer 100 mg of glycolic acid, previously dried overnight in a vacuum desiccator over phosphorus pentoxide, to a 100-mL volumetric flask, and dissolve in and dilute with water to volume.
Standard solution A: Transfer 0.5 mL of the *Standard stock solution* to a 100-mL volumetric flask. Add water to make 5 mL, add 5 mL of glacial acetic acid, then dilute with acetone to volume.
Standard solution B: Transfer 1.0 mL of the *Standard stock solution* to a 100-mL volumetric flask. Add water to make 5 mL, add 5 mL of glacial acetic acid, then dilute with acetone to volume.
Standard solution C: Transfer 1.5 mL of the *Standard stock solution* to a 100-mL volumetric flask. Add water to make 5 mL, add 5 mL of glacial acetic acid, then dilute with acetone to volume.
Standard solution D: Transfer 2.0 mL of the *Standard stock solution* to a 100-mL volumetric flask. Add water to make 5 mL, add 5 mL of glacial acetic acid, then dilute with acetone to volume.
Sample solution: Transfer 500 mg of Low-Substituted Carboxymethylcellulose Sodium to a beaker, moisten thoroughly with 5 mL of glacial acetic acid, add 5 mL of water, and stir with a glass rod to ensure proper hydration (about 30 min). Add 80 mL of acetone while stirring, add 2 g of sodium chloride, and stir for several minutes to ensure the complete precipitation of carboxymethylcellulose. Pass through a fast filter paper, previously wetted with a small amount of acetone, and collect the filtrate in a 100-mL volumetric flask. Rinse the beaker and filter with acetone, and add the washings to the flask. Dilute the filtrate with acetone to volume, and mix. Allow to stand for 24 h without shaking, and use the clear supernatant.
Instrumental conditions
Mode: Vis
Analytical wavelength: 540 nm
Blank: Use acetone solution containing 5% of glacial acetic acid and 5% of water.
Analysis: Transfer 2.0 mL of the *Sample solution* and 2.0 mL of each *Standard solution* to separate 25-mL volumetric flasks. Place the uncovered flasks in a boiling water bath for 20 min, accurately timed, to remove the acetone, then remove from the bath, and cool. Add to each flask 5.0 mL of 2,7-dihydroxynaphthalene TS, mix, add an additional 15 mL, and again mix. Cover the mouth of each flask with a small piece of aluminum foil. Place the flasks upright in a boiling water bath for 20 min, then remove from the bath, cool, and dilute with sulfuric acid to volume.
Determine the absorbances of the solutions against the *Blank*, and prepare a standard curve using the absorbances obtained from the solutions prepared from the *Standard solutions*. From the standard curve and the absorbance of the *Sample solution*, determine the concentration, in mg/mL, of glycolic acid in the *Sample solution*, and calculate the percentage of sodium glycolate in the sample taken:

$$\text{Result} = \{[(C \times V)/(W \times F)] \times (M_{r1}/M_{r2}) \times [100/(100 - b)]\} \times 100$$

C = concentration of glycolic acid in the sample, determined from the standard curve (mg/mL)
V = volume of *Sample solution* (mL)
W = weight of sample (g)
F = unit conversion factor, 1000 mg/g
M_{r1} = molecular weight of sodium glycolate, 98.03
M_{r2} = molecular weight of glycolic acid, 76.05
b = percentage obtained from the test for *Loss on Drying*, determined separately

Acceptance criteria: The sum of the percentages obtained from the tests for *Sodium chloride* and *Sodium glycolate* is NMT 0.5%.

SPECIFIC TESTS

• **pH** ⟨791⟩
Sample: 1 g
Analysis: Shake the *Sample* with 100 mL of carbon dioxide-free water, centrifuge, and test the suspension.
Acceptance criteria: 6.0–8.5

• **Loss on Drying** ⟨731⟩
Sample: 1.0 g
Analysis: Dry the *Sample* at 105° for 3 h.
Acceptance criteria: NMT 10.0%

• **Water-Soluble Substances**
Sample: 5.0 g
Analysis: Disperse the *Sample* in 400 mL of water, and during the first 30 min, stir for 1 min every 10 min. Allow to stand for 1 h, and centrifuge, if necessary. Decant 100.0 mL of the supernatant onto a fast filter paper in a vacuum filtration funnel, apply a vacuum, and collect

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75.0 mL of the filtrate. Evaporate in a tared platinum or porcelain dish, and dry at 105° for 4 h.
Acceptance criteria: NMT 70%

• **SETTLING VOLUME**
[NOTE—The following test, which can relate to excipient function, may be carried out depending on the intended use in the formulation. In cases where there are no concerns regarding the settling volume of this article, this test may be omitted. Where the labeling states the settling volume, determine the settling volume as follows.]
Sample: 5.0 g
Analysis: In a 100-mL graduated cylinder, transfer 20 mL of isopropyl alcohol, add the *Sample*, and shake vigorously. Dilute with isopropyl alcohol to 30 mL, then with water to 50 mL, and shake vigorously. Within 15 min, repeat the shaking three times. Allow to stand for 4 h, and determine the volume of the settled mass.
Acceptance criteria: 15.0–35.0 mL

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.
• **LABELING:** When the settling volume is determined, label it to indicate the settling volume value.

Add the following:

▲• **USP REFERENCE STANDARDS** ⟨11⟩
USP Low-Substituted Carboxymethylcellulose Sodium RS▲ (NF 1-May-2020)

Carboxymethylcellulose Sodium—*see Carboxymethylcellulose Sodium General Monographs*

Carboxymethylcellulose Sodium 12

DEFINITION
Carboxymethylcellulose Sodium 12 is the sodium salt of a polycarboxymethyl ether of cellulose. Its degree of substitution is NLT 1.15 and NMT 1.45, corresponding to a sodium (Na) content of NLT 10.4% and NMT 12.0%, calculated on the dried basis.

IDENTIFICATION
• **A.**
Sample solution: Add 1 g of powdered Carboxymethylcellulose Sodium 12 to 50 mL of water, while stirring to produce a uniform dispersion. Continue the stirring until a clear solution is produced. [NOTE—Save the unused portion of this solution for use in *Identification* tests *B* and *C*.]
Analysis: To 1 mL of the *Sample solution*, diluted with an equal volume of water, in a small test tube, add 5 drops of 1-naphthol TS. Incline the test tube, and carefully introduce down the side of the tube 2 mL of sulfuric acid so that it forms a lower layer.
Acceptance criteria: A red-purple color develops at the interface.
• **B.**
Analysis: To 5 mL of the *Sample solution* prepared for *Identification* test *A* add an equal volume of barium chloride TS.
Acceptance criteria: A fine, white precipitate is formed.
• **C. IDENTIFICATION TESTS—GENERAL,** *Sodium* ⟨191⟩**:** A portion of the *Sample solution* prepared for *Identification* test *A* meets the requirements.

ASSAY
• **DEGREE OF SUBSTITUTION**
Sample: 200 mg, previously dried at 105° for 3 h
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.1 N perchloric acid in dioxane VS
Endpoint detection: Potentiometric
Electrode system: A pH meter equipped with a standard glass electrode and a calomel electrode modified as follows. Discard the aqueous potassium chloride solution contained in the electrode, rinse, and fill with the supernatant obtained by shaking thoroughly 2 g each of potassium chloride and silver chloride (or silver oxide) with 100 mL of methanol, then add a few crystals of potassium chloride and silver chloride (or silver oxide) to the electrode.
Analysis: Weigh the *Sample*, and transfer to a glass-stoppered, 250-mL conical flask. Add 75 mL of glacial acetic acid, connect the flask to a water-cooled condenser, and reflux gently on a hot plate for 2 h. Cool, and transfer the solution to a 250-mL beaker with the aid of 50 mL of glacial acetic acid. Titrate with *Titrant* while stirring with a magnetic stirrer. Record the amount, in mL, of *Titrant* versus mV (0- to 700-mV range), and continue the titration to a few mL beyond the endpoint. Plot the titration curve, and read the volume of *Titrant* at the inflection point.
Calculate the degree of substitution in the Carboxymethylcellulose Sodium 12 taken:

$$\text{Result} = (M_{r1} \times N \times V)/(G - \Delta M_{r2} \times N \times V)$$

M_{r1} = molecular weight of 1 anhydroglucose unit, 162
N = *Titrant* normality
V = volume of *Titrant* (mL)
G = weight of Carboxymethylcellulose Sodium 12 taken (mg)
ΔM_{r2} = increase in molecular mass of 1 anhydroglucose unit for each sodium carboxymethyl group added, 80

Acceptance criteria: 1.15–1.45, corresponding to 10.4%–12.0% sodium content, on the dried basis

IMPURITIES
• **LIMIT OF SODIUM CHLORIDE**
Sample: 5 g
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.05 N silver nitrate VS
Endpoint detection: Potentiometric
Electrode system: A silver electrode and a mercurous sulfate electrode having a potassium sulfate bridge
Analysis: Transfer the *Sample* to a 250-mL beaker, add 50 mL of water and 5 mL of 30% hydrogen peroxide, and heat on a water bath for 20 min, stirring occasionally to ensure hydration. Cool, add 100 mL of water and 10 mL of nitric acid, and titrate with *Titrant*, determining the endpoint while stirring constantly.
Calculate the percentage of NaCl in the sample taken:

$$\text{Result} = (M_r \times V \times N \times F)/\{[(100 - b)/100] \times W\} \times 100$$

M_r = molecular weight of sodium chloride, 58.44
V = volume of *Titrant* (mL)

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N = normality of *Titrant*
F = conversion factor, 10^{-3} g/mg
b = percentage obtained from the test for *Loss on Drying*, determined separately
W = *Sample* weight (g)

Acceptance criteria: See *Limit of Sodium Glycolate*.

• **LIMIT OF SODIUM GLYCOLATE**

Standard stock solution: Transfer 100 mg of glycolic acid, previously dried in a desiccator at room temperature overnight, to a 100-mL volumetric flask. Dissolve in and dilute with water to volume, and mix. [NOTE—Use this solution within 30 days.]

Standard solutions: Into four separate 100-mL volumetric flasks, transfer 1.0-, 2.0-, 3.0-, and 4.0-mL portions of the *Standard stock solution*, respectively. To each flask, add water to make 5 mL, add 5 mL of glacial acetic acid, dilute with acetone to volume, and mix.

Sample solution: Transfer 500 mg to a 100-mL beaker, moisten thoroughly with 5 mL of glacial acetic acid, followed by 5 mL of water, and stir with a glass rod to ensure proper hydration (usually about 15 min). Slowly add 50 mL of acetone, with stirring, then add 1 g of sodium chloride, and stir for several min to ensure complete precipitation of the carboxymethylcellulose. Filter through a soft, open-textured paper, previously wetted with a small amount of acetone, and collect the filtrate in a 100-mL volumetric flask. Use an additional 30 mL of acetone to facilitate the transfer of the solids and to wash the filter cake, then dilute with acetone to volume.

Blank: Prepare a 25-mL blank flask containing 2.0 mL of a solution containing 5% each of glacial acetic acid and water in acetone.

Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: Spectrophotometry
Analytical wavelength: 540 nm

Analysis: Transfer 2.0 mL of the *Sample solution* and 2.0 mL of each *Standard solution* to separate 25-mL volumetric flasks. The 25-mL volumetric flask containing the *Blank* is included in the following tests. Place the uncovered flasks in a boiling water bath for 20 min, accurately timed, to remove the acetone, remove from the bath, and cool. Add to each flask 5.0 mL of 2,7-dihydroxynaphthalene TS, mix, add an additional 15 mL, and again mix. Cover the mouth of each flask with a small piece of aluminum foil. Place the flasks upright in a boiling water bath for 20 min, then remove from the bath, cool, dilute with sulfuric acid to volume, and mix.

Determine the absorbances of each solution against the *Blank*, and prepare a standard curve using the absorbances obtained from the solutions prepared from the *Standard solutions*. From the standard curve and the absorbance of the *Sample solution*, determine the weight (w), in mg, of glycolic acid in the *Sample solution*.

Calculate the percentage of sodium glycolate in the specimen taken:

$$\text{Result} = w \times F/\{[(100 - b)/100] \times W\} \times 100$$

w = weight of glycolic acid in the sample determined from the standard curve (mg)
F = factor converting glycolic acid to sodium glycolate, 1.29
b = percentage of *Loss on Drying*, determined separately
W = weight of the sample taken (mg)

Acceptance criteria: The sum of the percentages from the tests for *Limit of Sodium Chloride* and *Limit of Sodium Glycolate* is NMT 0.5%.

SPECIFIC TESTS

• **pH** ⟨791⟩: 6.5–8.5, in a 10-mg/mL solution

• **LOSS ON DRYING** ⟨731⟩: Dry a sample at 105° for 3 h: it loses NMT 10.0% of its weight.

• **VISCOSITY–ROTATIONAL METHODS** ⟨912⟩

Analysis: Determine the viscosity in a water solution at the concentration stated on the label. Using undried Carboxymethylcellulose Sodium 12, weigh the amount which, on the dried basis, will provide 200 g of solution of the stated concentration. Add the substance in small amounts to 180 mL of stirred water contained in a tared, wide-mouth bottle, continue stirring rapidly until the powder is well wetted, add sufficient water to make the mixture weigh 200 g, and allow to stand, with occasional stirring, until solution is complete. Adjust the temperature to 25 ± 0.2°, and determine the viscosity, using a rotational type of viscometer, making certain that the system reaches equilibrium before taking the final reading.

Acceptance criteria: The viscosity of solutions of 2% concentration is NLT 80.0% and NMT 120.0% of that stated on the label; the viscosity of solutions of 1% concentration is NLT 75.0% and NMT 140.0% of that stated on the label or it is between the maximum and minimum values, where stated as a range of viscosities.

ADDITIONAL REQUIREMENTS

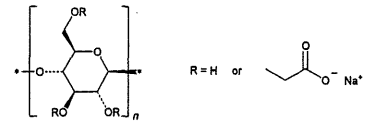
• **PACKAGING AND STORAGE:** Preserve in tight containers.

• **LABELING:** Label it to indicate the nominal viscosity in solutions of stated concentrations of either 1% (w/w) or 2% (w/w). The indicated viscosity may be in the form of a range encompassing 80.0%–120.0% of the nominal viscosity, where the solution concentration is 2% (w/w); or 75.0%–140.0% of the nominal viscosity, where the solution concentration is 1% (w/w).

Carboxymethylcellulose Sodium Paste—

see Carboxymethylcellulose Sodium Paste General Monographs

Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium



Sodium salts of polymers containing substituted anhydroglucose units with the general formula:

$[C_6H_7O_2(OH)_x(OCH_2COONa)_y]_n$

where n is the degree of polymerization;
y is the degree of substitution;
x is between 1.50 and 2.80;
y is between 0.20 and 1.50;
and x + y = 3.0

Carboxymethyl cellulose, sodium, partially hydrolyzed enzymatically.

DEFINITION

Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium is the sodium salt of a polycarboxymethyl ether of cellulose,

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which has been partially hydrolyzed by enzymatic treatment with food grade *Trichoderma reesei cellulase*. Its degree of substitution is NLT 0.20 and NMT 1.50, corresponding to a sodium (Na) content of NLT 2.6% and NMT 12.2%, calculated on the dried basis.

IDENTIFICATION

- **A.** Vigorously shake a 0.1% solution of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium: no layer of foam appears.
- **B.** To 5 mL of a 0.5% solution of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium add 5 mL of a 5% solution of copper or aluminium sulfate: a precipitate appears.
- **C.** Add 0.5 g of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium to 50 mL of water, while stirring, to produce a uniform dispersion. Continue stirring until a clear solution of 1% is produced. Dilute 1 mL of this solution with 1 mL of water in a small test tube. Add 5 drops of 1-naphthol TS. Incline the tube, and carefully introduce down the side of the tube 2 mL of sulfuric acid, so that it forms a lower layer: a red-purple color develops at the interface.
- **D. IDENTIFICATION TESTS—GENERAL,** *Sodium* ⟨191⟩**:** A portion of the 1% solution obtained from *Identification* test C meets the requirements.
- **E.** It meets the requirements of the test for *Viscosity*.

ASSAY

- **DEGREE OF SUBSTITUTION**

Sample: 2.0 g of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium

Analysis: Ignite a clean and dry quartz crucible with a Bunsen burner, cool to room temperature in a desiccator, and weigh the crucible accurately. Transfer the *Sample* to the crucible. Carefully ignite on a small flame for about 10 min, and make sure that the *Sample* does not burn or excessively glow. Cool, and moisten the residue with 3–5 mL of sulfuric acid. Heat the crucible cautiously until the fuming is complete, and heat further until the *Sample* turns grayish white. Place the crucible in an oven at about 600°, until no black spots are visible. Cool the crucible in a desiccator to room temperature, and weigh. Place the crucible again in an oven at about 600° for 1 h, cool the crucible to room temperature in a desiccator, and weigh. This last step is repeated until a constant weight is achieved. Calculate the percentage of sodium, P_{Na}, in the portion of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium taken:

$$\text{Result} = 100 \times N_{Na} \times A_{Na} \times (W_2 - W_1)/\{M_r \times W[(100 - \text{LOD})/100]\}$$

N_{Na} = number of sodium atoms/molecule of sodium sulfate, 2
A_{Na} = atomic weight of sodium, 22.99
W_2 = weight of the crucible with ash residue (g)
W_1 = weight of the crucible (g)
M_r = molecular weight of sodium sulfate, 142.04
W = weight of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium taken (g)
LOD = *Loss on Drying* value of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium (%)

Calculate the *Degree of Substitution* (DS):

$$\text{Result} = M_{r1} \times (P_{Na} - P_{NaCl} - P_{NaG})/\{100 \times A_{Na} - [(M_{r2} - A_H) \times (P_{Na} - P_{NaCl} - P_{NaG})]\}$$

M_{r1} = molar mass of one glucose unit, 162.14
P_{NaCl} = percentage of sodium chloride obtained in the test for *Limit of Sodium Chloride and Sodium Glycolate*
P_{NaG} = percentage of sodium glycolate obtained in the test for *Limit of Sodium Chloride and Sodium Glycolate*
A_{Na} = atomic weight of sodium, 22.99
M_{r2} = molar mass of one sodium carboxymethyl unit (CH_2COONa), 81.03
A_H = atomic weight of hydrogen, 1.01

Acceptance criteria: NLT 0.20 and NMT 1.50 carboxymethyl groups (CH_2COOH)/anhydroglucose unit on the dried basis

IMPURITIES

INORGANIC IMPURITIES

- **Limit of Lead**

Nitric acid solution: Dilute 10 mL of nitric acid with 20 mL of water. Boil this solution to remove nitrous fumes, and allow it to cool to room temperature.

Lead standard stock solution: Dissolve 1.60 g of lead nitrate ($Pb(NO_3)_2$) in about 30 mL of *Nitric acid solution* in a 1000-mL volumetric flask, dilute with water to volume, and mix. At 20°, transfer 10.0 mL of this lead solution to a 500-mL volumetric flask, and dilute with water to volume. The *Lead standard stock solution* contains 20 µg/mL of Pb.

Standard solutions: Transfer 0, 1, 2, 3, 4, and 5 mL of the *Lead standard stock solution* to six identical 100-mL volumetric flasks, and add 50 mL of water to each flask. To each flask, add 8 mL of sulfuric acid and 10 mL of hydrochloric acid, and mix well. After complete dissolution, dilute each flask with water to volume, and mix. Transfer 1 mL of each solution to a separate 50-mL volumetric flask, and dilute with water to a final concentration of 0, 4, 8, 12, 16, and 20 µg/L of Pb, respectively.

Sample solution: Transfer 1 g of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium to a 100-mL beaker, and add 5 mL of 30% hydrogen peroxide. While stirring, add 50 mL of water to the solution, and heat the beaker on a hot plate at 50°, until all solids are dissolved. Quantitatively transfer this solution to a 100-mL volumetric flask, add 300 µL of nitric acid, dilute with water to volume, and mix.

Blank solution: Prepare as directed in the *Sample solution*, but omit the test specimen.

Spectrometric conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩)

Mode: Graphite furnace atomic absorption spectrophotometer, equipped with a lead hollow-cathode lamp and an adequate means of background correction.

Analytical wavelength: Lead emission line of 283.3 nm

Analysis

Samples: *Standard solutions*, *Sample solution*, and *Blank solution*

Concomitantly determine the absorbances of the *Samples*. Optimize the instrument program for lead as recommended by the manufacturer. The strongest *Standard solution* is aspirated to optimize the instrument settings to give a full-scale reading on the detector. Correct the area responses of all *Sample solution* and *Standard solutions* for the *Blank solution* area response. Generate the appropriate lead calibration curve, and determine the lead concentration, C, in µg/L, in the *Sample solution*.

Calculate the content of lead, in µg, in each g of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium taken:

$$\text{Result} = (V \times C)/W$$

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V = volume of the *Sample solution*, 0.1 L
W = weight of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 3 µg/g of lead.

• Limit of Sodium Chloride and Sodium Glycolate

Sodium Chloride

Ferric solution: Dissolve 20 g of ferric ammonium sulfate ($FeNH_4(SO_4)_2 \cdot 12H_2O$) in a 100-mL volumetric flask containing 80 mL of water, add 0.3 mL of 10 N nitric acid (dilute 600 mL of nitric acid with water to 1000 mL), dilute with water to volume, and mix.

Analysis: Transfer an accurate quantity of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium, equivalent to 5.0 g on the dried basis, to a platinum or porcelain crucible. Heat the test specimen first with a small flame so that the test specimen does not ignite. When the charring is complete, heat further in an electric oven at about 600° for 15 min. After cooling, pulverize the ashes thus obtained and extract several times with water. Filter the extracts into a 500-mL volumetric flask, add 5 mL of 10 N nitric acid, and dilute with water to volume. Transfer 100 mL of this extract to a suitable flask, add 2.0 mL of 0.2 N silver nitrate and 3 g of potassium nitrate, and mix. [NOTE—Silver chloride precipitate may develop. The potassium nitrate will prevent the silver chloride from interfering with the indication reaction.] After complete dissolution of the potassium nitrate, titrate this mixture with 0.02 N ammonium thiocyanate VS by adding 3 mL of *Ferric solution* as an indicator. Titrate until a red color develops and persists for at least 30 s. Each mL of 0.02 N silver nitrate is equivalent to 1.169 mg of sodium chloride.

Calculate the percentage of sodium chloride, P_{NaCl}, in the portion of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium taken:

$$\text{Result} = (0.001169 \times D \times V/W) \times 100$$

D = dilution factor, 5
V = volume of the 0.02 N silver nitrate consumed (mL)
W = weight of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium taken, calculated on the dried basis (g)

Sodium Glycolate

Mobile phase: Prepare a filtered and degassed solution of 0.05% phosphoric acid in water.

Standard stock solution: Transfer 100 mg of glycolic acid, previously dried overnight in a vacuum desiccator over phosphorus pentoxide, and accurately weighed, to a 100-mL volumetric flask, dissolve in and dilute with *Mobile phase* to volume, and mix.

Standard solutions: Transfer 0.5-, 1.0-, 1.5-, 2.0-, and 2.5-mL portions of the *Standard stock solution*, respectively, into five identical 100-mL volumetric flasks. To each flask, dilute with *Mobile phase* to volume, and mix. The *Standard solutions* have concentrations of 5.0, 10.0, 15.0, 20.0, and 25.0 mg/L, respectively.

Sample solution: Transfer an accurate quantity of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium, equivalent to 200 mg on the dried basis, to a flask. Dissolve in and dilute with *Mobile phase* to 20 mL.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 205 nm

Column: 7.8-mm × 30-cm; packing L22. [NOTE—Two 7.8-mm × 15-cm columns can be used in place of one 7.8-mm × 30-cm column, provided that the *System suitability* requirements are met.]

Flow rate: 0.5 mL/min

Injection size: 50 µL

System suitability

Sample: *Standard solution* with the concentration of 15 mg/L

Suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solutions* and *Sample solution*

Separately inject equal volumes of the *Standard solutions* and the *Sample solution* into the chromatograph, record the chromatograms, and measure the responses for the major peaks. Plot the peak areas from the *Standard solutions* versus the concentration of glycolic acid, in mg/L, in the *Standard solutions*. From the standard curve and the peak area from the *Sample solution*, determine the concentration of glycolic acid, C, in mg/L, in the *Sample solution*.

Calculate the percentage of sodium glycolate, P_{NaG}, in the portion of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium taken:

$$\text{Result} = \{[V \times (M_{r1}/M_{r2}) \times C]/W\} \times 100$$

V = volume of the *Sample solution*, 0.02 L
M_{r1} = molecular weight of sodium glycolate, 98.03
M_{r2} = molecular weight of glycolic acid, 76.05
W = weight of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium taken to prepare the *Sample solution*, calculated on the dried basis (mg)

Acceptance criteria: The sum of the percentages from the tests for sodium chloride and sodium glycolate ($P_{NaCl} + P_{NaG}$) is NMT 0.5%.

ORGANIC IMPURITIES

• Procedure: Limit of Residual Enzyme

Sample: 20.0 g of the dry Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium

Analysis: Transfer the *Sample* to a 2000-mL beaker. Add 100 mL of sulfuric acid, and mix using a stirring plate, until all particles are wetted. Prevent excessive foam formation during mixing. Add some glass beads to prevent boiling delay, and slowly heat the solution to a temperature not exceeding 150°. Stir for 30 min, and carefully add 5 mL of hydrogen peroxide dropwise at interval steps. After each step, allow the reaction to subside, and ensure that the solution does not contain any particulates. [NOTE—It takes up to approximately 125 mL of hydrogen peroxide.] The temperature is gradually increased to about 300°, until strong fumes of sulfur trioxide appear. Stop the heating. If the solution darkens again during heating, carefully add some drops of hydrogen peroxide until a clear solution is obtained. Allow the solution to cool down, add 10 mL of water, and heat again until strong fumes of sulfur trioxide appear. After the solution becomes clear and is cooled down, flush the sides with water, and transfer the solution quantitatively to a 4000-mL round-bottom flask. Dilute with water to a volume of approximately 500 mL, and place the round-bottom flask under the distillation unit. Pipet 10 mL of 0.1 N sulfuric acid into a 300-mL conical flask, and dilute with water to 100 mL. Place the conical flask at the end of the distillation unit, taking care that the end of the distillation unit is below the surface of the liquid. Through a dropping funnel add 500 mL of a 32% sodium hydroxide solution to

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the round-bottom flask with heavy stirring. Slowly heat until the solution boils. Boil, and collect the distillate for 20 min. Remove the conical flask, and stop the heating. Flush the inside of the condenser with water into the conical flask. Titrate the solution with 0.1 M sodium hydroxide. Perform a blank determination.

Calculate the percentage of protein in the portion of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium taken:

$$\text{Result} = \{F \times A_N \times [V_A C_A - (V_{TB} - V_B) \times C_{TB}]/W\} \times 100$$

F	= calculation factor for the theoretical amount of nitrogen present in protein, 6.25
A_N	= atomic weight of nitrogen, 14.01 g/mol
V_A	= volume of 0.1 N sulfuric acid added to the conical flask (mL)
C_A	= exact concentration of sulfuric acid added to the conical flask (N)
V_{TB}	= volume of the sodium hydroxide solution consumed in the titration for the sample determination (mL)
V_B	= volume of the sodium hydroxide solution consumed in the titration for the blank determination (mL)
C_{TB}	= concentration of the sodium hydroxide solution used in the titration (N)
W	= weight of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium taken (mg)

Acceptance criteria: NMT 0.1% of protein

SPECIFIC TESTS

- **VISCOSITY—ROTATIONAL METHODS** ⟨912⟩

  Analysis: After determining the *Loss on Drying*, weigh a quantity of undried Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium, equivalent to 60.0 g on the dried basis. In a 400-mL beaker, transfer about 240 mL of water, minus the amount of water in the test specimen. Over a period of seconds, transfer the test specimen to the 400-mL beaker containing water, while slowly and continuously stirring the solution to make the mixture weigh 300 g. When the specimen is well wetted, increase the rate of stirring, taking care to avoid mixing in excess air. Dissolve the test specimen while stirring rapidly. Equilibrate the mixture in a water bath at 25° for 1 h, until all air bubbles dissipate. Stir the solution in the beaker for 5 min at 300 rpm, making sure that no air bubbles are incorporated. Transfer the solution to a 250-mL beaker of 5 cm in internal diameter and about 12 cm in height, for measurement. Determine its viscosity at 25 ± 0.1°, using a suitable rotational viscometer with a cylindrical spindle 1.9 cm in diameter and 6.5 cm high, attached to a shaft 0.3 cm in diameter.[1] The spindle rotates at 12 rpm at an immersion depth of 8.1 cm. Follow the instrument manufacturer's directions to measure the apparent viscosity.

  Acceptance criteria: The viscosity is between 200 and 500 mPa · s.
- **pH** ⟨791⟩**:** 6.0–8.5, in a solution prepared in carbon dioxide-free water (1 in 100)
- **LOSS ON DRYING** ⟨731⟩**:** Dry it at 105° for 3 h: it loses NMT 12.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store in a cool and dry place.
- **LABELING:** Label it to indicate the viscosity, giving the viscosity measurement parameters, concentration of the solution, and the type of equipment used. The labeling also indicates the value for *Degree of Substitution*.

[1] A suitable spindle is available from Brookfield as an LV1 spindle, or the equivalent.

Cardamom Oil

DEFINITION

Cardamom Oil is the volatile oil distilled from the seed of *Elettaria cardamomum* (L.) Maton var. *cardamomum* (Fam. Zingiberaceae).

IMPURITIES

- **ARSENIC,** *Method II* ⟨211⟩**:** NMT 3 µg/g
- **LEAD** ⟨251⟩

  Diluted standard lead solution: 10 mL

  Analysis: Proceed as directed in the chapter.

  Acceptance criteria: NMT 10 µg/g

SPECIFIC TESTS

- **SPECIFIC GRAVITY** ⟨841⟩**:** 0.917–0.947
- **OPTICAL ROTATION,** *Angular Rotation* ⟨781A⟩**:** +22° to +44°
- **REFRACTIVE INDEX** ⟨831⟩**:** 1.463–1.466 at 20°
- **SOLUBILITY IN 70% ALCOHOL:** One volume dissolves in 5 volumes of 70% alcohol.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant source from which the article was derived.

Cardamom Seed

DEFINITION

Cardamom Seed is the dried ripe seed of *Elettaria cardamomum* (L.) Maton var. *cardamomum* (Fam. Zingiberaceae).

[NOTE—Cardamom Seed should be recently removed from the capsule.]

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Pesticide Residue Analysis***:** Meets the requirements

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS**

  Macroscopic

  Unground Cardamom Seed: Seeds usually appear in agglutinated groups of 2–7 seeds and as separate seeds surrounded by an adhering membranous aril. The individual seeds are oblong-ovoid or irregularly three- to four-sided, 3–4 mm in length. They are convex on the dorsal side, strongly longitudinally grooved on the ventral side, coarsely tuberculated, and externally pale orange to dark brown. The odor is aromatic. The taste is aromatic, pungent, and slightly bitter.

  Powdered Cardamom Seed: Brown to weak yellow to light olive green

  Microscopic

  Unground Cardamom Seed: Sections show a loosely attached membranous aril; a brownish seed coat consisting of an epidermal layer of thick-walled cells, a pigment layer of small cells with red to orange contents, a layer of volatile oil cells with suberized walls and a single

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layer of radially elongated, strongly lignified stone cells with inner walls heavily thickened, and a minute lumen containing silica; and a large, colorless perisperm surrounding a central, orange to yellow endosperm in which a small, straight embryo is embedded.

Powdered Cardamom Seed: It consists chiefly of fragments of perisperm, endosperm, embryo, and seed coat. The endosperm and perisperm cells are filled with starch grains 1–4 µm in diameter, or may contain one or more prisms of calcium oxalate 10–25 µm in diameter. The seed coat is characterized by its red- to orange-colored cells, is polygonal in surface view, and is about 20 µm in diameter. Fragments of pericarp tissue with spiral vessels and with accompanying slightly lignified fibers are relatively few.

- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Acid-Insoluble Ash*: NMT 4.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve against attack by insects.
- **LABELING:** The label states the Latin binomial and the official name.

Compound Cardamom Tincture

DEFINITION

Prepare Compound Cardamom Tincture as follows (see *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩).

Cardamom Seed, in moderately coarse powder	20 g
Cinnamon, in fine powder	25 g
Caraway, in moderately coarse powder	12 g
Glycerin	50 mL
Diluted Alcohol	950 mL
Diluted Alcohol, a sufficient quantity to make	1000 mL

Macerate the mixed powders in 750 mL of a mixture of 50 mL of *Glycerin* and 950 mL of *Diluted Alcohol* in a container that can be closed, and put it in a warm place. Agitate it frequently during 3 days or until the soluble matter is dissolved. Transfer the mixture to a filter, and when most of the liquid has drained away, wash the residue on the filter by using first the remainder of the mixture of *Glycerin* and *Diluted Alcohol* prepared as directed above, followed by *Diluted Alcohol*, combining the filtrates to produce 1000 mL, and mix. [NOTE—Compound Cardamom Tincture may be colored with one or more colors.]

OTHER COMPONENTS

- **ALCOHOL DETERMINATION,** *Method II* ⟨611⟩: 43.0%–47.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Package in tight, light-resistant containers, and avoid exposure to direct sunlight and excessive heat.

Carmellose

Attributes	EP	JP	USP
Definition	+	+	+
Identification A*	+	+	+
Identification B	+	+	+
Chloride	+	+	+
Sulfate	+	+	+
Loss on Drying	+	+	+
Residue on Ignition	+	+	+

* EP and USP will adopt Carmellose Reference Standard; JP will adopt a Reference spectrum.

Legend: + will adopt and implement; – will not stipulate
Nonharmonized attributes: Heavy Metals and Packaging and Storage (USP)
Reagents and reference materials: Each pharmacopeia will adapt the text to take account of local reference substances and spectra and reagent specifications.

Carboxymethylcellulose [9000-11-7].

DEFINITION

Carmellose is a carboxymethyl ether of cellulose.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K▲** (CN 1-May-2020)
- **B. pH** ⟨791⟩

Sample solution: 10 mg/mL of Carmellose suspension
Acceptance criteria: 3.5–5.0

IMPURITIES

INORGANIC IMPURITIES

- **Residue on Ignition** ⟨281⟩: NMT 1.5% on 1 g, calculated on the dried basis
- **Chlorides**

Sample solution: Shake well 0.8 g of Carmellose with 50 mL of water, dissolve in 10 mL of sodium hydroxide TS, and add water to make 100 mL. Heat 20 mL of this solution with 10 mL of nitric acid, diluted on a water bath until a flocculent precipitate is produced. Cool, centrifuge, and take out the supernatant. Wash the precipitate with three 10-mL portions of water by centrifuging each time, combine the supernatant and the washings, and add water to make 100 mL. Take 25 mL of this solution, add 6 mL of nitric acid, diluted, and dilute with water to make 50 mL.
Control solution: 0.40 mL of 0.01 N hydrochloric acid VS and 6 mL of nitric acid, diluted. Add water to make 50 mL.
Analysis: Add 1 mL of silver nitrate TS to the *Sample solution* and to the *Control solution*, mix well, and allow to stand for 5 min protected from direct sunlight. Compare the opalescence developed in both solutions against a black background by viewing downward or transversely.
Acceptance criteria: The turbidity produced in the *Sample solution* is NMT that in the *Control solution* (NMT 0.36%).

- **Sulfates**

Sample solution: Shake well 0.40 g of Carmellose with 25 mL of water, dissolve in 5 mL of sodium hydroxide TS, and add 20 mL of water. Heat this solution with 2.5 mL of hydrochloric acid in a water bath until a flocculent precipitate is produced. Cool, centrifuge, and take out the supernatant. Wash the precipitate with three 10-mL portions of water by

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centrifuging each time, combine the supernatant and the washings, and add water to make 100 mL. Filter this solution, discard 5 mL of the first filtrate, take 25 mL of the subsequent filtrate, add 1 mL of hydrochloric acid, and dilute with water to make 50 mL.

Control solution: 1.5 mL of 0.01 N sulfuric acid VS and 1 mL of dilute hydrochloric acid. Add water to make 50 mL.

Analysis: Add 2 mL of barium chloride TS to the *Sample solution* and to the *Control solution,* mix well, and allow to stand for 10 min. Compare the turbidity developed in both solutions against a black background by viewing downward or transversely.

Acceptance criteria: The turbidity produced in the *Sample solution* is NMT that of the *Control solution* (NMT 0.72%).

SPECIFIC TESTS

- **Loss on Drying** ⟨731⟩**:** Dry 1 g at 105° for 4 h: it loses NMT 8.0% of its weight.

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in tight containers.
- **USP Reference Standards** ⟨11⟩
  USP Carmellose RS

Carrageenan

Carrageenan [9000-07-1].

DEFINITION

Carrageenan is the hydrocolloid obtained by extraction with water or aqueous alkali, from some members of the class Rhodophyceae (red seaweeds). Carrageenan consists chiefly of potassium, sodium, calcium, magnesium, and ammonium sulfate esters of galactose and 3,6-anhydrogalactose copolymers. These hexoses are alternately linked α-1,3 and β-1,4 in the polymer. The prevalent copolymers in the hydrocolloid are designated *kappa-*, *iota-*, and *lambda*-carrageenan. *kappa*-Carrageenan is mostly the alternating polymer of D-galactose-4-sulfate and 3,6-anhydro-D-galactose. *iota*-Carrageenan is similar, except that the 3,6-anhydrogalactose is sulfated at carbon 2. Between *kappa*-carrageenan and *iota*-carrageenan there is a continuum of intermediate compositions differing in degree of sulfation at carbon 2. In *lambda*-carrageenan, the alternating monomeric units are mostly D-galactose-2-sulfate (1,3-linked) and D-galactose-2,6-disulfate (1,4-linked). The ester sulfate content for Carrageenan ranges from 18% to 40%. In addition, it contains inorganic salts that originate from the seaweed and from the process of recovery from the extract.

Carrageenan is recovered by alcohol precipitation, by drum drying, or by freezing. The alcohols used during recovery and purification are restricted to methanol, alcohol, and isopropyl alcohol. Carrageenan that is recovered by drum-roll drying may contain mono- and di-glycerides or up to 5% of polysorbate 80 used as roll-stripping agents.

IDENTIFICATION

- **A.**

Sample solution: A 20-mg/mL solution prepared by heating a uniform dispersion in a hot water bath to 80°

Analysis: Cool the the *Sample solution.*

Acceptance criteria: The *Sample solution* becomes more viscous upon cooling and may form a gel.

- **B.**

Sample solution: Prepare as directed for the *Sample solution* in *Identification* test *A.*

Analysis: To 10 mL of the *Sample solution,* while still hot, add 4 drops of a 0.1-g/mL solution of potassium chloride, mix, and cool.

Acceptance criteria: A short-textured ("brittle") gel indicates a carrageenan of a predominantly *kappa* type; a compliant ("elastic") gel indicates a predominantly *iota* type. If the solution does not gel, the carrageenan is of a predominantly *lambda* type.

- **C.**

Analysis: Dilute a portion of the *Sample solution,* prepared as directed in *Identification* test *A,* with 4 parts of water, and add 2–3 drops of methylene blue TS.

Acceptance criteria: A blue, stringy precipitate is formed (also positive for furcellaran, a similar colloid).

- **D. Infrared Absorption**

Sample 1: Disperse 2 g in 200 mL of a 25-mg/mL solution of potassium chloride, and stir for 1 h. Allow to stand for 18 h, stir again for 1 h, and transfer to a centrifuge tube. (If the transfer cannot be made because the dispersion is too viscous, dilute with up to 200 mL of the potassium chloride solution.) Centrifuge at approximately 1000 × *g* for 15 min. Remove the clear supernatant, resuspend the residue in 200 mL of a 25-mg/mL solution of potassium chloride, and centrifuge again. Coagulate the combined supernatants by adding 2 volumes of dilute alcohol (9 in 10). (Retain the sediment for use in preparing *Sample 2.*) Recover the coagulum, and wash with 250 mL of the dilute alcohol. Press the excess liquid from the coagulum, and dry it at 60° for 2 h. The material so obtained is the nongelling fraction (*lambda* carrageenan).

Sample 2: Disperse the sediment retained from the preparation of *Sample 1* in 250 mL of cold water, heat at 90° for 10 min, and cool to 60°. Coagulate the mixture, then recover, wash, and dry the coagulum as described above. The material so obtained is the gelling fraction (*kappa-* and *iota*-carrageenan).

Analysis: Prepare a 2-mg/mL solution of each *Sample,* cast films 5 µm thick (when dry) on a suitable nonsticking surface, and obtain the IR absorption spectrum of each film.

Acceptance criteria: Carrageenan has strong, broad absorption bands, typical of all polysaccharides, in the 1000 to 1100 cm^{-1} region. Absorption maxima are 1065 cm^{-1} and 1020 cm^{-1} for gelling and nongelling types, respectively. Other characteristic absorption bands and their intensities relative to the absorbance at 1050 cm^{-1} are as shown in *Table 1.*

Table 1

Wave Number (cm^{-1})	Molecular Assignment	Absorbance Relative to 1050 cm^{-1}		
		kappa	*iota*	*lambda*
1220–1260	Ester sulfate	0.7–1.2	1.2–1.6	1.4–2.0
928–933	3,6-Anhydro galactose	0.3–0.6	0.2–0.4	0–0.2
840–850	Galactose-4-sulfate	0.3–0.5	0.2–0.4	—
825–830	Galactose-2-sulfate	—	—	0.2–0.4
810–820	Galactose-6-sulfate	—	—	0.1–0.3
800–805	3,6-Anhydro galactose-2-sulfate	0–0.2	0.2–0.4	—

IMPURITIES

- **Arsenic** ⟨211⟩**:** NMT 3 ppm
- **Lead** ⟨251⟩**:** NMT 10 ppm

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SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩: The total bacterial count does not exceed 200 cfu/g, and the tests for *Salmonella* species and *Escherichia coli* are negative.
- **LOSS ON DRYING** ⟨731⟩: Dry a sample at a pressure not exceeding 10 mm of mercury at 70° for 18 h, cool in a desiccator, and weigh: it loses NMT 12.5% of its weight.
- **SOLUBILITY IN WATER:** NMT 30 mL of water is required to dissolve 1 g at a temperature of 80°.
- **ACID-INSOLUBLE MATTER**

  Sample: 2 g

  Analysis: Transfer the *Sample* to a 250-mL beaker containing 150 mL of water and 1.5 mL of sulfuric acid. Cover with a watch glass, and heat on a steam bath for 6 h, rubbing down the wall of the beaker frequently with a rubber-tipped stirring rod, and replacing any water lost by evaporation. Transfer 500 mg of a suitable filter aid to the beaker, and filter through a tared filtering crucible provided with a 2.4-cm glass fiber filter. Wash the residue several times with hot water, dry at 105° for 3 h, cool in a desiccator, and weigh. The difference between the total weight and the sum of the weights of the filter aid, crucible, and glass fiber filter is the weight of the acid-insoluble matter.

  Acceptance criteria: NMT 2.0% of the weight of Carrageenan taken
- **VISCOSITY–ROTATIONAL METHODS** ⟨912⟩

  Sample: 7.5 g

  Analysis: Transfer the *Sample* to a tared, tall-form, 600-mL beaker, add 450 mL of water, and disperse with agitation for 15 min. Add water to bring the weight to 500 g, and heat in a water bath, with continuous agitation, until a temperature of 80° is reached. Add water to adjust for loss by evaporation, cool to between 76° and 77°, and place in a constant-temperature bath maintained at 75°. Provide a suitable rotational viscometer with a spindle 1.88 cm in diameter and 6.51 cm high, using an immersion depth of 8.10 cm (No. 1 spindle). Allow the spindle to rotate in the solution at 30 rpm for 6 revolutions, then observe the scale reading. Convert the scale reading to centipoises by multiplying by the constant for the spindle and speed used.

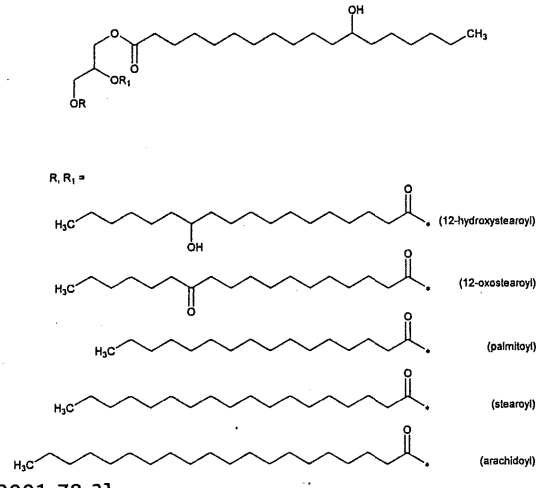
  Acceptance criteria: At 75° is NLT 5 centipoises
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩: NMT 35.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, preferably in a cool place.

Castor Oil—*see Castor Oil General Monographs*

Hydrogenated Castor Oil



[8001-78-3].

DEFINITION

Hydrogenated Castor Oil is refined, bleached, hydrogenated, and deodorized Castor Oil, consisting mainly of NLT 70.0% of the triglyceride of hydroxystearic acid.

IDENTIFICATION

- **A. IDENTITY BY FATTY ACID COMPOSITION**

  Diluent: Chloroform

  Standard solution 1: 0.8 mg/mL each of methyl palmitate, methyl arachidate, and methyl 12-ketostearate, 8 mg/mL of methyl stearate, and 0.4 mg/mL of methyl 12-hydroxystearate, from USP Methyl Palmitate RS, methyl arachidate, methyl 12-ketostearate, USP Methyl Stearate RS, and USP Methyl 12-Hydroxystearate RS, in *Diluent*

  Standard solution 2: 4 mg/mL each of methyl stearate and methyl 12-hydroxystearate, from USP Methyl Stearate RS and USP Methyl 12-Hydroxystearate RS, in *Diluent*

  Sample solution: Transfer 140 mg of Hydrogenated Castor Oil to a 10-mL screw-cap test tube, add 3.0 mL of *Diluent*, and mix well. Add 0.5 mL of 0.5 M sodium methoxide in methanol,[1] and mix with the sample. Allow the reaction to proceed at room temperature for 2 h. After 2 h, add 5 mL of water, and mix. Centrifuge the test tube at 1000 × g for 5–15 min at 4° until a clear organic layer forms. Separate the organic layer, and remove the aqueous layer. Place an aliquot of the organic layer into an autosampler vial.

  Chromatographic system

  (See *Chromatography* ⟨621⟩, *System Suitability*.)

  Mode: GC

  Detector: Flame ionization

  Column: 0.32-mm × 30-m fused silica capillary bonded with a 0.25-µm layer of phase G16

  Temperatures

  Injection port: 240°

  Detector: 250°

  Column: See *Table 1* for oven program.

[1] 0.5 M sodium methoxide in methanol is available from Sigma-Aldrich (www.sigmaaldrich.com), product #403067. Any other equivalent reagent can be used as well.

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Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)	Total Time (min)
80	0	80	1	1
80	30	140	0	3
140	20	180	5	10
180	2	250	10	55

Carrier gas: Hydrogen
Flow rate: 5.0 mL/min, constant flow mode
Injection volume: 1.0 µL
Injection type: Split ratio, 120:1 or 60:1
Liner: Single taper, low-pressure drop liner with deactivated wool
Run time: 55 min
System suitability
Sample: *Standard solution 1*
[NOTE—See *Table 2* for relative retention times.]

Table 2

Component	Relative Retention Time
Methyl palmitate (C16:0)	0.27
Methyl stearate (C18:0)	0.37
Methyl arachidate (C20:0)	0.54
Methyl 12-ketostearate	0.85
Methyl 12-hydroxystearate	1.00

Suitability requirements
Resolution: NLT 5 between any two adjacent peaks
Relative standard deviation: NMT 2.0% for the peak area ratio of methyl 12-hydroxystearate to methyl 12-ketostearate
Analysis
Samples: *Standard solution 1*, *Standard solution 2*, and *Sample solution*
Calculate the relative response factor, *F*, for methyl 12-hydroxystearate:

$$F = (r_S/r_R) \times (C_R/C_S)$$

r_S = peak area of methyl stearate from *Standard solution 2*
r_R = peak area of methyl 12-hydroxystearate from *Standard solution 2*
C_R = concentration of USP Methyl 12-Hydroxystearate RS in *Standard solution 2* (mg/mL)
C_S = concentration of USP Methyl Stearate RS in *Standard solution 2* (mg/mL)

Correct the peak area of methyl 12-hydroxystearate in the *Sample solution* by multiplying by *F*.
Calculate the percentage of each fatty acid component in the portion of sample taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak area of each individual fatty acid methyl ester, except for the uncorrected peak area of methyl 12-hydroxystearate (or the corrected peak area of methyl 12-hydroxystearate), from the *Sample solution*
r_T = sum of all the peak areas, excluding the solvent and methyl 12-hydroxystearate peaks and including the corrected peak area of methyl 12-hydroxystearate, from the *Sample solution*

Acceptance criteria: Hydrogenated Castor Oil exhibits the composition profile of fatty acids shown in *Table 3*.

Table 3

Component	Percentage (%)
Palmitic acid (C16:0)	≤2.0
Stearic acid (C18:0)	7.0–14.0
Arachidic acid (C20:0)	≤1.0
12-Ketostearic acid (or 12-oxostearic acid)	≤5.0
12-Hydroxystearic acid	78.0–91.0
Any other unspecified fatty acid or impurity	≤3.0

• **B. MELTING RANGE OR TEMPERATURE** ⟨741⟩, *Procedures, Procedure for Class II* : 85°–88°

ASSAY

• **TRIGLYCERIDE COMPOSITION**
Solution A: Methanol
Solution B: 2-Propanol
Mobile phase: See *Table 4*.

Table 4

Time (min)	Solution A (%)	Solution B (%)
0	100	0
20	50	50
23	0	100
25	100	0
35	100	0

Diluent: Methylene chloride
System suitability solution: 3.0 mg/mL of USP Hydrogenated Castor Oil RS in *Diluent*. [NOTE—Due to low solubility, sonicate the solution for about 2–3 min.]
Sample solution: 3.0 mg/mL of Hydrogenated Castor Oil in *Diluent*. [NOTE—Due to low solubility, sonicate the solution for about 2–3 min.]
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Evaporative light-scattering
Column: 4.6-mm × 25-cm; 5-µm packing L1
Temperatures
Column: 25°
Detector: 40°
Flow rate: 1.0 mL/min
Injection volume: 5 µL
Run time: 35 min
[NOTE—Depending on the different settings of the *Detector*, the *Temperatures* and *Flow rate* can be adjusted as long as system suitability requirements are met.]
System suitability
Sample: *System suitability solution*
[NOTE—See *Table 5* for relative retention times.]

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Table 5

Component	Relative Retention Time
Glyceryl tri(12-hydroxystearate) [or tri(12-hydroxystearoyl)-glycerol]	1.0
Di(12-hydroxystearoyl)-(12-oxostearoyl)-glycerol	1.1
Di(12-hydroxystearoyl)-palmitoyl-glycerol	1.7
Di(12-hydroxystearoyl)-stearoyl-glycerol	1.8
Di(12-hydroxystearoyl)-arachidoyl-glycerol	1.9

Suitability requirements

Resolution: NLT 1.5 between glyceryl tri(12-hydroxystearate) and di(12-hydroxystearoyl)-(12-oxostearoyl)-glycerol

Tailing factor: 0.8–1.8 for the glyceryl tri(12-hydroxystearate) peak

Relative standard deviation: NMT 2% for the peak area of glyceryl tri(12-hydroxystearate)

Analysis

Samples: *System suitability solution* and *Sample solution*

Calculate the percentage of each of the triglycerides in the portion of sample taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak area of each individual triglyceride from the *Sample solution*

r_T = sum of all the peak areas, excluding the solvent peak, from the *Sample solution*

Acceptance criteria: Hydrogenated Castor Oil exhibits the composition profile shown in *Table 6*.

Table 6

Component	Percentage (%)
Glyceryl tri(12-hydroxystearate) [or tri(12-hydroxystearoyl)-glycerol]	≥70.0
Di(12-hydroxystearoyl)-(12-oxostearoyl)-glycerol	≤14.0
Di(12-hydroxystearoyl)-palmitoyl-glycerol	≤2.0
Di(12-hydroxystearoyl)-stearoyl-glycerol	10.0–23.0
Di(12-hydroxystearoyl)-arachidoyl-glycerol	≤2.0

IMPURITIES

• LIMIT OF NICKEL

[CAUTION—When using closed high-pressure digestion vessels and laboratory microwave equipment, the safety precautions and operating instructions given by the manufacturer must be followed.]

[NOTE—If an alternative apparatus is used, adjustment of the instrument parameters may be necessary.]

Nickel standard stock solution: Dilute nickel standard solution TS two-fold with water. This solution contains the equivalent of 5 µg/mL of nickel.

Standard solutions: Transfer 25, 50, 75, and 100 µL of *Nickel standard stock solution* to four identical 25-mL volumetric flasks. To each flask add 0.5 mL of a 10-mg/mL solution of magnesium nitrate, 0.5 mL of a 100-mg/mL solution of monobasic ammonium phosphate, and 6.0 mL of nickel-free nitric acid. Dilute with water to volume, and mix well. [NOTE—Content of nickel in the nickel-free nitric acid is NMT 0.005 ppm.] The *Standard solutions* contain 0.005, 0.01, 0.015, and 0.02 µg/mL of nickel, respectively.

Sample solution: Transfer about 250 mg of Hydrogenated Castor Oil to a suitable high-pressure-resistant digestion vessel (fluoropolymer or quartz glass), and add 6.0 mL of nickel-free nitric acid and 2.0 mL of 30% hydrogen peroxide. Place the closed vessel in a laboratory microwave oven, and digest using an appropriate program (e.g., 1000 W for 40 min). Allow the digestion vessel to cool before opening. Add 2.0 mL of 30% hydrogen peroxide, and repeat the digestion step. Allow the digestion vessel to cool down before opening. Quantitatively transfer to a 25-mL volumetric flask, add 0.5 mL of a 10-mg/mL solution of magnesium nitrate and 0.5 mL of a 100-mg/mL solution of monobasic ammonium phosphate. Dilute with water to volume, and mix well.

Blank solution: Place 6.0 mL of nickel-free nitric acid and 2.0 mL of 30% hydrogen peroxide in a suitable high-pressure-resistant digestion vessel. Prepare as directed in *Sample solution*, beginning with "Place the closed vessel in a laboratory microwave oven, and digest using an appropriate program (e.g., 1000 W for 40 min)."

Zero solution: In a 50-mL volumetric flask, introduce 1.0 mL of a 10-mg/mL solution of magnesium nitrate, 1.0 mL of a 100-mg/mL solution of monobasic ammonium phosphate, and 12.0 mL of nickel-free nitric acid. Dilute with water to volume, and mix well.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption, equipped with a graphite furnace, a background compensation system, and a coated tube resistant to pyrolysis

Analytical wavelength: 232.0 nm

Lamp: Nickel hollow-cathode

Temperature: Maintain the drying temperature of the furnace at 120° for 35 s after a 5-s ramp; maintain the ashing temperature at 1100° for 10 s after a 30-s ramp; maintain the cooling temperature at 800° for 5 s after a 5-s decrease; and maintain the atomization temperature at 2600° for 7 s. [NOTE—The temperature program may be modified to obtain optimum furnace temperatures.]

Analysis

Samples: *Standard solutions*, *Sample solution*, and *Blank solution*

Concomitantly determine the absorbances of the *Samples* using the *Instrumental conditions* described above. Use the *Zero solution* to set the instrument to zero. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of nickel, and draw the straight line best fitting the plotted points. From this graph, determine the concentration, C_T, in µg/mL, of nickel in the *Sample solution*, and determine the concentration, C_B, in µg/mL, of nickel in the *Blank solution*. If necessary, dilute with the *Zero solution* to obtain a reading within the calibrated absorbance range.

Calculate the quantity, in µg, of nickel in each g of Hydrogenated Castor Oil taken:

$$\text{Result} = V \times (C_T - C_B)/W$$

V = volume of the *Sample solution* and the *Blank solution*, 25 mL

C_T = concentration of nickel in the *Sample solution* (µg/mL)

C_B = concentration of nickel in the *Blank solution* (µg/mL)

W = weight of Hydrogenated Castor Oil taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 20 µg/g

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• **ALKALINE IMPURITY**
Sample: 1.0 g
Analysis: Dissolve the *Sample* by gently heating in a mixture of 1.5 mL of alcohol and 3.0 mL of toluene. Add 0.05 mL of bromophenol blue TS, and titrate with 0.01 N hydrochloric acid VS to a yellow endpoint.
Acceptance criteria: NMT 0.2 mL of 0.01 N hydrochloric acid.

SPECIFIC TESTS
• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Iodine Value*: NMT 5
• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Saponification Value*: 176–182
• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Acid Value*
Sample solution: Melt 20 g in a conical flask on a steam bath, add 75 mL of hot alcohol that has previously been neutralized with 0.1 N sodium hydroxide to phenolphthalein TS, swirl, and add 1 mL of phenolphthalein TS.
Analysis: Titrate with 0.1 N sodium hydroxide VS, swirling vigorously, until the solution remains faintly pink after being shaken for 60 s.
Acceptance criteria: NMT 11.0 mL of 0.1 N sodium hydroxide VS, corresponding to NMT 3.1 for acid value
• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Hydroxyl Value*
Sample solution: 2 g in a glass-stoppered, 250-mL conical flask. Add 5.0 mL of a freshly prepared mixture of acetic anhydride and pyridine (1:3), and swirl to mix. Connect the flask to a reflux condenser, and heat on a steam bath for 2 h. Add 10 mL of water through the condenser, swirl to mix, heat on a steam bath for an additional 10 min. The titration can be performed on a warm solution of 50°–65° to avoid a flocculation of the substance. Add through the condenser 15 mL of normal butyl alcohol that has previously been neutralized to phenolphthalein, remove the condenser, wash the tip of the condenser and the sides of the flask with an additional 10 mL of neutralized normal butyl alcohol, and add 1 mL of phenolphthalein TS.
Analysis: Titrate with 0.5 N alcoholic potassium hydroxide VS to a faint pink endpoint. Perform a blank determination on a 5.0-mL portion of the acetic anhydride–pyridine mixture. To determine the amount of free acid in Hydrogenated Castor Oil, weigh 10 g into a 250-mL conical flask, add 10 mL of pyridine that has previously been neutralized to phenolphthalein, swirl to mix, add 1 mL of phenolphthalein TS, and titrate with 0.5 N alcoholic potassium hydroxide VS to a faint pink endpoint. Calculate the hydroxyl value in the portion of Hydrogenated Castor Oil taken:

$$\text{Result} = M_r \times N \times [A + (B \times W/D) - C]/W$$

M_r = molecular weight of potassium hydroxide, 56.1
N = normality of the alcoholic potassium hydroxide solution
A = volume of 0.5 N alcoholic potassium hydroxide consumed by the blank (mL)
B = volume consumed in the free-acid titration (mL)
W = weight of Hydrogenated Castor Oil taken (g)
D = weight of Hydrogenated Castor Oil used in the free-acid titration (g)
C = volume consumed in the sample titration (mL)

Acceptance criteria: 154–163

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight containers, and avoid exposure to excessive heat.

• **USP REFERENCE STANDARDS** ⟨11⟩
USP Hydrogenated Castor Oil RS
USP Methyl 12-Hydroxystearate RS
USP Methyl Palmitate RS
USP Methyl Stearate RS

Cellaburate

Cellulose, acetate butanoate;
Cellulose, acetate butyrate;
Acetylbutyrylcellulose;
Cellulose butyrate acetate;
Cellulose acetate butyrate [9004-36-8].

DEFINITION
Cellaburate is a reaction product of cellulose, acetic anhydride or acetic acid, and butyric acid or butyric anhydride. It contains NLT 1.0% and NMT 41.0% of acetyl (C_2H_3O) groups, by weight, and NLT 5.0% and NMT 56.0% of butyryl (C_4H_7O) groups, by weight, calculated on the previously dried, acid-free basis.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F**▲ (CN 1-May-2020)
Sample solution: Dissolve 150 mg in 1 mL of acetone. Evenly cast 1 drop of the solution on a sodium chloride plate. Heat the plate at 105° for 10 min.
Acceptance criteria: Meets the requirements

ASSAY
• **ACETYL AND BUTYRYL CONTENT**
Internal standard solution: 4.6 mg/mL of isovaleric acid in pyridine. Store it in a tightly closed container.
Saponification solution: Place 250 mL of *n*-propyl alcohol in a 500-mL volumetric flask, add 65.5 g of potassium hydroxide, and mix to dissolve. Dilute with *n*-propyl alcohol to volume, and mix.
Acid solution: Place 250 mL of *n*-propyl alcohol in a 500-mL volumetric flask, add 166 mL of hydrochloric acid, and mix. Dilute with *n*-propyl alcohol to volume, and mix.
Standard solution: Transfer 0.20 g of glacial acetic acid and 0.31 g of butyric acid to a 50-mL volumetric flask. Dilute with *Internal standard solution* to volume, and mix.
Sample solution: Transfer 0.15 g of Cellaburate, previously dried at 105° for 1 h, into a 25-mm × 160-mm test tube. Pipet 10 mL of *Internal standard solution* into the test tube, and dissolve by stirring and heating at 110° for 30 min. While stirring, add 5 mL of *Saponification solution* slowly into the tube. Heat at 110° for 10 min. Cool, and add 5 mL of the *Acid solution*. Mix on a vortex mixer, and allow the precipitate to settle.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.53-mm × 30-m fused silica bonded with a 1-µm layer of phase G35
Temperatures
Column: 125°
Injector: 250°
Detector: 250°
Carrier gas: Helium
Flow rate: 8 mL/min
Injection volume: 1 µL

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Injection type: Split ratio, 35:1
System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for acetic acid, butyric acid, and isovaleric acid are about 0.45, 0.85, and 1.00, respectively.]
Suitability requirements
Tailing factor: NMT 1.5 for the butyric acid peak
Relative standard deviation: NMT 3.0%
Calibration
Sample: *Standard solution*
Number of injections: 3
Calculate the average unit weight response, F_{SA}, of acetic acid per 10 mL of the *Internal standard solution*:

$$F_{SA} = (W_{RA}/R_{SA}) \times F_I$$

W_{RA} = weight of acetic acid in the *Standard solution* (g)
R_{SA} = average peak response ratio of acetic acid to isovaleric acid
F_I = volume ratio of the *Internal standard solution* in the *Sample solution* to that in the *Standard solution*, 10:50

Similarly, calculate the average unit weight response, F_{SB}, of butyric acid per 10 mL of the *Internal standard solution*:

$$F_{SB} = (W_{RB}/R_{SB}) \times F_I$$

W_{RB} = weight of butyric acid in the *Standard solution* (g)
R_{SB} = average peak response ratio of butyric acid to isovaleric acid

Analysis
Sample: Upper clear solution from the *Sample solution*
Calculate the percentage of acetyl in the portion of Cellaburate taken:

$$\text{Result} = [(M_{r1}/M_{r2}) \times R_{UA} \times F_{SA}]/W_U \times 100$$

M_{r1} = formula weight of acetyl, 43
M_{r2} = formula weight of acetic acid, 60
R_{UA} = peak response ratio of acetic acid to isovaleric acid in the *Sample solution*
W_U = weight of Cellaburate taken to prepare the *Sample solution* (g)

Calculate the percentage of butyryl in the portion of Cellaburate taken:

$$\text{Result} = [(M_{r3}/M_{r4}) \times R_{UB} \times F_{SB}]/W_U \times 100$$

M_{r3} = formula weight of butyryl, 71
M_{r4} = formula weight of butyric acid, 88
R_{UB} = peak response ratio of butyric acid to isovaleric acid in the *Sample solution*

Acceptance criteria: 1.0%–41.0% of acetyl (C_2H_3O) groups and 5.0%–56.0% of butyryl (C_4H_7O) groups on the previously dried, acid-free basis

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 0.1%
- **LIMIT OF FREE ACID**

Indicator solution: In a 1-L volumetric flask dissolve 0.675 g of bromocresol purple in 25 mL of 0.10 N sodium hydroxide. Dilute with water to volume, and mix.

Calibration solutions: Pipet 1, 2, 3, and 4 mL of 0.001 N acetic acid VS into four 100-mL volumetric flasks, respectively. Pipet 4 mL of the *Indicator solution* into each flask and into an empty 100-mL volumetric flask, and dilute each flask with water to volume to obtain solutions containing 0.0, 0.60, 1.20, 1.80, and 2.40 µg/mL of acetic acid.
Control solution: Place 96 mL of water in a suitable bottle, add a stirring bar, cap the bottle, and stir for 75 min at room temperature. Pipet 4 mL of the *Indicator solution* into the bottle, and mix.
Sample solution: Transfer 1–2 g of Cellaburate to a bottle, and add 96 mL of water. Add a stirring bar, cap the bottle, and stir for 75 min at room temperature. Pipet 4 mL of the *Indicator solution* into the bottle, stir to mix, and allow the solid to settle for 2 min.
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: Vis
Analytical wavelength: Determine the maximum absorption of the basic form of bromocresol purple in the *Calibration* at about 589 nm.
Cell: 1 cm
Blank: Water
Calibration: Determine the absorbances of the *Calibration solutions*. The absorbance difference between the 0.0-µg/mL solution and the other solutions adheres to Beer's law over the range stated in the *Calibration solutions*. Plot the absorbance difference versus the concentration of the acetic acid, in µg/mL, on linear graph paper, and draw the straight line best fitting the points, including the origin.
Analysis
Samples: *Control solution* and *Sample solution*
Pass 10 mL of the *Sample solution* through a polytef syringe filter that has been presoaked in isopropyl alcohol. Determine the absorbance of the filtered *Sample solution*. In the same manner, determine the absorbance of the *Control solution*.
Calculate the percentage of free acid, as acetic acid, in the portion of Cellaburate taken:

$$\text{Result} = (V \times C_U/W_U)/F \times 100$$

V = total volume of the *Sample solution*, 100 mL
C_U = concentration of free acid, calculated as acetic acid (µg/mL), based on the absorbance difference between the *Control solution* and the *Sample solution* read directly from the calibration plot
W_U = weight of Cellaburate taken to prepare the *Sample solution* (g)
F = unit conversion, 10^6 µg/g

[NOTE—If the C_U value is more than 2.8 µg/mL, reduce the sample size by half in the *Sample solution*, and repeat the determination.]
Acceptance criteria: NMT 0.1%

SPECIFIC TESTS

- **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 5.0%, using a mixture of methylene chloride and methanol (2:1) in place of the methanol solvent

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.
- **LABELING:** The labeling indicates the nominal percentage ranges of the acetyl and butyryl groups.

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• **USP REFERENCE STANDARDS** ⟨11⟩
USP Cellaburate RS

Cellacefate

Portions of the monograph text that are national *USP* text, and are not part of the harmonized text, are marked with symbols (• •) to specify this fact.

Cellulose, acetate, 1,2-benzenedicarboxylate;
Cellulose acetate phthalate [9004-38-0].

DEFINITION
Cellacefate is a reaction product of phthalic anhydride and a partial acetate ester of cellulose. It contains NLT 21.5% and NMT 26.0% of acetyl (C_2H_3O) groups and NLT 30.0% and NMT 36.0% of phthalyl (o-carboxybenzoyl) ($C_8H_5O_3$) groups, calculated on the anhydrous, acid-free basis.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K.▲** (CN 1-May-2020) Do not dry specimens.

ASSAY
• **PHTHALYL CONTENT**
Sample solution: Transfer 1 g to a conical flask, dissolve in 50 mL of a mixture of alcohol and acetone (3:2), and add phenolphthalein TS.
Analysis: Titrate the *Sample solution* with 0.1 N sodium hydroxide VS. Perform a blank determination, and make any necessary correction (see *Titrimetry* ⟨541⟩).
Calculate the percentage of phthalyl on the acid-free basis:

$$\text{Result} = \{[(1.491 \times A/W) - (1.795 \times B)]/(100 - B)\} \times 100$$

A = volume of 0.1 N sodium hydroxide consumed, corrected for the blank (mL)
W = weight of Cellacefate taken, calculated on the anhydrous basis (g)
B = percentage of acid found in the test for *Limit of Free Acid*

Acceptance criteria: 30.0%–36.0% of phthalyl ($C_8H_5O_3$) on the anhydrous, acid-free basis
• **CONTENT OF ACETYL**
Sample solution: Transfer 100 mg to a glass-stoppered flask, and add 25.0 mL of 0.1 N sodium hydroxide VS. Connect the flask to a reflux condenser, and reflux for 30 min. Cool, and add phenolphthalein TS.
Analysis: Titrate the *Sample solution* with 0.1 N hydrochloric acid VS. Perform a blank determination (see *Titrimetry* ⟨541⟩).
Calculate the free and combined acids as acetyl:

$$\text{Result} = 0.4305 \times (A/W)$$

A = volume of 0.1 N sodium hydroxide consumed, corrected for the blank (mL)
W = weight of Cellacefate taken, calculated on the anhydrous basis (g)

Calculate the percentage of acetyl on the acid-free basis:

$$\text{Result} = 100 \times [P - (0.5182 \times B)]/(100 - B) - (0.5772 \times C)$$

P = free and combined acids, as acetyl
B = percentage of acid found in the test for *Limit of Free Acid*
C = percentage of phthalyl found in the test for *Phthalyl Content*

Acceptance criteria: 21.5%–26.0% of acetyl (C_2H_3O) on the anhydrous, acid-free basis

IMPURITIES
• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%
• **LIMIT OF FREE ACID**
Sample solution: Transfer 3.0 g to a glass-stoppered flask, add 100 mL of dilute methanol (1 in 2), insert the stopper in the flask, and shake for 2 h. Filter, and wash the flask and the filter with two 10-mL portions of the methanol solution, adding the washings to the filtrate.
Analysis: Titrate the combined filtrate and washings from the *Sample solution* with 0.1 N sodium hydroxide VS to a phenolphthalein endpoint. Perform a blank determination on 120 mL of the dilute methanol (1 in 2) (see *Titrimetry* ⟨541⟩).
Calculate the percentage of free acid, B:

$$\text{Result} = 0.8306 \times A/W$$

A = volume of 0.1 N sodium hydroxide consumed, corrected for the blank (mL)
W = weight of Cellacefate taken, calculated on the anhydrous basis (g)

Acceptance criteria: NMT 3.0%, calculated as phthalic acid

SPECIFIC TESTS
• **WATER DETERMINATION,** *Method I* ⟨921⟩
Sample: 0.5 g
Analysis: Dissolve the *Sample* in a mixture of dehydrated alcohol and methylene chloride (3:2) instead of methanol as the solvent.
Acceptance criteria: NMT 5.0%
• **VISCOSITY—CAPILLARY METHODS** ⟨911⟩
Sample: 15 g, calculated on the anhydrous basis
Analysis: Dissolve the *Sample* in 85 g of a mixture of 249 parts of anhydrous acetone and 1 part of water, by weight.
Acceptance criteria: The apparent viscosity (see *Viscosity—Capillary Methods* ⟨911⟩, *Method I*) is between 45 and 90 centipoises, determined at 25 ± 0.2°.

ADDITIONAL REQUIREMENTS
• **•PACKAGING AND STORAGE:** Preserve in tight containers.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Cellacefate RS

Microcrystalline Cellulose

Add the following:

▲Portions of this monograph that are national *USP* text, and are not part of the harmonized text, are marked with symbols (• •) to specify this fact.▲ (NF 1-Dec-2019)

Cellulose [9004-34-6].

DEFINITION
Microcrystalline Cellulose is purified, partially depolymerized cellulose prepared by treating alpha cellulose, obtained as a pulp from fibrous plant material, with mineral acids.

Supplement 1

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IDENTIFICATION

Add the following:

▲[NOTE—Compliance is determined by meeting the requirements of *Identification A, B,* and *C.*]▲ (NF 1-Dec-2019)

Add the following:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197A** or **197K:**▲ (CN 1-May-2020) ▲
[NOTE—Disregard any peak between 800 and 825 cm^{-1} as well as those between 950 and 1000 cm^{-1}.]▲ (NF 1-Dec-2019)

Change to read:

- **▲B.**▲ (NF 1-Dec-2019)
Iodinated zinc chloride solution: Dissolve 20 g of zinc chloride and 6.5 g of potassium iodide in 10.5 mL of water. Add 0.5 g of iodine, and shake for 15 min.
Sample: 10 mg
Analysis: Place the *Sample* on a watch glass, and disperse in 2 mL of *Iodinated zinc chloride solution.*
Acceptance criteria: The substance takes on a violet-blue color.

Change to read:

- **▲C.**▲ (NF 1-Dec-2019)
Sample: 1.3 g of Microcrystalline Cellulose, accurately weighed to 0.1 mg
Analysis: Transfer the *Sample* to a 125-mL conical flask. Add 25.0 mL of water and 25.0 mL of 1.0 M cupriethylenediamine hydroxide solution. Immediately purge the solution with nitrogen, insert the stopper, and shake on a wrist-action shaker, or other suitable mechanical shaker, until completely dissolved. Transfer an appropriate volume of the sample solution to a calibrated number 150 Cannon-Fenske, or equivalent, viscometer. Allow the solution to equilibrate at 25 ± 0.1° for NLT 5 min. Time the flow between the 2 marks on the viscometer, and record the flow time, t_1, in seconds. Calculate the kinematic viscosity, $(KV)_1$, of Microcrystalline Cellulose taken:

$$\text{Result} = t_1 \times k_1$$

t_1 = flow time (s)
k_1 = viscometer constant (see *Viscosity—Capillary Methods* ⟨911⟩)

Obtain the flow time, t_2, for 0.5 M cupriethylenediamine hydroxide solutions using a number 100 Cannon-Fenske, or equivalent, viscometer. Calculate the kinematic viscosity, $(KV)_2$, of the solvent:

$$\text{Result} = t_2 \times k_2$$

t_2 = flow time for 0.5 M cupriethylenediamine hydroxide solutions (s)
k_2 = viscometer constant

Determine the relative viscosity, η_{rel}, of the Microcrystalline Cellulose specimen taken:

$$\text{Result} = (KV)_1/(KV)_2$$

$(KV)_1$ = kinematic viscosity of Microcrystalline Cellulose taken
$(KV)_2$ = kinematic viscosity of the solvent

Determine the intrinsic viscosity, $[\eta]_c$, by interpolation, using the *Intrinsic Viscosity Table* in the *Reference Tables* section.
Calculate the degree of polymerization, *P*:

$$\text{Result} = [(95) \times [\eta]_c/\{W_s \times [(100 - \%\text{LOD})/100]\}$$

$[\eta]_c$ = intrinsic viscosity
W_s = weight of Microcrystalline Cellulose taken (g)
%LOD = value obtained from the test for *Loss on Drying*

Acceptance criteria: The degree of polymerization is NMT 350.

IMPURITIES

INORGANIC IMPURITIES

- **Residue on Ignition** ⟨281⟩: NMT 0.1%

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩: The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g. It meets the requirements of the tests for absence of *Staphylococcus aureus* and *Pseudomonas aeruginosa* and for the absence of *Escherichia coli* and *Salmonella species.*
- **CONDUCTIVITY**
Sample: 5 g
Analysis: Shake the *Sample* with 40 mL of water for 20 min, and centrifuge. Retain the supernatant for use in the *pH* test. Using an appropriate conductivity meter that has been standardized with a potassium chloride conductivity calibration standard having a conductivity of 100 µS/cm, measure the conductivity of the supernatant after a stable reading is obtained, and measure the conductivity of the water used to prepare the test specimen.
Acceptance criteria: The conductivity of the supernatant does not exceed the conductivity of the water by more than 75 µS/cm.
- **pH** ⟨791⟩: 5.0–7.5 in the supernatant obtained in the *Conductivity* test

Change to read:

- **LOSS ON DRYING** ⟨731⟩
Analysis: Dry a sample at 105° for 3 h.
Acceptance criteria: NMT 7.0%▲◆▲ (NF 1-Dec-2019) or some other lower percentage, or is within a percentage range, as specified in the labeling.▲◆▲ (NF 1-Dec-2019)
- **BULK DENSITY**
Analysis: Use a volumeter that has been fitted with a 10-mesh screen. The volumeter is freestanding of the brass or stainless steel cup, which is calibrated to a capacity of 25.0 ± 0.05 mL and has an inside diameter of 30.0 ± 2.0 mm. Weigh the empty cup, position it under the chute, and slowly pour the powder from a height of 5.1 cm (2 in) above the funnel through the volumeter, at a rate suitable to prevent clogging, until the cup overflows. [NOTE—If excessive clogging of the screen occurs, remove the screen.] Level the excess powder, and weigh the filled cup. Calculate the bulk density by dividing the weight of the powder in the cup by the volume of the cup.
Acceptance criteria: The bulk density is within the labeled specification.

Change to read:

- **▲◆▲** (NF 1-Dec-2019)**PARTICLE SIZE DISTRIBUTION**

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[NOTE—In cases where there are no functionality-related concerns regarding the particle size distribution of the article, this test may be omitted.]

Where the labeling states the particle size distribution, determine the particle size distribution as directed in *Particle Size Distribution Estimation by Analytical Sieving* ⟨786⟩, or by a suitable validated procedure. ▲◆▲ (NF 1-Dec-2019)

• **WATER-SOLUBLE SUBSTANCES**

Sample: 5.0 g

Analysis: Shake the *Sample* with 80 mL of water for 10 min, and pass with the aid of a vacuum through filter paper (Whatman No. 42 or equivalent) into a vacuum flask. Transfer the filtrate to a tared beaker, evaporate to dryness without charring, dry at 105° for 1 h, cool in a desiccator, and weigh.

Acceptance criteria: The difference between the weight of the residue and the weight obtained from a blank determination does not exceed 12.5 mg (0.25%).

• **ETHER-SOLUBLE SUBSTANCES**

Sample: 10.0 g

Analysis: Place the *Sample* in a chromatographic column having an internal diameter of about 20 mm, and pass 50 mL of peroxide-free ether through the column. Evaporate the eluate to dryness in a previously dried and tared evaporating dish with the aid of a current of air in a fume hood. After all the ether has evaporated, dry the residue at 105° for 30 min, cool in a desiccator, and weigh.

Acceptance criteria: The difference between the weight of the residue and the weight obtained from a blank determination does not exceed 5.0 mg (0.05%).

ADDITIONAL REQUIREMENTS

Change to read:

• ▲◆▲ (NF 1-Dec-2019)**PACKAGING AND STORAGE:** Preserve in tight containers. ▲◆▲ (NF 1-Dec-2019)

Change to read:

• ▲◆▲ (NF 1-Dec-2019)**LABELING:** The labeling indicates the nominal loss on drying, bulk density, and degree of polymerization values. Degree of polymerization compliance is determined using *Identification* ▲C.▲ (NF 1-Dec-2019) Where the particle size distribution is stated in the labeling, proceed as directed in the test for *Particle Size Distribution*. The labeling indicates with which technique the particle size distribution was determined if a technique other than analytical sieving was used; and the labeling indicates the d_{10}, d_{50}, and d_{90} values and the range for each. ▲◆▲ (NF 1-Dec-2019)

Add the following:

▲• **USP REFERENCE STANDARDS** ⟨11⟩

USP Microcrystalline Cellulose RS ▲ (NF 1-Dec-2019)

Microcrystalline Cellulose and Carboxymethylcellulose Sodium

DEFINITION

Microcrystalline Cellulose and Carboxymethylcellulose Sodium is a colloid-forming, attrited mixture of Microcrystalline Cellulose and Carboxymethylcellulose Sodium. It contains NLT 75.0% and NMT 125.0% of the labeled amount of carboxymethylcellulose sodium, calculated on the dried basis. The viscosity of its aqueous dispersion of percent by weight stated on the label is NLT 60.0% and NMT 140.0% of that stated on the label in centipoises.

IDENTIFICATION

• **A.**

Sample: 6 g

Analysis: Mix the *Sample* with 300 mL of water in a blender at 18,000 rpm for 5 min.

Acceptance criteria: A white, opaque dispersion is produced that does not settle on standing.

• **B.**

Sample: The dispersion obtained in *Identification* test *A*

Analysis: Add several drops of the *Sample* to a solution of aluminum chloride (100 mg/mL).

Acceptance criteria: Each drop forms a white, opaque globule that does not disperse on standing.

• **C.**

Sample: The dispersion obtained in *Identification* test *A*

Analysis: Add 3 mL of iodine TS to the *Sample*.

Acceptance criteria: No blue or purplish-blue color is produced.

ASSAY

• **CARBOXYMETHYLCELLULOSE SODIUM**

Sample solution: Transfer 2000 mg of Microcrystalline Cellulose and Carboxymethylcellulose Sodium to a glass-stoppered, 250-mL conical flask. Add 75 mL of glacial acetic acid, attach a condenser, and reflux for 2 h. Cool, and transfer the mixture to a 250-mL beaker with the aid of small volumes of glacial acetic acid.

Titrimetric conditions

Mode: Direct titration

Titrant: 0.1 N perchloric acid in dioxane VS

Endpoint detection: Potentiometry

Analysis: Titrate the *Sample solution* and calculate the percentage of carboxymethylcellulose sodium in the sample taken:

$$\text{Result} = [(V_S \times N \times F)/W] \times 100$$

V_S = *Titrant* volume consumed by the *Sample* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 296.0 mg/mEq
W = *Sample* weight (mg)

Acceptance criteria: 75.0%–125.0% on the dried basis

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩: NMT 5.0%

SPECIFIC TESTS

• **VISCOSITY—ROTATIONAL METHODS** ⟨912⟩

Analysis: Determine the amounts of Microcrystalline Cellulose and Carboxymethylcellulose Sodium needed to prepare 600 g of a suitable dispersion, calculated on the dried basis. Transfer an amount of water to a 1000-mL blender bowl. Begin stirring with an 18,000 rpm blender at a reduced speed obtained by adjusting the voltage to 30 volts by means of a suitable transformer, and immediately add the accurately weighed portion of Microcrystalline Cellulose and Carboxymethylcellulose Sodium, taking care to avoid contacting the sides of the bowl with the powder. Continue stirring at this speed for 15 s following the addition of the powder, then increase the transformer setting to 115 volts, and mix for 2 min, accurately timed, at 18,000 rpm. Stop the blender, and lower the appropriate spindle of a suitable rotational viscometer into the dispersion. Thirty s after cessation of mixing, start the

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viscometer, and determine the viscosity using the appropriate spindle to obtain a scale reading between 10% and 90% of full-scale at a speed of 20 rpm. Determine the scale reading after 30 s of rotation, and calculate the viscosity, in centipoises, by multiplying the scale reading by the constant for the spindle used at 20 rpm.
Acceptance criteria: 60.0%–140.0% of that stated on the label, in centipoises
- **pH** ⟨791⟩
Sample solution: The dispersion prepared in the test for *Viscosity—Rotational Methods* ⟨912⟩
Acceptance criteria: 6.0–8.0
- **Loss on Drying** ⟨731⟩
Analysis: Dry a sample at 105° to constant weight.
Acceptance criteria: NMT 8.0%

ADDITIONAL REQUIREMENTS
- **Packaging and Storage:** Preserve in tight containers, store in a dry place, and avoid exposure to excessive heat.
- **Labeling:** Label it to indicate the percentage content of carboxymethylcellulose sodium and the viscosity of the dispersion in water of the designated weight percentage composition.

Silicified Microcrystalline Cellulose

DEFINITION
Silicified Microcrystalline Cellulose is composed of intimately associated microcrystalline cellulose and colloidal silicon dioxide particles, derived from aqueous coprocessing prior to drying the material during manufacture. The microcrystalline cellulose component is purified, partially depolymerized cellulose, prepared by treating alpha cellulose, obtained as a pulp from fibrous plant material, with mineral acids. The colloidal silicon dioxide is a submicroscopic fumed silica prepared by the vapor-phase hydrolysis of a silicon compound. The *Residue on Ignition* result indicates the percentage of colloidal silicon dioxide; the remainder is microcrystalline cellulose.

IDENTIFICATION

Change to read:

- **A. ▲Spectroscopic Identification Tests** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
- **B.**
Sample: 10 mg
Iodinated zinc chloride solution: Dissolve 20 g of zinc chloride and 6.5 g of potassium iodide in 10.5 mL of water. Add 0.5 g of iodine, and shake for 15 min.
Analysis: Place the *Sample* on a watch glass, and disperse in 2 mL of *Iodinated zinc chloride solution*.
Acceptance criteria: The substance takes on a violet-blue color.
- **C.**
Sample: 5 mg of residue from the test for *Residue on Ignition*
Analysis: Transfer the *Sample* to a platinum crucible, and mix with about 200 mg of anhydrous potassium carbonate. Ignite at a red heat over a burner for about 10 min, and cool. Dissolve the melt in 2 mL of freshly distilled water, warming if necessary, and slowly add 2 mL of ammonium molybdate TS to the solution.
Acceptance criteria: A deep yellow color is produced.
- **D. Silica Dispersion Uniformity Test**
Conditioned test substance: Pass Silicified Microcrystalline Cellulose through an 850-µm sieve, disperse it into a suitable scale blender,[1] and tumble/mix the test substance for a minimum of 20 min to condition the material in preparation.
Analysis: Assemble a sieve stack composed of the following nested sieves: 60-, 80-, 120-, 200-, 325-, and 400-US mesh, plus pan. Tare each sieve to the nearest 0.1 g. Weigh 200.0 g of the *Conditioned test substance*, and transfer to the top sieve. Agitate the sieve stack on a suitable sieve shaker for 20 min. Separate and record the weight of each sieve, including the *Conditioned test substance* fraction. Determine the *Conditioned test substance* fraction mass by difference. Analyze a test substance from each sieve fraction, using *Residue on Ignition* ⟨281⟩. Obtain the *Residue on Ignition* (ROI) value in percentage, P_i, for each sieve fraction, excluding any fraction weighing less than 0.5 g. Calculate the average percentage of ROI value, P_A, for P_i (i = 1–6).
Calculate the variance for the sieve fraction, excluding the pan and any fraction weighing less than 0.5 g:

$$\text{Result} = \sum_{i=1}^{n} (P_i - P_A)^2/(n-1)$$

Acceptance criteria: NMT 0.02

SPECIFIC TESTS
- **Residue on Ignition** ⟨281⟩: 1.8%–2.2%
- **Microbial Enumeration Tests** ⟨61⟩ and **Tests for Specified Microorganisms** ⟨62⟩: The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.
- **Conductivity**
Sample: 5 g
Analysis: Shake the *Sample* with 40 mL of water for 20 min, and centrifuge. Retain the supernatant for use in the *pH* test. Using an appropriate conductivity meter that has been standardized with a potassium chloride conductivity calibration standard having a conductivity of 100 µS/cm, measure the conductivity of the supernatant after a stable reading is obtained, and measure the conductivity of the water used to prepare the test specimen.
Acceptance criteria: The conductivity of the supernatant does not exceed the conductivity of the water by more than 75 µS/cm.
- **pH** ⟨791⟩: 5.0–7.5 for the supernatant obtained in the *Conductivity* test
- **Loss on Drying** ⟨731⟩
Analysis: Dry at 105° for 3 h.
Acceptance criteria: NMT 7.0%, within a percentage range, as specified in *Labeling*
- **Degree of Polymerization**
Sample: 1.3 g, weighed to 0.1 mg
Analysis: Transfer the *Sample* to a 125-mL conical flask. Add 25.0 mL of water and 25.0 mL of 1.0 M cupriethylenediamine hydroxide solution. Immediately purge the solution with nitrogen, insert the stopper, and shake on a wrist action shaker or other suitable mechanical shaker until completely dissolved. Transfer an appropriate volume of the solution to a calibrated number 150 Cannon-Fenske, or equivalent, viscometer. Allow the solution to equilibrate at 25 ± 0.1° for NLT 5 min. Time the flow between the two marks on the viscometer. Calculate the kinematic viscosity of the *Sample* taken:

$$v_1 = t_1 \times k_1$$

[1] Planetary mixer, Turbula T2F mixer, or V-blender.

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t_1 = time of flow between the two marks on the viscometer (s)
k_1 = viscometer constant (see *Viscosity—Capillary Methods* ⟨911⟩)

Obtain the flow time for a 0.5 M cupriethylenediamine hydroxide solution, using a number 100 Cannon-Fenske, or equivalent, viscometer.
Calculate the kinematic viscosity of the solvent:

$$v_2 = t_2 \times k_2$$

t_2 = time of flow for a 0.5 M cupriethylenediamine hydroxide solution (s)
k_2 = viscometer constant

Determine the relative viscosity of the Silicified Microcrystalline Cellulose taken:

$$\text{Result} = v_1/v_2$$

Calculate the degree of polymerization:

$$\text{Result} = 95 \times [\eta]_c/\{W_S \times [(100 - ROI)/100] \times [(100 - LOD)/100]\}$$

$[\eta]_c$ = value from interpolation of the relative viscosity, using the *Intrinsic Viscosity Table* in the *Reference Tables* section
W_S = weight of the sample taken (g)
ROI = value from the test for *Residue on Ignition* (%)
LOD = value from the test for *Loss on Drying* (%)

Acceptance criteria: NMT 350

- **BULK DENSITY**

Sample: Silicified Microcrystalline Cellulose powder
Analysis: Use a volumeter that has been fitted with a 10-mesh screen. The volumeter is freestanding of the brass or stainless steel cup, which is calibrated to a capacity of 25.0 ± 0.05 mL and has an inside diameter of 30.0 ± 2.0 mm. Weigh the empty cup, position it under the chute, and slowly pour the powder from a height of 5.1 cm (2 inches) above the funnel through the volumeter, at a rate suitable to prevent clogging, until the cup overflows. [NOTE—If excessive clogging of the screen occurs, remove the screen.] Level the excess powder, and weigh the filled cup. Calculate the bulk density by dividing the weight of the powder in the cup by the volume of the cup.
Acceptance criteria: Within the labeled specification

- **PARTICLE SIZE DISTRIBUTION:** Where the labeling states the particle size distribution, determine the particle size distribution as directed in a suitable validated procedure.

- **WATER-SOLUBLE SUBSTANCES**

Sample: 5.0 g
Analysis: Shake the *Sample* with 80 mL of water for 10 min, and filter with the aid of vacuum through filter paper (Whatman No. 42 or equivalent) into a vacuum flask. Transfer the filtrate to a tared beaker, evaporate to dryness without charring, dry at 105° for 1 h, cool in a desiccator, and weigh.
Acceptance criteria: NMT 0.25%; the difference between the weight of the residue and the weight obtained from a blank determination is NMT 12.5 mg.

- **ETHER-SOLUBLE SUBSTANCES**

Sample: 10.0 g
Analysis: Place the *Sample* in a chromatographic column having an internal diameter of about 20 mm, and pass 50 mL of peroxide-free ether through the column. Evaporate the eluate to dryness in a previously dried and tared evaporating dish with the aid of a current of air in a fume hood. After all the ether has evaporated, dry the residue at 105° for 30 min, cool in a desiccator, and weigh.
Acceptance criteria: NMT 0.05%; the difference between the weight of the residue and the weight obtained from a blank determination is NMT 5.0 mg.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.
- **LABELING:** Label it to indicate the nominal *Loss on Drying*, *Bulk Density*, and *Degree of Polymerization* values. Where the particle size distribution is stated in the labeling, proceed as directed in *Particle Size Distribution*. The labeling indicates the technique with which the particle size distribution was determined, if a technique other than analytical sieving was used. The labeling also indicates the d_{10}, d_{50}, and d_{90} values and the range for each.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Silicified Microcrystalline Cellulose RS

Cellulose, Oxidized—*see Oxidized Cellulose General Monographs*

Cellulose, Oxidized Regenerated—*see Oxidized Regenerated Cellulose General Monographs*

Powdered Cellulose

DEFINITION
Powdered Cellulose is purified, mechanically disintegrated cellulose prepared by processing alpha cellulose obtained as a pulp from fibrous plant materials.

IDENTIFICATION

- **A. PROCEDURE**

Iodinated zinc chloride solution: Dissolve 20 g of zinc chloride and 6.5 g of potassium iodide in 10.5 mL of water. Add 0.5 g of iodine, and shake for 15 min.
Sample: 10 mg
Analysis: Place the *Sample* on a watch glass, and disperse in 2 mL of *Iodinated zinc chloride solution*.
Acceptance criteria: The substance takes on a violet-blue color.

- **B. PROCEDURE**

Sample: 0.25 g of Powdered Cellulose, accurately weighed to 0.1 mg
Analysis: Transfer the *Sample* to a 125-mL conical flask. Add 25.0 mL of water and 25.0 mL of 1.0 M cupriethylenediamine hydroxide solution. Immediately purge the solution with nitrogen, insert the stopper, and shake on a wrist action shaker, or other suitable mechanical shaker, until completely dissolved. Transfer an appropriate volume of the solution to a calibrated number 150 Cannon-Fenske, or equivalent, viscometer. Allow the solution to equilibrate at 25 ± 0.1° for NLT 5 min. Time the flow between the two marks on the viscometer, and record the flow time, t_1, in s.
Calculate the kinematic viscosity, $(KV)_1$, of the Powdered Cellulose taken:

$$\text{Result} = t_1 \times k_1$$

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k_1 = viscometer constant (see *Viscosity—Capillary Methods* ⟨911⟩)

Obtain the flow time, t_2, for a 0.5 M cupriethylenediamine hydroxide solution using a number 100 Cannon-Fenske, or equivalent, viscometer.
Calculate the kinematic viscosity, $(KV)_2$, of the solvent:

$$\text{Result} = t_2 \times k_2$$

k_2 = viscometer constant

Determine the relative viscosity, η_{rel}, of the Powdered Cellulose specimen taken:

$$\text{Result} = (KV)_1/(KV)_2$$

$(KV)_1$ = kinematic viscosity of the Powdered Cellulose
$(KV)_2$ = kinematic viscosity of the solvent

Determine the intrinsic viscosity, $[\eta]_c$, by interpolation, using the *Intrinsic Viscosity Table* in the *Reference Tables* section.
Calculate the degree of polymerization, P:

$$\text{Result} = [95 \times [\eta]_c]/\{W_S \times [(100 - \%LOD)/100]\}$$

W_S = weight of the Powdered Cellulose taken (g)
%LOD = value obtained from the test for *Loss on Drying*

Acceptance criteria: The degree of polymerization is greater than 440.

IMPURITIES

INORGANIC IMPURITIES

- **Residue on Ignition** ⟨281⟩: NMT 0.3%, calculated on the dried basis

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩: The total aerobic microbial count does not exceed 1000 cfu/g, the total combined molds and yeasts count does not exceed 100 cfu/g, and it meets the requirements of the tests for absence of *Staphylococcus aureus and Pseudomonas aeruginosa* and for absence of *Escherichia coli* and *Salmonella* species.
- **pH** ⟨791⟩: The pH of the supernatant is 5.0–7.5. Mix 10 g with 90 mL of water, and allow to stand with occasional stirring for 1 h.
- **LOSS ON DRYING** ⟨731⟩: Dry a sample at 105° for 3 h: it loses NMT 6.5% of its weight.
- **WATER-SOLUBLE SUBSTANCES**
  Sample: 6.0 g
  Analysis: Mix the *Sample* with 90 mL of recently boiled and cooled water, and allow to stand with occasional stirring for 10 min. Filter, with the aid of vacuum, discard the first 10 mL of the filtrate, and pass the filtrate through the same filter a second time, if necessary, to obtain a clear filtrate. Evaporate a 15.0-mL portion of the filtrate in a tared evaporating dish to dryness without charring, dry at 105° for 1 h, cool in a desiccator, and weigh.
  Acceptance criteria: The difference between the weight of the residue and the weight obtained from a blank determination does not exceed 15.0 mg (1.5%).
- **ETHER-SOLUBLE SUBSTANCES**
  Sample: 10.0 g
  Analysis: Place the *Sample* in a chromatography column having an internal diameter of about 20 mm, and pass 50 mL of peroxide-free ether through the column. Evaporate the eluate to dryness in a previously dried and tared evaporating dish with the aid of a current of air in a fume hood. After all the ether has evaporated, dry the residue at 105° for 30 min, cool in a desiccator, and weigh.
  Acceptance criteria: The difference between the weight of the residue and the weight obtained from a blank determination does not exceed 15.0 mg (0.15%).

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **LABELING:** The labeling indicates the nominal degree of polymerization value. Degree of polymerization compliance is determined using *Identification* test *B*.

Cellulose Acetate

Portions of this monograph that are national *USP* text, and are not part of the harmonized text, are marked with symbols (‣ ◂) to specify this fact.

Cellulose acetate;
Cellulose, diacetate [9035-69-2].
Cellulose, triacetate [9012-09-3].

DEFINITION

Cellulose Acetate is partially or completely acetylated cellulose.

It contains NLT 29.0% and NMT 44.8%, by weight, of acetyl (C_2H_3O) groups, calculated on the dried basis. Its acetyl content is NLT 90.0% and NMT 110.0% of that indicated on the label.

IDENTIFICATION

- **A. INFRARED ABSORPTION**
  Sample solution: Prepare a 20 mg/mL solution of Cellulose Acetate, previously dried, in acetone (mono- and diester) or in methylene chloride (di- and triester).
  Analysis: Spread 1 drop of the *Sample solution* on a sodium chloride plate, place a second sodium chloride plate over it, and spread the specimen between the plates. Separate the plates, heat them both at 105° for 1 h, and reassemble the dried plates.
  Acceptance criteria: The IR absorption spectrum exhibits maxima only at the same wavelengths as those of a similar preparation of USP Cellulose Acetate RS, treated in the same manner.

ASSAY

- **CONTENT OF ACETYL**
  For Cellulose Acetate labeled to contain NMT 42.0% of acetyl groups
  Sample: 2 g of Cellulose Acetate
  Titrimetric system
  (See *Titrimetry* ⟨541⟩.)
  Mode: Residual titration
  Titrant: 1.0 N sodium hydroxide VS
  Back-titrant: 1.0 N sulfuric acid VS
  Endpoint detection: Visual
  Analysis: Transfer the *Sample* to a 500-mL flask. Add 100 mL of acetone and 5–10 mL of water to the flask, insert the stopper into the flask, and stir with a magnetic stirrer until solution is complete. Pipet 30 mL of *Titrant* to the solution, with constant stirring. A finely divided precipitate of regenerated cellulose, free from lumps, is obtained. Insert the stopper into the flask, and stir with a magnetic stirrer for 30 min. Add 100 mL of water that has been preheated to 80°, washing down the sides of the flask. Stir for 2 min, and cool to room temperature. Titrate the excess sodium hydroxide solution with *Back-titrant* to a phenolphthalein endpoint. Treat a blank in the same manner.

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Calculate the percentage of acetyl in the portion of Cellulose Acetate taken:

$$\text{Result} = (V_B - V_S)/W \times 4.305$$

V_B = *Back-titrant* volume consumed by the blank (mL)
V_S = *Back-titrant* volume consumed by Cellulose Acetate (mL)
W = weight of Cellulose Acetate taken, calculated on the dried basis (g)

Acceptance criteria: 29.0%–44.8% by weight of acetyl (C_2H_3O) groups on the dried basis

For Cellulose Acetate labeled to contain more than 42.0% of acetyl groups

Sample: 2 g of Cellulose Acetate

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Residual titration

Titrant: 0.5 N hydrochloric acid VS

Back-titrant: 0.5 N sodium hydroxide VS

Endpoint detection: Visual

Analysis: Transfer the *Sample* to a 500-mL conical flask. Add 30.0 mL of dimethyl sulfoxide and 100 mL of acetone, and stir for 16 h with the aid of a magnetic stirrer. Pipet 30 mL of 1 N sodium hydroxide VS slowly into the flask, with constant stirring. Insert the stopper into the flask, and stir for 6 min. Allow to stand without stirring for 60 min. Resume stirring, and add 100 mL of water that has been preheated to 80°, washing down the sides of the flask. Stir for 2 min, and cool to room temperature. Add 4–5 drops of phenolphthalein TS, and titrate the excess sodium hydroxide solution with *Titrant*. Add an excess of 0.5 mL of *Titrant*. Stir for 5 min. Allow to stand for 30 min. Titrate with *Back-titrant* to a persistent pink endpoint, using a magnetic stirrer for agitation. Calculate the net number of milliequivalents of sodium hydroxide consumed, and correct this value by use of the average of two blank determinations run concomitantly through the entire procedure.

Calculate the percentage of acetyl in the portion of Cellulose Acetate taken:

$$\text{Result} = (n/W) \times 4.305$$

n = corrected value of the net number of milliequivalents of sodium hydroxide consumed
W = weight of Cellulose Acetate taken, calculated on the dried basis (g)

Acceptance criteria: 29.0%–44.8% by weight of acetyl (C_2H_3O) groups on the dried basis

IMPURITIES

- **Residue on Ignition** ⟨281⟩: NMT 0.1%
- **Limit of Free Acid**

Sample: 5 g

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Direct titration

Titrant: 0.01 N sodium hydroxide VS

Endpoint detection: Visual

Analysis: Transfer the *Sample* to a 250-mL flask. Add 150 mL of freshly boiled, cooled water. Insert the stopper into the flask, swirl the suspension gently, and allow it to stand for 3 h. Filter through paper, and wash the flask and the filter with freshly boiled, cooled water, adding the washings to the filtrate. Add phenolphthalein TS, and titrate the combined filtrate and washings with the *Titrant*.

Calculate the percentage of free acid in the portion of Cellulose Acetate taken:

$$\text{Result} = (V/W) \times 0.06005$$

V = *Titrant* volume consumed (mL)
W = weight of Cellulose Acetate taken, calculated on the dried basis (g)

Acceptance criteria: NMT 0.1%, calculated as acetic acid

SPECIFIC TESTS

- **Loss on Drying** ⟨731⟩

Analysis: Dry at 105° for 3 h.

Acceptance criteria: NMT 5.0%

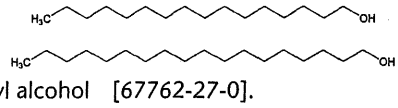
- **Microbial Enumeration Tests** ⟨61⟩ and **Tests for Specified Microorganisms** ⟨62⟩: The total aerobic microbial count is NMT 10^3 cfu/g, and the total combined molds and yeasts count is NMT 10^2 cfu/g. It meets the requirements of the tests for absence of *Escherichia coli* and *Salmonella* species.

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in tight containers.
- **Labeling:** The labeling states the nominal percentage content of acetyl.
- **USP Reference Standards** ⟨11⟩

USP Cellulose Acetate RS

Cetostearyl Alcohol



Cetylstearyl alcohol [67762-27-0].

DEFINITION

Cetostearyl Alcohol contains NLT 40.0% of stearyl alcohol ($C_{18}H_{38}O$), and the sum of the stearyl alcohol content and the cetyl alcohol ($C_{16}H_{34}O$) content is NLT 90.0% and NMT 102.0%. It is obtained from sources of vegetable, animal, or synthetic origin.

IDENTIFICATION

- **A. Chromatographic Identity**

Analysis: Proceed as directed in the *Assay*.

Acceptance criteria: The retention times of the major peaks of the *Sample solution*, excluding the solvent and internal standard peaks, correspond to the cetyl alcohol and stearyl alcohol peaks of the *System suitability solution*.

ASSAY

- **Procedure**

Internal standard solution: 1 mg/mL of 1-pentadecanol (internal standard) in ethanol

System suitability solution: Prepare 1 mg/mL each of USP Cetyl Alcohol RS, USP Stearyl Alcohol RS, and USP Oleyl Alcohol RS in *Internal standard solution*. Heat the solution in a sealed container in a 50° water bath until all fatty alcohols are dissolved. Allow the solution to cool to room temperature, and mix well.

Standard solution: To match the cetyl alcohol and stearyl alcohol ratio in the test sample, prepare the sum of 2.0 mg/mL of USP Cetyl Alcohol RS and USP Stearyl Alcohol RS in *Internal standard solution*. Heat the solution in a sealed container in a 50° water bath until cetyl alcohol and stearyl alcohol are dissolved. Allow the solution to cool to room temperature, and mix well.

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Sample solution: Prepare 2.0 mg/mL of Cetostearyl Alcohol in *Internal standard solution*, and heat the solution in a sealed container in a 50° water bath until cetostearyl alcohol is dissolved. Allow the solution to cool to room temperature, and mix well.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.25-mm × 30-m fused-silica capillary; coated with a 0.25-µm layer of phase G7
Temperatures
Detector: 280°
Injection port: 270°
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
60	20	180	—
180	10	220	5

Carrier gas: Hydrogen
Flow rate: 2.0 mL/min, constant flow mode
Injection volume: 1 µL
Injection type: Split injection; split ratio, is 100:1
Liner: Single taper, low pressure drop liner with deactivated wool
Run time: 15 min

System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—See *Table 2* for the relative retention times.]

Table 2

Component	Relative Retention Time
1-Pentadecanol (internal standard)	1.00
Cetyl alcohol	1.09
Stearyl alcohol	1.25
Oleyl alcohol	1.28

Suitability requirements
Resolution: NLT 30 between the cetyl alcohol and stearyl alcohol peaks; NLT 2.0 between the stearyl alcohol and oleyl alcohol peaks, *System suitability solution*
Tailing factor: 0.8–1.8 for the stearyl alcohol and 1-pentadecanol peaks, *Standard solution*
Relative standard deviation: NMT 1%, using the area ratio of stearyl alcohol to 1-pentadecanol, *Standard solution*

Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentages of cetyl alcohol ($C_{16}H_{34}O$) or stearyl alcohol ($C_{18}H_{38}O$) in the portion of Cetostearyl Alcohol taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of cetyl alcohol (or stearyl alcohol) to the internal standard [peak response of cetyl alcohol (or stearyl alcohol)/peak response of the internal standard] from the *Sample solution*

R_S = peak response ratio of cetyl alcohol (or stearyl alcohol) to the internal standard [peak response of cetyl alcohol (or stearyl alcohol)/peak response of the internal standard] from the *Standard solution*

C_S = concentration of USP Cetyl Alcohol RS (or USP Stearyl Alcohol RS) in the *Standard solution* (mg/mL)

C_U = concentration of Cetostearyl Alcohol in the *Sample solution* (mg/mL)

Acceptance criteria
Stearyl alcohol ($C_{18}H_{38}O$): NLT 40.0%
Sum of stearyl alcohol ($C_{18}H_{38}O$) and cetyl alcohol ($C_{16}H_{34}O$): 90.0%–102.0%

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%, determined on 2 g
- **LIMIT OF RELATED FATTY ALCOHOLS**

Solution A: 1 mg/mL of 1-pentadecanol in ethanol
Resolution solution: Prepare 1 mg/mL each of USP Lauryl Alcohol RS, USP Myristyl Alcohol RS, USP Cetyl Alcohol RS, USP Stearyl Alcohol RS, USP Oleyl Alcohol RS, USP Linolenyl Alcohol RS, and USP Arachidyl Alcohol RS in *Solution A*. Heat the solution in a sealed container in a 50° water bath until all fatty alcohols are dissolved. Allow the solution to cool to room temperature, and mix well. Dilute the solution with ethanol to obtain a solution containing 0.05 mg/mL each of USP Lauryl Alcohol RS, USP Myristyl Alcohol RS, USP Cetyl Alcohol RS, 1-pentadecanol, USP Stearyl Alcohol RS, USP Oleyl Alcohol RS, USP Linolenyl Alcohol RS, and USP Arachidyl Alcohol RS.
Sample solution: 1 mg/mL of Cetostearyl Alcohol in ethanol. Heat the solution in a sealed container in a 50° water bath until cetostearyl alcohol is dissolved. Allow the solution to cool to room temperature, and mix well.
Chromatographic system: Proceed as directed in the *Assay*, except for the split ratio.
Injection type: Split injection; split ratio is 5:1

System suitability
Sample: *Resolution solution*
[NOTE—See *Table 3* for the relative retention times.]

Table 3

Component	Relative Retention Time
Lauryl alcohol	0.79
Myristyl alcohol	0.93
1-Pentadecanol	1.00
Cetyl alcohol	1.09
Stearyl alcohol	1.25
Oleyl alcohol	1.28
Linolenyl alcohol	1.36
Arachidyl alcohol	1.44

Suitability requirements
Resolution: NLT 15 between the myristyl alcohol and 1-pentadecanol peaks; NLT 30 between the cetyl alcohol and stearyl alcohol peaks; NLT 2.0 between the stearyl alcohol and oleyl alcohol peaks

Analysis
Samples: *Resolution solution* and *Sample solution*
Identify each related fatty alcohol peak in the *Sample solution* based on that in the *Resolution solution*.

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Calculate the percentage of each related fatty alcohol or any unspecified impurity in the portion of Cetostearyl Alcohol taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of each related fatty alcohol (or any unspecified impurity) from the *Sample solution*

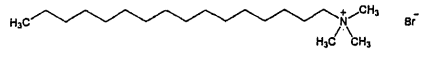
r_T = sum of all the peak responses excluding peak responses due to solvent from the *Sample solution*

Acceptance criteria: Disregard peaks that are less than 0.05% for any unspecified impurities, and any peaks due to solvent.
Sum of unspecified impurities: NMT 1%
Sum of related fatty alcohols and unspecified impurities: NMT 10.0%

SPECIFIC TESTS
- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩**:** NMT 2
- **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩**:** 208–228
- **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩**:** NMT 4
- **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 0.5%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **LABELING:** Label it to indicate whether it is derived from vegetable, animal, or synthetic sources.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Arachidyl Alcohol RS
  USP Cetyl Alcohol RS
  USP Lauryl Alcohol RS
  USP Linolenyl Alcohol RS
  USP Myristyl Alcohol RS
  USP Oleyl Alcohol RS
  USP Stearyl Alcohol RS

Cetrimonium Bromide



$C_{19}H_{42}BrN$ 364.45
Hexadecyltrimethylammonium bromide [57-09-0].

DEFINITION
Cetrimonium Bromide contains NLT 96.0% and NMT 101.0% of hexadecyltrimethylammonium bromide, calculated as $C_{19}H_{42}BrN$, on the dried basis.

IDENTIFICATION
- **A. ULTRAVIOLET ABSORPTION**
  Sample solution: 10 mg/mL in alcohol
  Analytical wavelength: 260–280 nm
  Cell: 1 cm
  Acceptance criteria: After correcting for the blank, the absorbance is NMT 0.05.
- **B.**
  Sample solution: Dissolve 5 mg of Cetrimonium Bromide in 5 mL of phosphate buffer pH 8.0.
  Blank: 5 mL of phosphate buffer pH 8.0
  Analysis: Add a strip of methyl green–iodomercurate paper to the *Sample solution* and *Blank*.
  Acceptance criteria: After 5 min, the *Sample solution* shows a more intense greenish-blue color than the *Blank*.
- **C.**
  Sample: 2.0 g
  Analysis: Transfer the *Sample* to a 100-mL flask, and dissolve in and dilute with previously boiled and cooled water to volume.
  Acceptance criteria: The solution froths copiously when shaken.
- **D. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩
  Solution A: 270 mg/mL of sodium acetate trihydrate
  Standard solution: 20 mg/mL of USP Cetrimonium Bromide RS
  Sample solution: 20 mg/mL of Cetrimonium Bromide
  Chromatographic system
  Developing solvent system: Methanol, acetone, and *Solution A* (45:20:35)
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Proceed as directed in the chapter. Remove the plate from the developing chamber, and dry the plate in a current of hot air. Allow to cool. Expose the plate to iodine vapor, and examine in daylight.
  Acceptance criteria: The principal spot of the *Sample solution* is similar in position, color, and size to that of the *Standard solution*.
- **E. IDENTIFICATION TESTS—GENERAL,** *Bromide* ⟨191⟩**:** A solution of it meets the requirements.

ASSAY
- **PROCEDURE**
  Sample solution: 20 mg/mL of Cetrimorium Bromide
  Blank: Combine 10.0 mL of freshly prepared 50-mg/mL potassium iodide, 20 mL of water, and 40 mL of hydrochloric acid.
  Titrimetric system
  (See *Titrimetry* ⟨541⟩.)
  Mode: Residual titration
  Titrant: 0.05 M potassium iodate VS
  Analysis
  Samples: *Sample solution* and *Blank*
  Transfer 25.0 mL of the *Sample solution* to a separatory funnel, and add 25 mL of chloroform, 10 mL of 0.1 N sodium hydroxide VS, and 10.0 mL of a freshly prepared solution of potassium iodide (50 mg/mL). Shake well, allow to separate, and discard the chloroform layer. Wash the aqueous layer with three 10-mL portions of chloroform, and discard the washings. Transfer the aqueous layer to a stoppered conical flask, add 40 mL of hydrochloric acid, and allow to cool. Titrate with *Titrant* until the deep brown color is almost discharged. Add 2 mL of chloroform, and continue the titration, shaking vigorously, until the color of the chloroform layer no longer changes. Perform a blank determination. Each mL of *Titrant* is equivalent to 36.45 mg of Cetrimonium Bromide ($C_{19}H_{42}BrN$).
  Acceptance criteria: 96.0%–101.0% on the dried basis

IMPURITIES
- **RESIDUE ON IGNITION** ⟨281⟩
  Sample: 1.0 g
  Acceptance criteria: NMT 0.5%
- **LIMIT OF AMINES AND AMINE SALTS**
  Sample: 5.0 g
  Analysis: Dissolve the *Sample* in 30 mL of a mixture of methanol and 1 N hydrochloric acid VS (99:1), and add 100 mL of isopropyl alcohol. Pass a stream of nitrogen slowly through the solution. Gradually add 15.0 mL of 0.1 N tetrabutylammonium hydroxide VS, recording the potentiometric titration curve.
  Acceptance criteria: If the curve shows two inflection points, the volume of titrant added between the two points is NMT 2.0 mL.

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SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩: The total aerobic microbial count is NMT 10^3 cfu/g. The total combined molds and yeasts count is NMT 10^1 cfu/g.
- **APPEARANCE OF SOLUTION**
  Analysis: Transfer 2.0 g of Cetrimonium Bromide to a 100-mL flask, and dissolve in and dilute with previously boiled and cooled water to volume.
  Acceptance criteria: The solution is clear and colorless.
- **ACIDITY OR ALKALINITY**
  Sample solution: 50 mL of the solution obtained in the *Appearance of Solution* test
  Analysis: To the *Sample solution* add 0.1 mL of bromocresol purple TS.
  Acceptance criteria: NMT 0.1 mL of 0.1 N sodium hydroxide or 0.1 N hydrochloric acid is required to change the color of the indicator.
- **LOSS ON DRYING** ⟨731⟩
  Sample: 1.0 g
  Analysis: Dry at 105° for 2 h.
  Acceptance criteria: NMT 2.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Cetrimonium Bromide RS

Cetyl Alcohol



$C_{16}H_{34}O$ 242.44
1-Hexadecanol [36653-82-4].

DEFINITION

Cetyl Alcohol contains NLT 90.0% and NMT 102.0% of cetyl alcohol ($C_{16}H_{34}O$), the remainder consisting chiefly of related alcohols. It is obtained from sources of vegetable, animal, or synthetic origin.

IDENTIFICATION

- **A. CHROMATOGRAPHIC IDENTITY**
  Analysis: Proceed as directed in the *Assay*.
  Acceptance criteria: The retention time of the major peak of the *Sample solution*, excluding the solvent and internal standard peaks, corresponds to the cetyl alcohol peak of the *System suitability solution*.

ASSAY

- **PROCEDURE**
  Internal standard solution: 1 mg/mL of 1-pentadecanol (internal standard) in ethanol
  System suitability solution: Prepare 1 mg/mL each of USP Cetyl Alcohol RS, USP Stearyl Alcohol RS, and USP Oleyl Alcohol RS in *Internal standard solution*, and heat the solution in a sealed container in a 50° water bath until all fatty alcohols are dissolved. Allow the solution to cool to room temperature, and mix well.
  Standard solution: Prepare 1.0 mg/mL of USP Cetyl Alcohol RS in *Internal standard solution*, and heat the solution in a sealed container in a 50° water bath until cetyl alcohol is dissolved. Allow the solution to cool to room temperature, and mix well.
  Sample solution: Prepare 1.0 mg/mL of Cetyl Alcohol in *Internal standard solution*, and heat the solution in a sealed container in a 50° water bath until cetyl alcohol is dissolved. Allow the solution to cool to room temperature, and mix well.
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: GC
  Detector: Flame ionization
  Column: 0.25-mm × 30-m fused-silica capillary; coated with a 0.25-µm layer of phase G7
  Temperatures
  Injection port: 270°
  Detector: 280°
  Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
60	20	180	—
180	10	220	5

  Carrier gas: Hydrogen
  Flow rate: 2.0 mL/min, constant flow mode
  Injection volume: 1 µL
  Injection type: Split injection; split ratio is 100:1
  Liner: Single taper, low pressure drop liner with deactivated wool
  Run time: 15 min
  System suitability
  Samples: *System suitability solution* and *Standard solution*
  [NOTE—See *Table 2* for the relative retention times.]

Table 2

Name	Relative Retention Time
1-Pentadecanol (internal standard)	1.00
Cetyl alcohol	1.09
Stearyl alcohol	1.25
Oleyl alcohol	1.28

  Suitability requirements
  Resolution: NLT 30 between the cetyl alcohol and stearyl alcohol peaks; NLT 2.0 between the stearyl alcohol and oleyl alcohol peaks, *System suitability solution*
  Tailing factor: 0.8–1.8 for the cetyl alcohol and 1-pentadecanol peaks, *Standard solution*
  Relative standard deviation: NMT 1%, using the area ratio of cetyl alcohol to 1-pentadecanol, *Standard solution*
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Calculate the percentage of cetyl alcohol ($C_{16}H_{34}O$) in the portion of Cetyl Alcohol taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of cetyl alcohol to the internal standard (peak response of cetyl alcohol/peak response of the internal standard) from the *Sample solution*

R_S = peak response ratio of cetyl alcohol to the internal standard (peak response of cetyl alcohol/peak response of the internal standard) from the *Standard solution*

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C_S = concentration of USP Cetyl Alcohol RS in the *Standard solution* (mg/mL)
C_U = concentration of Cetyl Alcohol in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–102.0%

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%, determined on 2 g
- **LIMIT OF RELATED FATTY ALCOHOLS**

Solution A: 1 mg/mL of 1-pentadecanol in ethanol
Resolution solution: Prepare 1 mg/mL of USP Lauryl Alcohol RS, 1 mg/mL of USP Myristyl Alcohol RS, 1 mg/mL of USP Cetyl Alcohol RS, 1 mg/mL of USP Stearyl Alcohol RS, and 1 mg/mL of USP Oleyl Alcohol RS in *Solution A*. Heat the solution in a sealed container in a 50° water bath until all fatty alcohols are dissolved. Allow the solution to cool to room temperature, and mix well. Dilute the solution with ethanol to have a solution containing 0.05 mg/mL each of USP Lauryl Alcohol RS, USP Myristyl Alcohol RS, USP Cetyl Alcohol RS, 1-pentadecanol, USP Stearyl Alcohol RS, and USP Oleyl Alcohol RS.
Sample solution: Prepare 1.0 mg/mL of Cetyl Alcohol in ethanol, and heat the solution in a sealed container in a 50° water bath until cetyl alcohol is dissolved. Allow the solution to cool to room temperature, and mix well.
Chromatographic system: Proceed as directed in the *Assay*, except for the split ratio.
Injection type: Split injection; split ratio is 5:1
System suitability
Sample: *Resolution solution*
[NOTE—See *Table 3* for the relative retention times.]

Table 3

Name	Relative Retention Time
Lauryl alcohol	0.79
Myristyl alcohol	0.93
1-Pentadecanol	1.00
Cetyl alcohol	1.09
Stearyl alcohol	1.25
Oleyl alcohol	1.28

Suitability requirements
Resolution: NLT 15 between the myristyl alcohol and 1-pentadecanol peaks; NLT 30 between the cetyl alcohol and stearyl alcohol peaks; NLT 2.0 between the stearyl alcohol and oleyl alcohol peaks
Analysis
Samples: *Resolution solution* and *Sample solution*
Identify each related fatty alcohol peak in the *Sample solution* based on that in the *Resolution solution*.
Calculate the percentage of each related fatty alcohol or an unspecified impurity in the portion of Cetyl Alcohol taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of each related fatty alcohol (or any unspecified impurity) from the *Sample solution*
r_T = sum of all the peak responses excluding peak responses due to solvent from the *Sample solution*

Acceptance criteria: Disregard peaks that are less than 0.05% for any unspecified impurities and any peaks due to solvent.

Sum of unspecified impurities: NMT 1%
Sum of related fatty alcohols and unspecified impurities: NMT 10.0%

SPECIFIC TESTS

- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩: NMT 2
- **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩: 218–238
- **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩: NMT 5
- **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 0.5%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **LABELING:** Label it to indicate whether it is derived from vegetable, animal, or synthetic sources.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Cetyl Alcohol RS
  USP Lauryl Alcohol RS
  USP Myristyl Alcohol RS
  USP Oleyl Alcohol RS
  USP Stearyl Alcohol RS

Cetyl Esters Wax

DEFINITION

Cetyl Esters Wax is a mixture consisting primarily of esters of saturated fatty alcohols (C_{12} to C_{18}) and saturated fatty acids (C_{12} to C_{20}). It contains NLT 90.0% of total saturated fatty esters and NLT 10.0% of fatty esters with carbon-chain length of carbon number 32 that consist chiefly of cetyl palmitate.

IDENTIFICATION

- **A. CHROMATOGRAPHIC IDENTITY**

Analysis: Proceed as directed in the test for *Total Fatty Esters and Cetyl Palmitate* in the *Assay*.
Acceptance criteria: The retention times of the four major fatty ester peaks (fatty esters with carbon-chain length of carbon number 28, fatty esters with carbon-chain length of carbon number 30, fatty esters with carbon-chain length of carbon number 32, and fatty esters with carbon-chain length of carbon number 34) of the *Sample solution* correspond to those of *Reference solution D*.

- **B. FATS AND FIXED OILS** ⟨401⟩, *Procedures, Saponification Value*: 109–120

ASSAY

- **TOTAL FATTY ESTERS AND CETYL PALMITATE**

Reference solution A: 1 mg/mL of USP Lauryl Alcohol RS, 1 mg/mL of USP Myristyl Alcohol RS, 1 mg/mL of USP Cetyl Alcohol RS, and 1 mg/mL of USP Stearyl Alcohol RS in *n*-hexane
Reference solution B: 1 mg/mL of USP Palmitic Acid RS in *n*-hexane
Reference solution C: 1 mg/mL of USP Cetyl Palmitate RS in *n*-hexane
Reference solution D: 1 mg/mL of USP Cetyl Esters Wax RS in *n*-hexane
Sample solution: 1 mg/mL of Cetyl Esters Wax in *n*-hexane
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.53-mm × 10-m fused-silica capillary; 2.65-µm layer of phase G2
Temperatures
Injection port: 350°
Detector: 350°

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Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
100	20	300	15

Carrier gas: Helium
Flow rate: 6.5 mL/min
Injection volume: 1 µL
Injection type: Split ratio, 10:1
Run time: 25 min

System suitability
Samples: *Reference solution A, Reference solution B, Reference solution C,* and *Reference solution D*
[NOTE—For relative retention times, see *Table 2*.]

Table 2

Component	Relative Retention Times
Lauryl alcohol	0.31
Lauric acid	0.34
Myristyl alcohol	0.39
Myristic acid	0.42
Cetyl alcohol	0.46
Palmitic acid	0.49
Stearyl alcohol	0.53
Stearic acid	0.55
Fatty esters with carbon-chain length of carbon number 26	0.72
Fatty esters with carbon-chain length of carbon number 28	0.79
Fatty esters with carbon-chain length of carbon number 30	0.87
Fatty esters with carbon-chain length of carbon number 32 that consist chiefly of cetyl palmitate	1.00
Fatty esters with carbon-chain length of carbon number 34	1.18
Fatty esters with carbon-chain length of carbon number 36	1.44

Suitability requirements
Resolution: NLT 2.0 between fatty esters with carbon-chain length of carbon number 32 and fatty esters with carbon-chain length of carbon number 34, *Reference solution D*

Analysis
Samples: *Reference solution A, Reference solution B, Reference solution C, Reference solution D,* and *Sample solution*
Identify fatty alcohols, fatty acids, and fatty esters based on the chromatograms from *Reference solution A, Reference solution B, Reference solution C,* and *Reference solution D,* and also according to *Table 2*.
Calculate the percentage of each individual group of fatty esters (fatty esters with carbon-chain length of carbon number 26, fatty esters with carbon-chain length of carbon number 28, fatty esters with carbon-chain length of carbon number 30, fatty esters with carbon-chain length of carbon number 32, fatty esters with carbon-chain length of carbon number 34, or fatty esters with carbon-chain length of carbon number 36) (P_{FE}) in the portion of Cetyl Esters Wax taken:

$$\text{Result } (P_{FE}) = (r_U / r_T) \times 100$$

r_U = peak area of each individual group of fatty esters (fatty esters with carbon-chain length of carbon number 26, fatty esters with carbon-chain length of carbon number 28, fatty esters with carbon-chain length of carbon number 30, fatty esters with carbon-chain length of carbon number 32, fatty esters with carbon-chain length of carbon number 34, or fatty esters with carbon-chain length of carbon number 36) from the *Sample solution*
r_T = sum of the total peak areas from the *Sample solution*, except for the peak areas from solvent

Calculate the percentage of total saturated fatty esters in the portion of Cetyl Esters Wax taken:

$$\text{Result} = \Sigma P_{FE}$$

Acceptance criteria
Total saturated fatty esters: NLT 90.0%
Fatty esters with carbon-chain length of carbon number 32 that consist chiefly of cetyl palmitate: NLT 10.0%

IMPURITIES

• **LIMIT OF TOTAL FREE FATTY ALCOHOLS AND FREE FATTY ACIDS**
Reference solution A, Reference solution B, Reference solution D, Sample solution, Chromatographic system, and **System suitability:** Proceed as directed in the test for *Total Fatty Esters and Cetyl Palmitate*.
Analysis
Samples: *Reference solution A, Reference solution B, Reference solution D,* and *Sample solution*
Identify fatty alcohols, fatty acids, and fatty esters based on the chromatograms from *Reference solution A, Reference solution B,* and *Reference solution D,* and also according to *Table 2*.
Calculate the percentage of each individual fatty alcohol (lauryl alcohol, myristyl alcohol, cetyl alcohol, or stearyl alcohol) or fatty acid (lauric acid, myristic acid, palmitic acid, or stearic acid) (P_{FA}) in the portion of Cetyl Esters Wax taken:

$$\text{Result } (P_{FA}) = (r_U / r_T) \times 100$$

r_U = peak area of each individual fatty alcohol (lauryl alcohol, myristyl alcohol, cetyl alcohol, or stearyl alcohol) or fatty acid (lauric acid, myristic acid, palmitic acid, or stearic acid) from the *Sample solution*
r_T = sum of the total peak areas from the *Sample solution*, except for the peak areas from solvent

Calculate the percentage of total fatty alcohols and fatty acids in the portion of Cetyl Esters Wax taken:

$$\text{Result} = \Sigma P_{FA}$$

Acceptance criteria
Total free fatty alcohols and free fatty acids: NMT 10.0%

SPECIFIC TESTS

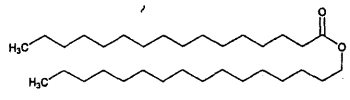
• **MELTING RANGE OR TEMPERATURE** ⟨741⟩, *Procedures, Procedure for Class II*: 43°–47°
• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Acid Value*: NMT 5

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- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Iodine Value*: NMT 1
- **PARAFFIN AND FREE ACIDS**
  Sample: 1 g
  Analysis: Dissolve the *Sample* in 50 mL of boiling alcohol.
  Acceptance criteria: The *Sample* dissolves completely, and the resulting solution is neutral or acidic to moistened litmus paper.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers, in a dry place, and prevent exposure to excessive heat.
- **LABELING:** Label to indicate the percentages of fatty esters with carbon-chain length of carbon number 26, fatty esters with carbon-chain length of carbon number 28, fatty esters with carbon-chain length of carbon number 30, fatty esters with carbon-chain length of carbon number 32, fatty esters with carbon-chain length of carbon number 34, and fatty esters with carbon-chain length of carbon number 36.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Cetyl Alcohol RS
  USP Cetyl Esters Wax RS
  USP Cetyl Palmitate RS
  USP Lauryl Alcohol RS
  USP Myristyl Alcohol RS
  USP Palmitic Acid RS
  USP Stearyl Alcohol RS

Cetyl Palmitate



$C_{32}H_{64}O_2$ 480.85
Hexadecanoic acid hexadecyl ester;
Cetyl palmitate [540-10-3].

DEFINITION
Cetyl Palmitate consists of esters of cetyl alcohol and saturated high molecular weight fatty acids, principally palmitic acid.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F**▲ (CN 1-May-2020)
  Sample: Use a thin film of melted test specimen.
  Acceptance criteria: Meets the requirements
- **B.** The retention times of cetyl alcohol and palmitic acid from the *Sample solution* correspond to those from the *System suitability solution*, as obtained in the test for *Content of Palmitic Acid*.

ASSAY
- **CONTENT OF PALMITIC ACID**
  System suitability solution: Transfer 20 mg each of cetyl alcohol, stearic acid, palmitic acid, and oleic acid to a 25-mL conical flask fitted with a suitable water-cooled reflux condenser and a magnetic stir bar, and proceed as directed for *Test solution* in *Fats and Fixed Oils, Fatty Acid Composition* ⟨401⟩, beginning with "Add 5.0 mL of a solution prepared by dissolving".
  Sample solution: Proceed as directed for *Test solution* in *Fats and Fixed Oils, Fatty Acid Composition* ⟨401⟩.
  Chromatographic system: Prepare as directed in *Fats and Fixed Oils, Fatty Acid Composition* ⟨401⟩.
  Injection volume: 1 µL
  System suitability
  Sample: *System suitability solution*
  [NOTE—The relative retention times for methyl palmitate, cetyl alcohol, methyl stearate, and methyl oleate are about 0.87, 0.96, 0.99, and 1.0, respectively.]
  Suitability requirements
  Resolution: NLT 1.5 between the methyl stearate and methyl oleate peaks
  Relative standard deviation: NMT 6.0% for the palmitate and stearate peaks; NMT 2.0% for the ratio of the palmitate peak to the stearate peak
  Analysis
  Samples: *System suitability solution* and *Sample solution*
  Identify the methyl palmitate peak of the *Sample solution* by comparing the retention times of the *Sample solution* peaks with those of the *System suitability solution* peaks.
  Calculate the percentage of palmitic acid in the portion of Cetyl Palmitate taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak area of methyl palmitate
r_T = sum of all the peak areas, excluding the solvent and cetyl alcohol peaks

Acceptance criteria: The palmitate peak is NLT 90% of the total area for all the peaks.

IMPURITIES
- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.05%

SPECIFIC TESTS
- **MELTING RANGE OR TEMPERATURE,** *Class II* ⟨741⟩: 46°–53°
- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩: NMT 1
- **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩: NMT 6
- **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩: NMT 1
- **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩: 110–130
- **LOSS ON DRYING** ⟨731⟩
  Analysis: Dry at 105° for 1 h.
  Acceptance criteria: NMT 3.0%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers at controlled room temperature, and avoid exposure to excessive heat.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Cetyl Palmitate RS

Cetylpyridinium Chloride—*see Cetylpyridinium Chloride General Monographs*

Activated Charcoal—*see Activated Charcoal General Monographs*

NF Monographs

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Cherry Juice

DEFINITION

Cherry Juice is the liquid expressed from the fresh ripe fruit of *Prunus cerasus* L. (Fam. Rosaceae). It contains NLT 1.0% malic acid ($C_4H_6O_5$).

Coarsely crush washed, stemmed, unpitted, sour cherries in a grinder to break the pits but not mash the kernels. Dissolve 0.1% Benzoic Acid in the mixture, and allow to stand at room temperature until a small portion of the filtered Juice, when mixed with one-half of its volume of alcohol, does not become cloudy within 30 min. Press the Juice from the mixture, and filter it.

IDENTIFICATION

- **A.**

Sample: 5 mL

Analysis: Add lead acetate TS to the *Sample* until the mixture, when filtered, gives no further precipitation with the lead acetate solution. Filter, and to the clear filtrate add 5 mL of a 100-mg/mL sodium oxalate solution to remove the excess lead. Filter, add 5 mL of alkaline cupric tartrate TS to 5 mL of the clear filtrate, and warm.

Acceptance criteria: A red precipitate is formed.

OTHER COMPONENTS

- **MALIC ACID**

Solution A: 6 N ammonium hydroxide and water (2:98)

Sample: 10.0 mL

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Direct titration

Titrant: 0.1 N potassium permanganate VS

Endpoint detection: Visual

Analysis: Place the *Sample* in a 125-mL flask, and add 1 g of calcium carbonate. Heat on a steam bath for 15 min, mixing occasionally, and pass through a filter. Wash the filter with five 5-mL portions of water, and to the combined filtrate and washings add 1 mL of 6 N ammonium hydroxide and 15 mL of ammonium oxalate TS. Heat on a steam bath for 15 min, pass through hardened filter paper, and wash the flask and the filter with five 5-mL portions of *Solution A*. Puncture the filter paper, and wash the precipitate into the same flask by means of hot water, followed by 30 mL of 12 N sulfuric acid. Heat the solution to 80°. Titrate with *Titrant*. Each mL of *Titrant* is equivalent to 6.704 mg of malic acid ($C_4H_6O_5$).

Acceptance criteria: NLT 1.0%

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩: 0.35%–0.55%
- **ARSENIC,** *Method I* ⟨211⟩

Sample solution: To 10 mL in a Kjeldahl flask add 5 mL of nitric acid and 5 mL of sulfuric acid, and heat the mixture until the volume is reduced to 5 mL and the color becomes brownish or black. Add a small portion of nitric acid, and continue the heating, adding small portions of nitric acid as often as browning recurs, until the organic matter is destroyed and dense, white fumes are liberated. Dilute the solution with about 10 mL of water, add 200 mg of ammonium oxalate, and continue the heating until dense, white fumes again are evolved and the solution is colorless to pale yellow. Cool, cautiously add water to make 20 mL, and mix.

Acceptance criteria: NMT 0.3 µg/g

- **LEAD** ⟨251⟩

Test preparation: Add 1.0 mL of Juice to 10 mL of nitric acid in a 250-mL conical flask, and boil for 5–10 min. Cool in an ice bath, and transfer to a separator with the aid of 5 mL of lead-free water.

Analysis: Proceed as directed in the chapter, except use 15 mL of *Ammonium Citrate Solution*, 500 µL of *Hydroxylamine Hydrochloride Solution*, and 3 mL of *Potassium Cyanide Solution*.

Acceptance criteria: NMT 5 µg/mL

SPECIFIC TESTS

- **SPECIFIC GRAVITY** ⟨841⟩: 1.045–1.075
- **REFRACTIVE INDEX** ⟨831⟩: NLT 1.350
- **pH** ⟨791⟩: 3.0–4.0
- **LIMIT OF NONVOLATILE RESIDUE**

Sample: 5.0 mL

Analysis: Evenly spread the *Sample* over the bottom of a tared half Petri dish, and place on a steam bath for 1 h. Dry in a vacuum desiccator over silica gel for 16 h.

Acceptance criteria: The weight of the residue is NLT 500 mg. NMT 9.5% is found.

- **LIMIT OF VOLATILE ACIDS**

Sample: 25 mL

Analysis: Distill the *Sample* with steam to obtain 100 mL of distillate. Add phenolphthalein TS, and titrate with 0.10 N sodium hydroxide.

Acceptance criteria: NMT 1.5 mL is required.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Package in tight, light-resistant containers, and prevent exposure to excessive heat.
- **LABELING:** The label states the Latin binomial name and, following the official name, the part of the plant source from which the article was derived.

Cherry Syrup

DEFINITION

Prepare Cherry Syrup as follows (see *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩).

Cherry Juice	475 mL
Sucrose	800 g
Alcohol	20 mL
Purified Water, a sufficient quantity to make	1000 mL

Dissolve *Sucrose* in *Cherry Juice* by gently heating on a steam bath, cool, and remove the foam and floating solids. Add *Alcohol* and sufficient *Purified Water* to make 1000 mL, and mix.

OTHER COMPONENTS

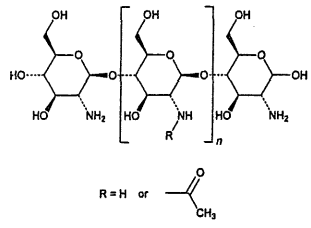
- **ALCOHOL DETERMINATION,** *Method I* ⟨611⟩: 1.0%–2.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Package in tight, light-resistant containers, and prevent exposure to excessive heat.
- **LABELING:** The label states the Latin binomial name and, following the official name, the part of the plant source from which the article was derived.

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Chitosan



Poly-β-(1,4)-2-amino-2-deoxy-D-glucose [9012-76-4].

DEFINITION

Chitosan is an unbranched binary polysaccharide consisting of *N*-acetyl-D-glucosamine and D-glucosamine units linked in a β(1→4) manner. Chitosan is obtained by partial deacetylation of chitin, which is extracted from the shells of edible shrimps and crabs suitable for human use. Its degree of deacetylation is NLT 70.0% and NMT 95.0%.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS ⟨197⟩, *Infrared Spectroscopy*: 197A▲** (CN 1-May-2020)
- **B.**

Sample: 0.2 g of Chitosan powder

Analysis: Add 80 g of water to the *Sample,* and stir briefly to obtain a dispersion. Separately prepare a glycolic acid solution by dissolving 0.1 g of glycolic acid in 20 g of water. Add the solution in one step to the dispersion. Stir the mixture gently at room temperature until it becomes a clear solution. [NOTE—It takes approximately 30–60 min to obtain a clear solution.] Add 5 g of a 0.5% sodium lauryl sulfate aqueous solution to the clear solution.

Acceptance criteria: A gelatinous mass is formed.

ASSAY

Change to read:

- **DEGREE OF DEACETYLATION**

[NOTE—If tetramethylsilane is not used as the NMR reference, a suitable signal of the solvent itself can be used as a reference.]

Solvent: Deuterated formic acid

NMR reference: Tetramethylsilane

Sample solution: Into a 20-mL scintillation vial with a screw cap, dissolve 5–10 mg of Chitosan in deuterated formic acid, containing 0.5%–1.0% of tetramethylsilane, to obtain 1 mL of solution. Tightly close the vial, and dissolve Chitosan using a magnetic stirrer. To completely dissolve takes it about 48 h; the stirring is stopped when a clear solution with a high viscosity is obtained. Break up any clumps formed during the dissolution process with a spatula, if necessary.

Analysis

Sample: *Sample solution*

Transfer 0.5–1.0 mL of the *Sample solution* to a standard 5-mm NMR spinning tube. Proceed as directed in *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩, *Relative Method of Quantitation,* scanning the region from 0–7 ppm, and using the calculation formulas below. Record as A_1 the average area of the composite band from about 6–3 ppm, representing the seven protons with oxygen neighbors in the sugar ring. Record as A_2 the average area of the signals at about 2 ppm, due to the methyl groups of the acetyl units, with reference to the tetramethylsilane singlet at 0 ppm.

Calculate the percentage of deacetylation degree, by weight, in the Chitosan taken:

$$\text{Result} = \{1 - [(7 \times A_2)/(3 \times A_1)]\}_{\blacktriangle \text{(ERR 1-Jul-2019)}} \times 100$$

Acceptance criteria: The degree of deacetylation is NLT 70.0% and NMT 95.0%.

IMPURITIES

- **RESIDUE ON IGNITION ⟨281⟩:** NMT 1.0%
- **LIMIT OF LEAD, MERCURY, CHROMIUM, NICKEL, CADMIUM, AND ARSENIC**

65% Nitric acid: Use ultratrace nitric acid, 65%–70% HNO_3, ACS reagent grade.

Internal standard: Transfer 0.2 mL of a solution containing 1000 ppm of yttrium [NOTE—Yttrium ICP standard solutions are commercially available.[1]] and 0.2 mL of a solution containing 1000 ppm of lutetium [NOTE—Lutetium ICP standard solutions are commercially available.[2]] to a 100-mL volumetric flask, add 1 mL of *65% Nitric acid,* dilute with water to volume, and mix.

Blank standard: Transfer 1.0 mL of the *Internal standard* to a 100-mL volumetric flask, add 1 mL of *65% Nitric acid,* dilute with water to volume, and mix.

Standard solutions: Transfer 0.1 mL of a solution containing 10 ppm of each of lead, mercury, chromium, nickel, cadmium, and arsenic [NOTE—Multi-element ICP standard solutions are commercially available.[3]] to a 100-mL volumetric flask, add 1 mL of the *Internal standard* and 1 mL of *65% Nitric acid,* and dilute with water to volume (*Standard solution 0.01 ppm*). Transfer 0.1 mL of a solution containing 10 ppm of each of lead, mercury, chromium, nickel, cadmium, and arsenic to a 1000-mL volumetric flask, add 10 mL of the *Internal standard* and 10 mL of *65% Nitric acid,* and dilute with water to volume (*Standard solution 0.001 ppm*).

Sample solution: Transfer 1.0 g of Chitosan to a clean, dry, 100-mL Kjeldahl flask. [NOTE—A 300-mL flask may be used if the reaction foams excessively.] Clamp the flask at an angle of 45°, and add a sufficient quantity of a mixture of 8 mL of sulfuric acid and 10 mL of nitric acid to moisten the substance thoroughly. Warm gently until the reaction commences, allow the reaction to subside, and add portions of the same acid mixture, heating after each addition, until a total of 18 mL of the acid mixture has been added. Increase the amount of heat, and boil gently until the solution darkens. Cool, add 2 mL of nitric acid, and heat again until the solution darkens. Continue the heating, followed by addition of nitric acid until no further darkening occurs, then heat strongly to the production of dense, white fumes. Cool, cautiously add 5 mL of water, boil gently to the production of dense, white fumes, and continue heating until the volume is reduced to a few mL. Cool, cautiously add 5 mL of water, and examine the color of the solution. If the color is yellow, cautiously add 1 mL of 30% hydrogen peroxide, and again evaporate to the production of dense, white fumes and a volume of 2–3 mL. If the solution is still yellow, repeat the addition of 5 mL of water and the peroxide treatment. Cool, dilute cautiously with a

[1] A suitable yttrium ICP standard is available from LGC Promochem (http://www.lgcstandards.com) or from Merck KGaA, Frankfurter Str. 250, 64293 Darmstadt, Germany.

[2] A suitable lutetium ICP standard is available from LGC Promochem (http://www.lgcstandards.com) or from Merck KGaA, Frankfurter Str. 250, 64293 Darmstadt, Germany.

[3] Suitable multi-element ICP standard solutions are available from LGC Promochem (http://www.lgcstandards.com) or from Merck KGaA, Frankfurter Str. 250, 64293 Darmstadt, Germany.

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few mL of water, rinse into a 20-mL volumetric flask, and dilute with water to volume. Transfer 5.0 mL of digestion solution to a 100-mL volumetric flask, and retain the remaining digestion solution for use in the *Limit of Iron*. Add 1 mL of the *Internal standard* to the 100-mL volumetric flask, dilute with water to volume, and mix.

Blank solution: Prepare the blank digestion solution, following the preparation procedure for the *Sample solution*, but without using Chitosan. Transfer 5.0 mL of blank digestion solution to a 100-mL volumetric flask, and retain the remaining digestion solution for use in the *Limit of Iron*. Add 1 mL of the *Internal standard* to the 100-mL volumetric flask, dilute with water to volume, and mix.

Instrumental conditions

(See *Plasma Spectrochemistry* ⟨730⟩.)

Mode: Inductively coupled plasma–mass spectrometer (ICP–MS)

Spectrometer: Quadrupole mass spectrometer

Detector: Ion detector maintained under vacuum

Other requirements: The instrument should read all isotopes for the following elements shown in *Table 1*, for the yttrium internal standard (89 amu) and the lutetium internal standard (175 and 176 amu), and should report the total element contents using the most naturally abundant isotopes.

Table 1

Element	Isotope (amu)
Lead	208
Mercury	201
	202
Chromium	53
Nickel	58
	60
Cadmium	114
Arsenic	75

Analysis

Samples: *Blank standard, Standard solutions, Sample solution,* and *Blank solution*

Instrument performance must be verified to conform to the manufacturer's specifications for resolution and sensitivity. Before analyzing samples, the instrument must pass a suitable performance check. Perform the evaluation using instrument software such as correction equations for interferences and taking the *Internal standard* into account. Generate the calibration curve using the *Blank standard, Standard solution 0.001 ppm,* and *Standard solution 0.01 ppm*: the linear regression coefficient is NLT 0.999.

Aspirate the *Blank solution* and *Sample solution,* respectively, at least in duplicate, and report the average reading as each element content of the sample. Determine the concentration C_B, in µg/mL, of each element in the *Blank solution,* and also determine the concentration C_S, in µg/mL, of each element in the *Sample solution* using the calibration curve.

Calculate the quantity, in µg/g, of each element in the portion of Chitosan taken:

$$\text{Result} = [(C_S - C_B)/W] \times F \times V$$

F = dilution factor, 4

V = volume of the *Sample solution,* 100 mL

W = weight of the Chitosan taken to prepare the *Sample solution* (g)

Acceptance criteria: See *Table 2*.

Table 2

Element	Acceptance Criteria (ppm)
Lead	NMT 0.5
Mercury	NMT 0.2
Chromium	NMT 1.0
Nickel	NMT 1.0
Cadmium	NMT 0.2
Arsenic	NMT 0.5

• **LIMIT OF IRON**

65% Nitric acid: Use ultratrace nitric acid, 65%–70% HNO_3, ACS reagent grade.

Blank standard: Prepared as directed in the *Limit of Lead, Mercury, Chromium, Nickel, Cadmium, and Arsenic.*

Standard stock solution 100 ppm: Immediately before use, dilute an appropriate amount of iron standard[4] with a solution of *65% Nitric acid* (1 in 100) to prepare an acidic solution containing the equivalent of 100 µg/mL of iron.

Standard solutions: Separately transfer 0.1 and 0.5 mL of *Standard stock solution 100 ppm* to 100-mL volumetric flasks, dilute with a solution of *65% Nitric acid* (1 in 100) to volume, and mix. These solutions contain, respectively, 0.1 and 0.5 µg of iron/mL (*Standard solution 0.1 ppm* and *Standard solution 0.5 ppm*).

Sample solution: Transfer 10.0 mL of the digestion solution from the test for *Limit of Lead, Mercury, Chromium, Nickel, Cadmium, and Arsenic* to a 100-mL volumetric flask, dilute with water to volume, and mix.

Blank solution: Transfer 10.0 mL of the blank digestion solution from the test for *Limit of Lead, Mercury, Chromium, Nickel, Cadmium, and Arsenic* to a 100-mL volumetric flask, dilute with water to volume, and mix.

Instrumental conditions

(See *Plasma Spectrochemistry* ⟨730⟩.)

Mode: Inductively coupled plasma–atomic emission spectrometer (ICP–AES)

Analytical wavelength: 238.040 and 239.562 nm with the settings optimized as directed by the manufacturer

Analysis

Samples: *Blank standard, Standard solutions, Sample solution,* and *Blank solution*

Instrument performance must be verified to conform to the manufacturer's specifications for resolution and sensitivity. Before analyzing samples, the instrument must pass a suitable performance check. Generate the calibration curve using the *Blank standard, Standard solution 0.1 ppm,* and *Standard solution 0.5 ppm*: the linear regression coefficient is NLT 0.999.

Aspirate the *Blank solution* and *Sample solution,* respectively, at least in duplicate, and report the average reading as the iron content of the sample. Determine the concentration C_B, in µg/mL, of iron in the *Blank solution,* and also determine the concentration C_S, in µg/mL, of iron in the *Sample solution* using the calibration curve.

[4] Suitable iron standards are available from LGC Promochem (http://www.lgcstandards.com) (Single element standard for ICP, Iron 10,000 µg/mL dilute nitric acid) or from Merck KGaA, Frankfurter Str. 250, 64293 Darmstadt, Germany (iron ICP standard, 10000 mg/L in 10% nitric acid).

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Calculate the quantity, in µg/g, of iron in the portion of Chitosan taken:

$$\text{Result} = [(C_S - C_B)/W] \times F \times V$$

F = dilution factor, 2
V = volume of the *Sample solution*, 100 mL
W = weight of the Chitosan taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 10 ppm of iron

• **LIMIT OF PROTEIN CONTENT**

Control solution A: 1.0 mg/mL of bovine serum albumin

Control solution B: 0.1 mg/mL of bovine serum albumin

Sample solution C: Dissolve 100 mg of Chitosan in 4 mL of 100% formic acid, mix, and stir for about 48 h at room temperature using a magnetic stirrer. This solution contains 25 mg/mL of Chitosan in 100% formic acid.

Sample solutions D, E, F: Dilute aliquots of the *Sample solution C* with water to obtain the following solutions with concentrations of 2.5 mg/mL of Chitosan in 10% formic acid, 0.5 mg/mL of Chitosan in 2% formic acid, and 0.25 mg/mL of Chitosan in 1% formic acid, respectively.

Molecular weight standard preparation G: Use a commercially available preparation of apparent molecular weight protein standards of 10,000–190,000 Da dissolved in the loading buffer consisting of 50 mM of tris(hydroxymethyl)aminomethane hydrochloride (pH 6.8), 5 mM of ethylenediaminetetraacetic acid, 10 mM of dithiothreitol [NOTE—A 2%–5% solution of beta-mercaptoethanol can be used to replace dithiothreitol.], 1% (w/v) sodium dodecyl sulfate, and 10% (w/v) glycerol.[5] [NOTE—A protein ladder containing the following molecular weight standards in 10, 15, 20, 25, 40, 50, 60, 85, 120, and 190 KDa or other appropriate combinations can be used.]

Sample buffer: Transfer 666 mg of tris(hydroxymethyl) aminomethane hydrochloride, 682 mg of tris(hydroxymethyl)aminomethane, 800 mg of lithium dodecyl sulfate, 6 mg of ethylenediaminetetraacetic acid, 4 g of glycerol, 0.75 mL of 1% solution of Coomassie blue G250, and 0.25 mL of 1% solution of phenol red to a 10-mL volumetric flask, add 8 mL of water to the flask, and mix. If necessary, adjust with hydrochloric acid or sodium hydroxide to pH of 7.2. Dilute with water to volume. [NOTE—Store the buffer at 4°. It is stable for 6 months when stored at 4°.] An equivalent commercially available buffer can also be used.[6]

Running buffer: Prepare a solution containing 1 M of tris(hydroxymethyl)aminomethane, 1 M of 2-(4-morpholinyl) ethanesulfonic acid, 20.5 mM ethylenediaminetetraacetic acid, and 69.3 mM dodecyl sodium sulfate in water. If necessary, adjust with hydrochloric acid or sodium hydroxide to pH of 7.3. An equivalent commercially available appropriate SDS running buffer can be used.[7]

Fixing solution: A solution containing 40% ethanol and 10% acetic acid

Sensitizing solution: Transfer 10 mL of a solution mainly containing 10%–20% of 2-(4-morpholinyl) ethanesulfonic acid, 0.1%–1.0% of sodium hydroxide, and 7%–13% of *N,N*-dimethylformamide into a 100-mL volumetric flask, add 30 mL of alcohol, and dilute with water to volume. Alternatively, follow the instructions of a commercially available silver staining kit[8] to prepare the *Sensitizing solution, Staining solution,* and *Developing solution.*

Staining solution: Transfer 1.0 mL of a solution mainly containing 10%–30% of silver nitrate into a 100-mL volumetric flask, and dilute with water to volume.

Developing solution: Transfer 10 mL of a solution mainly containing 10%–30% of sodium carbonate into a 100-mL volumetric flask, add 1 drop of a solution containing 30%–60% of formaldehyde, and dilute with water to volume.

Stopping solution: A solution containing 10%–30% of ethylenediaminetetraacetic acid and 10%–30% of tris(hydroxymethyl)-aminomethane. A *Stopping solution* is commercially available and included in a silver staining kit.

Analysis: Mix 75 µL each of *Sample solutions C, D, E,* and *F* with 25 µL of the *Sample buffer,* and incubate at 70° for 10 min. In a suitable device for polyacrylamide gel electrophoresis (see *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* ⟨1056⟩) add appropriate volumes of the *Running buffer* in the upper and the lower buffer chambers. Attach a 4%–12% gradient Bis-Tris ready-made polyacrylamide gel sandwiched between two glass plates, such that the wells for sample application are exposed to the *Running buffer* in the upper buffer chamber. Separately apply equal volumes (about 20 µL) of each of the treated *Sample solutions C, D, E,* and *F, Control solution A,* and *Control solution B* onto separate lanes; apply equal volumes (about 10 µL) of the *Molecular weight standard preparation G* to both sides of the gel. [NOTE—Do not apply any solution in the outside lanes.] Connect the lower buffer chamber electrode to the positive terminal and the upper buffer chamber electrode to the negative terminal of a suitable power supply unit, and carry out the electrophoresis at a constant voltage of about 100 V for about 100 min.

Remove the gel from the gel assembly. [NOTE—Do not touch the gel with bare hands. Use gloves.]

Place the gel in a clean staining tray of appropriate size. Rinse the gel briefly with water. Fix the gel in 100 mL of *Fixing solution* for 20 min with gentle rotation. [NOTE—The gel can be stored in the *Fixing solution* overnight.]

Decant the *Fixing solution,* and wash the gel in 30% ethanol for 10 min. Decant the ethanol, and add 100 mL of *Sensitizing solution* to the washed gel in the staining container. Incubate the gel in the *Sensitizing solution* for about 10 min. [NOTE—All incubations should be performed on a rotary shaker rotating at a speed of 1 revolution/s at room temperature.]

Decant the *Sensitizing solution,* and wash the gel in 100 mL of 30% ethanol for 10 min. Wash the gel in 100 mL of water for 10 min. Incubate the gel in 100 mL of *Staining solution* for 15 min. After staining is complete, decant the *Staining solution,* and wash the gel with 100 mL of water for 20–60 s. [NOTE—Washing the gel for more than 1 min will remove the silver ion from the gel resulting in decreased sensitivity.]

Incubate the gel in 100 mL of *Developing solution* for 4–8 min until bands start to appear and the desired band intensity is reached.

Once the appropriate staining intensity is achieved, immediately add 10 mL of *Stopping solution* directly to the gel still immersed in the *Developing solution.* Gently agitate the gel for 10 min. The color changes from pink to colorless indicating that the development has stopped.

[5] A suitable molecular weight standard preparation is available as BenchMark Prestained Protein Ladder from Invitrogen, product number: 10748010.

[6] A suitable sample buffer is available as 4X NuPAGE LDS sample buffer from Invitrogen, product number: NP0007.

[7] A suitable running buffer is available as NuPAGE MES SDS Running Buffer from Invitrogen, product number: NP0002.

[8] A suitable silver staining kit is available as NuPAGE Silver Staining Kit from Invitrogen, product number: LC6070.

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Decant the colorless solution, and wash the gel with 100 mL of water for 10 min.

Use a gel imaging system, ideally with a CCD camera, to record the results.

Acceptance criteria: NMT 0.2% of protein

SPECIFIC TESTS

- **BACTERIAL ENDOTOXINS TEST** ⟨85⟩

Sample stock solution: Transfer 0.5 g of Chitosan to a 50-mL volumetric flask, add LAL Reagent Water[9] and 4.6 mL of 1 N hydrochloric acid, dilute with the LAL Reagent Water to volume, and mix well. Incubate this solution in a water bath at 40° for 48 h.

Sample solution: Dilute the *Sample stock solution* to 1:50 with LAL Reagent Water, including dilution 1:2 with a β-glucan blocker.[10]

Acceptance criteria: The level of bacterial endotoxins is such that the requirement under the relevant dosage form monograph(s) in which Chitosan is used can be met. Where the label states that Chitosan must be subjected to further processing during the preparation of wound dressing dosage forms, the level of bacterial endotoxins is such that the requirement under the relevant dosage form monograph(s) in which Chitosan is used can be met.

- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩

Sample: Prepare a solution (1 in 50).

Acceptance criteria: The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g. It meets the requirements of the tests for absence of *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

- **APPARENT AVERAGE MOLECULAR WEIGHT AND MOLECULAR WEIGHT DISTRIBUTION**

[NOTE—This test is applicable to Chitosan of an average molecular weight of NMT 1,000,000 Da. In the following test an apparent average molecular weight is determined.]

Mobile phase: Transfer 12.75 g of sodium nitrate to a 1000-mL volumetric flask containing 800 mL of water, add a suitable amount of formic acid, dilute with water to volume, mix well, and make a concentration of formic acid of 0.5 M. The *Mobile phase* contains 0.15 M sodium nitrate in 0.5 M aqueous formic acid.

System suitability solution: 1.0 mg/mL of ethylene glycol in *Mobile phase*

Standard solutions: Prepare several sets of mixtures, containing ten polyethylene glycol (PEG) standards of different known molecular weight, which are used to cover the molecular weight range from about 200 to 1,100,000 g/mol.[11] [NOTE—These standards could be mixtures of polyethylene glycols and polyethylene oxides.] Prepare each set of PEG molecular weight standards to have a known concentration of about 1.0 mg/mL for each standard in *Mobile phase*. Allow the *Standard solutions* to stand at room temperature for at least 8 h. Do not filter before use.

Sample solution: Prepare a solution containing 1.0 mg/mL of Chitosan in *Mobile phase*. Cap, and mix well. Allow the solution to stand at room temperature for at least 12 h. Pass the chitosan solution through a membrane filter of 0.45-µm pore size, discard an appropriate volume of the initial filtrate, and use the rest of the filtered solution for analysis.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Refractive index

Detector temperature: 35°

Columns: 7.5-mm × 30-cm analytical column, 10-µm packing L38; and two 7.5-mm × 30-cm analytical columns; 17-µm packing L38

Flow rate: 1.0 mL/min for *System suitability solution*; and 0.5 mL/min for *Standard solutions* and *Sample solution*

Injection size: 20 µL for *System suitability solution*; and 100 µL for *Standard solutions* and *Sample solution*

System suitability

Samples: *System suitability solution* and *Standard solution*

Suitability requirements

Plate count: NLT 80% of the value that is certified by the column manufacture for new columns, determining the plate count for the ethylene glycol peak, *System suitability solution*

Resolution: NLT 1.7 between the PEG standards, *Standard solution*. [NOTE—The resolution between the PEG standard with a molecular weight of 1,000,000 Da and its adjacent PEG standard should be NLT 1.0 if 1.7 cannot be met.]

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine elution peak maxima and corresponding retention volumes (elution volume), V_p, for the ten PEG standards, corresponding to the specified molecular weight, M_p, at the peak maximum of the standards.

Molecular weight calibration: Analyze each polyethylene glycol standard, and use a suitable gel-permeation chromatography or size-exclusion chromatography (GPC/SEC) software, or an equivalent data handling system, to compute the data and calibration. Plot $\log M_p$ for each standard in the *Standard solutions* versus its retention volume, V_p, in mL, at each standard peak maximum, and construct the best polynomial line fitting the ten points.

Data analysis for sample: [NOTE—Based on the PEG molecular weight calibration curve, calculate the molecular weight and molecular weight distribution of Chitosan using the slice by slice method.]

Analyze the Chitosan sample by identifying retention volumes V_a and V_b corresponding to the beginning and end of the Chitosan chromatogram. The baseline between V_a and V_b is assumed to be linear. [NOTE—Draw a straight line between V_a and V_b.] Data analysis is based on a suitable GPC/SEC computer software or a real-time data acquisition system with either off-line or on-line data processing that is able to provide a means of determining chromatographic peak heights or integrated area segments as prescribed intervals under the SEC chromatogram and handling and reporting the data. The following describes the data processes which can either be computed by the GPC/SEC software or by an equivalent data processing system. Upon acquisition, handle the data under the Chitosan elution peak in discrete segments A_i, integrated area slices, or as digitized chromatogram heights H_i by recording the vertical displacements between the chromatogram trace and the baseline at elution volume, V_i, over designated intervals. A minimum of 40 area segments or heights are required. Obtain the corresponding value of M_i for Chitosan based on its elution volume, V_i, from the molecular

[9] Use Sterile Water for Injection or other water that shows no reaction with the specific LAL Reagent with which it is to be used, at the limit of sensitivity of such reagent.

[10] Available from Cambrex Europe s.p.r.l., Verviers, Belgium; Charles River, www.criver.com; or all the major LAL manufacturers.

[11] Suitable polyethylene glycol molecular weight standards are available as ReadyCal kits from Polymer Standards Service (PSS), www.polymer.de.

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weight calibration curve obtained in *Molecular weight calibration*.

Calculate the number-, and weight-average molecular weights, M_n and M_w in g/mol, respectively, using the following formulas.

$$M_n = \frac{\sum_{i=1}^{N} A_i}{\sum_{i=1}^{N}\left(\frac{A_i}{M_i}\right)} \qquad M_w = \frac{\sum_{i=1}^{N}(A_i \bullet M_i)}{\sum_{i=1}^{N} A_i}$$

If the elution volume internal ΔV_i, for instance, $V_2 - V_1 = V_3 - V_2$, etc, is constant; parameters A_i and M_i are the chromatographic peak slice area and Chitosan molecular weight associated with the elution volume, V_i; and N is the number of data points obtained from the chromatogram between V_a and V_b ($N \geq 40$). [NOTE—If N is sufficiently large, the use of area segments A_i or peak heights H_i will yield equivalent results.]

Calculate the molecular weight distribution or polydispersity for Chitosan using the following expression:

$$\text{Result} = M_w/M_n$$

Acceptance criteria: The values of apparent weight-average molecular weight and polydispersity are NLT 85% and NMT 115% of their respective values stated on the label.

- **LOSS ON DRYING** ⟨731⟩**:** Dry 1.0 g in an oven at 100°–105° for 7 h: it loses NMT 5.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in light-resistant and well-closed containers in a dry place, and store at a temperature below 30°.
- **LABELING:** Label it to indicate its apparent weight-average molecular weight, M_w, and polydispersity (M_w/M_n). Where Chitosan is intended for use in the manufacture of wound dressings, it is so labeled. Where Chitosan must be subjected to further processing during the preparation of wound dressings, it is so labeled. Label to indicate the natural source from which Chitosan is derived.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Chitosan RS

Chlorobutanol



$C_4H_7Cl_3O$ 177.46

$C_4H_7Cl_3O \cdot \frac{1}{2}H_2O$ 186.46

2-Propanol, 1,1,1-trichloro-2-methyl-; 1,1,1-Trichloro-2-methyl-2-propanol [57-15-8]. Hemihydrate [6001-64-5].

DEFINITION

Chlorobutanol is anhydrous or contains NMT one-half molecule of water of hydration. It contains NLT 98.0% and NMT 100.5% of chlorobutanol ($C_4H_7Cl_3O$), calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

- **A.** ▲**SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
- **B.** The retention time of the chlorobutanol peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

ASSAY

- **PROCEDURE**

Standard solution: 10.0 mg/mL of USP Chlorobutanol RS and 15.0 mg/mL of 2,2,2-trichloroethanol (internal standard) in *n*-hexane

Sample solution: 10.0 mg/mL of Chlorobutanol and 15.0 mg/mL of 2,2,2-trichloroethanol (internal standard) in *n*-hexane

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.32-mm × 30-m fused silica; coated with a 0.25-µm layer of stationary phase G16
Temperatures
Injection port: 260°
Detector: 280°
Column: 135°
Carrier gas: Helium
Flow rate: 1.0 mL/min
Injection volume: 1 µL
Injection type: Split injection, split ratio 10:1
Run time: 12 min

System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for chlorobutanol and 2,2,2-trichloroethanol are 1.0 and 1.3, respectively.]
Suitability requirements
Resolution: NLT 5 between the chlorobutanol and 2,2,2-trichloroethanol peaks
Tailing factor: NMT 1.5 for the chlorobutanol peak
Relative standard deviation: NMT 0.3% for peak area ratio of chlorobutanol to the internal standard

Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of chlorobutanol ($C_4H_7Cl_3O$) in the portion of Chlorobutanol taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times P \times 100$$

R_U = peak area ratio of chlorobutanol to the internal standard from the *Sample solution*
R_S = peak area ratio of chlorobutanol to the internal standard from the *Standard solution*
C_S = concentration of USP Chlorobutanol RS in the *Standard solution* (mg/mL)
C_U = concentration of Chlorobutanol in the *Sample solution* (mg/mL)
P = labeled purity of USP Chlorobutanol RS

Acceptance criteria: 98.0%–100.5% on the anhydrous basis

IMPURITIES

- **CHLORIDE**

Control solution: 0.50 mL of 0.020 N hydrochloric acid in a mixture of 25 mL of diluted alcohol and 1 mL of nitric acid
Sample solution: 0.50 g of Chlorobutanol in a mixture of 25 mL of diluted alcohol and 1 mL of nitric acid

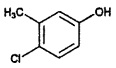
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Analysis: To the *Control solution* and *Sample solution* add 2 mL of silver nitrate TS.
Acceptance criteria: 0.07%; any turbidity produced in the *Sample solution* is NMT that produced in the *Control solution*.

SPECIFIC TESTS
- **WATER DETERMINATION** ⟨921⟩, *Method I*: NMT 1.0% (anhydrous form) and NMT 6.0% (hydrous form)
- **REACTION**
  Sample: 0.5 g
  Analysis: Shake the *Sample* thoroughly with 25 mL of water.
  Acceptance criteria: The water remains neutral to litmus.
- **BACTERIAL ENDOTOXINS TEST** ⟨85⟩: If labeled for use in preparing parenteral dosage forms, it also meets the following requirements. The level of bacterial endotoxins is such that the requirement in the relevant dosage form monograph(s) in which Chlorobutanol is used can be met. Where the label states that Chlorobutanol must be subjected to further processing during the preparation of injectable dosage forms, the level of bacterial endotoxins is such that the requirement in the relevant dosage form monograph(s) in which Chlorobutanol is used can be met.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **LABELING:** Label it to indicate whether it is anhydrous or hydrous. Where Chlorobutanol is intended for use in the manufacture of injectable dosage forms, it is so labeled. Where Chlorobutanol must be subjected to further processing during the preparation of injectable dosage forms to ensure acceptable levels of bacterial endotoxins, it is so labeled.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Chlorobutanol RS

Chlorocresol



C_7H_7ClO 142.58
Phenol, 4-chloro-3-methyl-;
4-Chloro-*m*-cresol [59-50-7].

DEFINITION
Chlorocresol contains NLT 99.0% and NMT 101.0% of C_7H_7ClO (4-chloro-3-methylphenol).

IDENTIFICATION
- **A.**
  Sample solution: Dissolve 40 mg of Chlorocresol in 10 mL of water.
  Analysis: Add 1 drop of ferric chloride TS to the *Sample solution*.
  Acceptance criteria: A blue color develops.
- **B.**
  Sample: 50 mg
  Analysis: Transfer the *Sample* to a crucible, add 500 mg of anhydrous sodium carbonate, and mix. Heat the mixture until fused. Cool, add 5 mL of water, and boil. Acidify with 1 mL of nitric acid, filter, and add 1 mL of silver nitrate TS to the filtrate.
  Acceptance criteria: A white precipitate is formed.

ASSAY
- **PROCEDURE**
  Sample: 70 mg

  Titrimetric system
  (See *Titrimetry* ⟨541⟩.)
  Mode: Residual titration
  Titrant: 0.1 N sodium thiosulfate VS
  Endpoint detection: Visual
  Analysis: Transfer the *Sample* to an iodine flask, add 30 mL of glacial acetic acid, 25.0 mL of 0.1 N bromine VS, 10 mL of potassium bromide solution (150 mg/mL), and 10 mL of hydrochloric acid.
  Immediately insert the stopper, and allow to stand for 15 min, protected from light. Quickly add 10 mL of potassium iodide solution (100 mg/mL) and 100 mL of water, taking precautions against the escape of bromine vapor. Immediately insert the stopper, and shake the mixture thoroughly. Remove the stopper, and rinse it and the neck of the flask with a small quantity of water so that the washing flows into the flask. Add 1 mL of chloroform, and shake the mixture thoroughly.
  Titrate the liberated iodine with *Titrant*, adding 3 mL of starch TS as the endpoint is approached. Perform a blank determination. Each mL of 0.1 N bromine VS is equivalent to 3.565 mg of C_7H_7ClO (4-chloro-3-methylphenol).
  Acceptance criteria: 99.0%–101.0%

SPECIFIC TESTS
- **MELTING RANGE OR TEMPERATURE** ⟨741⟩: 63°–66°
- **LIMIT OF NONVOLATILE RESIDUE**
  Sample: 1.0 g
  Analysis: Heat the *Sample* in a tared crucible on a steam bath until it has evaporated, and dry the residue at 105° for 1 h.
  Acceptance criteria: NMT 0.1%
- **COMPLETENESS OF SOLUTION**
  Sample: 1 g
  Analysis: Transfer the *Sample* to a test tube, add 0.4 mL of alcohol, and shake.
  Acceptance criteria: The solution is complete.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

Chocolate

DEFINITION
Chocolate is a powder prepared from the roasted, cured kernels of the ripe seed of *Theobroma cacao* L. (Fam. Sterculiaceae). Chocolate yields NLT 10.0% and NMT 22.0% of nonvolatile ether-soluble extractive.

COMPOSITION
- **CONTENT OF NONVOLATILE ETHER-SOLUBLE EXTRACTIVE**
  Sample: 10 g of Chocolate
  Analysis: Extract the *Sample* with anhydrous ethyl ether in a continuous-extraction apparatus for 8 h. Allow the ether solution to evaporate spontaneously in a suitable tared container, dry at 105° for 1 h, and weigh the nonvolatile ether-soluble extractive.
  [NOTE—Retain the ether-insoluble residue for the test for *Botanical Characteristics* and the tests for *Articles of Botanical Origin, Methods of Analysis, Total Ash; Acid-Insoluble Ash;* and *Crude Fiber*.]
  Acceptance criteria: 10.0%–22.0%

CONTAMINANTS
- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩: The total bacterial count does not exceed 5×10^3 cfu/g, and it meets the

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requirements of the tests for absence of *Escherichia coli*, a 10-g specimen being used.

• **Articles of Botanical Origin** ⟨561⟩, *Pesticide Residue Analysis*: Meets the requirements

SPECIFIC TESTS

• **Botanical Characteristics**

Microscopic: It shows numerous broken parenchyma cells of the cotyledons containing a reddish brown or purplish brown to yellowish orange pigment; numerous starch grains; oil globules; aleurone grains; and occasionally acicular or prismatic crystals of fat. The starch grains are simple and two- to three-compound, the single grains up to 15 µm in diameter, and they stain slowly with iodine TS. The ether-insoluble residue retained from the *Content of Nonvolatile Ether-Soluble Extractive* shows few or no fragments of cocoa shells and no cereal starch grains.

• **Articles of Botanical Origin** ⟨561⟩, *Methods of Analysis, Total Ash*

Sample: Use the ether-insoluble residue from the *Content of Nonvolatile Ether-Soluble Extractive.*

Acceptance criteria: NMT 8.0%

• **Articles of Botanical Origin** ⟨561⟩, *Methods of Analysis, Acid-Insoluble Ash*

Sample: Use the ether-insoluble residue from the *Content of Nonvolatile Ether-Soluble Extractive.*

Acceptance criteria: NMT 0.4%

• **Articles of Botanical Origin** ⟨561⟩, *Methods of Analysis, Crude Fiber*

Sample: Use the ether-insoluble residue from the *Content of Nonvolatile Ether-Soluble Extractive.*

Acceptance criteria: NMT 7.0%

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Preserve in well-closed containers.

Chocolate Syrup

DEFINITION

Prepare Chocolate Syrup as follows (see *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩).

Chocolate	180 g
Sucrose	600 g
Liquid Glucose	180 g
Glycerin	50 mL
Sodium Chloride	2 g
Vanillin	0.2 g
Sodium Benzoate	1 g
Purified Water, a sufficient quantity to make	1000 mL

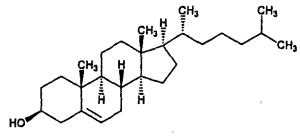
Mix *Chocolate* and *Sucrose*, and to this mixture gradually add a solution of *Liquid Glucose, Glycerin, Sodium Chloride, Vanillin*, and *Sodium Benzoate* in 325 mL of hot *Purified Water*. Bring the entire mixture to a boil, and maintain at boiling temperature for 3 min. Allow to cool to room temperature, and add sufficient *Purified Water* to make the product measure 1000 mL.

[Note—Chocolate containing NMT 12% of nonvolatile, ether-soluble extractive ("fat") yields a Syrup having a minimum tendency to separate. "Breakfast Chocolate" contains over 22% of "fat".]

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Package in tight containers, and avoid exposure to excessive heat.

Cholesterol



$C_{27}H_{46}O$ 386.65

Cholest-5-en-3-ol, (3β)-;
Cholest-5-en-3β-ol [57-88-5].

DEFINITION

Cholesterol is a steroid alcohol containing NLT 95.0% and NMT 102.0% of cholest-5-en-3β-ol ($C_{27}H_{46}O$), calculated on the dried basis. It may contain suitable antioxidants.

IDENTIFICATION

Change to read:

• **A.** ▲**Spectroscopic Identification Tests** ⟨197⟩, *Infrared Spectroscopy*: **197A** or **197K**▲ (CN 1-May-2020)

• **B.** It meets the requirements of the test for *Optical Rotation* ⟨781S⟩, *Procedures, Specific Rotation.*

• **C.** It meets the requirements of the test for *Melting Range or Temperature* ⟨741⟩.

ASSAY

• **Procedure**

Standard solution: 1.0 mg/mL of USP Cholesterol RS and 1.0 mg/mL of pregnenolone isobutyrate (internal standard) in heptane

Sample solution: 1.0 mg/mL of Cholesterol and 1.0 mg/mL of pregnenolone isobutyrate (internal standard) in heptane

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability.*)

Mode: GC

Detector: Flame ionization

Column: 0.25-mm × 30-m capillary bonded with a 0.25-µm layer of phase G2

Temperatures

Injection port: 285°

Column: 275°

Detector: 300°

Carrier gas: Helium

Flow rate: 2.0 mL/min

Injection volume: 1.0 µL

Injection type: Split ratio, 25:1

Liner: Cup splitter liner (4 mm × 6.3 × 78.5) with deactivated glass wool

System suitability

Sample: *Standard solution*

[Note—The relative retention times for pregnenolone isobutyrate and cholesterol are 1.0 and 1.2, respectively.]

Suitability requirements

Resolution: NLT 10 between pregnenolone isobutyrate and cholesterol

Relative standard deviation: NMT 2.0% for the peak response ratio of cholesterol to the internal standard

Analysis

Samples: *Standard solution* and *Sample solution*

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Calculate the percentage of cholesterol in the portion of sample taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of cholesterol to the internal standard (peak response of cholesterol/peak response of the internal standard) from the *Sample solution*

R_S = peak response ratio of cholesterol to the internal standard (peak response of cholesterol/peak response of the internal standard) from the *Standard solution*

C_S = concentration of USP Cholesterol RS in the *Standard solution* (mg/mL)

C_U = concentration of Cholesterol in the *Sample solution* (mg/mL)

Acceptance criteria: 95.0%–102.0% on the dried basis

IMPURITIES

- **Residue on Ignition** ⟨281⟩: NMT 0.1%
- **Limit of Related Sterols and Other Organic Impurities**

Internal standard solution: 0.02 mg/mL of pregnenolone isobutyrate (internal standard) in heptane

System suitability solution: 0.02 mg/mL of USP Cholesterol RS, 0.04 mg/mL of desmosterol, and 0.02 mg/mL of lathosterol in *Internal standard solution*

Sample solution: 2.0 mg/mL of Cholesterol in *Internal standard solution*

Chromatographic system: Proceed as directed in the *Assay*.

System suitability

Sample: *System suitability solution*

[Note—See *Table 1* for the relative retention times.]

Table 1

Name	Relative Retention Time
Pregnenolone isobutyrate (internal standard)	1.00
Cholesterol	1.23
Desmosterol (cholesta-5,24-dien-3β-ol)	1.31
Lathosterol (5α-cholest-7-en-3β-ol)	1.34

Suitability requirements

Resolution: NLT 2.0 between desmosterol and lathosterol

Relative standard deviation: NMT 5.0% for peak response ratio of desmosterol to the internal standard

Analysis

Samples: *System suitability solution* and *Sample solution*

Three more related sterols may be observed (see *Table 2*).

Table 2

Name	Relative Retention Time
Pregnenolone isobutyrate (internal standard)	1.00
β-Cholestanol (5α-cholestan-3β-ol, dihydrocholesterol)	1.24
24-Dehydrolathosterol (5α-cholesta-7,24-dien-3β-ol)	1.42
4-Methylcholest-5-en-3β-ol	1.51

Calculate the percentage of desmosterol or lathosterol in the portion of Cholesterol taken:

$$\text{Result} = (R_{U1}/R_{S1}) \times (C_{S1}/C_U) \times 100$$

R_{U1} = peak response ratio of desmosterol or lathosterol to the internal standard (peak response of desmosterol or lathosterol/peak response of the internal standard) from the *Sample solution*

R_{S1} = peak response ratio of desmosterol or lathosterol to the internal standard (peak response of desmosterol or lathosterol/peak response of the internal standard) from the *System suitability solution*

C_{S1} = concentration of desmosterol or lathosterol in the *System suitability solution* (mg/mL)

C_U = concentration of Cholesterol in the *Sample solution* (mg/mL)

Calculate the percentage of β-cholestanol, 24-dehydrolathosterol, 4-methylcholest-5-en-3β-ol, or any other unspecified organic impurity in the portion of Cholesterol taken:

$$\text{Result} = (R_{U2}/R_{S2}) \times (C_{S2}/C_U) \times 100$$

R_{U2} = peak response ratio of β-cholestanol, 24-dehydrolathosterol, 4-methylcholest-5-en-3β-ol, or any other unspecified impurity to the internal standard (peak response of β-cholestanol, 24-dehydrolathosterol, 4-methylcholest-5-en-3β-ol, or any other unspecified impurity/peak response of the internal standard) from the *Sample solution*

R_{S2} = peak response ratio of cholesterol to the internal standard (peak response of cholesterol/peak response of the internal standard) from the *System suitability solution*

C_{S2} = concentration of USP Cholesterol RS in the *System suitability solution* (mg/mL)

C_U = concentration of Cholesterol in the *Sample solution* (mg/mL)

Acceptance criteria: See *Table 3*. Disregard peaks less than 0.05% for any unspecified impurities and any peaks due to solvent.

Table 3

Name	Acceptance Criteria (%)
β-Cholestanol (5α-cholestan-3β-ol, dihydrocholesterol)	≤0.6
Desmosterol	≤4
Lathosterol	≤2
24-Dehydrolathosterol (5α-cholesta-7,24-dien-3β-ol)	≤0.2
4-Methylcholest-5-en-3β-ol	≤0.5
Total impurities including related sterols	≤5

SPECIFIC TESTS

- **Melting Range or Temperature** ⟨741⟩: 147°–150°
- **Optical Rotation** ⟨781S⟩, *Procedures, Specific Rotation*

Sample solution: 20 mg/mL, undried, in dioxane

Acceptance criteria: −34° to −38°

- **Acidity**

Sample: 1.0 g

Analysis: Dissolve the *Sample* in 10 mL of ether in a small flask, add 10.0 mL of 0.10 N sodium hydroxide, and shake for about 1 min. Heat gently to expel the ether, then boil for 5 min. Cool, dilute with 10 mL of water, add

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phenolphthalein TS, and titrate with 0.10 N sulfuric acid until the pink color just disappears, stirring the solution vigorously throughout the titration. Perform a blank determination (see *Titrimetry* ⟨541⟩, *Residual Titrations*).
Acceptance criteria: The difference between the number of mL of 0.10 N sulfuric acid consumed in the blank and the number of mL consumed in the *Sample* is NMT 0.3 mL.

- **Loss on Drying** ⟨731⟩
Analysis: Dry under vacuum at 60° for 4 h.
Acceptance criteria: NMT 0.3%
- **Solubility in Alcohol**
Sample: 500 mg
Analysis: Dissolve the *Sample* in 50 mL of warm alcohol in a stoppered flask or cylinder, and allow to stand at room temperature for 2 h.
Acceptance criteria: No deposit or turbidity is formed.

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in well-closed, light-resistant containers.
- **Labeling:** Label it to indicate whether cholesterol is derived from animal, synthetic, or vegetable sources. For animal-derived sources, indicate the species and tissue used (for example, bovine brain and spinal cord, wool fat, or chicken eggs). Indicate the names and amounts of any added antioxidants.
- **USP Reference Standards** ⟨11⟩
USP Cholesterol RS

Clove Oil

DEFINITION

Clove Oil is the volatile oil distilled with steam from the dried flower buds of *Syzygium aromaticum* (L.) Merr. and L. M. Perry (Fam. Myrtaceae). It contains NLT 85.0% of total phenolic substances, chiefly eugenol ($C_{10}H_{12}O_2$).

ASSAY

- **Procedure**
Sample: 10 mL of Clove Oil
Analysis: Pipet the *Sample* into a suitable cassia flask, the neck of which is graduated from 0–6 mL at intervals of 0.1 mL. Add 75 mL of 1 N potassium hydroxide, and shake the mixture for 5 min. Heat for 10 min in boiling water, shaking the flask at least 3 times during the heating. Remove the flask from the bath, and cool to room temperature. When the liquids have separated completely, add sufficient 1 N potassium hydroxide to raise the lower level of the oily layer so that it is within the graduated portion of the neck. Measure the volume of the oily layer after standing for 18 h.
Acceptance criteria: NMT 1.5 mL, indicating the presence of NLT 85.0%, by volume, of total phenolic substances in the Oil

IMPURITIES

- **Limit of Phenol**
Sample: 1 mL of Clove Oil
Analysis: Shake the *Sample* with 20 mL of hot water: the water shows not more than a scarcely perceptible acid reaction with blue litmus paper. Cool the mixture, pass the water layer through a wetted filter, and treat the clear filtrate with 1 drop of ferric chloride TS.
Acceptance criteria: The mixture exhibits only a transient, grayish-green color, but not a blue or violet color.

SPECIFIC TESTS

- **Specific Gravity** ⟨841⟩: 1.038–1.060
- **Optical Rotation,** *Angular Rotation* ⟨781A⟩: It is optically inactive or slightly levorotatory. The angular rotation is not grater than –1.5°.
- **Refractive Index** ⟨831⟩: 1.527–1.535 at 20°
- **Solubility in 70% Alcohol:** One volume dissolves in 2 volumes of 70% alcohol.

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in well-filled, tight containers, and avoid exposure to excessive heat.
- **Labeling:** The label states the Latin binomial name and, following the official name, the part of the plant source from which the article was derived.

Cocoa Butter

DEFINITION

Cocoa Butter is the fat obtained from the seed of *Theobroma cacao* L. (Fam. Sterculiaceae).

SPECIFIC TESTS

- **Fatty Acid Composition**
Sample solution: Place 100–150 mg of Cocoa Butter in a 50-mL round-bottom flask, and add 4 mL of 0.5 N sodium hydroxide solution, prepared in methanol. Add a few boiling chips to the flask, connect the round-bottom flask to a condenser, and boil the mixture under total reflux until the fat globules go into solution. Add 5.0 mL of a 2.0 M borontrifluoride in methanol solution to the boiling mixture via the condenser, and continue boiling for 2 min. Add 2–5 mL of chromatographic *n*-heptane to the boiling mixture via the condenser, and boil for another min. Remove the flask from the source of heat, and remove the reflux condenser. Add saturated sodium chloride solution, and swirl the flask gently. Add more of the saturated sodium chloride solution to bring the liquid level into the neck of the round-bottom flask. Transfer 1 mL of the organic layer into a glass-stoppered test tube, add some anhydrous sodium sulfate to remove the last traces of water, and filter. Use the filtrate.
System suitability solution: 1 mg/mL each of methyl stearate and methyl oleate, in *n*-heptane
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.25-mm × 15-m fused-silica capillary; 0.25-µm stationary phase G19 coating
Temperature
Detector: 250°
Injection port: 250°
Column: See *Table 1*.
[Note—The components of interest elute during the temperature program. The final hold at a temperature of 240° serves only to facilitate elution of higher boiling components.]

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
180	10	240	5

Carrier gas: Helium
Linear velocity: 48 cm/s
Injection size: 0.1 µL

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Injection type: Split
Split ratio: 60:1
System suitability
Sample: *System suitability solution*
[NOTE—The relative retention times for stearate and oleate are about 0.97 and 1.0, respectively.]
Suitability requirements
Resolution: NLT 1.5 between the stearate and oleate peaks
Relative standard deviation: NMT 5.0%
Analysis
Sample: *Sample solution*
Measure the areas for the peaks of the methyl esters of the fatty acids. [NOTE—The relative retention times for palmitate, stearate, oleate, linoleate, linolenate (if present), and arachidate are about 1.0, 1.55, 1.60, 1.72, 1.89, and 2.30, respectively.]
Calculate the percentage of each fatty acid methyl ester in the portion of Cocoa Butter taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = response of each peak
r_T = sum of the responses of all of the peaks

Acceptance criteria: See *Table 2*.

Table 2

Fatty Acid Methyl Ester	Relative Retention Time	Percentage (%)
Palmitate	1.0	23–30
Stearate	1.55	31–37
Oleate	1.60	31–38
Linoleate	1.72	1.6–4.8
Linolenate (if present)	1.89	0–1.5
Arachidate	2.30	0–1.5

• **MELTING RANGE**
Analysis: Melt the material to be tested at a temperature of 50°–60°. Place 50 g of the melted material in a beaker, and cool in a water bath at 25°. Stir continuously until it assumes a pasty consistency, taking care to avoid the inclusion of air bubbles. Place the beaker in a water bath maintained at a temperature of 32°–33°. Continue stirring until the specimen reaches the temperature of the water bath and changes to a liquid cream (about 30 min). Pour the contents into another beaker, and allow it to solidify at room temperature for at least 2 h. Press one side of a U-shaped capillary tube, 1.5 mm in diameter and 80 mm in length with a distance of 10 mm between both capillaries, into the solidified specimen. Using a very fine metal rod, push the column down to 10 mm before the bend of the U-tube. Then attach the other arm of the U-tube to a precision thermometer (having 0.1° graduations) by suitable means, with the U-tube bend at the level of the thermometer bulb. Insert the thermometer into a water bath so that the upper edge of the material is at least 20 mm below the surface, and heat as directed under *Melting Range or Temperature* ⟨741⟩, *Class I* except, within 5° of the expected melting temperature, regulate the rate of the temperature rise so that it does not exceed 0.2°/min.
Acceptance criteria: The slip point (temperature at which the column visibly flows toward the bend in the tube) is 30°–34°. The clear melting point (clarity via magnifying glass) is 31°–35°.
• **REFRACTIVE INDEX** ⟨831⟩**:** 1.454–1.459 at 40°
• **FATS AND FIXED OILS,** *Free Fatty Acids* ⟨401⟩**:** The free fatty acids in 10.0 g of it require for neutralization NMT 5.0 mL of 0.10 N sodium hydroxide (1.4% as oleic acid).
• **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩**:** 33–42
• **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩**:** 188–198

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Coconut Oil

Coconut oil [8001-31-8].

DEFINITION
Coconut Oil is the refined fixed oil of the seeds of *Cocos nucifera* L. (Fam. Palmae).

IDENTIFICATION
• **A.** It meets the requirements in *Specific Tests* for *Fats and Fixed Oils, Fatty Acid Composition* ⟨401⟩.

IMPURITIES
• **ARSENIC,** *Method II* ⟨211⟩**:** NMT 0.5 µg/g
• **ALKALINE IMPURITIES**
Sample: 10 mL of Coconut Oil
Analysis: Mix 10 mL of freshly distilled acetone and 0.3 mL of water, and add 0.05 mL of bromophenol blue TS. Neutralize the solution to a green color if necessary with 0.01 N hydrochloric acid or 0.01 N sodium hydroxide. Add the *Sample*, shake, and allow to stand. Titrate with 0.01 N hydrochloric acid VS to change the color of the upper layer to yellow.
Acceptance criteria: NMT 0.1 mL of 0.01 N hydrochloric acid VS is required.

SPECIFIC TESTS
• **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩**:** NMT 0.5, determined on 20.0 g
• **FATS AND FIXED OILS,** *Fatty Acid Composition* ⟨401⟩**:** Coconut Oil exhibits the composition profile of fatty acids, shown in *Table 1*.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
6	0	≤1.5
8	0	5.0–11.0
10	0	4.0–9.0
12	0	40.0–50.0
14	0	15.0–20.0
16	0	7.0–12.0
18	0	1.5–5.0
20	0	≤0.2
16	1	≤1.0
18	1	4.0–10.0
18	2	1.0–3.0
18	3	≤0.2
20	1	≤0.2

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- **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩: NMT 5.0
- **FATS AND FIXED OILS,** *Unsaponifiable Matter* ⟨401⟩: NMT 1.0%
- **MELTING RANGE OR TEMPERATURE** ⟨741⟩: 23°–26°
- **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 0.1%, 50 mL of chloroform being used as the solvent instead of 35–40 mL of methanol

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant, well-filled containers. No storage requirement specified.

Hydrogenated Coconut Oil

Hydrogenated coconut oil [84836-98-6].

DEFINITION

Hydrogenated Coconut Oil is the product obtained by refining and hydrogenating the oil obtained from the seeds of *Cocos nucifera* L. (Fam. Palmae).

IDENTIFICATION

- **A.** It meets the requirements in *Specific Tests* for *Fats and Fixed Oils, Fatty Acid Composition* ⟨401⟩.
- **B.** It meets the requirements in *Specific Tests* for *Melting Range or Temperature* ⟨741⟩.

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩

Sample: 5 g

Acceptance criteria: NMT 0.1%

- **LIMIT OF NICKEL**

Nickel standard solution: Immediately before use, prepare the equivalent of 0.2 µg/mL of nickel by diluting 10 mL of nickel standard solution TS with water to 500 mL.

Sample solution: Weigh 5.0 g of Hydrogenated Coconut Oil into a previously tared platinum or silica crucible. Cautiously heat the substance, and introduce into it a wick formed from twisted, ashless filter paper. Ignite the wick. When the substance ignites, stop heating. After combustion, ignite in a muffle furnace at about 600°. Continue the incineration until a white ash is obtained. After cooling, with the aid of two 2-mL portions of diluted hydrochloric acid, transfer the residue to a 25-mL volumetric flask, add 0.3 mL of nitric acid, and dilute with water to volume.

Standard solutions: Into four separate identical 10-mL volumetric flasks, introduce respectively 0, 1.0, 2.0, and 4.0 mL of *Nickel standard solution*. To each flask add a 2.0-mL portion of the *Sample solution*, and dilute with water to volume to obtain four *Standard solutions* containing an added quantity of nickel of 0, 0.2, 0.4, and 0.8 µg, respectively.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometer, equipped with a graphite furnace

Analytical wavelength: 232.0 nm

Lamp: Nickel hollow-cathode

Analysis

Samples: *Standard solutions*

Concomitantly determine the absorbances of the *Standard solutions* at least three times each. Record the average of the steady readings for each of the *Standard solutions*. Plot the absorbances of the *Standard solutions* versus the added quantity, in µg, of nickel. Extrapolate the line joining the points on the graph until it meets the quantity axis. The distance between this point and the intersection of the axes represents the quantity of nickel, in µg, in the 2-mL portion of the *Sample solution*.

Calculate the content of nickel in the portion of the sample taken:

$$\text{Result} = [V \times (A/V_A)]/W$$

V = volume of the *Sample solution*, 25 mL
A = nickel, as determined above
V_A = volume of the *Sample solution* added to the *Standard solutions*, 2 mL
W = weight of Hydrogenated Coconut Oil taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 1 µg/g

- **ALKALINE IMPURITIES**

Sample: 2.0 g

Analysis: Dissolve the *Sample* by gently heating in a mixture of 1.5 mL of alcohol and 3.0 mL of toluene. Add 0.05 mL of bromophenol blue TS, and titrate with 0.01 N hydrochloric acid VS to a yellow endpoint.

Acceptance criteria: NMT 0.4 mL of 0.01 N hydrochloric acid VS is required.

SPECIFIC TESTS

- **MELTING RANGE OR TEMPURATURE** ⟨741⟩: 30°–38°
- **FATS AND FIXED OILS,** *Acid Value, Method II* ⟨401⟩: NMT 2.0
- **FATS AND FIXED OILS,** *Fatty Acid Composition* ⟨401⟩: Hydrogenated Coconut Oil exhibits the following composition profile of fatty acids in *Table 1*.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
6	0	≤1.5
8	0	5.0–9.0
10	0	4.0–9.0
12	0	44.0–52.0
14	0	15.0–20.0
16	0	8.0–11.0
18	0	8.0–14.0
20	0	≤0.2
>20	0	≤0.5
16	1	≤1.0
18	1	≤1.5
18	2	≤0.5
18	3	≤0.2
20	1	≤0.2

- **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩: NMT 5.0
- **FATS AND FIXED OILS,** *Unsaponifiable Matter* ⟨401⟩: NMT 0.8%
- **LOSS ON DRYING** ⟨731⟩

Analysis: Dry a sample at 105° for 4 h.

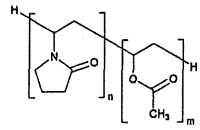
Acceptance criteria: NMT 0.1%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at a temperature not exceeding 55°.

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Copovidone



$(C_6H_9NO)_n + (C_4H_6O_2)_m$

Acetic acid ethenyl ester polymer with 1-ethenyl-2-pyrrolidone;

1-Vinyl-2-pyrrolidone polymer with vinyl acetate [25086-89-9].

DEFINITION

Copovidone is a copolymer of 1-vinyl-2-pyrrolidone and vinyl acetate in the mass proportion of 3:2. The nominal K-value of copovidone as stated in the labeling is NLT 90.0% and NMT 110.0%.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
- **B.**

Sample solution: 20 mg/mL

Analysis: To 5 mL of the *Sample solution*, add a few drops of iodine TS.

Acceptance criteria: A deep red color is produced.

ASSAY

- **PROCEDURE 1: CONTENT OF COPOLYMERIZED VINYL ACETATE**

Analysis: Determine the saponification value as directed under *Fats and Fixed Oils* ⟨401⟩, *Saponification Value*.

Calculate the percentage of copolymerized vinyl acetate in the portion of Copovidone taken:

$$\text{Result} = 0.1 \times (M_{r1}/M_{r2}) \times S$$

M_{r1}	= molecular weight of vinyl acetate, 86.09
M_{r2}	= molecular weight of potassium hydroxide, 56.11
S	= saponification value

Acceptance criteria: 35.3%–41.4% of the copolymerized vinyl acetate component, calculated on the dried basis

- **PROCEDURE 2: NITROGEN DETERMINATION,** *Method II* ⟨461⟩

Analysis: Proceed as directed using 0.1 g of Copovidone. In the procedure, use 5 g of a powdered mixture of potassium sulfate, cupric sulfate, and titanium dioxide (33:1:1) instead of potassium sulfate and cupric sulfate (10:1); omit the use of hydrogen peroxide; and heat until the solution has a clear, yellow-green color and the sides of the flask are free from carbonaceous material. Then heat for a further 45 min; add 20 mL of water, instead of 70 mL, after the second heating; and use bromocresol green–methyl red TS instead of methyl red–methylene blue TS. Titrate the distillate with 0.05 N sulfuric acid VS until the color of the solution changes from green through pale grayish blue to pale grayish red-purple.

Acceptance criteria: 7.0%–8.0% on the dried basis

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%
- **LIMIT OF ALDEHYDES**

Solution A: 17.4 mg/mL of monobasic potassium phosphate, adjusted if necessary, with 1 N potassium hydroxide to a pH of 9.0

Solution B: Transfer a quantity of lyophilized aldehyde dehydrogenase equivalent to 70 units to a glass vial, and dissolve in 10.0 mL of water. [NOTE—This solution is stable for 8 h at 4°.]

Solution C: 40 mg of nicotinamide adenine dinucleotide in 10 mL of *Solution A*, in a glass vial. [NOTE—This solution is stable for 4 weeks at 4°.]

Blank solution: Water

Standard solution: Transfer 2 mL of water at 4° to a glass weighing bottle, and weigh. Add 100 mg of freshly distilled acetaldehyde, and weigh. Transfer this solution to a 100-mL volumetric flask. Rinse the weighing bottle with several portions of water at 4°, and transfer each rinsing to the 100-mL volumetric flask. Dilute the solution in the 100-mL volumetric flask with water at 4° to volume. Store at 4° for 20 h. Transfer 1 mL of this solution to a 100-mL volumetric flask, and dilute with *Solution A* to volume.

Sample solution: 10 mg/mL of Copovidone in *Solution A*, in a 100-mL volumetric flask. Insert a stopper into the flask, heat at 60° for 1 h, and cool to room temperature.

Analysis: Pipet 0.5 mL each of the *Standard solution*, *Sample solution*, and *Blank solution* into separate 1-cm cells. Add 2.5 mL of *Solution A* and 0.2 mL of *Solution C* to each cell. Cover the cells to exclude oxygen. Mix by inversion, and allow to stand for 2–3 min at 22 ± 2°. Determine the absorbances of the solutions at a wavelength of 340 nm. Add 0.05 mL of *Solution B* to each cell. Cover the cells to exclude oxygen. Mix by inversion, and allow to stand for 5 min at 22 ± 2°. Determine the absorbances of the solutions at a wavelength of 340 nm.

Calculate the percentage of aldehydes, expressed as acetaldehyde, in the portion of Copovidone taken:

$$\text{Result} = \{[(A_{U2} - A_{U1}) - (A_{B2} - A_{B1})]/[(A_{S2} - A_{S1}) - (A_{B2} - A_{B1})]\} \times (C/W) \times 10$$

A_{U2}	= absorbance of the solution from the *Sample solution*, after the addition of *Solution B*
A_{U1}	= absorbance of the solution from the *Sample solution*, before the addition of *Solution B*
A_{B2}	= absorbance of the solution from the *Blank solution*, after the addition of *Solution B*
A_{B1}	= absorbance of the solution from the *Blank solution*, before the addition of *Solution B*
A_{S2}	= absorbance of the solution from the *Standard solution*, after the addition of *Solution B*
A_{S1}	= absorbance of the solution from the *Standard solution*, before the addition of *Solution B*
C	= concentration of acetaldehyde in the *Standard solution* (mg/mL)
W	= weight, calculated on the dried basis, of Copovidone taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.05%

- **LIMIT OF HYDRAZINE**

Standard solution: 9 µg/mL of salicylaldazine and 10 mg/mL of salicylaldehyde in toluene

Sample solution: Transfer the equivalent of 2.5 g of dried Copovidone to a 50-mL centrifuge tube, add 25 mL of water, and mix to dissolve. Add 500 µL of a 50-mg/mL solution of salicylaldehyde in methanol, adjust the solution with 0.25 N sulfuric acid to a pH of about 2, swirl, and heat in a water bath at 60° for 15 min. Allow to cool, add 2.0 mL of toluene, insert a stopper in the tube, shake vigorously for 2 min, and centrifuge. Use the clear upper toluene layer.

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

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Adsorbent: 0.25-mm layer of dimethylsilanized chromatographic silica gel mixture
Application volume: 10 µL
Developing solvent system: Acetonitrile and water (17:3)

Analysis
Samples: *Standard solution* and *Sample solution*
Proceed as directed in the chapter. Allow the spots to dry, and develop the chromatogram in the *Developing solvent system* until the solvent front has moved about three-fourths of the length of the plate. Locate the spots on the plate by examination under UV light at a wavelength of 365 nm: salicylaldazine appears as a fluorescent spot having an R_F value of about 0.6–0.7, and the fluorescence of any salicylaldazine spot from the *Sample solution* is not more intense than that produced by the spot from the *Standard solution*.

Acceptance criteria: NMT 1 ppm

• **LIMIT OF PEROXIDES**
Copovidone solution: 40 mg/mL of Copovidone in water calculated on the dried basis
Sample solution: Transfer 25.0 mL of *Copovidone solution* to a 50-mL beaker, and add 2 mL of titanium trichloride–sulfuric acid TS. Allow to stand for 30 min at room temperature.
Blank solution: Transfer 25.0 mL of *Copovidone solution* to a 50-mL beaker, and add 2 mL of 13% sulfuric acid.
Spectrometric conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: UV-Vis
Analytical wavelength: 405 nm
Cell: 1 cm
Blank: *Blank solution*
Analysis: Determine the absorbance of the *Sample solution*.
Acceptance criteria: The absorbance is NMT 0.35 (corresponding to NMT 0.04%, expressed as hydrogen peroxide).

• **LIMIT OF MONOMERS (1-VINYL-2-PYRROLIDONE, VINYL ACETATE, AND 2-PYRROLIDONE)**
Solution A: Water, acetonitrile, and methanol (90:5:5)
Solution B: Water, acetonitrile, and methanol (50:45:5)
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
2	100	0
26	80	20
27	0	100
36	0	100
38	100	0

Standard stock solution: 0.50 mg/mL of 1-vinyl-2-pyrrolidone, 0.50 mg/mL of vinyl acetate, and 3.0 mg/mL of 2-pyrrolidone in methanol
Standard solution: *Standard stock solution* in *Solution A* (1 in 2000)
Sample solution: Dissolve 250 mg of Copovidone in 1 mL of methanol, mix ultrasonically, dilute with water to 10 mL. If necessary, filter to remove undissolved particles.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 205 nm and 235 nm
Column
Guard: 4.0-mm × 2.5-cm; packing L1
Analytical: 4.0-mm × 25-cm; 5-µm packing L1
Column temperature: 30°
Injection size: 10 µL
Flow rate: 1.0 mL/min
System suitability
Sample: *Standard solution*
Suitability requirements
Resolution: NLT 2.0 between the 2-pyrrolidone and vinyl acetate peaks, and NLT 2.0 between the vinyl acetate and 1-vinyl-2-pyrrolidone peaks. [NOTE—According to the above operating conditions, the order of elution is 2-pyrrolidone, vinyl acetate, and 1-vinyl-2-pyrrolidone.]
Relative standard deviation: NMT 2.0% for each analyte, on replicate injections

Analysis
Samples: *Standard solution* and *Sample solution*
[NOTE—After each injection of the *Sample solution* wash the polymeric material of Copovidone from the guard column by passing the *Mobile phase* through the column backwards for 30 min at the same flow rate.]
Calculate the content of 1-vinyl-2-pyrrolidone in the portion of Copovidone taken:

$$\text{Result} = (A_{TA}/A_{SA}) \times (C_{SA}/C_T) \times 100$$

A_{TA} = 1-vinyl-2-pyrrolidone peak response from the *Sample solution*
A_{SA} = 1-vinyl-2-pyrrolidone peak response from the *Standard solution*
C_{SA} = concentration of 1-vinyl-2-pyrrolidone in the *Standard solution* (mg/mL)
C_T = concentration of Copovidone in the *Sample solution* on the dried basis (mg/mL)

Calculate the content of vinyl acetate in the portion of Copovidone taken:

$$\text{Result} = (A_{TB}/A_{SB}) \times (C_{SB}/C_T) \times 100$$

A_{TB} = vinyl acetate peak response from the *Sample solution*
A_{SB} = vinyl acetate peak response from the *Standard solution*
C_{SB} = concentration of vinyl acetate in the *Standard solution* (mg/mL)
C_T = concentration of Copovidone in the *Sample solution* on the dried basis (mg/mL)

Calculate the content of 2-pyrrolidone in the portion of Copovidone taken:

$$\text{Result} = (A_{TC}/A_{SC}) \times (C_{SC}/C_T) \times 100$$

A_{TC} = 2-pyrrolidone peak response from the *Sample solution*
A_{SC} = 2-pyrrolidone peak response from the *Standard solution*
C_{SC} = concentration of 2-pyrrolidone in the *Standard solution* (mg/mL)
C_T = concentration of Copovidone in the *Sample solution* on the dried basis (mg/mL)

Acceptance criteria: NMT 0.001% of 1-vinyl-2-pyrrolidone, NMT 0.001% of vinyl acetate, and NMT 0.5% of 2-pyrrolidone

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SPECIFIC TESTS

- **Loss on Drying** ⟨731⟩: Dry a sample at 105° for 3 h: it loses NMT 5.0% of its weight.
- **Clarity and Color of Solution**

  Sample: 1.0 g

  Analysis: Dissolve the *Sample* in 10 mL of water.

  Acceptance criteria: The solution is clear or slightly opalescent and colorless to pale yellow or pale red.
- **K-Value**

  Sample solution: Transfer a quantity of undried Copovidone, equivalent to 1.0 g on the dried basis, to a 100-mL volumetric flask, and dissolve in and dilute with water to volume. Allow to stand for 1 h.

  Analysis: Determine the viscosity, using a capillary-tube viscometer (see *Viscosity—Capillary Methods* ⟨911⟩), of this solution at 25 ± 0.2°.

  Calculate the relative K-value of Copovidone:

$$\text{Result} = \left[\sqrt{300c\,\log z + (c + 1.5c\,\log z)^2} + 1.5c\,\log z - c\right] \Big/ \left(0.15\,c + 0.003\,c^2\right) \times \left(100/K_U\right)$$

c = weight on the dried basis, of the specimen tested in each 100.0 mL of solution (g)

z = viscosity of the *Sample solution* relative to that of water

K_U = nominal K-value stated on the label

Acceptance criteria: 90.0%–110.0%

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in tight containers. No storage requirements specified.
- **Labeling:** Label it to indicate its nominal K-value.
- **USP Reference Standards** ⟨11⟩

  USP Copovidone RS

Coriander Oil

DEFINITION

Coriander Oil is the volatile oil obtained by steam distillation from the dried ripe fruit of *Coriandrum sativum* L. (Fam. Apiaceae).

SPECIFIC TESTS

- **Specific Gravity** ⟨841⟩: Between 0.863 and 0.875
- **Optical Rotation,** *Angular Rotation* ⟨781A⟩: Between +8° and +15°
- **Refractive Index** ⟨831⟩: Between 1.462 and 1.472 at 20°
- **Solubility in 70% Alcohol:** One volume dissolves in 3 volumes of 70% alcohol.

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in tight, light-resistant containers, protect from light, and store at controlled room temperature. Avoid exposure to excessive heat.
- **Labeling:** The label states the Latin binomial and, following the official name, the part of the plant source from which the article was derived.

Corn Oil

[8001-30-7].

DEFINITION

Corn Oil is the refined fixed oil obtained from the embryo of *Zea mays* L. (Fam. Gramineae).

IDENTIFICATION

- **A. Identity by Fatty Acid Composition**

  Analysis: Proceed as directed in the test for *Fats and Fixed Oils* ⟨401⟩, *Procedures, Fatty Acid Composition.*

  Acceptance criteria: Meets the composition profile of fatty acids in *Table 1*
- **B. Identity by Triglyceride Profile**

  Analysis: Proceed as directed in *Identification of Fixed Oils by Thin-Layer Chromatography* ⟨202⟩, *Identification, Method I* or *Method II.*

  Acceptance criteria: Meets the requirements in the chapter

IMPURITIES

- **Alkaline Impurities**

  Sample: 10 mL of Corn Oil

  Analysis: Mix 10 mL of acetone and 0.3 mL of water, and add 0.05 mL of bromophenol blue TS. Neutralize the solution to a green color if necessary with 0.01 N hydrochloric acid or 0.01 N sodium hydroxide. Add the *Sample*, shake, and allow to stand. Titrate with 0.01 N hydrochloric acid VS to change the color of the upper layer to yellow.

  Acceptance criteria: NMT 0.1 mL of 0.01 N hydrochloric acid is required.

SPECIFIC TESTS

- **Fats and Fixed Oils** ⟨401⟩, *Procedures, Acid Value*: NMT 0.2
- **Fats and Fixed Oils** ⟨401⟩, *Procedures, Fatty Acid Composition*: Corn Oil exhibits the composition profile of fatty acids in *Table 1*.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
<14	0	≤0.1
14	0	≤0.1
16	0	8.6–16.5
16	1	≤0.5
18	0	1.0–3.3
18	1	20.0–42.2
18	2	39.4–62.0
18	3	0.5–1.5
20	0	≤0.8
20	1	≤0.5
22	0	≤0.3
22	1	≤0.1
24	0	≤0.4

- **Fats and Fixed Oils** ⟨401⟩, *Procedures, Peroxide Value*: NMT 10.0
- **Fats and Fixed Oils** ⟨401⟩, *Procedures, Sterol Composition*: The sterol fraction of the Corn Oil contains NMT 0.3% of brassicasterol.
- **Fats and Fixed Oils** ⟨401⟩, *Procedures, Unsaponifiable Matter*: NMT 1.5%
- **Water Determination** ⟨921⟩, *Method I*: NMT 0.1%. Use a mixture of equal volumes of decanol and anhydrous methanol as the solvent.

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ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and avoid exposure to excessive heat.
- **LABELING:** Where Corn Oil is intended for use in the manufacture of injectable dosage forms, it is so labeled.
- **OTHER REQUIREMENTS:** For Corn Oil intended for use in injectable dosage forms, specified in *Labeling*, the requirements for *Acid Value, Peroxide Value, Unsaponifiable Matter*, and *Water* under *Injections and Implanted Drug Products* ⟨1⟩, *Product Quality Tests Common to Parenteral Dosage Forms, Specific Tests, Vehicles and added substances, Nonaqueous vehicles* must be met.

Corn Syrup

[8029-43-4].

DEFINITION

Corn Syrup is an aqueous solution of saccharides obtained by partial hydrolysis of edible corn starch by food grade acids and/or enzymes. It contains NLT 20.0% reducing sugar content (dextrose equivalent) expressed as D-glucose, calculated on the dried basis.

IDENTIFICATION

- **A.**

Analysis: Add a few drops of a solution of Syrup (1 in 20) to 5 mL of hot, alkaline cupric tartrate TS.

Acceptance criteria: A copious, red precipitate of cuprous oxide is formed.

ASSAY

- **REDUCING SUGARS (DEXTROSE EQUIVALENT)**

Apparatus: Mount a ring support on a ring stand 1–2 in above a gas burner, and mount a second ring 6–7 in above the first. Place 6-in open-wire gauze on the lower ring to support a 250-mL conical flask, and place a 4-in watch glass with a center hole on the upper ring to deflect heat. Attach a 25-mL buret to the ring stand so that the tip just passes through the watch glass centered above the flask. Place an indirectly lighted white surface behind the assembly for observing the endpoint.

Standard solution: 6 mg/mL of USP Dextrose RS

Sample solution: 10 mg/mL of Corn Syrup

Analysis: Transfer 25.0-mL portions of alkaline cupric tartrate TS to each of two flasks, and boil. Immediately place one flask on the wire gauze of the *Apparatus*, and adjust the burner so that the boiling point is reached in about 2 min. Titrate with the *Standard solution* to within 0.5 mL of the anticipated endpoint. Heat the flask, with swirling, boil moderately for 2 min, and add 2 drops of methylene blue solution (1 in 100). Immediately add 2 drops of the *Standard solution* from the buret, and bring to a boil. Allow the cuprous oxide to settle slightly, and observe the color of the supernatant. Complete the titration within 1 min by adding the *Standard solution* dropwise, and boil after each addition to the disappearance of the blue color, as determined by viewing against a white background in daylight or under equivalent illumination. If more than 0.5 mL of the titrant is required after the addition of the indicator, repeat the titration, adding the necessary volume of titrant before adding the indicator. Bring the contents of the second flask to a boil, and similarly titrate with the *Sample solution*.

Calculate the percentage of reducing sugars as D-glucose, calculated on the dried basis, in the portion of Corn Syrup taken:

$$\text{Result} = (C_S/C_U) \times (V_S/V_U) \times [1/(0.01 \times A)] \times 100$$

C_S = concentration of USP Dextrose RS in the *Standard solution* (mg/mL)

C_U = concentration of Corn Syrup in the *Sample solution* (mg/mL)

V_S = titrated volume of the *Standard solution* (mL)

V_U = titrated volume of the *Sample solution* (mL)

A = percentage of dry solids in Corn Syrup measured by the refractive index

Acceptance criteria: NLT 20.0% reducing sugar content on the dried basis

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩

Sample: 20 g

Acceptance criteria: NMT 0.5%

- **LIMIT OF LEAD**

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glassware before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin. For digestion, use acid-cleaned, high-density polyethylene, polypropylene, polytef, or quartz tubes. Select all reagents to have as low a content of lead as practicable, and store all reagent solutions in borosilicate glass containers. Cleanse glassware before use by soaking in warm 8 N nitric acid for 30 min and rinsing with deionized water. Store final diluted solutions in acid-cleaned plastic or polytef tubes or bottles.]

Modifier solution: 200 mg/mL of magnesium nitrate. Just before use, transfer 1.0 mL of this solution to a 10-mL volumetric flask, and dilute with 5% nitric acid to volume.

Standard stock solution: Transfer 10.0 mL of lead nitrate stock solution TS to a 100-mL volumetric flask, add 40 mL of water and 5 mL of nitric acid, and dilute with water to volume. Transfer 1.0 mL of this solution to a second 100-mL volumetric flask, dilute with 5% nitric acid to volume, and mix. This solution contains 0.1 µg/mL of lead.

Standard solutions: Transfer portions of *Standard stock solution* to four suitable containers, and dilute with 5% nitric acid to obtain *Standard solutions* having lead concentrations of 100, 50, 25, and 10 ng/mL, respectively.

Sample solution: [NOTE—Perform this procedure in a fume hood.] Transfer 1.5 g of Corn Syrup to a digestion tube, and add 0.75 mL of nitric acid to the tube. Warm the solution slowly (to avoid spattering) to 90°–95°. Heat until all brown vapors have dissipated and any rust-colored tint has disappeared from the tube (20–30 min). Cool, add 0.5 mL of 50% hydrogen peroxide, dropwise, to the solution, heat to 90°–95° for 5 min, and cool. Add a second 0.5-mL portion of 50% hydrogen peroxide dropwise to the solution, and heat to 90°–100° until clear (5–10 min). Cool, and transfer the solution to a 10-mL volumetric flask. Rinse the digestion tube with 5% nitric acid, add the rinse to the volumetric flask, dilute with 5% nitric acid to volume, and mix.

Standard blank: 5% Nitric acid

Sample blank: Transfer 1.5 g of water to a digestion tube, and proceed as directed for the *Sample solution*, beginning with "add 0.75 mL of nitric acid".

Instrumental conditions

Mode: Graphite furnace atomic absorption with pyrolytically coated graphite tubes and adequate means of background correction

Lamp: Lead hollow-cathode

Analytical wavelength: Lead emission line of 283.3 nm

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Furnace program: See *Table 1*. [NOTE—The temperature program may be modified to obtain optimum furnace temperatures.]

Table 1

Step	Dry	Ash	Purge	Atomize
Temperature (°)	200	750	Cool down, and purge the air from the furnace 20	1800
Ramp time(s)	20	40	—	0
Hold time(s)	30	40	60	10
Argon flow rate (mL/min)	300	300	300	Argon gas flow stopped

Injection volume: 20 µL

Analysis

[NOTE—Use peak area measurements for all quantitations.]

Samples: Add 5 µL of the *Modifier solution* to 20 µL each of the *Standard solutions*, the *Sample solution*, the *Standard blank*, and the *Sample blank*, and mix.

Separately inject equal volumes (about 20 µL) of the *Samples* into the instrument for analysis. Using the *Standard blank* to set the instrument to zero, determine the integrated absorbances of the *Standard solutions*. Plot the integrated absorbances of the *Standard solutions* versus their contents of lead, in ng/mL, and draw the line best fitting the four points to determine the calibration curve. Similarly determine the integrated absorbances of the *Sample solution* and the *Sample blank*. Correct the absorbance value of the *Sample solution* by subtracting from it the absorbance value obtained from the *Sample blank*.

Calculate the concentration of lead in the portion of Corn Syrup taken:

$$\text{Result} = (C_L \times V/W) \times F$$

C_L = concentration of lead in the *Sample solution*, as determined from the calibration curve (ng/mL)
V = volume of the *Sample solution*, 10 mL
W = weight of Corn Syrup taken to prepare the *Sample solution* (g)
F = conversion factor, 10^{-3} µg/ng

Acceptance criteria: NMT 0.5 µg/g

• **LIMIT OF SULFUR DIOXIDE**

Starch indicator solution: Mix 10 g of soluble starch with 50 mL of cold water, transfer to 1000 mL of boiling water, stir until completely dissolved, cool, and add 1 g of salicylic acid preservative. [NOTE—Discard this solution after 1 month.]

Sample: 100 g

Blank: 200 mL of water

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Direct titration

Titrant: 0.005 N iodine VS

Endpoint detection: Visual

Analysis: Transfer the *Sample* to a 250-mL conical flask, add 100 mL of water, and mix. Cool to 5°–10°. While stirring with a magnetic stirrer, add 10 mL of cold (5°–10°) 1.5 N sodium hydroxide. Stir for an additional 20 s, and add 10 mL of *Starch indicator solution*. Add 10 mL of cold (5°–10°) 2.0 N sulfuric acid, and titrate immediately with *Titrant* until a light blue color persists for 1 min. Perform a blank determination, and make any necessary correction.

Calculate the concentration, in ppm (µg/g), of sulfur dioxide (SO_2) in the *Sample* taken:

$$\text{Result} = [(V_S - V_B) \times N \times F_1/W] \times F_2$$

V_S = *Titrant* volume consumed by the *Sample* (mL)
V_B = *Titrant* volume consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F_1 = equivalency factor, 32.0 mg/mEq
W = *Sample* weight (g)
F_2 = conversion factor, 10^3 µg/mg

Acceptance criteria: NMT 40 µg/g

• **ABSENCE OF SOLUBLE STARCH**

Analysis: Dissolve 1 g in 10 mL of water, and add 1 drop of iodine TS.

Acceptance criteria: A yellow color indicates the absence of soluble starch.

SPECIFIC TESTS

• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.

• **TOTAL SOLIDS**

Instrumental conditions

(See *Refractive Index* ⟨831⟩.)

Mode: Refractometer equipped with a jacket for water circulation or some other mechanism for maintaining the sample at 20 ± 0.1° or 45 ± 0.1°

Before proceeding with measurements, ensure that the sample and the prism have reached the equilibrium temperature and that the instrument has been properly checked and calibrated against a standard provided by the manufacturer.

Analysis: Measure the refractive index of Corn Syrup, and convert the value to approximate percent solids value using *Table 2* and *Table 3*. [NOTE—*Table 2* covers the approximate total solids levels of these products in commerce. If the ash or dextrose equivalent of the sample differs from that of the product in *Table 2*, use *Table 3* for the ash and dextrose equivalent correction.]

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Table 2. Reference for Converting the Refractive Index to Approximate Percent Solids (Corn Syrup)

Dextrose Equivalent (DE)	Dry Substance (DS) (%)	Refractive Index at 20°	Refractive Index at 45°	Degrees Baumé at 140° F (60° C) + 1
28 DE Corn syrup—0.3% ash	76.0	1.4888	1.4837	40.98
	77.0	1.4915	1.4864	41.49
	78.0	1.4943	1.4892	42.00
	79.0	1.4971	1.4919	42.51
	80.0	1.4999	1.4947	43.01
34 DE High-maltose corn syrup—0.3% ash	78.6	1.4933	1.4882	41.99
	79.6	1.4960	1.4909	42.49
	80.6	1.4988	1.4936	42.99
	81.6	1.5015	1.4964	43.49
	82.6	1.5043	1.4992	43.99
36 DE Corn syrup—0.3% ash	78.4	1.4938	1.4887	42.01
	79.4	1.4965	1.4914	42.52
	80.4	1.4993	1.4941	43.02
	81.4	1.5021	1.4969	43.52
	82.4	1.5049	1.4997	44.02
43 DE High-maltose corn syrup—0.3% ash	78.9	1.4934	1.4883	42.00
	79.9	1.4961	1.4910	42.51
	80.9	1.4988	1.4937	43.01
	81.9	1.5016	1.4964	43.51
	82.9	1.5044	1.4992	44.01
43 DE Corn syrup—0.3% ash	78.7	1.4933	1.4882	42.01
	79.7	1.4960	1.4909	42.51
	80.7	1.4988	1.4936	43.02
	81.7	1.5015	1.4964	43.52
	82.7	1.5043	1.4992	44.01
43 DE Corn syrup (ion-exchanged)—0.03% ash	78.8	1.4935	1.4884	41.99
	79.8	1.4962	1.4911	42.50
	80.8	1.4990	1.4938	43.00
	81.8	1.5018	1.4966	43.50
	82.8	1.5045	1.4994	43.99
53 DE Corn syrup—0.3% ash	80.5	1.4962	1.4911	42.64
	81.5	1.4989	1.4938	43.14
	82.5	1.5016	1.4965	43.64
	83.5	1.5044	1.4992	44.13
	84.5	1.5072	1.5020	44.63
63 DE Corn syrup—0.3% ash	81.0	1.4955	1.4904	42.53
	82.0	1.4982	1.4931	43.02
	83.0	1.5009	1.4958	43.52
	84.0	1.5037	1.4985	44.01
	85.0	1.5064	1.5012	44.50

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Table 2. Reference for Converting the Refractive Index to Approximate Percent Solids (Corn Syrup) *(continued)*

Dextrose Equivalent (DE)	Dry Substance (DS) (%)	Refractive Index at 20°	Refractive Index at 45°	Degrees Baumé at 140° F (60° C) + 1
63 DE Corn syrup (ion-exchanged)—0.03% ash	81.3	1.4963	1.4912	42.60
	82.3	1.4990	1.4939	43.10
	83.3	1.5017	1.4965	43.59
	84.3	1.5044	1.4993	44.09
	85.3	1.5072	1.5020	44.58
66 DE Corn syrup—0.3% ash	81.0	1.4949	1.4898	42.36
	82.0	1.4975	1.4924	42.86
	83.0	1.5002	1.4951	43.36
	84.0	1.5029	1.4978	43.85
	85.0	1.5056	1.5005	44.35
95 DE Corn syrup—0.3% ash	69.0	1.4598	1.4550	35.46
	70.0	1.4621	1.4573	35.96
	71.0	1.4644	1.4596	36.46
	72.0	1.4668	1.4619	36.96
	73.0	1.4692	1.4643	37.45
95 DE Corn syrup (ion-exchanged)—0.03% ash	69.0	1.4597	1.4549	35.39
	70.0	1.4620	1.4572	35.89
	71.0	1.4644	1.4595	36.39
	72.0	1.4667	1.4619	36.89
	73.0	1.4691	1.4642	37.38

Acceptance criteria: The total solids value is NLT 70.0%.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.
- **LABELING:** Label it to indicate its nominal dextrose equivalent. Label it also to indicate the presence of sulfur dioxide if the residual concentration is greater than 10 ppm (µg/g).
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Dextrose RS

High Fructose Corn Syrup

DEFINITION

High Fructose Corn Syrup is a clear aqueous solution of saccharides prepared from high-dextrose-equivalent corn starch hydrolysate by the partial enzymatic conversion of dextrose to fructose, using an insoluble glucose isomerase enzyme preparation that complies with 21 CFR 184.1372. It is available in two types, 42% and 55%, based on fructose as a proportion of total saccharides. High Fructose Corn Syrup 42% contains NLT 97.0% of total saccharides, expressed as a percentage of total solids, of which NLT 92.0% consists of monosaccharides, including 41.0%–50.0% of fructose, and NMT 8.0% consists of other saccharides. High Fructose Corn Syrup 55% contains NLT 95.0% of total saccharides, expressed as a percentage of total solids, of which NLT 94.0% consists of monosaccharides, including 54.0%–60.0% of fructose, and NMT 6.0% consists of other saccharides.

IDENTIFICATION

- **A.**
  Analysis: Add a few drops of a solution (1 in 10) of Syrup to 5 mL of hot, alkaline cupric tartrate TS.
  Acceptance criteria: A copious, red precipitate of cuprous oxide is formed (distinction from sucrose).
- **B.** It meets the requirements of the *Assay*.

ASSAY

- **PROCEDURE**
  Mobile phase: Water
  Standard solution: Prepare a solution in water, which contains a total of 10% (w/v) saccharide solids, using USP Dextrose RS, USP Fructose RS, and USP Maltose Monohydrate RS, in proportions that approximate, on the *Total Solids* basis, the composition of High Fructose Corn Syrup to be analyzed (see *Table 1*).

Table 1

Component	High Fructose Corn Syrup 42%	High Fructose Corn Syrup 55%
USP Fructose RS	4.2%	5.5%
USP Dextrose RS	5.0%	4.0%
USP Maltose Monohydrate RS	0.8%	0.5%

Sample solution: Dilute a known weight of Syrup, determined from the result of the test for *Total Solids*, with water to have 10% (w/v) solids.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Refractive index

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Table 3. Ash and Dextrose Equivalent (DE) Corrections for Corn Syrup: Changes in Refractive Index for an Increase in Dry Substance (DS) (Corn Syrup)

Dry Substance (DS) (%)	1% Ash	1 Dextrose Equivalent (DE)
2	0.000000	–0.000001
4	0.000000	–0.000003
6	0.000001	–0.000005
8	0.000002	–0.000007
10	0.000003	–0.000010
12	0.000004	–0.000012
14	0.000006	–0.000015
16	0.000008	–0.000017
18	0.000010	–0.000020
20	0.000013	–0.000023
22	0.000016	–0.000026
24	0.000019	–0.000029
26	0.000022	–0.000033
28	0.000026	–0.000036
30	0.000030	–0.000040
32	0.000034	–0.000044
34	0.000039	–0.000048
36	0.000044	–0.000052
38	0.000049	–0.000057
40	0.000055	–0.000061
42	0.000061	–0.000066
44	0.000068	–0.000071
46	0.000074	–0.000076
48	0.000082	–0.000081
50	0.000089	–0.000087
52	0.000097	–0.000093
54	0.000105	–0.000099
56	0.000114	–0.000105
58	0.000123	–0.000112
60	0.000133	–0.000118
62	0.000143	–0.000125
64	0.000153	–0.000132
66	0.000164	–0.000140
68	0.000175	–0.000147
70	0.000187	–0.000155
72	0.000199	–0.000163
74	0.000212	–0.000172
76	0.000225	–0.000181
78	0.000239	–0.000190
80	0.000253	–0.000199
82	0.000268	–0.000208
84	0.000283	–0.000218

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Column: 7.8-mm × 30-cm; packing L19
Temperatures
Detector: 45°
Column: 85°
Flow rate: 0.6 mL/min
Injection volume: 10 µL
Run time: 20 min
System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for maltose, dextrose, and fructose are about 0.83, 1.00, and 1.32, respectively.]
Suitability requirements
Resolution: NLT 1.5 between maltose and dextrose, and NLT 1.5 between dextrose and fructose
Relative standard deviation: NMT 1.0% for the fructose peak
Analysis
Samples: *Standard solution* and *Sample solution*
Inject a volume (about 10 µL) of the *Sample solution*, and measure all the peak areas. The elution pattern includes the higher degree of polymerized saccharides (DP4+), followed by tri-saccharides (DP3), maltose, dextrose, and fructose. The higher-molecular-weight polysaccharides containing more than 4 D-glucopyranosyl units can be integrated into one peak represented by the peak of DP4+.
[NOTE—The relative retention times for higher degrees of polymerized saccharides (DP4+), tri-saccharides (DP3), maltose, dextrose, and fructose are about 0.66, 0.74, 0.82, 1.00, and 1.32, respectively, from the *Sample solution*.]
Calculate the percentage of monosaccharides, P_{MS}, expressed in terms of fructose (P_F) and dextrose (P_D), in the total solid portion of Syrup taken:

$$P_{MS} = P_F + P_D$$

$$P_F = (r_{FU}/r_{FS}) \times [C_{FS} \times V_U/(W_U \times 0.01 \times P_{Solid})] \times 100$$

$$P_D = (r_{DU}/r_{DS}) \times [C_{DS} \times V_U/(W_U \times 0.01 \times P_{Solid})] \times 100$$

r_{FU} = peak area of fructose from the *Sample solution*
r_{FS} = peak area of fructose from the *Standard solution*
C_{FS} = concentration of USP Fructose RS in the *Standard solution* (mg/mL)
V_U = volume of the *Sample solution* (mL)
W_U = weight of Syrup taken to prepare the *Sample solution* (mg)
P_{Solid} = percentage of total solids in the Syrup as determined in the test for *Total Solids*
r_{DU} = peak area of dextrose from the *Sample solution*
r_{DS} = peak area of dextrose from the *Standard solution*
C_{DS} = concentration of USP Dextrose RS in the *Standard solution* (mg/mL)

Calculate the percentage of other saccharides, P_{OS}, expressed in terms of maltose (P_{DP2}), tri-saccharides (DP3) (P_{DP3}), and higher degree of polymerized saccharides (DP4+) (P_{DP4+}), in the total solid portion of Syrup taken:

$$P_{OS} = P_{DP2} + P_{DP3} + P_{DP4+} = [(r_{U1} + r_{U2} + r_{U3})/r_S] \times [C_S \times V_U/(W_U \times 0.01 \times P_{Solid})] \times 100$$

r_{U1} = peak area of maltose from the *Sample solution*
r_{U2} = peak area of tri-saccharides (DP3) from the *Sample solution*
r_{U3} = peak area of higher degree of polymerized saccharides (DP4+) from the *Sample solution*
r_S = peak area of maltose from the *Standard solution*
C_S = concentration of USP Maltose Monohydrate RS in the *Standard solution* (mg/mL)
V_U = volume of the *Sample solution* (mL)
W_U = weight of Syrup taken to prepare the *Sample solution* (mg)
P_{Solid} = percentage of total solids in the Syrup as determined in the test for *Total Solids*

Calculate the percentage of total saccharides, P_{TS}, expressed as a percentage of total solids:

$$P_{TS} = P_{MS} + P_{OS}$$

Calculate the percentage of monosaccharides in total saccharides:

$$\text{Result} = (P_{MS}/P_{TS}) \times 100$$

Calculate the percentage of fructose in total saccharides:

$$\text{Result} = (P_F/P_{TS}) \times 100$$

Calculate the percentage of other saccharides in total saccharides:

$$\text{Result} = (P_{OS}/P_{TS}) \times 100$$

Acceptance criteria
For High Fructose Corn Syrup 42%
Total saccharides: NLT 97.0%, expressed as a percentage of total solids. Total saccharides contain monosaccharides and other saccharides as follows.
Monosaccharides: NLT 92.0%
Fructose: 41.0%–50.0%
Other saccharides: NMT 8.0%
For High Fructose Corn Syrup 55%
Total saccharides: NLT 95.0%, expressed as a percentage of total solids. Total saccharides contain monosaccharides and other saccharides as follows.
Monosaccharides: NLT 94.0%
Fructose: 54.0%–60.0%
Other saccharides: NMT 6.0%

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.05%
- **LIMIT OF LEAD**

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glassware before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin. For digestion, use acid-cleaned, high-density polyethylene, polypropylene, polytef, or quartz tubes. Select all reagents to have as low a content of lead as practicable, and store all reagent solutions in borosilicate glass containers. Cleanse glassware before use by soaking in warm 8 N nitric acid for 30 min and rinsing with deionized water. Store final diluted solutions in acid-cleaned plastic or polytef tubes or bottles.]
Matrix modifier solution: 200 mg/mL of magnesium nitrate. Just before use, transfer 1.0 mL of this solution to a 10-mL volumetric flask, and dilute with 5% nitric acid to volume.
Alternate matrix modifier solution: Just before use, add 0.3 mL of commercially available 10,000 µg/mL palladium standard solution and 5 mL of commercially available 10,000 µg/mL magnesium nitrate standard solution to 9.7 mL of 5% nitric acid, and mix well. [NOTE—*Alternate matrix modifier solution* can be used to replace the *Matrix*

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modifier solution. Then the air ashing step in the furnace program (see *Table 2*) can be omitted.]

Standard stock solution: Transfer 10.0 mL of lead nitrate stock solution TS to a 100-mL volumetric flask, add 40 mL of water and 5 mL of nitric acid, and dilute with water to volume. Transfer 1.0 mL of this solution to a second 100-mL volumetric flask, dilute with 5% nitric acid to volume, and mix. This solution contains 0.1 µg/mL of lead.

Standard solutions: Transfer portions of *Standard stock solution* to four suitable containers, and dilute with 5% nitric acid to obtain *Standard solutions* having lead concentrations of 100, 50, 25, and 10 ng/mL, respectively.

Sample solution: [NOTE—It is recommended to perform this procedure in a fume hood. To ensure that a representative subsample of Syrup is used for analysis, ultrasonic and/or vortex mixing of Syrup samples prior to weighing is recommended.] Transfer 1.5 g of Syrup to two digestion tubes, labeled "Sample solution" and "Temperature monitor solution," and add 0.75 mL of nitric acid to each tube. Place a thermometer in the tube labeled "Temperature monitor solution," then use the *Temperature monitor solution* solely to monitor the temperature to be within ranges specified by the method. Warm both solutions slowly to 90°–95° to avoid spattering. Heat until all brown vapors have dissipated and the samples no longer have a rust-colored tint. This typically takes 20–30 min. Allow the samples to cool. Add 0.5 mL of 50% hydrogen peroxide dropwise to both solutions, heat to 90°–95° for 5 min, and cool. Add a second 0.5-mL portion of 50% hydrogen peroxide dropwise to both solutions, and heat to 90°–100° for 5–10 min or until the solutions are clear. Cool, and transfer the *Sample solution* to a 10-mL volumetric flask. Rinse the tube labeled "Sample solution" with 5% nitric acid, add the rinsing to the volumetric flask, dilute with 5% nitric acid to volume, and mix.

Standard blank: 5% Nitric acid

Sample blank: Transfer 1.5 g of water to a digestion tube, and proceed as directed for the *Sample solution*, beginning with "add 0.75 mL of nitric acid".

Instrumental conditions

Mode: Graphite furnace atomic absorption with pyrolytically coated graphite tubes and adequate means of background correction

Lamp: Lead hollow-cathode

Analytical wavelength: Lead emission line of 283.3 nm

Furnace program: See *Table 2*. [NOTE—The temperature program may be modified to obtain optimum furnace temperatures.]

Table 2

Step	Temperature (°)	Ramp (s)	Hold Time (s)	Gas	Gas Flow Rate (mL/min)	Read (s)
Dry	200	20	30	Argon	300	—
Char (Ash)	750	40	40	Air[a]	300	—
Cool down	20	1	60	Argon	300	—
Atomize	1800	0	10	Argon	Stop flow	10
Clean	2600	1	7	Argon	300	—
Cool down	20	1	5	Argon	300	—

[a] If the *Matrix modifier solution* is used, air ashing must be used in the experiment. If the *Alternate matrix modifier solution* is used, air can be substituted with argon.

If the *Matrix modifier solution* is used, the furnace controller must be able to handle two gas flows to facilitate air ashing. Argon is used as the purge gas for the furnace for all steps but the char. Oxygen ashing is used to avoid build-up of residue during the char step. Breathing quality air is used as the alternative gas for the air ashing. The long (60 s) "Cool down" step prior to atomization ensures that the air used for the oxygen ashing (char) is cleared from the furnace.

Autosampler

Sample volume: 20 µL

Alternative volume: 5 µL of *Matrix modifier solution* (or *Alternate matrix modifier solution*)

Analysis

Samples: Add 5 µL of the *Matrix modifier solution* (or *Alternate matrix modifier solution*) to each 20-µL aliquot of the four *Standard solutions*, add 5 µL of the *Matrix modifier solution* (or *Alternate matrix modifier solution*) to 20 µL of the *Sample solution*, add 5 µL of the *Matrix modifier solution* (or *Alternate matrix modifier solution*) to 20 µL of the *Standard blank*, and add 5 µL of the *Matrix modifier solution* (or *Alternate matrix modifier solution*) to 20 µL of the *Sample blank*.

Use peak area measurements for all quantitations.

Using the *Standard blank* to set the instrument to zero, determine the integrated absorbances of the *Standard solutions*. Plot the integrated absorbances of the *Standard solutions* versus their contents of lead, in ng/mL, and draw the line best fitting the four points to determine the calibration curve. Similarly determine the integrated absorbances of the *Sample solution* and the *Sample blank*. Correct the absorbance value of the *Sample solution* by subtracting from it the absorbance value obtained from the *Sample blank*.

Calculate the concentration of lead, in µg/g, in the portion of Syrup taken:

$$\text{Result} = (V \times C_L/W) \times F$$

V = volume of the *Sample solution*, 10 mL

C_L = concentration of lead in the *Sample solution*, as determined from the calibration curve (ng/mL)

W = weight of Syrup taken to prepare the *Sample solution* (g)

F = conversion factor, 10^{-3} µg/ng

Acceptance criteria: NMT 0.1 µg/g

• **LIMIT OF SULFUR DIOXIDE**

Starch indicator solution: Mix 10 g of soluble starch with 50 mL of cold water. Transfer to 1000 mL of boiling water, stir until completely dissolved, cool, and add 1 g of salicylic acid preservative. [NOTE—Discard the solution after 1 month.]

Sample: 100 g

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Direct titration

Titrant: 0.005 N iodine VS

Blank: 200 mL of water

Endpoint detection: Visual

Analysis: Transfer the *Sample* to a 250-mL conical flask, add 100 mL of water, and mix. Cool to 5°–10°. While stirring with a magnetic stirrer, add 10 mL of cold (5°–10°) 1.5 N sodium hydroxide. Stir for an additional 20 s, and add 10 mL of *Starch indicator solution*. Add 10 mL of cold (5°–10°) 2.0 N sulfuric acid, and titrate immediately with *Titrant* until a light blue color persists for 1 min. Perform a blank determination, and make any necessary correction.

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Calculate the concentration, in µg/g, of sulfur dioxide (SO_2) in the *Sample* taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F_1]/W\} \times F_2$$

V_S = *Titrant* volume consumed by the *Sample* (mL)
V_B = *Titrant* volume consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F_1 = equivalency factor, 32.0 mg/mEq
W = *Sample* weight (g)
F_2 = conversion factor, 10^3 µg/mg

Acceptance criteria: NMT 30 µg/g

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.
- **TOTAL SOLIDS**

Analysis: Determine the refractive index of the Syrup at 20° or 45° (see *Refractive Index* ⟨831⟩). Use *Table 3* and *Table 4* for calculating the percentage of total solids on a weight/weight basis. If necessary, interpolate between the refractive index values to find the percentage of total solids to the nearest 0.1%.

Table 3. High Fructose Corn Syrup 42%

Percentage of Total Solids (P_{solid})(%)	Refractive Index at 20°	Refractive Index at 45°
69.0	1.4597	1.4543
70.0	1.4620	1.4565
70.5	1.4632	1.4577
71.0	1.4643	1.4589
72.0	1.4667	1.4612
73.0	1.4691	1.4635
74.0	1.4715	1.4658
75.0	1.4738	1.4683
76.0	1.4763	1.4707
77.0	1.4787	1.4731
78.0	1.4811	1.4755
79.0	1.4836	1.4779
80.0	1.4861	1.4804
81.0	1.4886	1.4829

Table 4. High Fructose Corn Syrup 55%

Percentage of Total Solids (P_{solid})(%)	Refractive Index at 20°	Refractive Index at 45°
75.0	1.4738	1.4680
76.0	1.4762	1.4704
76.5	1.4774	1.4716
77.0	1.4786	1.4728
78.0	1.4811	1.4752
79.0	1.4835	1.4776
80.0	1.4860	1.4801
81.0	1.4885	1.4826

Acceptance criteria
High Fructose Corn Syrup 42%: NLT 70.5%
High Fructose Corn Syrup 55%: NLT 76.5%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirement specified.
- **LABELING:** Label it to state, as part of the official title, the nominal percentage of fructose. Label it to indicate the presence of sulfur dioxide if the residual concentration is greater than 10 µg/g.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Dextrose RS
  USP Fructose RS
  USP Maltose Monohydrate RS

Corn Syrup Solids

DEFINITION

Corn Syrup Solids (Dried Glucose Syrup) is a dried mixture of saccharides obtained by partial hydrolysis of edible corn starch by food-grade acids and/or enzymes. It contains NLT 20.0% reducing sugar content (dextrose equivalent) expressed as D-glucose, calculated on the dried basis.

IDENTIFICATION

- **A.**

Sample solution: 50 mg/mL
Analysis: Add a few drops of the *Sample solution* to 5 mL of hot, alkaline cupric tartrate TS.
Acceptance criteria: A copious, red precipitate of cuprous oxide is formed (distinction from sucrose).

ASSAY

- **REDUCING SUGARS (DEXTROSE EQUIVALENT)**

Apparatus: Mount a ring support on a ring stand 1–2 in above a gas burner, and mount a second ring 6–7 in above the first. Place a 6-in open-wire gauze on the lower ring to support a 250-mL conical flask, and place a 4-in watch glass with a center hole on the upper ring to deflect heat. Attach a 25-mL buret to the ring stand so that the tip just passes through the watch glass centered above the flask. Place an indirectly lighted white surface behind the assembly for observing the endpoint.
Standard solution: 6 mg/mL of USP Dextrose RS
Sample solution: Transfer 4 g of Corn Syrup Solids to a 500-mL volumetric flask, and dilute with water to volume.
Analysis: Transfer 25.0-mL portions of alkaline cupric tartrate TS to each of two flasks, and boil. Immediately place one flask on the wire gauze of the *Apparatus*, and adjust the burner so that the boiling point will be reached in 2 min. Titrate with the *Standard solution* to within 0.5 mL of the anticipated endpoint. Heat the flask, with swirling, boil moderately for 2 min, and add 2 drops of a 10-mg/mL methylene blue solution. Immediately add 2 drops of the *Standard solution* from the buret, and bring to a boil. Allow the cuprous oxide to settle slightly, and observe the color of the supernatant. Complete the titration within 1 min by adding the *Standard solution* dropwise and boiling after each addition to the disappearance of the blue color, as determined by viewing against a white background in daylight or under equivalent illumination. If more than 0.5 mL of the titrant is required after the addition of the indicator, repeat the titration, adding the necessary volume of titrant before adding the indicator. Bring the contents of the second flask to a boil, and similarly titrate with the *Sample solution*.

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Calculate the percentage of reducing sugars as D-glucose, calculated on the dried basis, in the portion of Corn Syrup Solids taken:

$$\text{Result} = (C_S/C_U) \times (V_S/V_U) \times [1/(0.01 \times A)] \times 100$$

C_S = concentration of USP Dextrose RS in the *Standard solution* (mg/mL)
C_U = concentration of Corn Syrup Solids taken to prepare the *Sample solution* (mg/mL)
V_S = titrated volumes of the *Standard solution* (mL)
V_U = titrated volumes of the *Sample solution* (mL)
A = percentage of dry solids in Corn Syrup Solids, as determined in the test for *Total Solids*

Acceptance criteria: NLT 20.0% reducing sugar content (dextrose equivalent) expressed as D-glucose on the dried basis

IMPURITIES

- **Residue on Ignition** ⟨281⟩: NMT 0.5%
- **Limit of Sulfur Dioxide**

Starch indicator solution: Mix 10 g of soluble starch with 50 mL of cold water. Transfer to 1000 mL of boiling water, and stir until completely dissolved. Cool, and add 1 g of salicylic acid preservative. [NOTE—Discard the solution after 1 month.]
Sample: 78 g
Blank: 200 mL of water
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.005 N iodine VS
Endpoint detection: Visual
Analysis: Transfer the *Sample* to a 250-mL conical flask. Dilute with 122 mL of water, and mix to dissolve. Cool to 5°–10°. While stirring with a magnetic stirrer, add 10 mL of cold (5°–10°) 1.5 N sodium hydroxide. Stir for an additional 20 s, and add 10 mL of *Starch indicator solution*. Add 10 mL of cold (5°–10°) 2.0 N sulfuric acid, and titrate immediately with *Titrant* until a light blue color persists for 1 min. Perform a blank determination, and make any necessary correction.
Calculate the amount of sulfur dioxide (SO_2) in the *Sample* taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F_1] \times F_2\}/W$$

V_S = *Titrant* volume consumed by the *Sample* (mL)
V_B = *Titrant* volume consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F_1 = equivalency factor, 32.0 mg/mEq
F_2 = conversion factor, 10^3 µg/mg
W = *Sample* weight (g)

Acceptance criteria: NMT 40 µg/g

- **Limit of Lead**

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glassware before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin. For digestion, use acid-cleaned, high-density polyethylene, polypropylene, polytef, or quartz tubes. Select all reagents to have as low a content of lead as practicable, and store all reagent solutions in borosilicate glass containers. Cleanse glassware before use by soaking in warm 8 N nitric acid for 30 min and rinsing with deionized water. Store final diluted solutions in acid-cleaned plastic or polytef tubes or bottles.]
Modifier solution: 200 mg/mL of magnesium nitrate. Just before use, transfer 1.0 mL of this solution to a 10-mL volumetric flask, and dilute with 5% nitric acid to volume.
Standard solutions: Transfer 10.0 mL of lead nitrate stock solution TS to a 100-mL volumetric flask. Add 40 mL of water and 5 mL of nitric acid, dilute with water to volume, and mix. Transfer 1.0 mL of this solution to a 100-mL volumetric flask, and dilute with 5% nitric acid to volume. This solution contains 0.1 µg/mL of lead. Transfer portions of this solution to four suitable containers, and dilute with 5% nitric acid to obtain *Standard solutions* having lead concentrations of 100, 50, 25, and 10 ng/mL, respectively.
Sample solution: [NOTE—Perform this procedure in a fume hood.] Transfer 1.2 g of Corn Syrup Solids to two digestion tubes labeled *Sample solution* and temperature monitor solution, and add 0.75 mL of nitric acid to each tube. Warm both solutions slowly to 90°–95° to avoid spattering. Heat until all brown vapors have dissipated and any rust-colored tint is gone from the tube labeled *Sample solution* (20–30 min). Cool, then add 0.5 mL of 50% hydrogen peroxide dropwise to both solutions, and heat to between 90° and 95° for 5 min. Cool, then add a second 0.5-mL portion of 50% hydrogen peroxide dropwise to each solution, and heat to 90°–100° until clear (5–10 min). Cool, and transfer the solution labeled *Sample solution* to a 10-mL volumetric flask. Rinse the *Sample solution* digestion tube with 5% nitric acid, add the rinse to the volumetric flask, and dilute with 5% nitric acid to volume.
Standard blank: 5% Nitric acid
Sample blank: Transfer 1.5 g of water to a digestion tube, and proceed as directed for the *Sample solution*, beginning with "add 0.75 mL of nitric acid".
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometer equipped with pyrolytically coated graphite tubes and adequate means of background correction
Analytical wavelength: 283.3 nm
Injection volume: 20 µL
Furnace program: See *Table 1*.
[NOTE—The temperature program may be modified to obtain optimum furnace temperatures.]

Table 1

Step	Dry	Ash	Purge	Atomize
Temperature (°)	200	750	Cool down, and purge the air from the furnace 20	1800
Ramp time(s)	20	40	—	0
Hold time(s)	30	40	60	10
Argon flow rate (mL/min)	300	300	300	Argon gas flow stopped

Analysis
Samples: *Standard solutions*, *Sample solution*, *Standard blank*, *Sample blank*
Add 5 µL of the *Modifier solution* to 20 µL each of the *Standard solutions*, the *Sample solution*, the *Standard blank*, and the *Sample blank*, and mix. Separately inject each into the atomic absorption spectrophotometer.
Using the *Standard blank* to set the instrument to zero, determine the integrated absorbances of the *Standard*

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solutions. Plot the integrated absorbances of the *Standard solutions* versus their contents of lead, and draw the line best fitting the four points to determine the calibration curve. Similarly, determine the integrated absorbances of the *Sample solution* and the *Sample blank*. Correct the absorbance value of the *Sample solution* with the *Sample blank*.

Calculate the concentration of lead in the portion of Corn Syrup Solids taken:

$$\text{Result} = [V \times (C/W)] \times F$$

V = volume of the *Sample solution*, 10 mL
C = concentration of lead in the *Sample solution*, as determined from the calibration curve (ng/mL)
W = weight of Corn Syrup Solids taken to prepare the *Sample solution* (g)
F = conversion factor, 10^{-3} µg/ng

Acceptance criteria: NMT 0.5 µg/g

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined yeasts and molds count does not exceed 10^2 cfu/g.
- **STARCH**

Sample solution: 100 mg/mL

Analysis: Add 1 drop of iodine TS to 10 mL of the *Sample solution*.

Acceptance criteria: A yellow color indicates the absence of soluble starch.

- **TOTAL SOLIDS**

Analysis: To determine the water content, proceed as directed in *Water Determination* ⟨921⟩, *Method Ia*, except use a weighed amount of Corn Syrup Solids, W_U, for the *Test Preparation*. In *Standardization of the Reagent*, proceed as directed, except use the formula for significant amounts of water (1% or more). [NOTE—Pure methanol can make the detector overly sensitive, particularly at low ppm levels of water, causing it to deflect to dryness and slowly recover with each addition of reagent. This slows down the titration and may allow the system to actually pick up ambient moisture during the resulting long titration. Adding chloroform or a similar nonconducting solvent will retard this sensitivity and can improve the analysis.] In the *Procedure* calculating the water content in the *Test Preparation*, use $W_W = S \times F$.

Calculate the percentage of total solids in the portion of the *Test Preparation* taken:

$$\text{Result} = (W_U - W_W)/W_U \times 100$$

W_U = weight of Corn Syrup Solids for the *Test Preparation* (mg)
W_W = weight of water determined (mg)

Acceptance criteria: NLT 90.0% when the reducing sugar content is NLT 88.0%; NLT 93.0% when the reducing sugar content is 20.0%–88.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tightly closed containers, and store in a cool, dry place.
- **LABELING:** Label it to indicate its nominal dextrose equivalent. Label it also to indicate the presence of sulfur dioxide if the residual concentration is greater than 10 µg/g.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Dextrose RS

Cottonseed Oil

[8001-29-4].

DEFINITION

Cottonseed Oil is the refined fixed oil obtained from the seed of cultivated plants of various varieties of *Gossypium hirsutum* L. or of other species of *Gossypium* (Fam. Malvaceae). It may contain suitable antioxidants.

IDENTIFICATION

- **A. IDENTITY BY FATTY ACID COMPOSITION:** Cottonseed Oil exhibits the composition profile of fatty acids, as determined in *Fats and Fixed Oils* ⟨401⟩, *Procedures, Fatty Acid Composition*. See *Table 1*.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
<14	0 or 1	≤0.2
14	0	0.3–1.0
16	0	18.0–26.4
16	1	≤1.2
18	0	2.1–3.3
18	1	14.0–21.7
18	2	46.7–58.3
18	3	≤1.0
20	0	≤1.0
20	1	≤0.5
22	0	≤0.6
22	1	≤0.5
24	0	≤0.5

- **B. IDENTITY BY TRIGLYCERIDE PROFILE**

Analysis: Proceed as directed in *Identification of Fixed Oils by Thin-Layer Chromatography* ⟨202⟩, *Identification, Method I* or *Method II*.

Acceptance criteria: Meets the requirements in the chapter

IMPURITIES

- **ALKALINE IMPURITIES**

Sample: 10 mL of Cottonseed Oil

Analysis: Mix 10 mL of acetone and 0.3 mL of water, and add 0.05 mL of bromophenol blue TS. If necessary, neutralize the solution to a green color with 0.01 N hydrochloric acid or 0.01 N sodium hydroxide. Add the *Sample*, shake, and allow to stand. Titrate with 0.01 N hydrochloric acid VS to change the color of the upper layer to yellow.

Acceptance criteria: NMT 0.1 mL of 0.01 N hydrochloric acid is required.

SPECIFIC TESTS

- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Acid Value*: NMT 0.2
- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Peroxide Value*: NMT 10.0
- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Unsaponifiable Matter*: NMT 1.5%

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• **WATER DETERMINATION** ⟨921⟩, *Method I, Method Ic*: NMT 0.1%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and avoid exposure to excessive heat.

• **LABELING:** Label it to indicate the name and quantity of any added antioxidants. Where Cottonseed Oil is intended for use in the manufacture of injectable dosage forms, it is so labeled.

• **OTHER REQUIREMENTS:** For Cottonseed Oil intended for use in injectable dosage forms, which is specified in the *Labeling* section, the requirements under *Injections and Implanted Drug Products* ⟨1⟩, *Product Quality Tests Common to Parenteral Dosage Forms, Specific Tests, Vehicles and added substances, Nonaqueous vehicles* must be met.

Hydrogenated Cottonseed Oil

DEFINITION

Hydrogenated Cottonseed Oil is the product obtained by refining and hydrogenating oil obtained from seeds of cultivated plants of various varieties of *Gossypium hirsutum* L. or of other species of *Gossypium* (Fam. Malvaceae). The product consists mainly of triglycerides of palmitic and stearic acids.

IDENTIFICATION

• **A.** It meets the requirements in *Specific Tests* for *Fats and Fixed Oils, Fatty Acid Composition* ⟨401⟩.

• **B.** It meets the requirements in *Specific Tests* for *Melting Range or Temperature, Class II* ⟨741⟩.

IMPURITIES

• **LIMIT OF NICKEL**

Solution A: Immediately before use, dilute 10 mL of nickel standard solution TS with water to 500 mL. This solution contains the equivalent of 0.2 µg/g of nickel.

Sample solution: Weigh 5.0 g of Hydrogenated Cottonseed Oil into a previously tared platinum or silica crucible. Cautiously heat, and introduce into the substance a wick formed from twisted ashless filter paper. Ignite the wick. When the substance ignites, stop heating. After combustion, ignite in a muffle furnace at 600°. Continue the incineration until white ash is obtained. After cooling, transfer the residue, with the aid of two 2-mL portions of diluted hydrochloric acid, to a 25-mL volumetric flask, add 0.3 mL of nitric acid, and dilute with water to volume.

Standard solutions: Into three identical 10-mL volumetric flasks, introduce respectively 1.0, 2.0, and 4.0 mL of *Solution A*. To each flask add a 2.0-mL portion of the *Sample solution*, and dilute with water to volume.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry, equipped with a graphite furnace

Analytical wavelength: 232.0 nm

Lamp: Nickel hollow-cathode

Analysis: Concomitantly determine the absorbances of the *Sample solution* and each of the *Standard solutions* at least three times each, and record the average of the steady readings. Plot the absorbances of the *Sample solution* and the *Standard solutions* versus the added quantity of nickel. Extrapolate the line joining the points on the graph until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of nickel in the *Sample solution*.

Acceptance criteria: NMT 1 µg/g

• **ALKALINE IMPURITIES**

Sample solution: Dissolve by gentle heating 2.0 g of Hydrogenated Cottonseed Oil in a mixture of 1.5 mL of alcohol and 3.0 mL of toluene.

Analysis: To the *Sample solution* add 0.05 mL of bromophenol blue TS, and titrate with 0.01 N hydrochloric acid to a yellow endpoint.

Acceptance criteria: NMT 0.4 mL of 0.01 N hydrochloric acid is required.

SPECIFIC TESTS

• **FATS AND FIXED OILS,** *Fatty Acid Composition* ⟨401⟩: Hydrogenated Cottonseed Oil exhibits the composition profile of fatty acids in *Table 1*, as determined in the chapter.

Table 1

Carbon-Chain Length	No. of Double Bonds	Percentage (%)
<14	0	≤0.2
14	0	≤1.0
16	0	19–26
18	0	68–80
20	0	≤1.0
22	0	≤1.0
24	0	≤0.5
18	1	≤4.0
18	2	≤1.0

• **MELTING RANGE OR TEMPERATURE,** *Class II* ⟨741⟩: 57°–70°

• **FATS AND FIXED OILS,** *Acid Value (Free Fatty Acids)* ⟨401⟩

Sample: 10 g

Analysis: Dissolve the *Sample* in 50 mL of a hot mixture of neutralized alcohol and toluene (1:1), add 0.5 mL of phenolphthalein TS, and titrate with 0.1 N potassium hydroxide VS to produce a permanent, faint pink color.

Acceptance criteria: NMT 0.5

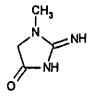
• **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩: NMT 5.0

• **FATS AND FIXED OILS,** *Unsaponifiable Matter* ⟨401⟩: NMT 1.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers. No storage requirements are specified.

Creatinine



$C_4H_7N_3O$ 113.12

DEFINITION

Creatinine contains NLT 98.5% and NMT 102.0% of $C_4H_7N_3O$, as Creatinine, calculated on the dried basis.

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IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

ASSAY

• **PROCEDURE**

Sample: 40 mg of Creatinine

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Direct titration

Titrant: 0.1 N perchloric acid VS

Endpoint detection: Potentiometric

Indicator electrode: Glass

Reference electrode: Silver–silver chloride

Reference electrode solution: Saturated lithium perchlorate and silver chloride in glacial acetic acid

Analysis: Dissolve the *Sample* in 10 mL of glacial acetic acid. Titrate the *Sample* with 0.1 N perchloric acid VS. Perform a blank determination, and make any necessary correction. Calculate the percentage of creatinine ($C_4H_7N_3O$) in the *Sample* taken:

$$\text{Result} = [(V - B) \times N \times F \times 100]/W$$

V = volume of the *Titrant* consumed by the *Sample* (mL)
B = volume of the *Titrant* consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 113.12 mg/mEq
W = weight of the *Sample* (mg)

Acceptance criteria: 98.5%–102.0% on the dried basis

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.2%

SPECIFIC TESTS

• **LOSS ON DRYING** ⟨731⟩: Dry a sample at 105° for 3 h: it loses NMT 3.0% of its weight.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Creatinine RS

Cresol

C_7H_8O 108.14

Phenol, methyl-;

Cresol [1319-77-3].

DEFINITION

Cresol is a mixture of isomeric cresols obtained from coal tar or from petroleum.

IDENTIFICATION

• **A.**

Sample solution: A saturated solution

Analysis: To the *Sample solution* add a few drops of ferric chloride TS.

Acceptance criteria: A bluish-violet color is produced.

IMPURITIES

• **LIMIT OF PHENOL**

Solution A: Bubble air through nitric acid until the acid is colorless, then mix 1 volume of the acid with 4 volumes of water.

Standard solution: Dissolve 1 g of phenol in 100 mL of water, and determine the actual C_6H_6O concentration as follows. Pipet 4 mL of the solution into an iodine flask, add 30.0 mL of 0.1 N bromine VS, then add 5 mL of hydrochloric acid, and immediately insert the stopper. Shake the flask repeatedly for 30 min, and allow to stand for 15 min. Add quickly 5 mL of a 200-mg/mL potassium iodide solution, taking precautions to prevent the escape of bromine vapor, and at once insert the stopper into the flask. Shake thoroughly, remove the stopper, and rinse it and the neck of the flask with a small quantity of water so that the washings flow into the flask. Add 1 mL of chloroform, shake the mixture, and titrate the liberated iodine with 0.1 N sodium thiosulfate VS, adding 3 mL of starch TS as the endpoint is approached. Perform a blank determination. Each mL of 0.1 N bromine is equivalent to 1.569 mg of C_6H_6O. Dilute a suitable volume of the solution with water to obtain a concentration of 250 µg/mL of C_6H_6O.

Sample solution: Place 2.5 g of Cresol in a 250-mL volumetric flask, add 10 mL of sodium hydroxide solution (100 mg/mL), and dilute with water to volume. Pipet 5 mL of this solution into a 200-mL volumetric flask, add 45 mL of water and 1 drop of methyl orange TS, neutralize with *Solution A* added dropwise, and then dilute with water to volume.

Analysis: Pipet 5.0 mL of the neutralized *Sample solution* into each of two 20- × 180-mm test tubes, graduated at the 25-mL mark, and pipet 5.0 mL of the *Standard solution* into each of two similar test tubes. To the contents of each tube add 5 mL of Millon's Reagent, allowing it to flow down the inner wall of the tube. Place the tubes simultaneously in a boiling water bath provided with a rack so that the tubes do not touch the bottom of the bath, and maintain the bath at boiling temperature for 30 min, accurately timed. At once remove the tubes from the bath, cool them immediately and thoroughly by placing them in a bath of cold water for NLT 10 min, and add 5 mL of *Solution A* to each tube. Add 3 mL of a 2% formaldehyde solution to one of each pair of tubes, add water to fill all tubes to volume, shake thoroughly, and allow to stand for 16 h, during which time the added formaldehyde imparts a yellow color while the contents of the other two tubes acquire an orange-red color.

Pipet 20 mL from each of the two tubes containing the *Standard solution* into separate 100-mL volumetric flasks, add 5 mL of *Solution A*, and then add water to volume. Transfer the solutions to burets marked *B1* and *B2*, representing, respectively, the solution not treated and the solution treated with formaldehyde.

Pipet 10 mL from each of the two tubes containing the *Sample solution* into separate 50-mL color-comparison tubes marked *N1* and *N2*, representing, respectively, the solution treated with formaldehyde and the solution not treated with formaldehyde.

Add to tube *N1* the orange-red colored solution from buret *B1*, and add to tube *N2* an equal volume of the yellow-colored solution from buret *B2*, until the colors in tubes *N1* and *N2* match when observed in a colorimeter.

Calculate the percentage of phenol (C_6H_6O) in the portion of the sample taken:

$$\text{Result} = V/W \times 5$$

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V = volume of the *Standard solution* taken from buret *B1* (mL)
W = weight of Cresol taken (g)

Acceptance criteria: NMT 5.0%

SPECIFIC TESTS
- **SPECIFIC GRAVITY** ⟨841⟩**:** 1.030–1.038
- **DISTILLING RANGE,** *Method II* ⟨721⟩**:** NLT 90% distills between 195° and 205°.
- **HYDROCARBONS**

Sample solution: 1 in 60

Standard solution: To 58 mL of water add 1.5 mL of 0.02 N sulfuric acid and 1 mL of barium chloride solution (100 mg/mL).

Analysis: Compare the turbidity of the *Sample solution* against the *Standard solution* after the *Standard solution* has been shaken and allowed to stand for 5 min.

Acceptance criteria: The *Sample solution* shows no more turbidity than the *Standard solution.*

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

Croscarmellose Sodium

Portions of this monograph that are national *USP* text, and are not part of the harmonized text, are marked with symbols (•,) to specify this fact.

DEFINITION
Croscarmellose Sodium is the sodium salt of a cross-linked, partly *O*-(carboxymethylated) cellulose.

IDENTIFICATION
- **A.** Mix 1 g with 100 mL of methylene blue solution (1 in 250,000), stir the mixture, and allow it to settle. The Croscarmellose Sodium absorbs the methylene blue and settles as a blue, fibrous mass.
- **B.** Mix 1 g with 50 mL of water. Transfer 1 mL of the mixture to a small test tube, and add 1 mL of water and 5 drops of 1-naphthol TS. Incline the test tube, and carefully add 2 mL of sulfuric acid down the side so that it forms a lower layer: a reddish-violet color develops at the interface.
- **•C.** A portion of the mixture of Croscarmellose Sodium with water, prepared as directed in *Identification B,* imparts an intense yellow color to a nonluminous flame.•

IMPURITIES
- **RESIDUE ON IGNITION** ⟨281⟩**:** 14.0%–28.0%, calculated on the dried basis. Use 1.0 g for the test, and use sufficient sulfuric acid to moisten the entire residue after the initial charring step, and additional sulfuric acid if an excessive amount of carbonaceous material remains after the initial complete volatilization of white fumes.
- **•SODIUM CHLORIDE AND SODIUM GLYCOLATE**

Sodium chloride

Sample: 5 g of Croscarmellose Sodium

Analysis: Transfer the *Sample* to a 250-mL beaker. Add 50 mL of water and 5 mL of 30% hydrogen peroxide, and heat on a steam bath for 20 min, stirring occasionally to ensure hydration. Cool, and add 100 mL of water and 10 mL of nitric acid. Titrate with 0.05 N silver nitrate VS, determining the endpoint potentiometrically, using a silver-based indicator electrode and a double-junction reference electrode containing 10% potassium nitrate filling solution in the outer jacket and a standard filling solution in the inner jacket, and stirring constantly (see *Titrimetry* ⟨541⟩).

Calculate the percentage of sodium chloride in the specimen taken:

$$\text{Result} = (F \times V \times N)/[(100 - b) \times W]$$

F = equivalence factor for sodium chloride, 584.4
V = volume of the silver nitrate (mL)
N = normality of the silver nitrate
b = percentage of loss on drying, determined separately
W = weight of the specimen (g)

Sodium glycolate

Standard stock solution: Transfer 100 mg of glycolic acid, previously dried in a desiccator at room temperature overnight, to a 100-mL volumetric flask. Dissolve in and dilute with water to volume, and mix. [NOTE—Use this solution within 30 days.]

Standard solution A: Transfer 1.0 mL of the *Standard stock solution* to a 100-mL volumetric flask. Add water to make 5 mL, then add 5 mL of glacial acetic acid. Dilute with acetone to volume, and mix.

Standard solution B: Transfer 2.0 mL of the *Standard stock solution* to a 100-mL volumetric flask. Add water to make 5 mL, then add 5 mL of glacial acetic acid. Dilute with acetone to volume, and mix.

Standard solution C: Transfer 3.0 mL of the *Standard stock solution* to a 100-mL volumetric flask. Add water to make 5 mL, then add 5 mL of glacial acetic acid. Dilute with acetone to volume, and mix.

Standard solution D: Transfer 4.0 mL of the *Standard stock solution* to a 100-mL volumetric flask. Add water to make 5 mL, then add 5 mL of glacial acetic acid. Dilute with acetone to volume, and mix.

Sample solution: Transfer 500 mg to a 100-mL beaker. Moisten thoroughly with 5 mL of glacial acetic acid, followed by 5 mL of water, and stir with a glass rod to ensure proper hydration (usually about 15 min). Slowly add 50 mL of acetone while stirring, then add 1 g of sodium chloride, and stir for several min to ensure complete precipitation of the carboxymethylcellulose. Filter through a soft, open-textured paper, previously wetted with a small amount of acetone, and collect the filtrate in a 100-mL volumetric flask. Use an additional 30 mL of acetone to facilitate the transfer of the solids and to wash the filter cake, then dilute with acetone to volume, and mix.

Analysis

Samples: *Standard solution A, Standard solution B, Standard solution C, Standard solution D,* and *Sample solution*

Transfer 2.0 mL of the *Sample solution* and 2.0 mL of each *Standard solution* to separate 25-mL volumetric flasks, and prepare a blank flask containing 2.0 mL of a solution containing 5% each of glacial acetic acid and water in acetone. Place the uncovered flasks in a boiling water bath for 20 min to remove the acetone. Remove from the bath, and cool. Add to each flask 5.0 mL of 2,7-dihydroxynaphthalene TS, mix, add an additional 15 mL, and again mix. Cover the mouth of each flask with a small piece of aluminum foil. Place the flasks upright in a boiling water bath for 20 min, then remove from the bath, cool, dilute with sulfuric acid to volume, and mix.

Determine the absorbance of each solution at 540 nm, with a suitable spectrophotometer, against the blank, and prepare a standard curve using the absorbances obtained from the *Standard solutions.*

Calculate the percentage of sodium glycolate in the specimen taken:

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$$\text{Result} = (F \times W_1)/[(100 - b) \times W_2]$$

F = factor converting glycolic acid to sodium glycolate, 12.9
W_1 = weight of glycolic acid in the specimen, determined from the standard curve and the absorbance of the *Sample solution* (mg)
b = percentage of loss on drying, determined separately
W_2 = weight of the specimen taken (g)

Acceptance criteria: The sum of the percentages of sodium chloride and sodium glycolate is NMT 0.5%.•

SPECIFIC TESTS

• **•CONTENT OF WATER-SOLUBLE MATERIAL**

Analysis: Disperse 10 g in 800 mL of water, and stir for 1 min every 10 min during the first 30 min. Allow to stand for an additional hour, or centrifuge, if necessary. Decant 200 mL of the aqueous slurry onto a rapid-filtering filter paper in a vacuum filtration funnel, apply vacuum, and collect about 150 mL of the filtrate. Pour the filtrate into a tared 250-mL beaker, weigh, and calculate the weight, in g, of the filtrate, W_3, by difference. Concentrate on a hot plate to a small volume, but not to dryness; dry at 105° for 4 h; and again weigh.

Calculate, in g, the weight of the residue by difference, W_1.

Calculate the percentage of water-soluble material in the specimen, on the dried basis, taken:

$$\text{Result} = [100 \times W_1 \times (800 + W_2)]/\{W_2 \times W_3 \times [1 - (0.01 \times b)]\}$$

W_1 = weight of residue by difference (g)
W_2 = weight of the specimen taken (g)
W_3 = weight of the filtrate by difference (g)
b = percentage loss on drying of the specimen taken

Acceptance criteria: NMT 10.0%•

• **DEGREE OF SUBSTITUTION**

Sample: 1 g

Analysis: Transfer the *Sample* to a glass-stoppered, 500-mL conical flask. Add 300 mL of sodium chloride solution (1 in 10), then add 25.0 mL of 0.1 N sodium hydroxide VS. Insert the stopper, and allow to stand for 5 min with intermittent shaking. Add 5 drops of *m*-cresol purple TS, and from a buret add 15 mL of 0.1 N hydrochloric acid VS. Insert the stopper in the flask, and shake. If the solution is violet, add 0.1 N hydrochloric acid VS in 1-mL portions until the solution becomes yellow, shaking after each addition. Titrate with 0.1 N sodium hydroxide VS to a violet endpoint.

Calculate the net number of milliequivalents, M, of base required for the neutralization of 1 g of Croscarmellose Sodium, on the dried basis.

Calculate the degree of acid carboxymethyl substitution, A:

$$\text{Result} = 1150 \times M/[7102 - (412 \times M) - (80 \times C)]$$

M = milliequivalents of base
C = percentage of residue on ignition of the Croscarmellose Sodium as determined in the test for *Residue on Ignition*

Calculate the degree of sodium carboxymethyl substitution, S:

$$\text{Result} = [162 + (58 \times A)] \times C/[7102 - (80 \times C)]$$

A = degree of acid carboxymethyl substitution, as determined above
C = percentage of residue on ignition of the Croscarmellose Sodium as determined in the test for *Residue on Ignition*

The degree of substitution is the sum of $A + S$.

Acceptance criteria: The degree of substitution is 0.60–0.85 on the dried basis.

• **LOSS ON DRYING** ⟨731⟩

Analysis: Dry at 105° for 6 h.

Acceptance criteria: NMT 10.0%

• **• MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic microbial count does not exceed 1000 cfu/g, and the total combined molds and yeasts count does not exceed 100 cfu/g. It meets the requirements of the test for absence of *Escherichia coli*.•

• **PH** ⟨791⟩

Analysis: Mix 1 g with 100 mL of water for 5 min.

Acceptance criteria: 5.0–7.0

• **SETTLING VOLUME**

Analysis: To 75 mL of water in a 100-mL graduated cylinder, add 1.5 g of it in 0.5-g portions, shaking vigorously after each addition. Add water to make 100 mL, shake again until all of the powder is homogeneously distributed, and allow to stand for 4 h. Note the volume of the settled mass.

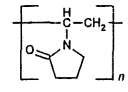
Acceptance criteria: The volume of the settled mass is 10.0–30.0 mL.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.

Crospovidone

Portions of the monograph text that are national *USP* text, and are not part of the harmonized text, are marked with symbols (• •) to specify this fact.



$(C_6H_9NO)_n$
1-Ethenyl-2-pyrrolidinone homopolymer;
1-Vinyl-2-pyrrolidinone homopolymer [9003-39-8].

DEFINITION

Crospovidone is a water-insoluble synthetic cross-linked homopolymer of *N*-vinyl-2-pyrrolidinone. It contains NLT 11.0% and NMT 12.8% of nitrogen (N), calculated on the dried basis. Two types of Crospovidone are available, depending on the particle size: Type A and Type B.

IDENTIFICATION

Change to read:

• **•A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K.**▲ (CN 1-May-2020) Previously dried in a vacuum at 105° for 1 h.•

• **B.**

Sample: 1 g

Analysis: Suspend the *Sample* in 10 mL of water, add 0.1 mL of 0.1 N iodine, and shake for 30 s. Add 1 mL of starch TS, and shake.

Acceptance criteria: No blue color develops.

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• **C.** To 10 mL of water add 0.1 g and shake. A suspension is formed, and no clear solution is obtained within 15 min.

• **D.**

Sample: 20 g of the dried substance

Analysis: Clean and dry the analytical sieves used in the analysis by washing the sieves in hot water. Allow to dry overnight in a drying cabinet at 105°. Place the *Sample* in a 1000-mL conical flask, add 500 mL of water, and shake the suspension for 30 min. Pour the suspension through a 63-µm analytical sieve, previously tared, and rinse the sieve with water until the filtrate is clear. Dry the sieve and sample residue at 105° for 5 h in a drying cabinet without circulating air. Cool in a desiccator for 30 min, and weigh. Calculate the percentage sieving residue fraction of sample particles having a diameter of more than 63 µm:

$$\text{Result} = [(m_1 - m_3) \times 100]/m_2$$

m_1 = mass of the sieve and sample residue, after drying for 5 h (g)

m_3 = mass of the sieve (g)

m_2 = initial mass of the sample, calculated on a dried basis (g)

Acceptance criteria: If the sieving residue fraction is more than 15%, the substance is classified as Type A; if the sieving residue fraction is NMT 15%, the substance is classified as Type B.

ASSAY

• **NITROGEN DETERMINATION,** *Method II* ⟨461⟩

Sample: 0.1 g

Analysis: Proceed as directed, using the *Sample*. In the *Procedure*, omit the use of hydrogen peroxide, and use 5 g of a powdered mixture of potassium sulfate, cupric sulfate, and titanium dioxide (33:1:1), instead of potassium sulfate and cupric sulfate (10:1). Heat until a clear, light green solution is obtained. Heat for an additional 45 min, and proceed as directed for *Procedure*, beginning with "Cautiously add to the digestion mixture 70 mL of water".

Acceptance criteria: 11.0%–12.8% on the dried basis

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 0.1%, determined on 1.0 g

• **PEROXIDES**

Sample suspension A: [NOTE—Use for Type A.] 40 mg/mL in water. To 25 mL of this suspension add 2 mL of titanium trichloride–sulfuric acid TS. Allow to stand for 30 min, and filter.

Sample suspension B: [NOTE—Use for Type B.] 16 mg/mL in water. To 25 mL of this suspension add 2 mL of titanium trichloride–sulfuric acid TS. Allow to stand for 30 min, and filter.

Compensation liquid A: [NOTE—Use for Type A.] 40 mg/mL in water. Filter, take 25 mL, and add 2 mL of a 13% solution of sulfuric acid.

Compensation liquid B: [NOTE—Use for Type B.] 16 mg/mL in water. Filter, take 25 mL, and add 2 mL of a 13% solution of sulfuric acid.

Analysis: Measure the absorbance of the filtrate at 405 nm against the appropriate compensation liquid.

Acceptance criteria: NMT 0.35. For Type A, this corresponds to NMT 400 ppm expressed as H_2O_2; for Type B, this corresponds to NMT 1000 ppm expressed as H_2O_2.

• **VINYLPYRROLIDINONE**

Mobile phase: Acetonitrile and water (1:9)

Sample solution: 25 mg/mL of suspension in methanol. Shake for 60 min. Leave the bulk to settle, and pass through a filter of 0.2-µm pore size.

Reference stock solution A: 5 µg/mL of vinylpyrrolidinone in methanol

Reference stock solution B: 100 µg/mL of vinylpyrrolidinone and 5 mg/mL of vinyl acetate in methanol

Reference solution A: A 1-in-20 solution of *Reference stock solution A* in *Mobile phase*

Reference solution B: A 1-in-100 solution of *Reference stock solution B* in *Mobile phase*

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV

Analytical wavelength: 235 nm

Precolumn: 4-mm × 2.5-cm; 5-µm packing L1

Column: 4-mm × 25-cm; 5-µm packing L1

Column temperature: 40°

Flow rate: 1 mL/min

Injection size: 50 µL. [NOTE—After each injection of the *Sample solution*, wash the precolumn by passing the *Mobile phase* backwards, at the same flow rate as applied in the test, for 30 min.]

System suitability

Samples: *Reference solution A* and *Reference solution B*

Suitability requirements

Resolution: NLT 2.0 between vinylpyrrolidinone and vinyl acetate, *Reference solution B*

Relative standard deviation: NMT 2.0% for 6 injections, *Reference solution A*

Analysis

Samples: *Sample solution* and *Reference solution A*

Record the chromatograms, and measure the responses for the vinylpyrrolidinone peak.

Acceptance criteria: The area of the peak from the *Sample solution* is NMT the area of the principal peak from *Reference solution A* (NMT 10 ppm).

SPECIFIC TESTS

• **LOSS ON DRYING** ⟨731⟩**:** Dry 0.5 g at 105° to constant weight: it loses NMT 5.0% of its weight.

• **WATER-SOLUBLE SUBSTANCES**

Sample: 25.0 g

Analysis: Transfer the *Sample* to a 400-mL beaker, add 200 mL of water, and stir on a magnetic stirrer, using a 5-cm stirring bar, for 1 h. Transfer to a 250-mL volumetric flask with the aid of 25 mL of water. Add water to volume. Allow the bulk of the solids to settle. Pass 100 mL of the relatively clear supernatant through a membrane filter of 0.45-µm pore size, protected against clogging by superimposing a membrane filter of 3-µm pore size. While filtering, stir the solution above the filter manually or with a mechanical stirrer, taking care not to physically damage the membrane filter. Transfer 50.0 mL of the clear filtrate to a tared 100-mL beaker, evaporate to dryness, and dry at 110° for 3 h.

Acceptance criteria: The weight of the residue does not exceed 75 mg (1.5%).

ADDITIONAL REQUIREMENTS

• **•PACKAGING AND STORAGE:** Preserve in tight containers.•

• **•LABELING:** The label states the type (Type A or Type B).•

• **• USP REFERENCE STANDARDS** ⟨11⟩

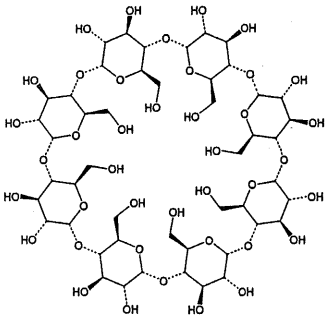
USP Crospovidone RS

•

NF Monographs

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Gamma Cyclodextrin



$(C_6H_{10}O_5)_8$ 1297.12
Cyclooctaamylose;
Cyclomaltooctaose [17465-86-0].

DEFINITION
Gamma Cyclodextrin is composed of 8 alpha-(1–4) linked D-glycopyranosyl units. It contains NLT 98.0% and NMT 102.0% of cyclooctaamylose $(C_6H_{10}O_5)_8$, calculated on the dried basis.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
- **B.** The retention time of the major peak from the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.
- **C.** It meets the requirements of the test for *Specific Rotation*.

ASSAY
- **PROCEDURE**

Mobile phase: Methanol and water (7:93)
System suitability solution: Prepare an aqueous solution containing 0.5 mg/mL each of USP Alpha Cyclodextrin RS, USP Beta Cyclodextrin RS, and USP Gamma Cyclodextrin RS.
Standard solution: 1.0 mg/mL of USP Gamma Cyclodextrin RS
Sample stock solution: Transfer 250 mg of Gamma Cyclodextrin to a 25-mL volumetric flask, and dissolve in water, with the aid of heat if necessary. Cool, and dilute with water to volume.
Sample solution: 1.0 mg/mL of Gamma Cyclodextrin, prepared from the *Sample stock solution*
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Refractive index
Column: 4.6-mm × 15-cm; 5-µm packing L1
Temperature
Detector: 40°
Column: 30°
Flow rate: 1.5 mL/min
Injection size: 50 µL
System suitability
Sample: *System suitability solution*
[NOTE—The relative retention times for gamma cyclodextrin, alfadex, and betadex are 0.8, 1.0, and 1.9, respectively.]
Suitability requirements
Resolution: NLT 1.5 between the gamma cyclodextrin and alfadex peaks
Tailing factors: 0.8–2.0 for the three cyclodextrins
Relative standard deviation: NMT 2.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of gamma cyclodextrin $[(C_6H_{10}O_5)_8]$ in the portion of sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of the *Standard solution* (mg/mL)
C_U = concentration of the *Sample solution* (mg/mL) corrected for water found in *Specific Tests, Loss on Drying*

Acceptance criteria: 98.0%–102.0% on the dried basis

IMPURITIES
- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%, determined on a 1.0-g specimen
- **RELATED COMPOUNDS**

Mobile phase, System suitability solution, and **Chromatographic system:** Proceed as directed in the *Assay*.
Standard solution: Transfer 5.0 mL of *System suitability solution* into a 50-mL volumetric flask, and dilute with water to volume.
Sample solution: Use the *Sample stock solution*, prepared as directed in the *Assay*.
Analysis
Samples: *Standard solution* and *Sample solution*
Acceptance criteria: For the *Sample solution*, the areas of any peaks corresponding to alfadex (alpha cyclodextrin) or to betadex (beta cyclodextrin) are not greater than the area of the corresponding peaks in the chromatogram of the *Standard solution* (0.5%); and the sum of the areas of all the peaks, excluding the principal peak, the peaks corresponding to alfadex or to betadex, and artifact peaks, is not greater than the area of the peak corresponding to gamma cyclodextrin in the chromatogram of the *Standard solution* (0.5%).
- **REDUCING SUBSTANCES**

Dextrose standard solution: 10.0 mg/mL of USP Dextrose RS, calculated on the anhydrous basis
Analysis: Transfer a quantity of Gamma Cyclodextrin, equivalent to 1.0 g on the dried basis, to a 500-mL conical flask. Dissolve in 10 mL of water, and add 25 mL of alkaline cupric citrate TS2. Cover the flask with aluminum foil, and boil the solution for 5 min. Cool in an ice bath to room temperature. Add 25 mL of 0.6 N acetic acid, 10 mL of 3 N hydrochloric acid, and 10 mL of 0.1 N iodine solution. [NOTE—The addition of these solutions must be in the order given.]
Titrate the solution with 0.1 N sodium thiosulfate VS, and determine the endpoint potentiometrically. Perform a blank determination (see *Titrimetry* ⟨541⟩, *Residual Titrations*). Calculate the difference in volumes required.
Create a calibration curve by similarly titrating 0.25, 0.5, 0.75, and 1.0 mL of *Dextrose standard solution*. Plot the amount, in mg, of dextrose in each titrated *Dextrose standard solution* versus the volume consumed, in mL, of 0.1 N sodium thiosulfate VS in the titration, and draw a straight line through the four points. From the line so obtained and the volume of 0.1 N sodium thiosulfate VS required in the titration of Gamma Cyclodextrin, determine the weight, *W*, in mg, of the reducing substances as dextrose in the portion of Gamma Cyclodextrin taken.

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Calculate the percentage of the reducing substances in the portion of Gamma Cyclodextrin taken:

$$\text{Result} = (W/W_G) \times F \times 100$$

W = weight of the reducing substances as dextrose in the portion of Gamma Cyclodextrin taken (mg)
W_G = weight of Gamma Cyclodextrin taken (g)
F = conversion factor, 10^{-3} g/mg

Acceptance criteria: NMT 0.5%

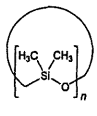
SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** It meets the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli.* The total aerobic microbial count does not exceed 1000 cfu/g, and the total combined molds and yeasts count does not exceed 100 cfu/g.
- **COLOR AND CLARITY OF SOLUTION**
  Sample solution: Transfer a quantity of Gamma Cyclodextrin, equivalent to 2.5 g on the dried basis, into a 25-mL volumetric flask, dissolve in and dilute with water that has been previously boiled and cooled to room temperature to volume, and mix.
  Analysis: Determine the absorbance of the *Sample solution* in a 1-cm cell at 420 nm, with a suitable spectrophotometer, after correcting for the blank.
  Acceptance criteria: At 420 nm, the absorbance is not greater than 0.20, and the solution is clear.
- **LOSS ON DRYING** ⟨731⟩**:** Dry a sample at 105° for 2 h: it loses NMT 11.0% of its weight.
- **OPTICAL ROTATION,** *Specific Rotation* ⟨781S⟩
  Sample solution: 10 mg/mL
  Analysis: Proceed as directed in the chapter.
  Acceptance criteria: +174° to +180°

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at room temperature.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Alpha Cyclodextrin RS
  USP Beta Cyclodextrin RS
  USP Dextrose RS
  USP Gamma Cyclodextrin RS

Cyclomethicone



$(C_2H_6OSi)_n$
Cyclopolydimethylsiloxane;
Cyclic polydimethylsiloxanes;
Cyclodimethicone [69430-24-6].

DEFINITION

Cyclomethicone is a fully methylated cyclic siloxane containing repeating units of $[-(CH_3)_2SiO-]_n$, in which n is 4, 5, 6, or a mixture of them. It contains NLT 98.0% of $(C_2H_6OSi)_n$, calculated as the sum of cyclomethicone 4, cyclomethicone 5, and cyclomethicone 6, and NLT 95.0% and NMT 105.0% of the labeled amount of any one or more of the individual cyclomethicone components.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy:* **197S▲** (CN 1-May-2020)
  Sample: Use neat liquids.
  Acceptance criteria: The IR absorption spectrum exhibits maxima only at the same wavelengths as that of a similar preparation of USP Cyclomethicone 4 RS, USP Cyclomethicone 5 RS, or USP Cyclomethicone 6 RS.

ASSAY

- **PROCEDURE**
  Standard solution A: USP Cyclomethicone 4 RS (neat)
  Standard solution B: USP Cyclomethicone 5 RS (neat)
  Standard solution C: USP Cyclomethicone 6 RS (neat)
  Sample solution: Cyclomethicone (neat)
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability.*)
  Mode: GC
  Detector: Flame ionization
  Column: 0.32-mm × 60-m fused silica; coated with a 1.0-µm film of phase G1
  Temperatures
  Injection port: 250°
  Detector: 300°
  Column: See *Table 1.*

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
60	—	60	5
60	10	200	15
200	5	225	5

Carrier gas: Helium
Flow rate: 1 mL/min
Injection volume: 1 µL
Injection type: Split ratio 1:20
System suitability
Samples: *Standard solution A*, *Standard solution B*, and *Standard solution C*
[NOTE—See *Table 2.*]

Table 2

Name	Relative Retention Time
Cyclomethicone 4	0.9
Cyclomethicone 5	1.0
Cyclomethicone 6	1.2

Suitability requirements
Relative standard deviation: NMT 2.0% for cyclomethicone 4, *Standard solution A*; NMT 2.0% for cyclomethicone 5, *Standard solution B*; NMT 2.0% for cyclomethicone 6, *Standard solution C*
Calculate the percentage of cyclomethicone 4, cyclomethicone 5, and cyclomethicone 6 by dividing 100 times the response of each peak at the retention time of the corresponding reference standard by the sum of all of the responses in the chromatogram. The percentages obtained from duplicate injections agree to within 1.0%.

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Analysis
Samples: *Standard solution A, Standard solution B, Standard solution C,* and *Sample solution*
Calculate the percentage of cyclomethicone 4 (cyclomethicone 5 or cyclomethicone 6) in the portion of Cyclomethicone taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of cyclomethicone 4 (cyclomethicone 5 or cyclomethicone 6)
r_T = sum of all the peak responses

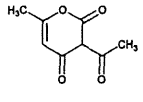
Calculate the percentage purity by adding the percentages of cyclomethicone 4, cyclomethicone 5, and cyclomethicone 6.

Acceptance criteria
Sum of cyclomethicone 4, cyclomethicone 5, and cyclomethicone 6: NLT 98.0% of $(C_2H_6OSi)_n$
Labeled amount: 95.0%–105.0% of the labeled amount of any one or more of the individual cyclomethicone components

IMPURITIES
• **LIMIT OF NONVOLATILE RESIDUE**
Sample: 2.0 g
Analysis: Transfer the *Sample* into an open, tared aluminum dish, and evaporate in a circulating air oven at 150° for 2 h. Allow to cool in a desiccator, and weigh.
Acceptance criteria: NMT 3.0 mg, corresponding to NMT 0.15% (w/w)

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight containers. Avoid exposure to excessive heat.
• **LABELING:** Label it to state, as part of the official title, the *n*-value of the Cyclomethicone. Where it is a mixture of two or three such cyclic siloxanes, the label states the *n*-value and percentage of each in the mixture.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Cyclomethicone 4 RS
USP Cyclomethicone 5 RS
USP Cyclomethicone 6 RS

Dehydroacetic Acid



$C_8H_8O_4$ 168.15
Keto form: 2*H*-Pyran-2,4(3*H*)-dione, 3-acetyl-6-methyl-; 3-Acetyl-6-methyl-2*H*-pyran-2,4(3*H*)-dione [520-45-6].
Enol form: 2*H*-Pyran-2-one, 3-acetyl-4-hydroxy-6-methyl-; 3-Acetyl-4-hydroxy-6-methyl-2*H*-pyran-2-one [771-03-9].

DEFINITION
Dehydroacetic Acid contains NLT 98.0% and NMT 100.5% of dehydroacetic acid ($C_8H_8O_4$), calculated on the dried basis.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

ASSAY
• **PROCEDURE**
Sample: 500 mg
Blank: 75 mL of neutralized alcohol
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.1 N sodium hydroxide VS
Endpoint detection: Visual
Analysis: Transfer the *Sample* into a 250-mL conical flask, dissolve it in 75 mL of neutralized alcohol, and add phenolphthalein TS. Titrate with *Titrant* to a pink endpoint that persists for NLT 30 s. Perform a blank determination.
Calculate the percentage of dehydroacetic acid ($C_8H_8O_4$) in the *Sample* taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

V_S = *Titrant* volume consumed by the *Sample* (mL)
V_B = *Titrant* volume consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 168.2 mg/mEq
W = *Sample* weight (mg)

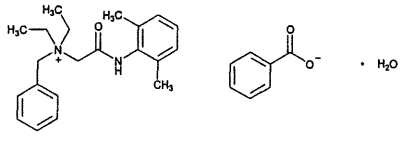
Acceptance criteria: 98.0%–100.5% on the dried basis

IMPURITIES
• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%

SPECIFIC TESTS
• **MELTING RANGE OR TEMPERATURE,** *Class I* ⟨741⟩: 109°–111°
• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry a sample at 80° for 4 h.
Acceptance criteria: NMT 1.0%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Dehydroacetic Acid RS

Denatonium Benzoate



$C_{28}H_{34}N_2O_3 \cdot H_2O$ 464.60
$C_{28}H_{34}N_2O_3$ 446.59
Benzenemethanaminium, *N*-[2-[(2,6-dimethylphenyl) amino]-2-oxoethyl]-*N*,*N*-diethyl-, benzoate, monohydrate; Benzyldiethyl[(2,6-xylylcarbamoyl)methyl]ammonium benzoate monohydrate [86398-53-0].
Anhydrous [3734-33-6].

DEFINITION
Denatonium Benzoate, dried at 105° for 2 h, contains one molecule of water of hydration or is anhydrous. When dried at 105° for 2 h, it contains NLT 99.5% and NMT 101.0% of denatonium benzoate ($C_{28}H_{34}N_2O_3$).

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IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

Change to read:

• **B. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Ultraviolet-Visible Spectroscopy*: **197U**▲ (CN 1-May-2020)
Analytical wavelength: 263 nm
Sample solution: 100 µg/mL
Medium: Water
Acceptance criteria: Absorptivities, calculated on the dried basis, do not differ by more than 3.0%.

• **C.**
Sample: 150 mg
Analysis: Dissolve the *Sample* in 10 mL of water, and add 15 mL of trinitrophenol TS.
Acceptance criteria: A yellow precipitate is formed.

• **D.**
Sample: 100 mg
Analysis: Dissolve the *Sample* in 10 mL of water, and add 20 mL of 2 N sulfuric acid and 15 mL of ammonium reineckate TS. Mix, filter through a sintered-glass crucible using gentle suction, and wash thoroughly with water. Remove as much water as possible with suction, and then dry in an oven at 105° for 1 h.
Acceptance criteria: The denatonium reineckate so obtained melts at about 170° (see *Melting Range or Temperature* ⟨741⟩).

ASSAY

• **PROCEDURE**
Sample: 900 mg, previously dried
Blank: 50 mL of glacial acetic acid
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.1 N perchloric acid VS
Endpoint detection: Visual
Analysis: Dissolve the *Sample* in 50 mL of glacial acetic acid, and add 1 drop of crystal violet TS. Titrate with *Titrant* to a green endpoint. Perform a blank determination, and make any necessary correction.
Calculate the percentage of denatonium benzoate ($C_{28}H_{34}N_2O_3$) in the portion of sample taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

V_S = *Titrant* volume consumed by the *Sample* (mL)
V_B = *Titrant* volume consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 446.6 mg/mEq
W = *Sample* weight (mg)

Acceptance criteria: 99.5%–101.0% on the dried basis

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%

• **CHLORIDE AND SULFATE,** *Chloride* ⟨221⟩
Standard solution: 0.10 mL of 0.020 N hydrochloric acid
Sample soluton: Dissolve 350 mg in 9 mL of water, add 1 mL of nitric acid, and filter.
Analysis: Use 1.0 mL of the *Sample solution*, and proceed as directed in the chapter
Acceptance criteria: 0.2%; the *Sample solution* shows no more chloride than the *Standard solution*.

SPECIFIC TESTS

• **MELTING RANGE OR TEMPERATURE** ⟨741⟩: 163°–170°, on a dried specimen

• **pH** ⟨791⟩
Sample solution: 30 mg/mL
Acceptance criteria: 6.5–7.5

• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry at 105° for 2 h.
Acceptance criteria
Monohydrate form: 3.5%–4.5%
Anhydrous form: NMT 1.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers.

• **LABELING:** Label it to indicate whether it is hydrous or anhydrous.

• **USP REFERENCE STANDARDS** ⟨11⟩
USP Denatonium Benzoate RS

Desoxycholic Acid—*see Desoxycholic Acid* General Monographs

Dextrates

DEFINITION

Dextrates is a purified mixture of saccharides resulting from the controlled enzymatic hydrolysis of starch. It is either anhydrous or hydrated. Its dextrose equivalent is NLT 93.0% and NMT 99.0%, calculated on the dried basis.

ASSAY

• **DEXTROSE EQUIVALENT**
Standard solution: 10 mg/mL of USP Dextrose RS
Sample solution: 10 mg/mL of Dextrates in hot water
Analysis: Transfer 25.0-mL portions of alkaline cupric tartrate TS to each of two boiling flasks. Bring the contents of one of the flasks to a boil, and titrate with the *Standard solution* to within 0.5 mL of the anticipated endpoint. Again heat the flask, with swirling, boil moderately for 2 min, add 2 drops of a 10-mg/mL methylene blue solution, immediately add 2 drops of the *Standard solution* from the buret, and again bring to a boil. Allow the cuprous oxide to settle slightly, and observe the color of the supernatant. Complete the titration within 3 min by adding the *Standard solution* dropwise, and boiling after each addition to the disappearance of the blue color, determined by viewing against a white background in daylight or under equivalent illumination. If more than 0.5 mL of the titrant was required after the addition of the indicator, repeat the titration, adding the necessary volume of titrant before adding the indicator. Bring the contents of the second flask to a boil, and similarly titrate with the *Sample solution*.
Calculate the percentage of dextrose equivalent taken on the dried basis:

$$\text{Result} = (C_S/C_U) \times (V_S/V_U) \times [1/(1 - 0.01 \times A)] \times 100$$

C_S = concentration of USP Dextrose RS in the *Standard solution* (mg/mL)
C_U = concentration of Dextrates in the *Sample solution* (mg/mL)
V_S = titrant volume consumed by the *Standard solution* (mL)
V_U = titrant volume consumed by the *Sample solution* (mL)

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A = loss on drying of Dextrates taken (%)

Acceptance criteria: 93.0%–99.0% on the dried basis

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 0.1%

SPECIFIC TESTS

• **pH** ⟨791⟩
Sample: 200 mg/mL in carbon dioxide-free water
Acceptance criteria: 3.8–5.8
• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry at 105° for 16 h in a convection oven.
Acceptance criteria: For the anhydrous form, NMT 2.0%; for the hydrated form, 7.8%–9.2%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store in a cool, dry place.
• **LABELING:** Label it to state whether it is anhydrous or hydrated.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Dextrose RS

Dextrin

DEFINITION

Dextrin is starch, or partially hydrolyzed starch, modified by heating in a dry state, with or without acids, alkalies, or pH control agents. During heating, moisture may be added.

IDENTIFICATION

• **A.**
Sample: 1 g
Analysis:
Suspend the *Sample* in 20 mL of water, and add a few drops of iodine TS.
Acceptance criteria: A blue to reddish-brown color results.
• **B.** Dextrin is very soluble in boiling water, forming a mucilaginous solution (difference from starch).

ASSAY

• **REDUCING SUGARS (DEXTROSE EQUIVALENT)**
Sample: A quantity of Dextrin equivalent to 2.0 g on the dried basis
Analysis: To the *Sample* add 100 mL of water, shake for 30 min, dilute with water to 200 mL, accurately measured, and filter. To 10.0 mL of alkaline cupric tartrate TS add 20.0 mL of the filtrate, mix, and heat on a hot plate adjusted to bring the solution to a boil in 3 min. Boil for 2 min, and cool quickly. Add 5 mL of potassium iodide solution (3 in 10) and 10 mL of 2 N sulfuric acid, mix, and titrate immediately with 0.1 N sodium thiosulfate VS, using starch TS, added toward the end of the titration, as an indicator. Repeat the procedure beginning with "To 10.0 mL of alkaline cupric tartrate TS" using, in place of the filtrate, 20.0 mL of a solution (1 in 1000) of anhydrous dextrose, accurately prepared. Perform a blank titration.
Compare $(V_B - V_U)$ and $(V_B - V_S)$.

$$\text{Result 1} = (V_B - V_U)$$

$$\text{Result 2} = (V_B - V_S)$$

V_B = volume of 0.1 N sodium thiosulfate consumed in the titrations of the blank
V_U = volume of 0.1 N sodium thiosulfate consumed in the titrations of the Dextrin
V_S = volume of 0.1 N sodium thiosulfate consumed in the titrations of the dextrose

Acceptance criteria: $(V_B - V_U)$ is NMT $(V_B - V_S)$, corresponding to NMT 10%, calculated as dextrose ($C_6H_{12}O_6$).

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 0.5%
• **CHLORIDE AND SULFATE,** *Chloride* ⟨221⟩
Sample: 3.0 g
Control: 2.8 mL of 0.020 N hydrochloric acid in 75 mL of water
Analysis: Dissolve the *Sample* in 75 mL of boiling water. Cool, dilute with water to 75 mL, and filter if necessary. To 25 mL of this solution and the *Control*, add 2 mL of nitric acid and 1 mL of silver nitrate TS.
Acceptance criteria: Any turbidity produced is NMT that of the *Control*, corresponding to NMT 0.2% of chloride.
• **LIMIT OF PROTEIN**
Sample: 10 g
Analysis: Proceed as directed in *Nitrogen Determination* ⟨461⟩, using the *Sample* instead of 1 g, 60 mL of sulfuric acid instead of 20 mL, and multiplying the percentage of nitrogen found by 6.25.
Acceptance criteria: NMT 1.0%

SPECIFIC TESTS

• **ACIDITY**
Sample: 10.0 g
Analysis: Add the *Sample* to 100 mL of 70% alcohol, previously neutralized to phenolphthalein. Shake for 1 h, filter, and titrate 50 mL of the filtrate with 0.10 N sodium hydroxide.
Acceptance criteria: NMT 3.0 mL
• **BOTANIC CHARACTERISTICS**
Microscopic: Granules are similar in appearance to the starch from which the Dextrin has been prepared, except that when prepared from corn starch, many of the granules show concentric striations, and when prepared from potato starch, concentric striations are not clearly visible; the hilum is frequently bicleft; and a small proportion of the granules are distorted.
• **LOSS ON DRYING** ⟨731⟩**:** Dry a sample at a pressure not exceeding 100 mm of mercury at 120° for 4 h: it loses NMT 13.0% of its weight.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Dextrose—*see Dextrose General Monographs*

Dextrose Excipient

$C_6H_{12}O_6 \cdot H_2O$ 198.17
D-Glucose, monohydrate;
D-Glucose monohydrate [77938-63-7].

DEFINITION

Change to read:

Dextrose Excipient is a sugar usually obtained by hydrolysis of starch. It contains 1 molecule of water of hydration. ▲It

NF Monographs

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contains NLT 97.5% and NMT 102.0% of dextrose ($C_6H_{12}O_6$), calculated on the anhydrous basis.▲ 2S (NF36)

IDENTIFICATION

Delete the following:

▲• **A.**
Sample solution: 50 mg/mL
Analysis: Add a few drops of the *Sample solution* to 5 mL of hot alkaline cupric tartrate TS.
Acceptance criteria: A copious red precipitate of cuprous oxide is formed.▲ 2S (NF36)

Change to read:

• ▲**A.** ▲**SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
Sample: Dry a test specimen per the conditions specified in the test for *Water Determination*.
Acceptance criteria: Meets the requirements▲ 2S (NF36)

Add the following:

▲• **B.**
Analysis: Examine the chromatograms obtained in the *Assay*.
Acceptance criteria: The principal peak obtained with the *Sample solution* is similar in retention time and size to the principal peak obtained with *Standard solution A*.▲ 2S (NF36)

Add the following:

▲• **C.** Meets the requirements for the water content in the test for *Water Determination*.▲ 2S (NF36)

ASSAY

Add the following:

▲• **PROCEDURE**
Mobile phase: Water
System suitability solution: 0.1 mg/mL each of USP Maltose Monohydrate RS, USP Maltotriose RS, and USP Fructose RS
Standard solution A: 30 mg/mL of USP Dextrose RS
Sample solution: Equivalent to 30 mg/mL of anhydrous dextrose
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Refractive index
Column: 7.8-mm × 30-cm; 9-µm packing L19
Temperatures
Column: 85 ± 1°
Detector: 40°
Flow rate: 0.3 mL/min
Injection volume: 20 µL
Run time: 1.5 times the retention time of dextrose
System suitability
Sample: *System suitability solution*
[NOTE—The relative retention times for maltotriose, maltose, isomaltose, dextrose, and fructose are 0.7, 0.8, 0.8, 1.0, and 1.3, respectively. The retention time for dextrose is about 21 min.]
Suitability requirements
Resolution: NLT 1.3 between maltotriose and maltose
Analysis
Samples: *Standard solution A* and *Sample solution*

Calculate the percentage of dextrose ($C_6H_{12}O_6$) in the portion of Dextrose Excipient taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of dextrose from the *Sample solution*
r_S = peak area of dextrose from *Standard solution A*
C_S = concentration of USP Dextrose RS in *Standard solution A* (mg/mL)
C_U = concentration of Dextrose Excipient in the *Sample solution* (mg/mL)

Acceptance criteria: 97.5%–102.0% on the anhydrous basis▲ 2S (NF36)

IMPURITIES

Add the following:

▲• **RELATED SUBSTANCES**
Mobile phase, System suitability solution, and **Chromatographic system:** Proceed as directed in the *Assay*.
Standard solution B: Dilute 1.0 mL of the *Sample solution* with water to 250.0 mL.
Standard solution C: Dilute 25.0 mL of *Standard solution B* with water to 200.0 mL.
Sample solution: Equivalent to 30 mg/mL of anhydrous dextrose
System suitability
Sample: *System suitability solution*
[NOTE—The relative retention times for maltotriose, maltose, isomaltose, dextrose, and fructose are 0.7, 0.8, 0.8, 1.0, and 1.3, respectively. The retention time for dextrose is about 21 min.]
Suitability requirements
Resolution: NLT 1.3 between maltotriose and maltose
Analysis
Samples: *Standard solution B*, *Standard solution C*, and *Sample solution*
The reporting threshold is 0.05%. Disregard any peak with an area less than the principal peak from *Standard solution C*.
Acceptance criteria
Maltose and isomaltose: NMT 0.4%; the sum is NMT the area of the principal peak from *Standard solution B*.
Maltotriose: NMT 0.2%; NMT 0.5 times the area of the principal peak from *Standard solution B*
Fructose: NMT 0.15%; NMT 3 times the area of the principal peak from *Standard solution C*
Unspecified impurities: NMT 0.10%; NMT twice the area of the principal peak from *Standard solution C*
Total impurities: NMT 0.5%; NMT 1.25 times the area of the principal peak from *Standard solution B*▲ 2S (NF36)

Delete the following:

▲• **HEAVY METALS** ⟨231⟩
Test preparation: 4.0 g of Dextrose Excipient in 25 mL of water
Acceptance criteria: NMT 5 ppm▲ (Official 1-Jan-2018)
• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%
• **SOLUBLE STARCH, SULFITES**
Sample solution: 1 g of Dextrose Excipient in 10 mL of water
Analysis: To the *Sample solution* add 1 drop of iodine TS.
Acceptance criteria: The liquid is colored yellow.

Automated Monographs

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SPECIFIC TESTS

Delete the following:

▲• **OPTICAL ROTATION** ⟨781S⟩, *Procedures, Specific Rotation*
Sample solution: 100 mg/mL of Dextrose Excipient in 0.012 N ammonium hydroxide
Acceptance criteria: +52.5° to +53.5° ▲ 2S (NF36)

Change to read:

• **WATER DETERMINATION** ⟨921⟩, *Method III*
Analysis: ▲Dry under vacuum at 70° to constant weight.▲ 2S (NF36)
Acceptance criteria: 7.5%–9.5%
• **COLOR OF SOLUTION**
Sample solution: Dissolve 25 g of Dextrose Excipient in water to make 50.0 mL.
Control solution: Mix 1.0 mL of cobaltous chloride CS, 3.0 mL of ferric chloride CS, and 2.0 mL of cupric sulfate CS with water to make 10 mL. Dilute 3 mL of this solution with water to 50 mL.
Analysis: Make the comparison by viewing the *Sample solution* and *Control solution* downward in matched color-comparison tubes against a white surface.
Acceptance criteria: The *Sample solution* has no more color than the *Control solution.*
• **ACIDITY**
Sample solution: 100 mg/mL in carbon dioxide-free water
Analysis: Add phenolphthalein TS to 50 mL of the *Sample solution,* and titrate with 0.020 N sodium hydroxide to the production of a distinct pink color.
Acceptance criteria: NMT 0.30 mL
• **CHLORIDE AND SULFATE** ⟨221⟩, *Chloride*
Standard solution: 0.50 mL of 0.020 N hydrochloric acid
Sample: 2.0 g
Acceptance criteria: 0.018%; the *Sample* shows no more chloride than the *Standard solution.*
• **CHLORIDE AND SULFATE** ⟨221⟩, *Sulfate*
Standard solution: 0.50 mL of 0.020 N sulfuric acid
Sample: 2.0 g
Acceptance criteria: 0.025%; the *Sample* shows no more sulfate than the *Standard solution.*
• **ARSENIC** ⟨211⟩, *Procedures, Method 1*: NMT 1 ppm
• **DEXTRIN**
Sample: 1 g of finely powdered Dextrose Excipient
Analysis: Reflux the *Sample* with 20 mL of alcohol.
Acceptance criteria: It dissolves completely.

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Change to read:

• **LABELING:** Label it to indicate that it is not intended for parenteral use. ▲Label it to indicate that it is dextrose monohydrate.▲ 2S (NF36)

Add the following:

▲• **USP REFERENCE STANDARDS** ⟨11⟩
USP Dextrose RS
USP Fructose RS
USP Maltose Monohydrate RS
USP Maltotriose RS▲ 2S (NF36)

Diacetylated Monoglycerides

DEFINITION
Diacetylated Monoglycerides is glycerin esterified with edible fat-forming fatty acids and acetic acid. It may be prepared by the interesterification of edible oils with triacetin in the presence of catalytic agents, followed by molecular distillation, or by the direct acetylation of edible monoglycerides with acetic anhydride without the use of catalyst or molecular distillation.

IDENTIFICATION

Change to read:

• **A.** ▲**SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F**▲ (CN 1-May-2020)
Sample: Undried specimen
Acceptance criteria: Meet the requirements

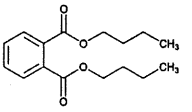
IMPURITIES
• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%

SPECIFIC TESTS
• **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩: NMT 3
• **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩: NMT 15
• **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩: 365–395

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Diacetylated Monoglycerides RS

Diatrizoic Acid—*see Diatrizoic Acid General Monographs*

Dibutyl Phthalate



$C_{16}H_{22}O_4$ 278.34
1,2-Benzenedicarboxylic acid, dibutyl ester [84-74-2].

DEFINITION
Dibutyl Phthalate contains NLT 99.0% and NMT 101.0% of dibutyl phthalate ($C_{16}H_{22}O_4$).

IDENTIFICATION

Change to read:

• **A.** ▲**SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F.**▲ (CN 1-May-2020) On undried specimen
• **B.** It meets the requirements in *Specific Tests* for *Refractive Index* ⟨831⟩.

ASSAY
• **PROCEDURE**
Sample: 0.75 g
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Residual titration

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Titrant: 0.5 N alcoholic potassium hydroxide VS
Back-titrant: 0.5 N hydrochloric acid VS
Endpoint detection: Visual
Analysis: Transfer the *Sample* to a flask, add 25.0 mL of *Titrant*, attach a reflux condenser to the flask, and boil in a water bath for 1 h. Add 1 mL of phenolphthalein TS. Titrate immediately with *Back-titrant*. Perform a blank determination. Each mL of *Titrant* is equivalent to 69.59 mg of dibutyl phthalate ($C_{16}H_{22}O_4$).
Acceptance criteria: 99.0%–101.0%

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩
Sample: 1.0 g
Acceptance criteria: NMT 0.1%

• **RELATED COMPOUNDS**
Internal standard solution: 3 mg/mL of dibenzyl in methylene chloride
Sample stock solution: 100 mg/mL of Dibutyl Phthalate in methylene chloride
Sample solution A: Dilute 5.0 mL of *Sample stock solution* with methylene chloride to 10 mL.
Sample solution B: Add 5.0 mL of *Sample stock solution*, 1.0 mL of *Internal standard solution*, and dilute with methylene chloride to 10 mL.
Standard solution: Add 1.0 mL of *Sample solution A*, 10.0 mL of *Internal standard solution*, and dilute with methylene chloride to 100 mL.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 4-mm × 1.5-m; packed with 3% liquid phase G3 on support S1A
Temperatures
Column: 190°
Injection port: 225°
Detector: 225°
Carrier gas: Nitrogen
Flow rate: 30 mL/min
Run time: Three times the retention time of dibutyl phthalate
Injection volume: 1 µL
System suitability
Samples: *Sample solution A* and *Standard solution*
Suitability requirements
[NOTE—The elution order is the internal standard peak followed by the dibutyl phthalate peak.]
Resolution: NLT 12 between the internal standard and the dibutyl phthalate peak, *Standard solution*
Additional requirements: Verify that there is no peak with the same retention time as the internal standard in *Sample solution A*. If such a peak is observed, make any necessary correction for factors of dilution, and then determine the area due to the interfering component that must be subtracted from the area of the internal standard peak appearing in the chromatogram recorded for *Sample solution B*.
Analysis
Samples: *Sample solution B* and *Standard solution*
Calculate the peak area ratio of dibutyl phthalate to the internal standard for the *Standard solution*. Calculate the peak area ratio of the sum of all peaks, excluding the main peak, the solvent peak, and the internal standard peak, to the internal standard peak for *Sample solution B*.
Acceptance criteria: NMT 1.0%; the ratio for *Sample solution B* is NMT the ratio for the *Standard solution*.

SPECIFIC TESTS

• **SPECIFIC GRAVITY** ⟨841⟩: 1.043–1.048 at 20°

• **REFRACTIVE INDEX** ⟨831⟩: 1.490–1.495 at 20°

• **ACIDITY**
Sample: 20.0 g
Analysis: Mix the *Sample* with 50 mL of alcohol that previously has been neutralized to a phenolphthalein endpoint. Add 0.2 mL of phenolphthalein TS, and titrate with 0.1 N sodium hydroxide VS.
Acceptance criteria: NMT 0.50 mL is required to change the color of the indicator.

• **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 0.2%

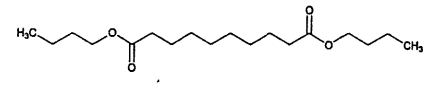
• **APPEARANCE**
Sample: Dibutyl Phthalate (neat)
Standard solution: Prepared immediately before use by mixing 2.4 mL of ferric chloride CS and 0.6 mL of cobaltous chloride CS with dilute hydrochloric acid (10 mg/mL) to make 10 mL, and diluting 5 mL of this solution with dilute hydrochloric acid (10 mg/mL) to make 100 mL
Analysis: Compare the *Sample* and the *Standard solution* by viewing the substance and the solution downward in matched color-comparison tubes against a white surface (see *Color and Achromicity* ⟨631⟩).
Acceptance criteria: The *Sample* is clear and not more intensely colored than the *Standard solution*.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.

• **USP REFERENCE STANDARDS** ⟨11⟩
USP Dibutyl Phthalate RS

Dibutyl Sebacate



$CH_3(CH_2)_3OOC(CH_2)_8\ COO(CH_2)_3CH_3$

$C_{18}H_{34}O_4$ 314.46

Decanedioic acid, 1,10-dibutyl ester;
Dibutyl 1,10-decanedioate;
Sebacic acid di-*n*-butyl ester [109-43-3].

DEFINITION
Dibutyl Sebacate consists of esters of *n*-butyl alcohol and saturated dibasic acids, principally sebacic acid. It contains NLT 92.0% and NMT 102.0% of dibutyl sebacate ($C_{18}H_{34}O_4$).

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F▲** (CN 1-May-2020)
Sample: Undried specimen
Acceptance criteria: Meets the requirements

• **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

ASSAY

• **PROCEDURE**
Internal standard solution: 0.9 mg/mL of methyl heptadecanoate in alcohol
Standard solution: 1.0 mg/mL of USP Dibutyl Sebacate RS in the *Internal standard solution*
Sample solution: 1.0 mg/mL of Dibutyl Sebacate in the *Internal standard solution*

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Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.32-mm × 30-m fused silica column, coated with a 0.25-µm film of phase G2
Temperature
Detector: 300°
Injection port: 300°
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
150	—	150	5
150	10	250	5

Carrier gas: Helium
Linear velocity: 50 cm/s
Injection volume: 1 µL
Injection type: Split injection, split ratio is about 30:1
Run time: 20 min
System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for dibutyl sebacate and methyl heptadecanoate are 1.0 and 0.9, respectively.]
Suitability requirements
Resolution: NLT 2.0 between methyl heptadecanoate and dibutyl sebacate
Relative standard deviation: NMT 2.0% for the peak response ratio of dibutyl sebacate to methyl heptadecanoate
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of dibutyl sebacate ($C_{18}H_{34}O_4$) in the portion of Dibutyl Sebacate taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

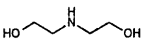
R_U = peak response ratio of dibutyl sebacate to methyl heptadecanoate from the *Sample solution*
R_S = peak response ratio of dibutyl sebacate to methyl heptadecanoate from the *Standard solution*
C_S = concentration of USP Dibutyl Sebacate RS in the *Standard solution* (mg/mL)
C_U = concentration of Dibutyl Sebacate in the *Sample solution* (mg/mL)

Acceptance criteria: 92.0%–102.0%

SPECIFIC TESTS
- **SPECIFIC GRAVITY** ⟨841⟩: 0.935–0.939 at 20°
- **REFRACTIVE INDEX** ⟨831⟩: 1.429–1.441
- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩: NMT 0.1
- **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩: 352–360

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers. Store at room temperature. Protect from moisture.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Dibutyl Sebacate RS

Diethanolamine



$C_4H_{11}NO_2$ 105.14
Ethanol, 2,2′-iminobis-;
2,2′-Iminodiethanol [111-42-2].

DEFINITION
Diethanolamine is a mixture of ethanolamines, consisting largely of diethanolamine. It contains NLT 98.5% and NMT 101.0% of ethanolamines, calculated on the anhydrous basis as $NH(C_2H_4OH)_2$.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F▲** (CN 1-May-2020)

ASSAY
- **PROCEDURE**
Sample: 2 g
Blank: 50 mL of water
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.5 N hydrochloric acid VS
Endpoint detection: Visual
Analysis: To the *Sample* add 50 mL of water and bromocresol green TS. Titrate with the *Titrant*. Perform a blank determination.
Calculate the percentage of diethanolamine, expressed as $NH(C_2H_4OH)_2$, in the *Sample* taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

V_S = *Titrant* volume consumed by the *Sample* (mL)
V_B = *Titrant* volume consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 105.14 (mg/mEq)
W = *Sample* weight (mg)

Acceptance criteria: 98.5%–101.0% on the anhydrous basis

IMPURITIES
- **LIMIT OF TRIETHANOLAMINE**
Mixed indicator: 1.5 mg/mL of methyl orange and 0.8 mg/mL of xylene cyanole FF in water
Sample: 20 g
Blank: 100 mL of methanol and 6–8 drops of *Mixed indicator*
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.5 N alcoholic sulfuric acid VS
Endpoint detection: Visual
Analysis: Place 100 mL of methanol and 6–8 drops of *Mixed indicator* in a 500-mL glass-stoppered conical flask, and neutralize with 0.1 N alcoholic sulfuric acid or 0.1 N alcoholic potassium hydroxide. The neutral solution is amber when viewed by transmitted light and is red-brown when viewed by reflected light. Add the *Sample*. Cautiously add 75 mL of acetic anhydride, and swirl to effect complete solution. Cool to room temperature, if necessary, and allow to stand at room temperature for 30 min. Titrate with the *Titrant*. Perform a blank determination.
Calculate the percentage of triethanolamine in the *Sample* taken:

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$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

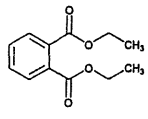
V_S = *Titrant* volume consumed by the *Sample* (mL)
V_B = *Titrant* volume consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 149.2 mg/mEq
W = *Sample* weight (mg)

Acceptance criteria: NMT 1.0% by weight

SPECIFIC TESTS
- **REFRACTIVE INDEX** ⟨831⟩: 1.473–1.476 at 30°
- **WATER DETERMINATION,** *Method I* ⟨921⟩
  Sample: 20 g
  Solvent: A mixture of methanol and glacial acetic acid (40:25)
  Acceptance criteria: NMT 0.15%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Diethanolamine RS

Diethyl Phthalate



$C_{12}H_{14}O_4$ 222.24
1,2-Benzenedicarboxylic acid, diethyl ester;
Diethyl phthalate [84-66-2].

DEFINITION
Diethyl Phthalate contains NLT 98.0% and NMT 102.0% of diethyl phthalate ($C_{12}H_{14}O_4$), calculated on the anhydrous basis. [**CAUTION**—Avoid contact.]

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F.**▲ (CN 1-May-2020) On undried specimen

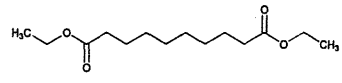
ASSAY
- **PROCEDURE**
  Sample: 1.5 g
  Titrimetric system
  (See *Titrimetry* ⟨541⟩.)
  Mode: Residual titration
  Titrant: 0.5 N alcoholic potassium hydroxide VS
  Back-titrant: 0.5 N hydrochloric acid VS
  Endpoint detection: Visual
  Analysis: Transfer the *Sample* to a flask, add 50.0 mL of the *Titrant*, and boil with a reflux condenser on a water bath for 1 h. Add 20 mL of water, then add phenolphthalein TS. Titrate the excess potassium hydroxide with the *Back-titrant*. Perform a blank determination. Each mL of 0.5 N potassium hydroxide is equivalent to 55.56 mg of diethyl phthalate ($C_{12}H_{14}O_4$).
  Acceptance criteria: 98.0%–102.0% on the anhydrous basis

IMPURITIES
- **RESIDUE ON IGNITION** ⟨281⟩
  Sample: 10 g
  Analysis: Gently heat the *Sample* until the liquid has evaporated, and ignite the residue to constant weight.
  Acceptance criteria: NMT 0.02%

SPECIFIC TESTS
- **SPECIFIC GRAVITY** ⟨841⟩: 1.118–1.122 at 20°
- **REFRACTIVE INDEX** ⟨831⟩: 1.500–1.505 at 20°
- **ACIDITY**
  Sample: 20.0 g
  Analysis: Mix the *Sample* with 50 mL of alcohol that previously has been neutralized to a phenolphthalein endpoint, and titrate with 0.1 N sodium hydroxide VS to a phenolphthalein endpoint.
  Acceptance criteria: NMT 0.50 mL is required for neutralization.
- **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 0.2%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Diethyl Phthalate RS

Diethyl Sebacate



$CH_3CH_2OOC(CH_2)_8COOCH_2CH_3$

$C_{14}H_{26}O_4$ 258.35
Decanedioic acid, 1,10-diethyl ester;
Diethyl 1,10-decanedioate [110-40-7].

DEFINITION
Diethyl Sebacate consists of the diester of alcohol (ethanol) and sebacic acid. It contains NLT 98.0% and NMT 102.0% of diethyl sebacate ($C_{14}H_{26}O_4$).

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F.**▲ (CN 1-May-2020)
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

ASSAY
- **PROCEDURE**
  Internal standard solution: 0.9 mg/mL of methyl heptadecanoate in *n*-heptane
  Standard solution: 1.0 mg/mL of USP Diethyl Sebacate RS in the *Internal standard solution*
  Sample solution: 1.0 mg/mL of Diethyl Sebacate in the *Internal standard solution*
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: GC
  Detector: Flame ionization
  Column: 0.53-mm × 30-m fused silica capillary; 1.5-µm layer of phase G1
  Temperatures
  Injection port: 300°
  Detector: 300°
  Column: See *Table 1*.

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Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
150	—	150	5
150	10	250	5

Carrier gas: Helium
Linear velocity: 50 cm/s
Injection volume: 1 µL
Injection type: Split injection; split ratio, 3:1
System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for diethyl sebacate and methyl heptadecanoate are 1.0 and 1.2, respectively.]
Suitability requirements
Resolution: NLT 2.0 between diethyl sebacate and methyl heptadecanoate
Relative standard deviation: NMT 2.0%, ratio of the peak response of diethyl sebacate to that of methyl heptadecanoate
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of diethyl sebacate ($C_{14}H_{26}O_4$) in the portion of Diethyl Sebacate taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of diethyl sebacate to methyl heptadecanoate from the *Sample solution*
R_S = peak response ratio of diethyl sebacate to methyl heptadecanoate from the *Standard solution*
C_S = concentration of USP Diethyl Sebacate RS in the *Standard solution* (mg/mL)
C_U = concentration of Diethyl Sebacate in the *Sample solution* (mg/mL)

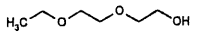
Acceptance criteria: 98.0%–102.0%

IMPURITIES
- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.10%

SPECIFIC TESTS
- **SPECIFIC GRAVITY** ⟨841⟩: 0.958–0.968 at 20°
- **REFRACTIVE INDEX** ⟨831⟩: 1.435–1.437 at 20°
- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Acid Value*: NMT 0.5
- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Iodine Value*: NMT 0.5

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers, and store in a cool, dry, and well-ventilated place.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Diethyl Sebacate RS

Diethylene Glycol Monoethyl Ether



$C_6H_{14}O_3$ 134.17

DEFINITION
Diethylene Glycol Monoethyl Ether contains NLT 99.0% and NMT 101.0% of $C_6H_{14}O_3$. It is produced by condensation of ethylene oxide and alcohol, followed by distillation.

IDENTIFICATION

Change to read:

- **A.** **▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F.▲** (CN 1-May-2020) Potassium bromide plates being used
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *System suitability solution*, as obtained in the *Assay*.

ASSAY
- **PROCEDURE**
Sample: Diethylene Glycol Monoethyl Ether (neat)
System suitability solution: 1 mg/mL each of 2-methoxyethanol, 2-ethoxyethanol, ethylene glycol, diethylene glycol, and USP Diethylene Glycol Monoethyl Ether RS in methanol
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.32-mm × 30-m fused-silica bonded with a 1.0-µm layer of phase G46
Temperature
Injector: 250°
Detector: 275°
Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
120	—	120	1
120	12	225	2

Carrier gas: Helium
Flow rate: 2.2 mL/min
Injection size: 0.5 µL
System suitability
Sample: *System suitability solution*
[NOTE—The relative retention times for 2-methoxyethanol, 2-ethoxyethanol, ethylene glycol, diethylene glycol monoethyl ether, and diethylene glycol are about 0.40, 0.43, 0.50, 0.93, and 1.0, respectively.]
Suitability requirements
Resolution: NLT 2.0 between 2-ethoxyethanol and ethylene glycol
Relative standard deviation: NMT 2.0%, determined from diethylene glycol monoethyl ether
Analysis
Sample: Diethylene Glycol Monoethyl Ether (neat)
Calculate the percentage of diethylene glycol monoethyl ether ($C_6H_{14}O_3$) in the portion of Diethylene Glycol Monoethyl Ether taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response for diethylene glycol monoethyl ether
r_T = sum of the responses of all the peaks

Acceptance criteria: 99.0%–101.0%

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IMPURITIES

ORGANIC IMPURITIES

• Procedure 1: Limit of Free Ethylene Oxide

Acetaldehyde solution: 10 µg/mL of acetaldehyde.
[NOTE—Prepare fresh just before use.]

Ethylene oxide stock solution
[**CAUTION**—Ethylene oxide is toxic and flammable. Prepare these solutions in a well-ventilated fume hood, using great care. Protect both hands and face by wearing polyethylene protective gloves and an appropriate face mask.]
[NOTE—Before using the polyethylene glycol 200 in this test, remove any volatile components from it by placing 500 mL of the polyethylene glycol 200 in a 1000-mL round-bottom flask, attaching the flask to a rotary evaporator, and evaporating at a temperature of 60° at a pressure of 1.5–2.5 kPa for 6 h.]
Fill a chilled pressure bottle with liquid ethylene oxide, and store in a freezer when not in use. Use a small piece of polyethylene film to protect the liquid from contact with the rubber gasket. Tare a glass-stoppered conical flask, add 50 mL of polyethylene glycol 200, and reweigh the flask. Transfer 5 mL of the liquid ethylene oxide to a 100-mL beaker chilled in a mixture of sodium chloride and wet ice (1:3). Using a gas-tight gas chromatographic syringe that has been previously cooled to –10°, transfer 300 µL (corresponding to about 250 mg) of liquid ethylene oxide to the polyethylene glycol 200, and swirl gently to mix. Replace the stopper, reweigh the flask, and determine the amount of ethylene oxide absorbed by weight difference. Adjust the weight of the mixture with polyethylene glycol 200 to 100.0 g, replace the stopper, and swirl gently to mix. This stock solution contains 2.5 mg/g of ethylene oxide.
[NOTE—Prepare this *Ethylene oxide stock solution* fresh just before use, and store in a refrigerator.]

Ethylene oxide standard solution A: Tare a glass-stoppered conical flask, and chill it in a refrigerator. Add 35 mL of polyethylene glycol 200, and reweigh the flask. Use a gas-tight gas chromatographic syringe that has been chilled in a refrigerator, and transfer 1 g of the chilled *Ethylene oxide stock solution* to the tared, conical flask. Adjust the weight of the solution with polyethylene glycol 200 to 50.0 g, replace the stopper, and swirl gently to mix. Transfer 10 g of this solution to a 50-mL volumetric flask. Add 30 mL of water, and mix. Dilute with water to volume, and mix to obtain a solution containing 10 µg/mL of ethylene oxide.
[NOTE—Prepare this solution fresh just before use, and store in a refrigerator.]

Ethylene oxide standard solution B: Transfer 10.0 mL of *Ethylene oxide standard solution A* to a 50-mL volumetric flask, and dilute with water to volume to obtain a solution containing 2 µg/mL of ethylene oxide. [NOTE—Prepare this solution fresh just before use, and store in a refrigerator.]

System suitability solution: Transfer 0.5 mL of *Ethylene oxide standard solution B* to a 10-mL pressure headspace vial, and add 0.1 mL of *Acetaldehyde solution* and 0.1 mL of water, seal the vial, and mix. Heat the mixture at 70° for 45 min.

Standard solution: Transfer 1 g of Diethylene Glycol Monoethyl Ether to a 10-mL pressure headspace vial, and add 0.5 mL of *Ethylene oxide standard solution B* and 0.5 mL of water. Seal the vial, and mix. Heat the mixture at 70° for 45 min.

Sample solution: Transfer 1 g of Diethylene Glycol Monoethyl Ether to a 10-mL pressure headspace vial, add 1 mL of water, seal the vial, and mix. Heat the mixture at 70° for 45 min.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
[NOTE—The use of a headspace apparatus that automatically transfers a measured amount of headspace is allowed.]
Mode: GC
Detector: Flame ionization
Column: 0.32-mm × 30-m glass or quartz capillary bonded with a 1.0-µm layer of phase G1
Temperature
Injector: 150°
Detector: 250°
Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
50	—	50	5
50	5	180	—
180	30	230	5

Carrier gas: Helium
Flow rate: 1 mL/min
Injection size: 1 mL

System suitability
Sample: Gaseous headspace of the *System suitability solution*
[NOTE—The relative retention times for acetaldehyde and ethylene oxide are about 0.94 and 1.0, respectively.]
Suitability requirements
Resolution: NLT 2.0 between the acetaldehyde and ethylene oxide peaks
Relative standard deviation: NMT 15%

Analysis
Samples: Gaseous headspace of the *Standard solution* and *Sample solution*
[NOTE—Use a heated, gas-tight gas chromatographic syringe.]
Calculate the amount of ethylene oxide in the portion of Diethylene Glycol Monoethyl Ether taken:

$$\text{Result} = r_U/[(r_S \times W_U) - (r_U \times W_S)]$$

r_U = ethylene oxide peak area from the *Sample solution*
r_S = ethylene oxide peak area from the *Standard solution*
W_U = weight of Diethylene Glycol Monoethyl Ether taken to prepare the *Sample solution* (g)
W_S = weight of Diethylene Glycol Monoethyl Ether taken to prepare the *Standard solution* (g)

Acceptance criteria: NMT 1 µg/g

• PROCEDURE 2: LIMIT OF 2-METHOXYETHANOL, 2-ETHOXYETHANOL, ETHYLENE GLYCOL, AND DIETHYLENE GLYCOL

Sample, System suitability solution, Chromatographic system, and **System suitability:** Proceed as directed in the *Assay*.

Analysis: Proceed as directed in the *Assay*.
Calculate the percentage of 2-methoxyethanol in the portion of Diethylene Glycol Monoethyl Ether taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response for 2-methoxyethanol
r_T = sum of all the peak responses

Calculate the percentage of 2-ethoxyethanol in the portion of Diethylene Glycol Monoethyl Ether taken:

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$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response for 2-ethoxyethanol
r_T = sum of all the peak responses

Calculate the percentage of ethylene glycol in the portion of Diethylene Glycol Monoethyl Ether taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response for ethylene glycol
r_T = sum of all the peak responses

Calculate the percentage of diethylene glycol in the portion of Diethylene Glycol Monoethyl Ether taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response for diethylene glycol
r_T = sum of all the peak responses

Acceptance criteria: See *Impurity Table 1*.

Impurity Table 1

Name	Acceptance Criteria, NMT (ppm)
2-Methoxyethanol	50
2-Ethoxyethanol	160
Ethylene glycol	620
Diethylene glycol	150

SPECIFIC TESTS
- **REFRACTIVE INDEX** ⟨831⟩: 1.426–1.428 at 20°
- **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 0.1%, determined on a 10-g specimen
- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩
  [NOTE—This test must be performed promptly after sampling to avoid oxidation of the sample specimen.]
  Analysis: Dissolve 30 g of Diethylene Glycol Monoethyl Ether in 30 mL of neutralized alcohol. Add 1 mL of phenolphthalein TS, and titrate with 0.01 N alcoholic potassium hydroxide VS to produce a permanent, faint pink color.
  Acceptance criteria: The acid value so obtained is NMT 0.1.
- **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩: NMT 8.0, 2 g being used

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers under an atmosphere of an inert gas, at a temperature not exceeding 35°.
- **LABELING:** Label it to indicate that it is intended for topical or transdermal use only and it is stored under an atmosphere of an inert gas. The material is not to be used for parenterals.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Diethylene Glycol Monoethyl Ether RS

Diethylene Glycol Stearates

DEFINITION
Diethylene Glycol Stearates is a mixture of diethylene glycol monoesters and diesters of stearic and palmitic acids. It contains NLT 45.0% of monoesters produced from the condensation of ethylene glycol and stearic acid of vegetable or animal origin.

IDENTIFICATION
- **A.** It meets the requirements in *Specific Tests* for *Melting Range or Temperature, Class II* ⟨741⟩.
- **B.** It meets the requirements in *Specific Tests* for *Fats and Fixed Oils, Fatty Acid Composition* ⟨401⟩.

ASSAY
- **PROCEDURE**
  Mobile phase: Tetrahydrofuran
  Sample solution: 40 mg/mL of Diethylene Glycol Stearates in tetrahydrofuran
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: LC
  Detector: Refractive index
  Column: 7.5-mm × 60-cm; containing 5-µm 100Å packing L21. [NOTE—Two or three 7.5-mm × 30-cm L21 columns may be used in place of the 60-cm column, provided that *System suitability* requirements are met.]
  Temperatures
  Detector: 40°
  Column: 40°. [NOTE—The column temperature may be lowered to ambient temperature, although working at 40° provides stable separation conditions and ensures better sample solubility.]
  Flow rate: 1 mL/min
  Injection volume: 40 µL
  System suitability
  Sample: *Sample solution*
  [NOTE—The relative retention times for diesters, monoesters, and diethylene glycol are 0.78, 0.84, and 1.0, respectively.]
  Suitability requirements
  Relative standard deviation: NMT 2.0% for the monoesters peak
  Analysis
  Sample: *Sample solution*
  Calculate the percentage of free fatty acids, *E*, in the portion of Diethylene Glycol Stearates taken:

$$E = I_A \times 270/561.1$$

I_A = acid value, determined in *Specific Tests* for *Fats and Fixed Oils, Acid Value (Free Fatty Acids)* ⟨401⟩

Calculate the percentage of monoesters in the portion of *Sample* taken:

$$\text{Result} = [r_M/(r_M + r_D)] \times (100 - D - E)$$

r_M = peak response for the monoesters
r_D = peak response for the diesters
D = percentage of free diethylene glycol in the portion of Diethylene Glycol Stearates taken, as determined in *Impurities* for *Limit of Free Diethylene Glycol*

Acceptance criteria: NLT 45.0% of monoesters

IMPURITIES
- **LIMIT OF FREE DIETHYLENE GLYCOL**
  Mobile phase, Sample solution, Chromatographic system, and System suitability: Proceed as directed in the *Assay*.
  Standard solution A: 0.5 mg/mL of diethylene glycol in tetrahydrofuran
  Standard solution B: 1.0 mg/mL of diethylene glycol in tetrahydrofuran

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Standard solution C: 2.0 mg/mL of diethylene glycol in tetrahydrofuran
Standard solution D: 4.0 mg/mL of diethylene glycol in tetrahydrofuran
Analysis
Samples: *Sample solution* and *Standard solutions*
Plot the diethylene glycol peak responses obtained versus the concentration of diethylene glycol in the *Standard solutions*. From the standard curve so obtained, determine the diethylene glycol concentration in the sample.
Calculate the percentage of free diethylene glycol in the portion of sample taken:

$$\text{Result} = (C/C_U) \times 100$$

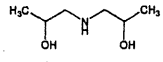
C = concentration of diethylene glycol, determined from the standard curve (mg/mL)
C_U = concentration of the *Sample solution* (mg/mL)

Acceptance criteria: NMT 8.0%

SPECIFIC TESTS
- **MELTING RANGE OR TEMPERATURE,** *Class II* ⟨741⟩**:** 43°–50°
- **FATS AND FIXED OILS,** *Acid Value (Free Fatty Acids)* ⟨401⟩
Sample: 10.0 g
Acceptance criteria: NMT 4.0
- **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩**:** NMT 3.0
- **FATS AND FIXED OILS,** *Saponification value* ⟨401⟩
Sample: 2.0 g
Acceptance criteria: 150–170
- **FATS AND FIXED OILS,** *Fatty Acid Composition* ⟨401⟩**:** 40.0%–60.0% of stearic acid, and the sum of palmitic and stearic acids is NLT 90.0%.
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩
Sample: 1.0 g
Acceptance criteria: NMT 0.1%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements are specified.
- **LABELING:** Label it to indicate that it is intended for topical and vaginal use only.

Diisopropanolamine



$C_6H_{15}NO_2$ 133.19
2-Propanol, 1,1′-iminobis-;
1,1′-Iminodi-2-propanol [110-97-4].

DEFINITION
Diisopropanolamine is a mixture of isopropanolamines, consisting largely of diisopropanolamine. It contains NLT 98.0% and NMT 102.0% of isopropanolamines, calculated as $NH(C_3H_6OH)_2$ on the anhydrous basis.

IDENTIFICATION
- **A.** The IR absorption spectrum of a thin film exhibits regions of absorption between 2.8 and 4.0 µm, between 6.7 and 7.1 µm, and between 8.5 and 9.4 µm; and several characteristic peaks, the most pronounced being at about 7.3, 7.5, 8.3, 9.6, 10.4, and 10.7 µm.

ASSAY
- **PROCEDURE**
Sample: 2 g
Blank: 50 mL of water
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.5 N hydrochloric acid VS
Endpoint detection: Visual
Analysis: Transfer the *Sample* to a 250-mL conical flask. Add 50 mL of water and bromocresol green TS. Titrate with *Titrant*. Perform a blank determination, and make any necessary correction.
Calculate the percentage of isopropanolamines, expressed as $NH(C_3H_6OH)_2$, in the portion of Diisopropanolamine taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

V_S = *Titrant* volume consumed by the *Sample* (mL)
V_B = *Titrant* volume consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 0.1332 g/mEq
W = *Sample* weight (g)

Acceptance criteria: 98.0%–102.0% on the anhydrous basis

IMPURITIES
- **LIMIT OF TRIISOPROPANOLAMINE**
Indicator solution: 1.5 mg/mL of methyl orange and 0.8 mg/mL of xylene cyanole FF
Sample: 20 g
Blank: 100 mL of methanol
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.5 N alcoholic sulfuric acid VS
Endpoint detection: Visual
Analysis: To a glass-stoppered, 500-mL conical flask add 100 mL of methanol and 6–8 drops of *Indicator solution*, and neutralize with 0.1 N alcoholic sulfuric acid or 0.1 N alcoholic potassium hydroxide. The neutral solution is amber when viewed by transmitted light and is red-brown when viewed by reflected light. Add the *Sample*, cautiously add 75 mL of acetic anhydride, and swirl to dissolve. Allow to stand at room temperature for 30 min. Cool to room temperature, if necessary. Titrate with *Titrant*. Perform a blank determination, and make any necessary correction.
Calculate the percentage of triisopropanolamine in the portion of Diisopropanolamine taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

V_S = *Titrant* volume consumed by the *Sample* (mL)
V_B = *Titrant* volume consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 0.1914 g/mEq
W = *Sample* weight (g)

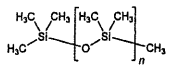
Acceptance criteria: NMT 1.0% by weight

SPECIFIC TESTS
- **WATER DETERMINATION,** *Method I* ⟨921⟩
Solvent: A mixture of 5.0 mL of glacial acetic acid and 25 mL of methanol
Acceptance criteria: NMT 0.50%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers. No specific storage conditions are required.

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Dimethicone



Dimethicone;
α-(Trimethylsilyl)-ω-methylpoly[oxy(dimethylsilylene)] [9006-65-9].

DEFINITION

Dimethicone is a mixture of fully methylated linear siloxane polymers containing repeating units of the formula:

$$[-(CH_3)_2SiO-]_n$$

stabilized with trimethylsiloxy end-blocking units of the formula:

$$[(CH_3)_3SiO-]$$

wherein *n* has an average value such that the corresponding nominal viscosity is in a discrete range between 20 and 30,000 centistokes. It contains NLT 97.0% and NMT 103.0% of polydimethylsiloxane ($[-(CH_3)_2SiO-]_n$).

The requirements for viscosity, specific gravity, refractive index, and loss on heating differ for the several types of Dimethicone, as set forth in *Table 1*.

IDENTIFICATION

• **A.** The IR absorption spectrum of the *Sample* exhibits maxima only at the same wavelengths as that of the *Standard*, obtained as directed in the *Assay*.

ASSAY

• **PROCEDURE**

Standard: USP Polydimethylsiloxane RS

Sample: Dimethicone

Instrumental conditions

(See *Mid-Infrared Spectroscopy* ⟨854⟩.)

Mode: IR, with a resolution of 4 cm^{-1} and fitted with an accessory for attenuated total reflectance and a germanium (Ge) sample trough (45° or 60°)

Wavelength range: 4000 to 700 cm^{-1}

Analysis

Samples: *Standard* and *Sample*

Using an empty and clean trough, determine the background spectrum. Fill the trough with the *Sample*, and record the spectrum. Clean the trough, fill it with the *Standard*, and determine the spectrum. Examine the spectra in the range between 1300 and 1200 cm^{-1}, and determine the absorbance of the peak in each spectrum at about 1259 cm^{-1}.

Calculate the percentage of polydimethylsiloxane ($[-(CH_3)_2SiO-]_n$) in the Dimethicone taken:

$$\text{Result} = (A_U/A_S) \times (D_S/D_U) \times 100$$

A_U = absorbance of the *Sample*
A_S = absorbance of the *Standard*
D_S = specific gravity of USP Polydimethylsiloxane RS
D_U = specific gravity of Dimethicone

Acceptance criteria: 97.0%–103.0%

SPECIFIC TESTS

• **SPECIFIC GRAVITY** ⟨841⟩: See *Table 1*.
• **REFRACTIVE INDEX** ⟨831⟩: See *Table 1*.
• **ACIDITY**

Sample: 15.0 g

Analysis: Dissolve the *Sample* in a mixture of 15 mL of toluene and 15 mL of butyl alcohol, previously neutralized to bromophenol blue TS, and titrate with 0.050 N alcoholic potassium hydroxide to a bromophenol blue endpoint.

Acceptance criteria: NMT 0.10 mL of 0.050 N alcoholic potassium hydroxide is required.

• **LOSS ON HEATING**

Sample: 1 g

Analysis: Preheat an open aluminum vessel (60 mm in diameter and 10 mm high) at 150° for 30 min, and allow to cool to room temperature. Tare the vessel, transfer the *Sample* to it, heat at 150° in a circulating air oven for 2 h, and allow to come to room temperature in a desiccator before weighing.

Acceptance criteria: See *Table 1*.

• **BACTERIAL ENDOTOXINS TEST** ⟨85⟩ (where it is intended for use in coating containers that come in contact with articles for parenteral use)

Sample: 1.0 mL

Analysis: Mix the *Sample* with 4.0 mL of polydimethylsiloxane having a viscosity of 0.65 centistokes, previously tested and shown to be negative for bacterial endotoxins, by mixing on a vortex mixer for 1 min in an extraction tube. Add 10 mL of water, and mix on a vortex mixer for NLT 60 min. Allow the layers to separate, and use the lower aqueous layer as the sample.

Acceptance criteria: It contains NMT 1.0 USP Endotoxin Unit/mL, equivalent to NMT 10 Endotoxin Units/mL of the Dimethicone taken.

• **VISCOSITY—CAPILLARY METHODS** ⟨911⟩ and **VISCOSITY—ROTATIONAL METHODS** ⟨912⟩

Analysis: Determine the viscosity of Dimethicone having a nominal viscosity of less than 1000 centistokes at 25 ± 0.1°, using a capillary viscometer. Determine the viscosity of Dimethicone having a nominal viscosity of 1000 centistokes or greater at 25 ± 0.1°, using a rotational viscometer.

Acceptance criteria: See *Table 1*.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers.
• **LABELING:** Label it to indicate its nominal viscosity value. Dimethicone intended for use in coating containers that come in contact with articles for parenteral use is so labeled.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Polydimethylsiloxane RS

Docusate Sodium—*see Docusate Sodium General Monographs*

Edetate Calcium Disodium—*see Edetate Calcium Disodium General Monographs*

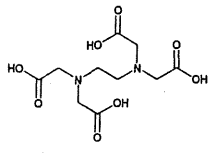
Edetate Disodium—*see Edetate Disodium General Monographs*

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Table 1 (Dimethicone)

Nominal Viscosity (centistokes)	Viscosity (centistokes)		Specific Gravity		Refractive Index		Loss on Heating (%)
	Min.	Max.	Min.	Max.	Min.	Max.	Max.
20	18	22	0.946	0.954	1.3980	1.4020	20.0
50	47.5	52.5	0.955	0.965	1.4005	1.4045	2.0
100	95	105	0.962	0.970	1.4005	1.4045	0.3
200	190	220	0.964	0.972	1.4013	1.4053	0.3
350	332.5	367.5	0.965	0.973	1.4013	1.4053	0.3
500	475	525	0.967	0.975	1.4013	1.4053	0.3
1000	950	1050	0.967	0.975	1.4013	1.4053	0.3
12,500	11,875	13,125	—	—	1.4015	1.4055	2.0
30,000	27,000	33,000	0.969	0.977	1.4010	1.4100	2.0

Edetic Acid



$C_{10}H_{16}N_2O_8$ 292.24

Glycine, *N*,*N*′-1,2-ethanediylbis[*N*-(carboxymethyl)-; (Ethylenedinitrilo)tetraacetic acid [60-00-4].

DEFINITION

Edetic Acid contains NLT 98.0% and NMT 100.5% of edetic acid ($C_{10}H_{16}N_2O_8$).

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

ASSAY

• **PROCEDURE**

Standard: 200 mg of chelometric standard calcium carbonate, previously dried to 110° for 2 h and cooled in a desiccator

Sample solution: Transfer 1.4 g of Edetic Acid to a 100-mL volumetric flask, dissolve in 11 mL of 1 N sodium hydroxide, dilute with water to volume, with cooling if necessary, and mix.

Analysis: Add 10 mL of water to the *Standard*, swirl to form a slurry, and cover the beaker with a watch glass. Without removing the watch glass, add 2 mL of 3 N hydrochloric acid from a pipet, and swirl to dissolve. Wash down the sides of the container, the outer surface of the pipet, and the watch glass with water, and dilute with water to 100 mL. While stirring with a magnetic stirrer, add 30 mL of the *Sample solution* from a 50-mL buret. Add 10 mL of 1 N sodium hydroxide and 300 mg of hydroxy naphthol blue, and continue the titration with the *Sample solution* to a blue endpoint.

Calculate the percentage of edetic acid ($C_{10}H_{16}N_2O_8$) in the portion of sample taken:

$$\text{Result} = [W/(V \times C)] \times (M_{r1}/M_{r2}) \times 100$$

W = weight of calcium carbonate (mg)
V = volume of the *Sample solution* consumed in the titration (mL)
C = concentration of the *Sample solution* (mg/mL)
M_{r1} = molecular weight of edetic acid, 292.24
M_{r2} = molecular weight of calcium carbonate, 100.09

Acceptance criteria: 98.0%–100.5%

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.2%

• **IRON**

Sample solution: Char 3.0 g of Edetic Acid thoroughly, and heat in an oven at 500° until most of the carbon is consumed. Cool, add 0.15 mL of nitric acid, and heat at 500° until all of the carbon is consumed. Dissolve the residue in 2 mL of a mixture of equal volumes of hydrochloric acid and water, digest in a covered dish on a steam bath for 10 min, remove the cover, and evaporate to dryness. Dissolve the residue in 1 mL of 1 N acetic acid and 20 mL of hot water, digest for 5 min on a steam bath, cool, and dilute with water to 30 mL.

Control solution: Dissolve 43.2 mg of ferric ammonium sulfate in 10 mL of 2 N sulfuric acid, and add water to make 1000 mL. Each mL contains 5 µg of Fe.

Analysis: To 2.0 mL each of *Sample solution* and *Control solution* add 2 mL of hydrochloric acid, and dilute with water to 50 mL. Add 50 mg of ammonium persulfate and 3 mL of ammonium thiocyanate solution (300 mg/mL), mix, and transfer to a color comparison tube.

Acceptance criteria: 0.005%; the color of the *Sample solution* is not deeper than that of the *Control solution*.

• **LIMIT OF NITRILOTRIACETIC ACID**

Mobile phase: Add 10 mL of 1.0 M tetrabutylammonium hydroxide in methanol to 200 mL of water, and adjust with 1 M phosphoric acid to a pH of 7.5 ± 0.1. Transfer the solution so obtained to a 1000-mL volumetric flask, add 90 mL of methanol, dilute with water to volume, mix, and pass through a filter of 0.5-µm pore size.

Solution A: 10 mg/mL of cupric nitrate [$Cu(NO_3)_2$]

Standard stock solution: Transfer 100 mg of nitrilotriacetic acid to a 10-mL volumetric flask, and add 0.5 mL of ammonium hydroxide. Dilute with water to volume.

Standard solution: Transfer 1.0 g of Edetic Acid to a 100-mL volumetric flask, add 300 µL of *Standard stock solution*, and dilute with *Solution A* to volume. Sonicate, if necessary, to completely dissolve.

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System suitability solution: Transfer 10 mg of Edetic Acid to a 100-mL volumetric flask, add 100 µL of *Standard stock solution*, and dilute with *Solution A* to volume. Sonicate, if necessary, to completely dissolve.
Sample solution: 10 mg/mL of Edetic Acid in *Solution A*. Sonicate, if necessary, to completely dissolve.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 15-cm; packing L7
Flow rate: 2 mL/min
Injection volume: 50 µL
System suitability
Samples: *Standard solution* and *System suitability solution*
[NOTE—The relative retention times for nitrilotriacetic acid, copper, and edetate are about 0.35, 0.65, and 1.0, respectively.]
Suitability requirements
Resolution: NLT 3 between the nitrilotriacetic acid and copper peaks, *System suitability solution*
Relative standard deviation: NMT 2.0%, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Acceptance criteria: 0.3%; the response of the nitrilotriacetic acid peak of the *Sample solution* does not exceed the difference between the nitrilotriacetic acid peak responses from the *Standard solution* and the *Sample solution*.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Edetic Acid RS

Egg Phospholipids

DEFINITION
Egg Phospholipids is a mixture of naturally occurring phospholipids obtained from the yolk of hens' eggs that is suitable for use as an emulsifying agent in injectable emulsions. The content of phosphatidylcholine, phosphatidylethanolamine, lysophosphatidylcholine, and other related phospholipids is to be reported in the certificate of analysis. It may also contain a suitable stabilizer.

ASSAY
- **CONTENT OF PHOSPHOLIPIDS**

Solution A: 1341.6 g of *n*-hexane, 334.1 g of 2-propanol, 39.4 g of glacial acetic acid, and 1.45 g of triethylamine (or 2.0 mL triethylamine)
Solution B: 663.5 g of 2-propanol, 140.0 g of water, 15.8 g of glacial acetic acid, and 0.58 g of triethylamine
Solvent: *n*-Hexane, 2-propanol, and water (23:23:4).
[NOTE—To avoid the formation of two phases, mix the 2-propanol and water first, and then add the *n*-hexane.]
Mobile phase: See the gradient table below.

Program Step	Time (min)	Flow (mL/min)	Solution A (%)	Solution B (%)
1	0	1.0	95	5
2	5.0	1.0	80	20
3	8.5	1.0	60	40
4	15.0	1.0	0	100
5	17.5	1.0	0	100
6	17.6	1.0	95	5
7	21.0	1.0	95	5
8	22.0	2.0	95	5
9	27.0	2.0	95	5
10	29.0	1.0	95	5

Standard solutions: Transfer USP Phosphatidylcholine RS, USP Phosphatidylethanolamine RS, and USP Lysophosphatidylcholine RS to separate flasks, dissolve each in *Solvent*, and dilute. *Standard solutions* of five different concentrations are prepared on the basis of the expected content of phosphatidylcholine, phosphatidylethanolamine, and lysophosphatidylcholine in the sample. The *Standard solutions* should cover a range of 60% to 140%. Calculate the concentrations of the Standards:

$$\text{Result} = WP/V$$

W = weight of the Standard (mg)
P = purity of the designated Reference Standard
V = volume of each of the *Standard solutions* (mL)

Sample solution: 100 mg of Egg Phospholipids in a 25-mL volumetric flask. Dissolve in *Solvent*, and dilute. Calculate the concentration, in mg/mL: this value is used as the sample amount.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: evaporative light-scattering detector
Column: 4-mm × 125-mm; 5-µm packing L20
Column temperature: 55°
Injection size: 20 µL
System suitability
Sample: *Standard solutions*
[NOTE—The relative retention times for phosphatidylcholine, phosphatidylethanolamine, and lysophosphatidylcholine are 1.00, 0.85, and 1.25, respectively.]
Suitability requirements
Relative standard deviation: NMT 5.0%
Analysis
Samples: Each of the *Standard solutions* and *Sample solution*
Identify the peaks of the relevant analytes in the chromatogram of the *Sample solution* by comparison with the chromatograms obtained from the *Standard solutions*. Measure the areas of the analyte peaks. Plot the logarithms of the relevant responses versus the logarithms of the concentrations, in mg/mL, of each analyte obtained from the *Standard solutions*, and determine the linear regression line using a least-squares analysis. The correlation coefficient for the linear regression line is NLT 0.995. From the graphs so obtained, determine the concentration, C, in mg/mL, of the relevant analyte in the *Sample solution*.
Separately calculate the percentages of phosphatidylethanolamine, phosphatidylcholine, and lysophosphatidylcholine in the portion of Egg Phospholipids taken:

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$$\text{Result} = (CV/W) \times 100$$

C = concentration of the relevant analyte in the *Sample solution* (mg/mL)
V = volume of the relevant analyte in the *Sample solution* (mL)
W = weight of Egg Phospholipids in the *Sample solution* (mg)

Acceptance criteria: NMT 3.0% of lysophosphatidylcholine

IMPURITIES

ORGANIC IMPURITIES

• **Procedure: Limit of Nonphosphatidyl Lipids**

Solvent: Diethyl ether

Sample solution: 500 mg of Egg Phospholipids, dissolved in 15 mL of *Solvent*, in a 50-mL conical flask

Chromatographic system

(See *Chromatography* ⟨621⟩, *Column Chromatography*.)

Mode: Column

Chromatographic column:

Transfer 1000 g of silica gel having a particle size of 0.05–0.2 mm into a container with well-closing screw caps. Add 150 g of water, shake well, and allow to stand for 24 h. Suspend 15 g of prepared adsorbent in 50 mL of *Solvent*, and introduce into a 1- to 2-cm chromatographic column. Drain the *Solvent* through the column to a level of about 1 cm above the silica gel bed.

Analysis

Sample: *Sample solution*

Transfer the *Sample solution* to the *Chromatographic column*. Rinse the column containing the *Sample solution* with two 15-mL portions of *Solvent*, allowing each rinse to pass through the column before adding the next. After rinsing, elute with 105 mL of *Solvent*. Evaporate the eluate (150 mL) in a tared, round–bottom, 250-mL conical flask to dryness, using a suitable rotary evaporator. The volatiles are blown out with a stream of nitrogen, and the residue is dried at 105° for 20 min. The weight of the residue gives the oil fraction, determined as nonpolar lipids, in Egg Phospholipids.

Calculate the percentage of the nonphosphatidyl lipids taken:

$$\text{Result} = A/W \times 100$$

A = weight of the residue (mg)
W = weight of Egg Phospholipids taken in the *Sample solution* (mg)

Acceptance criteria: NMT 7.0%

SPECIFIC TESTS

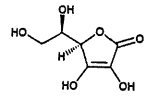
• **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩**:** NMT 20.0
• **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩**:** NMT 3
• **BACTERIAL ENDOTOXINS TEST** ⟨85⟩**:** NMT 6 USP Endotoxin Units/g
• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total microbial count does not exceed 100 cfu/g. It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.
• **WATER DETERMINATION,** *Method I* ⟨921⟩
Sample: 2 g in 50 mL of anhydrous methyl alcohol
Acceptance criteria: NMT 6.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve under nitrogen in a sealed container, and store at a temperature of –10° or below.

• **USP REFERENCE STANDARDS** ⟨11⟩
USP Phosphatidylcholine RS
USP Phosphatidylethanolamine RS
USP Lysophosphatidylcholine RS

Erythorbic Acid



$C_6H_8O_6$ 176.12

o-Araboascorbic acid;
D-Erythro-hex-2-enoic acid delta-lactone;
Isoascorbic acid, D-isoascorbic acid [89-65-6].

DEFINITION

Erythorbic Acid contains NLT 99.0% and NMT 100.5% of $C_6H_8O_6$, calculated on the dried basis.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K▲** (CN 1-May-2020)

• **B.**
Sample solution: 20 mg/mL of Erythorbic Acid and water
Analysis: To 2 mL of *Sample solution* add a few drops of sodium nitroferricyanide TS and then add 1 mL of 0.1 N sodium hydroxide.
Acceptance criteria: A transient blue color immediately appears.

• **C.**
Analysis: Dissolve about 15 mg of Erythorbic Acid in 15 mL of trichloroacetic acid (1:20). Add about 200 mg of activated charcoal, and shake the mixture vigorously for 1 min. Pass through a small fluted filter, refilter if necessary to obtain a clear filtrate, agitate the mixture until the pyrrole is dissolved, and heat in a water bath at 50°.
Acceptance criteria: A blue color appears.

ASSAY

• **PROCEDURE**
Sample: 400 mg
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.1 N iodine VS
Endpoint detection: Colorimetric
Analysis: Dissolve the *Sample* in a mixture of 100 mL of recently boiled and cooled water and 25 mL of 2 N sulfuric acid. Add 3 mL of starch TS, and titrate at once with 0.1 N iodine VS. Perform a blank determination.
Calculate the percentage of erythorbic acid ($C_6H_8O_6$) in the *Sample* taken:

$$\text{Result} = [(V - B) \times N \times F \times 100]/W$$

V = *Titrant* volume consumed by the *Sample* (mL)
B = *Titrant* volume consumed by the *Blank* (mL)
N = *Titrant* actual normality (mEq/mL)
F = equivalency factor, 88.06 mg/mEq
W = weight of the *Sample* (mg)

Acceptance criteria: 99.0%–100.5% on the dried basis

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IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.3%

• **LIMIT OF LEAD**

[NOTE—Select reagents having as low a lead content as practicable, and store all solutions in borosilicate glass containers. Rinse all glassware thoroughly with warm 8 N nitric acid followed by deionized water.]

Standard stock solution: Dissolve 160 mg of lead nitrate in 100 mL of water containing 1 mL of nitric acid. Dilute with water to 1000 mL. On the day of use, transfer 10.0 mL of the above solution to a 100-mL volumetric flask, and dilute with water to volume. Each mL of this solution contains the equivalent of about 10 µg of lead.

[NOTE—Prepare the *Standard solutions* on the day of use.]

Standard solution A: 1 µg/mL of lead from the *Standard stock solution*

Standard solution B: 2 µg/mL of lead from the *Standard stock solution*

Standard solution C: 5 µg/mL of lead from the *Standard stock solution*

Sample solution: Transfer a weighed portion of about 10 g of Erythorbic Acid to an evaporating dish. Add 5 mL of 25% sulfuric acid, and distribute the sulfuric acid uniformly through the sample. Within a hood, place the dish on a steam bath to evaporate most of the water. Place the dish on a burner, and slowly pre-ash the sample by expelling most of the sulfuric acid. Place the dish in a muffle furnace at 525°, and ash the sample until the residue appears free from carbon. Cool, and cautiously wash down the inside of the evaporation dish with water. Add 5 mL of 1 N hydrochloric acid. Place the dish on a steam bath, and evaporate to dryness. Add 1.0 mL of 3 N hydrochloric acid and approximately 5 mL of water, and heat briefly on a steam bath to dissolve any residue. Transfer quantitatively to a 10-mL volumetric flask, and dilute with water to volume.

Blank: To an evaporating dish add 5 mL of 25% sulfuric acid, and distribute the sulfuric acid uniformly. Within a hood, place the dish on a steam bath to evaporate most of the water. Place the dish on a burner, and expel most of the sulfuric acid. Then place the dish in a muffle furnace at 525° for about the same amount of time as in the *Sample solution* preparation. Cool, and cautiously wash down the inside of the evaporation dish with water. Add 5 mL of 1 N hydrochloric acid. Place the dish on a steam bath, and evaporate to dryness. Add 1.0 mL of 3 N hydrochloric acid and approximately 5 mL of water, and heat briefly on a steam bath to dissolve any residue. Transfer quantitatively to a 10-mL volumetric flask, and dilute with water to volume.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption

Lamp: Lead electrodeless discharge

Flame: Air–acetylene

Analytical wavelength: 283.3 nm

Slit width: 0.7 nm

Standard curve

Samples: *Standard solution A, Standard solution B, Standard solution C,* and *Blank*

Plot: Corrected absorbance values versus their corresponding concentration (µg/mL). [NOTE—Determine the corrected absorbance values by subtracting the absorbance of the *Blank* from the absorbance of the *Samples.*]

Analysis

Sample: *Sample solution*

From the *Standard curve,* determine the lead concentration in the *Sample solution.* Calculate the lead content, in ppm, in the portion of Erythorbic Acid taken:

$$\text{Result} = V \times C_S / W$$

V = volume of the *Sample solution* (mL)

C_S = concentration of lead in the *Sample solution* (µg/mL)

W = weight of Erythorbic Acid in the *Sample solution* (g)

Acceptance criteria: NMT 10 ppm

SPECIFIC TESTS

• **OPTICAL ROTATION,** *Specific Rotation* ⟨781S⟩: −16.5° to −18.0°

Sample solution: 100 mg/mL in water

• **LOSS ON DRYING** ⟨731⟩: Dry a sample in a vacuum over silica gel for 3 h: it loses NMT 0.4% of its weight.

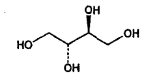
ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Erythorbic Acid RS

Erythritol



$C_4H_{10}O_4$ 122.12

1,2,3,4-Butanetetrol;

Butane 1,2,3,4-tetrol (*meso*-erythritol) [149-32-6].

DEFINITION

Erythritol is obtained by fermentation of starch enzyme hydrolysate (from starches such as wheat and corn). It is obtained from the fermentation broth of suitable osmophilic yeasts such as *Moniliella pollinis* or *Trichosporonoides megachiliensis.* It contains NLT 96.0% and NMT 102.0% of erythritol ($C_4H_{10}O_4$), calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

• **B. MELTING RANGE OR TEMPERATURE** ⟨741⟩: 119°–123°

ASSAY

• **PROCEDURE**

Mobile phase: 0.01% Sulfuric acid

System suitability solution: 0.05 mg/mL each of USP Erythritol RS and glycerol

Standard solution: 50 mg/mL of USP Erythritol RS

Sample solution: 50 mg/mL of Erythritol

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability.*)

Mode: LC

Detector: Refractive index

Column: 7.8-mm × 30-cm; packing L17

Column temperature: 70°

Flow rate: 0.8 mL/min

Injection volume: 10 µL

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System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The relative retention times for erythritol and glycerol are about 1.0 and 1.1, respectively.]
Suitability requirements
Resolution: NLT 2.0 between erythritol and glycerol, *System suitability solution*
Relative standard deviation: NMT 2.0%, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
[NOTE—Record chromatograms for over a period of 3 times the retention time of erythritol.]
Calculate the percentage of erythritol ($C_4H_{10}O_4$) in the portion of Erythritol taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP Erythritol RS in the *Standard solution* (mg/mL)
C_U = concentration of Erythritol in the *Sample solution* (mg/mL)

Acceptance criteria: 96.0%–102.0% on the anhydrous basis

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%
- **LIMIT OF LEAD**

Sample solution: Dissolve 20.0 g of Erythritol in diluted acetic acid, and dilute with the same medium to 100 mL. Add 2.0 mL of a saturated ammonium pyrrolidinedithiocarbamate solution (10 mg/mL of ammonium pyrrolidinedithiocarbamate) and 10.0 mL of methyl isobutyl ketone, and shake for 30 s. Protect from bright light. Allow the two layers to separate, and use the methyl isobutyl ketone layer.
Standard solutions: Prepare as directed for the *Sample solution*, except prepare three solutions by adding 0.5, 1.0, and 1.5 mL of standard lead solution TS in addition to the 20.0 g of Erythritol.
Blank solution: Prepare as directed for the *Sample solution*, omitting Erythritol.
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry, using methyl isobutyl ketone previously treated as described in the *Sample solution*, but without the sample added
Analytical wavelength: 283.3 nm
Lamp: Lead hollow-cathode
Flame: Air–acetylene
Analysis
Samples: *Sample solution* and *Standard solutions*
Introduce the *Sample solution* and each of the three *Standard solutions* into the instrument. Record the steady absorbance reading. Plot the absorbance readings against the known concentrations of added lead (in µg), and draw a straight line. Extrapolate the line until it meets the concentration axis, which is equal to the concentration, in ppm, of lead in the sample.
Acceptance criteria: NMT 0.5 µg/g

Change to read:

- **RELATED COMPOUNDS**

Mobile phase, System suitability solution, Standard solution, and **Sample solution:** Proceed as directed in the *Assay*.
Standard solution A: Transfer 2.0 mL of the *Standard solution* from the *Assay* to a 100-mL volumetric flask, and dilute with water (1 mg/mL of erythritol).
Chromatographic system: Proceed as directed in the *Assay*, except use an *Injection volume* of 20 µL.
Analysis
Samples: ▲*Standard solution A*▲ (ERR 1-Jun-2018) and *Sample solution*
Calculate the percentage of each impurity found:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response of erythritol from the ▲*Standard solution A*▲ (ERR 1-Jun-2018)
C_S = concentration of USP Erythritol RS in the ▲*Standard solution A* (mg/mL)▲ (ERR 1-Jun-2018)
C_U = concentration of Erythritol in the *Sample solution* (mg/mL)

Acceptance criteria
Individual impurities: NMT 2.0%
Total impurities: NMT 2.0%

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩: The total aerobic microbial count using the *Plate-Count Methods* is NMT 10^3 cfu/g, and the total combined molds and yeasts count is NMT 10^2 cfu/g. It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.
- **LOSS ON DRYING** ⟨731⟩
  Sample: 8 g
  Analysis: Dry the *Sample* at 105° for 4 h.
  Acceptance criteria: NMT 0.2%
- **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 0.5%
- **CONDUCTIVITY**

Sample solution: 200 mg/mL in water
Analysis: Using an appropriate conductivity meter, choose a conductivity cell that is appropriate for the properties and conductivity of the solution to be examined. Use a certified reference material,[1] for example, a solution of potassium chloride, that is appropriate for the measurement. The conductivity value of the certified reference material should be near the expected conductivity value of the solution to be examined. After calibrating the apparatus with a certified reference material solution, rinse the conductivity cell several times with water and at least twice with the aqueous solution to be examined. Measure the conductivity of the solution at a temperature of 20° while stirring gently with a magnetic stirrer.
Acceptance criteria: NMT 20 µS/cm

ADDITIONAL REQUIREMENTS

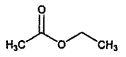
- **PACKAGING AND STORAGE:** Preserve in well-closed containers. Store at room temperature.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Erythritol RS
meso-Erythritol, 1,2,3,4-butanetetrol.
$C_4H_{10}O_4$ 122.12

[1] Commercially available conductivity calibration solutions for conductivity meter standardization, standardized by methods traceable to the National Institute of Standards and Technology (NIST), may be used. Solutions prepared according to instructions given in the American Society for Testing and Materials (ASTM) Standard D1125 may be used, provided that the conductivity of the resultant solution is the same as that of the solution prepared from the NIST-certified material.

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Ethyl Acetate



$C_4H_8O_2$ 88.11
Acetic acid, ethyl ester;
Ethyl acetate [141-78-6].

DEFINITION
Ethyl Acetate contains NLT 98.0% and NMT 102.0% of ethyl acetate ($C_4H_8O_2$).

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS ⟨197⟩, *Infrared Spectroscopy*: 197F** ▲ (CN 1-May-2020)

ASSAY
• **PROCEDURE**
Diluent: *N,N*-dimethylacetamide
System suitability solution: 2.0 mg/mL of USP Ethyl Acetate RS and 20 µg/mL of USP Methyl Ethyl Ketone RS in *Diluent*
Standard solution: 2.0 mg/mL of USP Ethyl Acetate RS in *Diluent*
Sample solution: 2.0 mg/mL of Ethyl Acetate in *Diluent*
Chromatographic system
(See *Chromatography ⟨621⟩, System Suitability.*)
Mode: GC
Detector: Flame ionization
Column: 0.32-mm × 60-m; coated with a 1.8-µm film of phase G43
Temperatures
Detector: 250°
Injection port: 210°
Column: See *Table 1.*

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
40	—	40	15
40	12	200	2

Carrier gas: Helium
Flow rate: 3.0 mL/min
Injection volume: 1 µL
Injection type: Split injection, split ratio 30:1
Run time: 30.3 min
System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The relative retention times for methyl ethyl ketone and ethyl acetate are 0.97 and 1.0, respectively.]
Suitability requirements
Resolution: NLT 2.0 between the methyl ethyl ketone and ethyl acetate peaks, *System suitability solution*
Tailing factor: NMT 1.5 for the ethyl acetate peak, *Standard solution*
Relative standard deviation: NMT 2.0%, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of ethyl acetate ($C_4H_8O_2$) in the portion of Ethyl Acetate taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times P \times 100$$

r_U = peak area of ethyl acetate from the *Sample solution*
r_S = peak area of ethyl acetate from the *Standard solution*
C_S = concentration of USP Ethyl Acetate RS in the *Standard solution* (mg/mL)
C_U = concentration of Ethyl Acetate in the *Sample solution* (mg/mL)
P = labeled purity of USP Ethyl Acetate RS

Acceptance criteria: 98.0%–102.0%

IMPURITIES
• **LIMIT OF NONVOLATILE RESIDUE**
Sample: Ethyl Acetate
Analysis: Evaporate the *Sample* in a tared porcelain dish on a steam bath, and dry at 105° for 1 h.
Acceptance criteria: NMT 0.02%

Change to read:

• **CHROMATOGRAPHIC PURITY**
Diluent and **Chromatographic system:** Proceed as directed in the *Assay.*
System suitability solution: 0.16 mg/mL each of acetaldehyde and methanol, 160 mg/mL of Ethyl Acetate, and 1.6 mg/mL of USP Methyl Ethyl Ketone RS in *Diluent*
Sensitivity solution: 0.08 mg/mL each of acetaldehyde, USP Ethyl Acetate RS, and USP 1-Ethoxy-2-methylpropane RS in *Diluent*
Methyl compounds identification solution: 0.16 mg/mL each of methanol, methyl acetate, and methyl isobutyrate in *Diluent*
Standard solution: 0.16 mg/mL each of acetaldehyde, USP Ethyl Acetate RS, and USP 1-Ethoxy-2-methylpropane RS in *Diluent*
Sample solution: 160 mg/mL of Ethyl Acetate in *Diluent*
System suitability
Samples: *System suitability solution, Sensitivity solution,* and *Standard solution*
[NOTE—The relative retention times for acetaldehyde, methanol, methyl ethyl ketone, ethyl acetate, and 1-ethoxy-2-methylpropane are 0.29, 0.31, 0.97, 1.0, and 1.1, respectively.]
Suitability requirements
Resolution: NLT 2.0 between the acetaldehyde and methanol peaks; NLT 2.0 between the methyl ethyl ketone and ethyl acetate peaks, *System suitability solution*
Signal-to-noise ratio: NLT 20 for the acetaldehyde, ethyl acetate, and 1-ethoxy-2-methylpropane peaks, *Sensitivity solution*
Tailing factor: NMT 1.5 for the acetaldehyde, ethyl acetate, and 1-ethoxy-2-methylpropane peaks, *Standard solution*
Relative standard deviation: NMT 5.0% for acetaldehyde, ethyl acetate, and 1-ethoxy-2-methylpropane, *Standard solution*
Analysis
Samples: *Methyl compounds identification solution, Standard solution,* and *Sample solution*
Calculate the percentage of acetaldehyde and 1-ethoxy-2-methylpropane in the portion of Ethyl Acetate taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of acetaldehyde or 1-ethoxy-2-methylpropane from the *Sample solution*

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r_S = peak area of acetaldehyde or 1-ethoxy-2-methylpropane from the *Standard solution*
C_S = concentration of acetaldehyde or USP 1-Ethoxy-2-methylpropane RS in the *Standard solution* (mg/mL)
C_U = concentration of Ethyl Acetate in the *Sample solution* (mg/mL)

Identify the methanol, methyl acetate, and methyl isobutyrate peaks in the *Sample solution* based on those in the *Methyl compounds identification solution.*
Calculate the content of methyl compounds in the portion of Ethyl Acetate taken:

$$\text{Result} = (r_T/r_S) \times F$$

r_T = sum of the peak areas of methanol, methyl acetate, and methyl isobutyrate from the *Sample solution*
r_S = peak area of ethyl acetate from the *Standard solution*
F = limit of methyl compounds, in percentage, 0.1

Acceptance criteria
Acetaldehyde: NMT 0.1%
▲1-Ethoxy-2-methylpropane:▲ (ERR 1-May-2018) NMT 0.1%
Methyl compounds: NMT 0.1%
Other impurities: NMT 0.3%; the sum of all other peaks areas in the chromatogram of the *Sample solution,* excluding the ethyl acetate, specified impurities, and solvent peaks areas, is not greater than three times the ethyl acetate peak area of the *Standard solution.*

SPECIFIC TESTS

- **ACIDITY**

Sample solution: 2.0 mL of Ethyl Acetate in 10 mL of neutralized alcohol
Analysis: Add 2 drops of phenolphthalein TS to the *Sample solution.* Neutralize with 0.10 N sodium hydroxide.
Acceptance criteria: NMT 0.10 mL of 0.10 N sodium hydroxide is required.

- **READILY CARBONIZABLE SUBSTANCES TEST** ⟨271⟩

Sample: 2 mL of Ethyl Acetate
Analysis: Add the *Sample* carefully to 10 mL of sulfuric acid to form separate layers.
Acceptance criteria: No dark zone is developed within 15 min.

- **SPECIFIC GRAVITY** ⟨841⟩: 0.894–0.898
- **BACTERIAL ENDOTOXINS TEST** ⟨85⟩: If labeled for use in preparing parenteral dosage forms, it also meets the following requirements. The level of bacterial endotoxins is such that the requirement in the relevant dosage form monographs(s) in which Ethyl Acetate is used can be met. Where the label states that Ethyl Acetate must be subjected to further processing during the preparation of injectable dosage forms, the level of bacterial endotoxins is such that the requirement in the relevant dosage form monograph(s) in which Ethyl Acetate is used can be met.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and avoid exposure to excessive heat.
- **LABELING:** Where Ethyl Acetate is intended for use in the manufacture of injectable dosage forms, it is so labeled. Where Ethyl Acetate must be subjected to further processing during the preparation of injectable dosage forms to ensure acceptable levels of bacterial endotoxins, it is so labeled.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Ethyl Acetate RS
USP 1-Ethoxy-2-methylpropane RS
USP Methyl Ethyl Ketone RS

Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion

[9010-88-2].

DEFINITION

Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion is an aqueous dispersion of a copolymer of ethyl acrylate and methyl methacrylate having an average molecular weight of about 800,000. It may contain suitable emulsifying agents.

IDENTIFICATION

Change to read:

- **▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy:* **197F▲** (CN 1-May-2020)

Analysis: Place 1 drop of Dispersion on a glass plate,[1] and cover the test substance with a water-resistant crystal disk (silver chloride or KRS-5).[2] Gently press on and then remove the crystal disk. Dry the crystal disk at 80° for approximately 15 min.
Acceptance criteria: The IR absorption spectrum of Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion exhibits maxima corresponding to the same wavelengths as those of a similar preparation of USP Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion RS treated in the same manner.

IMPURITIES

INORGANIC IMPURITIES

- **Residue on Ignition** ⟨281⟩: Using mild heating conditions (e.g., steam bath, sand bath) to avoid loss of material, evaporate the Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion to dryness before ignition: NMT 0.4% residue is obtained, calculated on the undried basis.

ORGANIC IMPURITIES

- **Procedure: Limit of Monomers**

Solution A: 35 mg/mL of sodium perchlorate
Solution B: Dilute phosphoric acid with water to obtain a solution having a pH of 2.0.
Mobile phase: *Solution B* and methanol (4:1)
Standard solution: Prepare a solution in tetrahydrofuran having a concentration of 2 µg/mL each of ethyl acrylate and methyl methacrylate. To 10.0 mL of this solution add 5.0 mL of *Solution A,* and mix. Dilute 5.0 mL of the mixture with water to 10.0 mL, and mix. The solution contains a concentration of 0.67 µg/mL each of ethyl acrylate and methyl methacrylate.
Sample stock solution: 20 mg/mL of Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion in tetrahydrofuran
Sample solution: To 5.0 mL of *Solution A* add 10.0 mL of *Sample stock solution,* dropwise, while stirring continuously. Centrifuge, and filter the clear supernatant. Dilute 5.0 mL of the clear supernatant with water to 10.0 mL, and mix.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability.*)
Mode: LC

[1] A simple glass microscope slide is suitable.
[2] KRS-5 consists of 42% thallium(I) bromide and 58% thallium(I) iodine by molecular weight. Suitable disks of silver chloride and of KRS-5 are available from www.photonic.saint-gobain.com, www.almazoptics.com, and www.internationalcrystal.net.

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Detector: UV 200 nm
Column: 4.6-mm × 12.0-cm; packing L1
Flow rate: 2 mL/min
Injection size: 50 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Resolution: NLT 2.0 between ethyl acrylate and methyl methacrylate
Relative standard deviation: NMT 2.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of each monomer in the portion of Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times D \times F \times 100$$

r_U = peak response of each monomer from the *Sample solution*
r_S = peak response of each monomer from the *Standard solution*
C_S = concentration of each monomer in the *Standard solution* (µg/mL)
C_U = concentration of Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion in the *Sample stock solution* (mg/mL)
D = dilution factor for the preparation of the *Sample solution* from the *Sample stock solution*, 3
F = unit conversion factor, 10^{-3} mg/µg

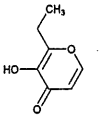
Total impurities: NMT 0.01% of total monomers

SPECIFIC TESTS
- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic microbial count does not exceed 1000 cfu/g, and the total yeasts and molds count does not exceed 100 cfu/g.
- **pH** ⟨791⟩**:** 5.5–8.6
- **LOSS ON DRYING** ⟨731⟩**:** Dry a sample at 110° for 3 h: it loses between 68.5% and 71.5% of its weight.
- **VISCOSITY—ROTATIONAL METHODS** ⟨912⟩**:** Equip a suitable rotational viscometer with an adapter comprising a cylindrical spindle rotating within an accurately machined chamber (or tube).[3] Mix the Dispersion, pipet the test specimen in the specified volume, which is recommended by the instrument manufacturer, into the chamber (or tube), and ensure that the temperature of the test specimen is at 20 ± 0.1°. The shear rate under the test condition is NLT 1 s^{-1} and NMT 100 s^{-1}.[4] Measure the apparent viscosity, following the instrument manufacturer's directions.
Acceptance criteria: The viscosity is between 2 and 20 mPa · s.
- **COAGULUM**
Sample: 100 g of Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion
Analysis: Weigh a stainless steel sieve having 125-µm openings or a suitable single-woven wire cloth with a mesh width of 125 µm, and filter the *Sample* through it. [NOTE—Suitable single-woven wire cloth mesh meets the requirements set in ISO 9044.]
Wash the sieve or the cloth with distilled water until a clear filtrate is obtained, and dry the sieve or the cloth to constant weight at 105°.
Acceptance criteria: The weight of the residue does not exceed 1000 mg (1%).

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers. Store between 5° and 25°, with excursions permitted up to 30°. Do not freeze.
- **LABELING:** Label it to indicate the name and quantity of any added emulsifiers.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion RS

[3] A commercial device is available from Brookfield as an ultra-low (UL) viscosity adapter. The adapter comprises a 0.4-cm diameter shaft, an accurately machined chamber (or tube) with an internal diameter of 2.8 cm and a depth of 13.5 cm, and a cylindrical spindle 2.5 cm in diameter and 9.1 cm in height.
[4] The cylindrical spindle rotates at 30 rpm.

Ethyl Maltol



$C_7H_8O_3$ 140.14
2-Ethyl-3-hydroxy-4-pyrone;
2-Ethyl-3-hydroxy-4*H*-pyran-4-one;
2-Ethyl pyromeconic acid [4940-11-8].

DEFINITION
Ethyl Maltol contains NLT 99.0% of $C_7H_8O_3$, calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197S.▲** (CN 1-May-2020) 1:50 solution in chloroform

ASSAY
- **PROCEDURE**
Standard solution: 10.0 µg/mL of USP Ethyl Maltol RS in 0.1 N hydrochloric acid
Sample solution: 10.0 µg/mL of Ethyl Maltol in 0.1 N hydrochloric acid
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: UV
Analytical wavelength: 276 nm
Cell: 1 cm
Blank: 0.1 N hydrochloric acid
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of ethyl maltol ($C_7H_8O_3$) in the portion of Ethyl Maltol taken:

$$\text{Result} = (A_U/A_S) \times (C_S/C_U) \times 100$$

A_U = absorbance of the *Sample solution*
A_S = absorbance of the *Standard solution*
C_S = concentration of USP Ethyl Maltol RS in the *Standard solution* (µg/mL)
C_U = concentration of Ethyl Maltol in the *Sample solution* (µg/mL)

Acceptance criteria: NLT 99.0% on the anhydrous basis

IMPURITIES
- **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 0.2% at 800° for 15 min

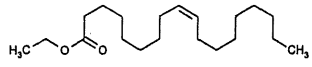
NF Monographs

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SPECIFIC TESTS
• **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 0.5%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight containers.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Ethyl Maltol RS

Ethyl Oleate



$C_{20}H_{38}O_2$ 310.51
9-Octadecenoic acid, (*Z*)-, ethyl ester;
Ethyl (*Z*)-9-octadecenoate;
Ethyl *cis*-9-octadecenoate [111-62-6].

DEFINITION
Ethyl Oleate consists of esters of ethyl alcohol and high molecular weight fatty acids, principally oleic acid. It contains NLT 65.0% of ethyl (*Z*)-9-octadecenoate ($C_{20}H_{38}O_2$). It may contain suitable stabilizers.

IDENTIFICATION
• **A. PRESENCE OF ESTER**
Analysis 1: Proceed as directed in *Fats and Fixed Oils, Saponification Value* ⟨401⟩.
Acceptance criteria: 177–188
Analysis 2: Proceed as directed in *Fats and Fixed Oils, Acid Value* ⟨401⟩.
Acceptance criteria: NMT 0.5
• **B. CHROMATOGRAPHIC IDENTITY**
Analysis: Proceed as directed in the *Assay*.
Acceptance criteria: The retention time of the major peak of the *Sample solution* corresponds to the ethyl oleate peak of the *System suitability solution*.

ASSAY
• **PROCEDURE**
System suitability solution: 5 mg/mL of USP Ethyl Oleate RS, 1.2 mg/mL of USP Ethyl Palmitate RS, 1.2 mg/mL of USP Ethyl Linoleate RS, and 0.5 mg/mL of USP Ethyl Stearate RS in *n*-hexane
Sample solution: 5 mg/mL of Ethyl Oleate in *n*-hexane
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.25-mm × 25-m capillary; bonded with a 0.2-µm layer of phase G16
Temperatures
Detector: 270°
Injection port: 250°
Column: See temperature program shown in *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
178	—	178	2
178	3.3	240	2.5

Carrier gas: Hydrogen
Flow rate: 0.7 mL/min
Injection volume: 1.0 µL
Injection type: Split injection; split ratio is 200:1
Run time: 23–24 min
System suitability
Sample: *System suitability solution*
[NOTE—See *Table 2* for the relative retention times.]

Table 2

Component	Relative Retention Time
Ethyl palmitate	0.65
Ethyl stearate	0.96
Ethyl oleate	1.00
Ethyl linoleate	1.08

Suitability requirements
Resolution: NLT 2.0 between the ethyl stearate and ethyl oleate peaks
Analysis
Samples: *System suitability solution* and *Sample solution*
Identify each ethyl ester (ethyl palmitate, ethyl stearate, ethyl oleate, or ethyl linoleate) peak in the *Sample solution* based on that in the *System suitability solution*.
Calculate the percentage of each ethyl ester (ethyl palmitate, ethyl stearate, ethyl oleate, or ethyl linoleate) in the portion of Ethyl Oleate taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of each ethyl ester (ethyl palmitate, ethyl stearate, ethyl oleate, or ethyl linoleate) from the *Sample solution*
r_T = sum of all the peak responses excluding peak responses due to solvent from the *Sample solution*

Acceptance criteria: Disregard peaks that are less than 0.05% for any unspecified impurities, and any peaks due to solvent.
Ethyl Oleate exhibits the composition profiles shown in *Table 3* below.

Table 3

Component	Percentage (%)
Ethyl palmitate	≤16.0
Ethyl stearate	≤6.0
Ethyl oleate	≥65.0
Ethyl linoleate	≤18.0

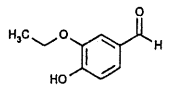
IMPURITIES
• **TOTAL ASH**
Sample: 2.0 g
Analysis: Heat a silica or platinum crucible to redness for 30 min, allow to cool in a desiccator, and weigh. Transfer the *Sample* to the crucible. Dry at 100°–105° for 1 h and ignite to constant weight in a muffle furnace at 600 ± 25°, allowing the crucible to cool in a desiccator after each ignition. Flames should not be produced at any time during the procedure. If after prolonged ignition the ash still contains black particles, take up with hot water, pass through an ashless filter paper, and ignite the residue and the filter paper. Combine the filtrate with the ash, carefully evaporate to dryness, and ignite to constant weight.
Acceptance criteria: NMT 0.1%

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SPECIFIC TESTS
- **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩: 75–85
- **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩: NMT 10.0
- **WATER DETERMINATION** ⟨921⟩: NMT 1.0%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** Label it to indicate whether oleic acid is derived from vegetable, animal, or synthetic sources. Indicate the names and amounts of any added stabilizers.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Ethyl Linoleate RS
  USP Ethyl Oleate RS
  USP Ethyl Palmitate RS
  USP Ethyl Stearate RS

Ethyl Vanillin



$C_9H_{10}O_3$ 166.17
Benzaldehyde, 3-ethoxy-4-hydroxy-;
3-Ethoxy-4-hydroxybenzaldehyde [121-32-4].

DEFINITION
Ethyl Vanillin, dried over phosphorus pentoxide for 4 h, contains NLT 98.0% and NMT 101.0% of $C_9H_{10}O_3$.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

Change to read:

- **B. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Ultraviolet-Visible Spectroscopy*: **197U**▲ (CN 1-May-2020)
  Sample solution: 8 µg/mL in methanol
  Acceptance criteria: Meets the requirements

ASSAY
- **PROCEDURE**
  Sample: 300 mg of Ethyl Vanillin (previously dried)
  Titrimetric system
  (See *Titrimetry* ⟨541⟩.)
  Mode: Direct titration
  Titrant: 0.1 N sodium methoxide VS
  Blank: 50 mL of dimethylformamide, accurately measured
  Endpoint detection: Visual
  Analysis: Transfer the *Sample solution* to a 125-mL conical flask, and dissolve in 50 mL of dimethylformamide. Add thymol blue TS and titrate, using a magnetic stirrer and taking precautions against the absorption of atmospheric carbon dioxide. Perform a blank determination. Calculate the percentage of ethyl vanillin ($C_9H_{10}O_3$) in the *Sample* taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

V_S = *Titrant* volume consumed by the *Sample* (mL)
V_B = *Titrant* volume consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 166.2 mg/mEq
W = *Sample* weight (mg)

Acceptance criteria: 98.0%–101.0% on the previously dried basis

IMPURITIES
- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%

SPECIFIC TESTS
- **MELTING RANGE OR TEMPERATURE** ⟨741⟩: 76°–78°
- **LOSS ON DRYING** ⟨731⟩: Dry a sample over phosphorus pentoxide for 4 h: it loses NMT 1.0% of its weight.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Ethyl Vanillin RS

Ethylcellulose

Portions of the monograph text that are national *USP* text, and are not part of the harmonized text, are marked with symbols (• •) to specify this fact.

Cellulose, ethyl ether;
Cellulose ethyl ether [9004-57-3].

DEFINITION
Ethylcellulose is a partly *O*-ethylated cellulose. It contains NLT 44.0% and NMT 51.0% of ethoxy ($-OC_2H_5$) groups, calculated on the dried basis. It may contain a suitable antioxidant.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, ***Infrared Spectroscopy***: **197F**▲ (CN 1-May-2020)
  Sample: Dissolve 40 mg in 1 mL of methylene chloride, spread 2 drops of this solution between two sodium chloride plates, then remove one of the plates to evaporate the solvent.
  Acceptance criteria: Meets the requirements

ASSAY
- **PROCEDURE**
  [**CAUTION**—Hydriodic acid and its reaction byproducts are highly toxic. Perform all steps of the *Standard solution* and the *Sample solution* in a properly functioning hood. Specific safety practices to be followed are to be identified to the analyst performing this test.]
  [NOTE—Prepare the solutions immediately before use.]
  Internal standard solution: To 10 mL of *o*-xylene add 0.5 mL of *n*-octane and dilute to 100.0 mL with *o*-xylene.
  Sample solution: To 30.0 mg of the substance to be examined, previously dried, add 60 mg of adipic acid in a 5-mL pressure-tight reaction vial equipped with a pressure-tight membrane stopper coated with polytetrafluoroethylene and secured with an aluminum crimped cap or any other sealing system providing a sufficient air-tightness. Add 2.00 mL of *Internal standard solution* and 1.0 mL of hydriodic acid and close immediately. Accurately weigh the vial (total mass before heating). Do not mix the contents of the vial by hand before heating. Place the vial in an oven or heat in a suitable heater, with continuous mechanical agitation, maintaining the

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internal temperature of the vial at 115 ± 2° for 70 min. Allow to cool and weigh accurately the vial (total mass after heating). If the difference of the total mass before heating to the total mass after heating is more than 10 mg, prepare a new test solution. After phase separation, pierce through the septum of the vial with a cooled syringe and withdraw a sufficient volume of the upper phase as the test solution.

Standard solution: Place 60 mg of adipic acid and 2.00 mL of *Internal standard solution* in another 5-mL reaction vial, add 1.0 mL of hydriodic acid and close immediately. Accurately weigh the vial, then inject 25 µL of iodoethane through the septum in the vial. Weigh again accurately and mix. After phase separation, pierce through the septum of the vial with a cooled syringe and withdraw a sufficient volume of the upper phase as the *Standard solution.*

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability.*)

Mode: GC

Detector: Flame ionization

Column: 0.53-mm × 30-m fused silica coated with 3-µm layer of phase G1. [NOTE—Use a guard column, if necessary.]

Temperatures

Injection port: 250°

Detector: 280°

Column: See *Table 1.*

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
50	0	50	3
50	10	100	0
100	34.9	250	8

Flow rate: 4.2 mL/min

Carrier gas: Helium

Split ratio: 40:1

Injection volume: 1 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention time is about 0.6 for iodoethane with reference to *n*-octane (retention time about 10 min).]

Suitability requirements

Resolution: NLT 5.0 between *n*-octane and iodoethane

Relative standard deviation: NMT 2.0%, using the response factor of the principal peak for six injections of the *Standard solution*

Analysis

Sample: *Sample solution*

[NOTE—Measure all of the peak areas, excluding the solvent peak.]

Calculate the response factor (*F*):

$$\text{Result} = (A_1 \times W_1 \times C)/(A_2 \times 100)$$

A_1 = peak area of the internal standard peak from the *Standard solution*

W_1 = weight of iodoethane in the *Standard solution* (mg)

C = content of iodoethane from the certificate of the manufacturer (%)

A_2 = peak area of the iodoethane peak from the *Standard solution*

Calculate the percentage content (m/m) of the ethoxy group:

$$\text{Result} = (A_4 \times F \times M_1 \times 100)/(A_3 \times W_2 \times M_2)$$

A_4 = peak area of iodoethane from the *Sample solution*

F = response factor calculated from above

M_1 = molar mass of ethoxy group, 45.1

A_3 = peak area of the internal standard from the *Sample solution*

W_2 = weight of the sample (dried substance) in the *Sample solution* (mg)

M_2 = molar mass of iodoethane, 156.0

Acceptance criteria: NLT 44.0% and NMT 51.0% of ethoxy groups on the dried basis

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩

Sample: 1.0 g

Acceptance criteria: NMT 0.5%

• **CHLORIDES**

Standard stock solution: 0.824 mg/mL of sodium chloride

Standard solution: 8.24 µg/mL of sodium chloride, prepared from the *Standard stock solution.* [NOTE—Prepare immediately before use.]

Sample solution: Disperse 250 mg in 50 mL of water, heat to boiling, and allow to cool, shaking occasionally. Filter, and discard the first 10 mL of the filtrate.

Analysis

Samples: *Standard solution* and *Sample solution*

Separately dilute 10 mL of the *Sample solution* and *Standard solution* with water to 15 mL, add 1 mL of diluted nitric acid (125 g/L), and pour the mixtures as a single addition into test tubes containing 1 mL of 0.1 N silver nitrate VS. Examine the tubes laterally against a black background.

Acceptance criteria: After standing for 5 min protected from light, any opalescence in the *Sample solution* is not more intense than that in the *Standard solution* (0.1%).

• **ACETALDEHYDE**

Solution A: 0.5 mg/mL of methylbenzothiazolone hydrazone hydrochloride

Solution B: 10 mg/mL of ferric chloride and 16 mg/mL of sulfamic acid

Standard stock solution: 10 mg/mL of acetaldehyde in water. [NOTE—Use immediately. Prepare the *Standard solution* and *Sample solution* at the same time.]

Standard solution: 3 µg/mL of acetaldehyde from the *Standard stock solution* in water. [NOTE—Use immediately.]

Sample solution: Dissolve 3.0 g of Ethylcellulose in 10 mL of water, stir by mechanical means for 1 h, allow to stand for 24 h, filter, and dilute the filtrate with water to 100.0 mL.

Analysis

Samples: *Standard solution* and *Sample solution*

Transfer 5.0 mL of the *Sample solution* and *Standard solution* to separate flasks. To each flask add 5 mL of *Solution A,* and heat in a water bath at 60° for 5 min. Add 2 mL of *Solution B,* and heat again at 60° for 5 min. Cool, and dilute with water to 25.0 mL.

Acceptance criteria: The *Sample solution* is not more intensely colored than the *Standard solution.*

SPECIFIC TESTS

• **VISCOSITY—CAPILLARY METHODS** ⟨911⟩

Solution A: Alcohol and toluene (1:4 w/w)

Sample solution: Shake a quantity of Ethylcellulose, equivalent to 5.00 g of the dried substance, with 95 g of *Solution A* until the substance is dissolved.

Analysis: Determine the viscosity using a capillary viscometer.

NF Monographs

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Acceptance criteria: The viscosity (mPa · s) determined at 25° is NLT 80.0% and NMT 120.0% of that stated on the label for a nominal viscosity greater than 6 mPa · s; and NLT 75.0% and NMT 140.0% of that stated on the label for a nominal viscosity of NMT 6 mPa · s.

• **ACIDITY OR ALKALINITY**

Solution A: Dissolve 100 mg of phenolphthalein in 80 mL of alcohol, and dilute with water to 100 mL.

Solution B: Dilute 50 mg of methyl red with 1.86 mL of 0.1 N sodium hydroxide and 50 mL of alcohol, and dilute with water to 100 mL.

Sample solution: To 0.5 g of Ethylcellulose add 25 mL of carbon dioxide-free water, and shake for 15 min. Pass through a sintered-glass filter with a maximum diameter of pores between 16 and 40 µm.

Analysis: To 10 mL of *Sample solution* add 0.1 mL of *Solution A* and 0.5 mL of 0.01 N sodium hydroxide (*Solution C*). To 10 mL of *Sample solution* add 0.1 mL of *Solution B* and 0.5 mL of 0.01 N hydrochloric acid (*Solution D*).

Acceptance criteria: *Solution C* is pink; *Solution D* is red.

• **LOSS ON DRYING** ⟨731⟩

Sample: 1 g

Analysis: Dry at 105° for 2 h.

Acceptance criteria: It loses NMT 3.0% of its weight.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

• **LABELING:** Label to indicate its nominal viscosity in mPa · s for a 5% m/m solution. The label states the name and amount of any added antioxidant.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Ethylcellulose RS

Ethylcellulose Aqueous Dispersion

DEFINITION

Ethylcellulose Aqueous Dispersion is a colloidal dispersion of Ethylcellulose in water. It contains NLT 90.0% and NMT 110.0% of the labeled amount of ethylcellulose. It contains suitable amounts of Cetyl Alcohol and Sodium Lauryl Sulfate, which assist in the formation and stabilization of the dispersion. It may contain suitable antifoaming and antimicrobial agents.

IDENTIFICATION

• **A.**

Diluent: Toluene and alcohol (80:20)

Standard solution: 250 mg of USP Ethylcellulose RS in 5 mL of *Diluent*

Sample solution: A small quantity of Dispersion in 5 mL of *Diluent*

Analysis: Transfer a few mL of the *Standard solution* and the *Sample solution* to two separate silver chloride plates, and evaporate.

Acceptance criteria: The IR absorption spectrum of the residue in the regions 3600 to 2600 cm^{-1} and 1500 to 800 cm^{-1} exhibits maxima only at the same wavenumbers as that of a film of USP Ethylcellulose RS.

• **B.**

Sample: 2 mL

Analysis: Transfer the *Sample* to a Petri dish, 100 mm in diameter, so that the bottom of the dish is covered uniformly. Place the dish in an oven or on a hot plate to evaporate the water.

Acceptance criteria: A transparent film results.

• **C.**

Sample solution: Dissolve the film formed in *Identification* test *B* in 20 mL of chloroform.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 2-mm × 1.8-m; 10% liquid phase G1 on support S1A

Temperatures

Injection port: 250°

Detector: 275°

Column: 220°

Injection volume: 2 µL

Acceptance criteria: The retention time of the major peak following the solvent peak from the sample corresponds to that from a similar solution of USP Cetyl Alcohol RS.

• **D.**

Indicator solution: To a 150-mL graduated beaker containing 0.7 mL of sulfuric acid and 5 g of anhydrous sodium sulfate slowly add water to the 90-mL mark. Add methylene blue solution (3 in 1000) to the 100-mL mark.

Analysis: To 1 mL of Dispersion in a 100-mL graduated mixing cylinder add 9 mL of water followed by 25 mL of *Indicator solution*. Add 15 mL of chloroform, and shake vigorously. Allow the two phases to separate.

Acceptance criteria: The lower phase is blue, indicating the presence of sodium lauryl sulfate.

ASSAY

• **PROCEDURE**

Sample solution: Dispersion equivalent to 25 mg of ethylcellulose

Analysis: Determine the ethoxy content, as directed in *Methoxy Determination* ⟨431⟩. Calculate the ethylcellulose content from the ethoxy content found, and calculate the ethoxy content of the ethylcellulose as declared in the labeling. Each mL of 0.1 N sodium thiosulfate is equivalent to 0.7510 mg of ($-OC_2H_5$).

Acceptance criteria: 90.0%–110.0%

SPECIFIC TESTS

• **VISCOSITY—ROTATIONAL METHODS** ⟨912⟩

Sample: Neat

Analysis: Use a rotational viscometer equipped with a low-viscosity adapter. Mix the *Sample*, and pipet 20 mL of it into the low-viscosity small sample adapter. Start the viscometer, and take readings after 60, 90, and 120 s at a temperature of 25 ± 2° and at a spindle speed that results in readings of 10%–90% of full-scale. Multiply the average of the three readings by the factor specified for the selected spindle speed to obtain the viscosity in centipoises.

Acceptance criteria: NMT 150 centipoises

• **pH** ⟨791⟩**:** 4.0–7.0

• **LOSS ON DRYING** ⟨731⟩

Sample: 5 mL

Analysis: Place 10 g of standard 20- to 30-mesh sand, previously dried for at least 30 min at 60°, into a tared Petri dish. Add the *Sample*, and again weigh. Dry at 60° to constant weight.

Acceptance criteria: NMT 71.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers, and protect from freezing.

• **LABELING:** The labeling states the ethoxy content of the ethylcellulose and the percentage of ethylcellulose. The labeling also states the names and quantities of any added antifoaming and antimicrobial agents.

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• **USP REFERENCE STANDARDS** ⟨11⟩
USP Cetyl Alcohol RS
USP Ethylcellulose RS

Ethylcellulose Dispersion Type B

DEFINITION
A stabilized dispersion of ethylcellulose in water. It contains NLT 90.0% and NMT 110.0% of the labeled amount of Ethylcellulose. It may contain suitable amounts of plasticizers, stabilizers, and glidants.

IDENTIFICATION
• **A. FILM FORMATION**
Analysis: Transfer an appropriate quantity of Ethylcellulose Dispersion Type B to a clear glass plate, distribute evenly, and place in a laboratory oven at about 60° until dry. [NOTE—It may take less than 60 min.]
Acceptance criteria: A continuous transparent or translucent film is formed.
• **B. INFRARED ABSORPTION**

Change to read:

Analysis: Use the film prepared in *Identification* test *A* and perform ▲*Spectroscopic Identification Tests* ⟨197⟩, *Infrared Spectroscopy: 197A*▲ (CN 1-May-2020)
Standard spectrum: Perform ▲*Spectroscopic Identification Tests* ⟨197⟩, *Infrared Spectroscopy: 197A*▲ (CN 1-May-2020) using USP Ethylcellulose RS.
Acceptance criteria: The IR absorption spectrum of the film so formed in the 3600–2600 cm^{-1} and 1500–800 cm^{-1} regions exhibits maxima corresponding to the same wave numbers as the *Standard spectrum*.
• **C.** The retention time of the ethylcellulose peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*. [NOTE—Plasticizer and/or stabilizer peaks may be present in the chromatogram.]

ASSAY
• **PROCEDURE**
Mobile phase: Tetrahydrofuran
Standard solution: Transfer 375 mg of USP Ethylcellulose RS to a 100-mL volumetric flask, and add 70 mL of tetrahydrofuran. Shake by mechanical means until the ethylcellulose is dissolved, and dilute with tetrahydrofuran to volume. The *Standard solution* contains 3.75 mg/mL of USP Ethylcellulose RS.
Sample solution: Add 30 mL of tetrahydrofuran to 1.0 g of Ethylcellulose Dispersion Type B in a 50-mL volumetric flask, and mix the mixture on a suitable shaker for 15 min. Dilute with tetrahydrofuran to volume, and mix. The *Sample solution* contains 20 mg/mL of Ethylcellulose Dispersion Type B in tetrahydrofuran. [NOTE—If Ethylcellulose Dispersion Type B contains inorganic insoluble material, a portion of the *Sample solution* should be centrifuged at 15,800 × *g* for NLT 15 min.]
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Refractive index
Column: 8.0-mm × 30-cm; 6-µm packing L21
Temperature
Detector: 45°
Column: 45°
Flow rate: 0.5 mL/min
Injection size: 20 µL
System suitability
Sample: *Standard solution*
Suitablity requirements
Relative standard deviation: NMT 5.0% determined for the ethylcellulose peak
Tailing factor: NMT 2.0 for the ethylcellulose peak
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of ethylcellulose in the portion of Ethylcellulose Dispersion Type B taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of ethylcellulose from the *Sample solution*
r_S = peak response of ethylcellulose from the *Standard solution*
C_S = concentration of USP Ethylcellulose RS in the *Standard solution* (mg/mL)
C_U = concentration of Ethylcellulose Dispersion Type B in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–110.0%

OTHER COMPONENTS
[NOTE—Perform the test for individual plasticizers, stabilizers, or glidants only if they are included in the *Labeling*.]
• **CONTENT OF MEDIUM-CHAIN TRIGLYCERIDES**
Mobile phase, Sample solution, and **Chromatographic system:** Proceed as directed in the *Assay*.
Standard solution: Transfer 375 mg of USP Ethylcellulose RS, 60 mg of medium-chain triglycerides, and 40 mg of oleic acid to a 100-mL volumetric flask. Add 70 mL of tetrahydrofuran, and shake by mechanical means until the ethylcellulose is dissolved. Dilute with tetrahydrofuran to volume. The *Standard solution* contains 3.75 mg/mL of USP Ethylcellulose RS, 0.6 mg/mL of medium-chain triglycerides, and 0.4 mg/mL of oleic acid. [NOTE—Oleic acid is included in the *Standard solution* to assist with consistent integration between *Standard* and *Sample solutions*.]
System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for ethylcellulose, medium-chain triglycerides, and oleic acid are 1.00, 1.18, and 1.25, respectively.]
Suitability requirements
Relative standard deviation: NMT 5.0% determined for the medium-chain triglycerides peak
Resolution: NLT 2.0 between ethylcellulose and medium-chain triglycerides
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of medium-chain triglycerides in the portion of Ethylcellulose Dispersion Type B taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of medium-chain triglycerides from the *Sample solution*
r_S = peak response of medium-chain triglycerides from the *Standard solution*
C_S = concentration of medium-chain triglycerides in the *Standard solution* (mg/mL)
C_U = concentration of Ethylcellulose Dispersion Type B in the *Sample solution* (mg/mL)

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Acceptance criteria: The percentage content of medium-chain triglycerides falls within the quantity range indicated by the *Labeling*. The ratio of medium-chain triglycerides to ethylcellulose is less than 0.25.

• **CONTENT OF OLEIC ACID**

Standard solution: 1.68 mg/mL of USP Oleic Acid RS in tetrahydrofuran

Sample solution: Add 15 mL of tetrahydrofuran to 2.0 g of Ethylcellulose Dispersion Type B in a 25-mL volumetric flask, and mix the mixture on a suitable shaker for 15 min. Dilute with tetrahydrofuran to volume, and mix. The *Sample solution* contains 80 mg/mL of Ethylcellulose Dispersion Type B in tetrahydrofuran.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.53-mm × 30-m capillary column; 0.25-µm layer of phase G25 (or G35)

Temperature

Detector: 280°

Injector port: 280°

Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
120	—	120	5
120	10	250	20

Carrier gas: Helium

Flow rate: 7.0 mL/min

Injection size: 1.0 µL

Injection type: Splitless

System suitability

Sample: *Standard solution*

[NOTE—The retention time for oleic acid is about 18.5 min.]

Suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of oleic acid in the portion of Ethylcellulose Dispersion Type B taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of oleic acid from the *Sample solution*

r_S = peak response of oleic acid from the *Standard solution*

C_S = concentration of USP Oleic Acid RS in the *Standard solution* (mg/mL)

C_U = concentration of Ethylcellulose Dispersion Type B in the *Sample solution* (mg/mL)

Acceptance criteria: The percentage content of oleic acid falls within the quantity range indicated by the *Labeling*. The ratio of oleic acid to ethylcellulose is less than 0.15.

• **CONTENT OF DIBUTYL SEBACATE AND OLEIC ACID**

Standard solution: 0.74 mg/mL of USP Dibutyl Sebacate RS and 0.48 mg/mL of USP Oleic Acid RS in tetrahydrofuran

Sample solution: Add 25 mL of tetrahydrofuran to 1.0 g of Ethylcellulose Dispersion Type B in a 50-mL volumetric flask, and mix the mixture on a suitable shaker for 15 min. Dilute with tetrahydrofuran to volume, and mix. The *Sample solution* contains 20 mg/mL of Ethylcellulose Dispersion Type B in tetrahydrofuran. [NOTE—If Ethylcellulose Dispersion Type B contains inorganic insoluble material, a portion of the *Sample solution* should be centrifuged at 15,800 × *g* for NLT 15 min.]

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.53-mm × 15-m capillary column; 0.1-µm layer of phase G25 (or G35)

Temperature

Detector: 280°

Injector port: 280°

Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
150	—	150	2
150	10	250	10

Carrier gas: Helium

Flow rate: 5.0 mL/min

Injection size: 0.5 µL

Injection type: Split (ratio about 10:1)

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for dibutyl sebacate and oleic acid are 1.00 and 1.45, respectively.]

Suitability requirements

Relative standard deviation: NMT 5.0%

Resolution: NLT 2.0 between dibutyl sebacate and oleic acid

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of each component (dibutyl sebacate or oleic acid) in the portion of Ethylcellulose Dispersion Type B taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of each component (dibutyl sebacate or oleic acid) from the *Sample solution*

r_S = peak response of each component (dibutyl sebacate or oleic acid) from the *Standard solution*

C_S = concentration of each component (USP Dibutyl Sebacate RS or USP Oleic Acid RS) in the *Standard solution* (mg/mL)

C_U = concentration of Ethylcellulose Dispersion Type B in the *Sample solution* (mg/mL)

Acceptance criteria

Dibutyl sebacate: The percentage content falls within the quantity range indicated by the *Labeling*. The ratio of dibutyl sebacate to ethylcellulose is less than 0.25.

Oleic acid: The percentage content falls within the quantity range indicated by the *Labeling*. The ratio of oleic acid to ethylcellulose is less than 0.15.

IMPURITIES

INORGANIC IMPURITIES

• **Residue on Ignition** ⟨281⟩: NMT 1.95%. [NOTE—Perform this test only if Ethylcellulose Dispersion Type B contains inorganic nonvolatile material.]

ORGANIC IMPURITIES

• **Procedure 1: Limit of Glycerin**

[NOTE—Perform this test only if Ethylcellulose Dispersion Type B contains glycerides.]

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Standard solution: 0.05 mg/mL of USP Glycerin RS in methanol
Sample solution: Add 15 mL of methanol to 2.0 g of Ethylcellulose Dispersion Type B in a 25-mL volumetric flask, and mix the mixture on a suitable shaker for 15 min. Dilute with methanol to volume, and mix. The *Sample solution* contains 80 mg/mL of Ethylcellulose Dispersion Type B in methanol.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.53-mm × 30-m capillary column; 3.0-µm layer of phase G43
Temperature
Detector: 280°
Injector port: 280°
Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
120	10	150	2
150	15	240	20

Carrier gas: Helium
Flow rate: 10.0 mL/min
Injection size: 1.0 µL
Injection type: Splitless
System suitability
Sample: *Standard solution*
[NOTE—The retention time for glycerin is about 3.8 min.]
Suitability requirements
Relative standard deviation: NMT 5.0%
Tailing factor: NMT 2.5
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of glycerin in the portion of Ethylcellulose Dispersion Type B taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of glycerin from the *Sample solution*
r_S = peak response of glycerin from the *Standard solution*
C_S = concentration of USP Glycerin RS in the *Standard solution* (mg/mL)
C_U = concentration of Ethylcellulose Dispersion Type B in the *Sample solution* (mg/mL)

Acceptance criteria: NMT 0.6%
• **Procedure 2: Limit of 1-Butanol**
[NOTE—Perform this test only if Ethylcellulose Dispersion Type B contains butyl esters.]
Standard solution: 0.1 mg/mL of USP 1-Butanol RS in methanol
Sample solution: Add 15 mL of methanol to 2.0 g of Ethylcellulose Dispersion Type B in a 25-mL volumetric flask, and mix the mixture on a suitable shaker for 15 min. Dilute with methanol to volume, and mix. The *Sample solution* contains 80 mg/mL of Ethylcellulose Dispersion Type B in methanol. [NOTE—If Ethylcellulose Dispersion Type B contains inorganic insoluble material, a portion of the *Sample solution* should be centrifuged at 15,800 × *g* for 30 min.]
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.53-mm × 30-m capillary column; 1.0-µm layer of phase G16
Temperature
Detector: 250°
Injector port: 250°
Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
45	—	45	5
45	10	80	—
80	20	220	10

Carrier gas: Helium
Flow rate: 10.0 mL/min
Injection size: 0.5 µL
Injection type: Splitless
System suitability
Sample: *Standard solution*
[NOTE—The retention time for 1-butanol is about 7.8 min.]
Suitability requirements
Relative standard deviation: NMT 5.0%
Tailing factor: NMT 2.0
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of 1-butanol in the portion of Ethylcellulose Dispersion Type B taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of 1-butanol from the *Sample solution*
r_S = peak response of 1-butanol from the *Standard solution*
C_S = concentration of USP 1-Butanol RS in the *Standard solution* (mg/mL)
C_U = concentration of Ethylcellulose Dispersion Type B in the *Sample solution* (mg/mL)

Acceptance criteria: NMT 0.2%

SPECIFIC TESTS

• **pH** ⟨791⟩: 9.5–11.5
• **TOTAL SOLIDS**
Analysis: Place 3 g (4 mm in diameter) of glass beads in an aluminum dish, and weigh. Add 10 g of Ethylcellulose Dispersion Type B, and again weigh. Dry at about 105° for 3 h. Determine the percentage of total solids in Ethylcellulose Dispersion Type B.
Acceptance criteria: 23.0%–26.0%
• **VISCOSITY—ROTATIONAL METHODS** ⟨912⟩
Sample: 500 mL of Ethylcellulose Dispersion Type B
Analysis: Transfer the *Sample* to a beaker with an inside diameter of 83 mm. Place the beaker in a water bath, cover with a watchglass, allow to equilibrate at 25 ± 0.1°, and let air bubbles dissipate. Keep the sample free from entrapped air bubbles and uniform in temperature. Stir the dispersion in the beaker at low agitation speed to ensure homogeneity, making sure that no air bubbles are incorporated. Determine viscosity at 25 ± 0.1°, using a suitable rotational viscometer with a spindle having a cylinder 4.7 cm in diameter and 0.2 cm high attached to a shaft 0.3 cm in diameter, the distance from the top of the

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cylinder to the lower tip of the shaft being 2.7 cm, and the immersion depth being 4.9 cm.[1] Operate the viscometer at 20 rpm. Follow the instrument manufacturer's directions to measure the apparent viscosity.

Acceptance criteria: 400–1500 mPa · s

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers at a temperature below 25°. Protect from freezing.
- **LABELING:** The labeling states the percentage of ethylcellulose. Label it to indicate the names and quantity ranges of any added plasticizers, stabilizers, and glidants. Label it to indicate whether any fatty acid components, or fatty acid-containing components are derived from animal, vegetable, or synthetic sources.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP 1-Butanol RS
1-Butanol.
$C_4H_{10}O$ 74.12

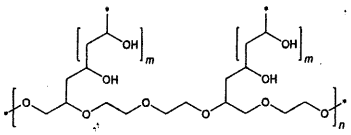
USP Dibutyl Sebacate RS
Decanedioic acid;
Dibutyl ester.
$C_{18}H_{34}O_4$ 314.46

USP Ethylcellulose RS
Cellulose, ethyl ether;
Cellulose ethyl ether.

USP Glycerin RS
1,2,3-Propanetriol;
Glycerol.

USP Oleic Acid RS
9-Octadecenoic acid, (*Z*)-;
Oleic acid.
$C_{18}H_{34}O_2$ 282.46

Ethylene Glycol and Vinyl Alcohol Graft Copolymer



Polyethylene glycol-graft-polyvinyl alcohol;
Graft-copoly(ethylene glycol-vinyl alcohol) [121786-16-1].

DEFINITION

Ethylene Glycol and Vinyl Alcohol Graft Copolymer is a graft copolymer of ethylene glycol and vinyl alcohol. It consists of about 75% of vinyl alcohol (–$CH_2CH(OH)$–) units and 25% of ethylene glycol (–CH_2CH_2O–) units. The Copolymer is produced by using polyethylene glycol and vinyl acetate as starting materials. Polyethylene glycol forms the polymer backbone on which polyvinyl acetate is grafted. Hydrolysis of polyvinyl acetate side chains leads to formation of polyvinyl alcohol grafted chains. Ethylene Glycol and Vinyl Alcohol Graft Copolymer may contain glidant to improve flowability.

IDENTIFICATION

- **INFRARED ABSORPTION**

Sample: 200 mg of Ethylene Glycol and Vinyl Alcohol Graft Copolymer

Analysis: Dissolve the *Sample* in 5–10 mL of water. Spread 1 mL of this solution on a thallium bromoiodide window.[1] Totally moisten the surface area of the window. Dry the window at 110° for approximately 30 min, and allow the window to cool to room temperature.

Acceptance criteria: The IR absorption spectrum of Ethylene Glycol and Vinyl Alcohol Graft Copolymer exhibits maxima corresponding to the same wavelengths as those of a similar preparation of USP Ethylene Glycol and Vinyl Alcohol Graft Copolymer RS treated in the same manner.

IMPURITIES

INORGANIC IMPURITIES

- **Residue on Ignition** ⟨281⟩

Sample: 10.0 g

Acceptance criteria: NMT 3.0%

Change to read:

ORGANIC IMPURITIES

- **Procedure 1: Ethylene Oxide and Dioxane**

[**CAUTION**—Ethylene oxide is toxic and flammable. Prepare these solutions in a well-ventilated fume hood, using great care. Protect both hands and face by wearing polyethylene protective gloves and an appropriate face mask. Store all solutions in hermetic containers, and refrigerate at a temperature between 4° and 8°.]

[NOTE—Before using polyethylene glycol 200 in this test, remove any volatile components from it by placing 500 mL of polyethylene glycol 200 in a 1000-mL round-bottom flask, and attaching the flask to a rotary evaporator maintained at a temperature of 60° and under a vacuum of 10–20 mm Hg for 6 h.]

Acetaldehyde solution: 10 µg/mL of acetaldehyde. [NOTE—Prepare the *Acetaldehyde solution* immediately before use.]

Ethylene oxide stock solution: Introduce 300 µL (corresponding to 250 mg) of gaseous ethylene oxide to the polyethylene glycol 200. Determine the absorbed mass of ethylene oxide via the weight of the solution before and after the absorption. Dilute the solution with the polyethylene glyol 200 to 100 g. This stock solution contains 2.5 mg/g of ethylene oxide. [NOTE—Prepare this stock solution immediately before use, and store in a refrigerator after preparation.]

Ethylene oxide solution: 100 µg/g of ethylene oxide in polyethylene glycol 200 from *Ethylene oxide stock solution*. Transfer 5 g of 100 µg/g ethylene oxide in polyethylene glycol 200 to a 50-mL volumetric flask filled with 30 mL of water. Dilute with water to volume, and mix to obtain a solution containing 10 µg/mL of ethylene oxide. [NOTE—Prepare this solution immediately before use, and use the solution directly after preparation.]

Dioxane solution: 500 µg/mL of dioxane

Standard solution A: Transfer 0.1 mL of *Ethylene oxide solution* to a 10-mL pressure headspace vial. [NOTE—Other sizes, such as 22-mL, may be used, depending on the operating conditions; however, the same size must be used for *Standard solution A*, *Standard solution B*, and the *Sample solution*.] Add 0.1 mL of *Acetaldehyde solution* and 0.1 mL of *Dioxane solution*, seal the vial, and mix.

Standard solution B: Transfer 1.0 g of Ethylene Glycol and Vinyl Alcohol Graft Copolymer to a 10-mL pressure headspace vial. Add 0.1 mL of *Ethylene oxide solution*, 0.1 mL of *Dioxane solution*, and 1.0 mL of *N*,*N*-dimethylacetamide, seal the vial, and mix.

[1] A commercial instrument is available as an RV2 spindle from Brookfield.

[1] Thallium bromoiodide, also known as KRS-5, consists of 42% thallium(I) bromide and 58% thallium(I) iodine by molecular weight. Suitable disks of KRS-5 are available from www.photonic.saint-gobain.com, www.almazoptics.com, and www.internationalcrystal.net.

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Sample solution: Transfer 1.0 g of Ethylene Glycol and Vinyl Alcohol Graft Copolymer to a 10-mL pressure headspace vial. Add 1.0 mL of *N,N*-dimethylacetamide and 0.2 mL of water, seal the vial, and mix.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

[NOTE—Use a headspace apparatus that automatically transfers a measured amount of headspace.]

Mode: GC

Detector: Flame ionization

Detector temperature: 250°

Column: 0.25-mm × 30-m glass or quartz capillary column; 1.0-µm layer of phase G1

Column temperature: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (min)	Hold Time at Final Temperature (min)
50	—	50	5
50	5	180	—
180	30	230	5

Carrier gas: Helium

Flow rate: 0.8 mL/min

Injection size: 1 mL (the gaseous headspace)

Injection type: Split ratio 20:1

Injection port temperature: 250°

Headspace sampler

Equilibration time: 45 min

Equilibration temperature

Standard solution A: 70°

Standard solution B: 90°

Sample solution: 90°

Transfer line temperature: 150°

Pressurization time: 1 min

Final headspace pressure: 0.7 bar

Injection time: 12 s

System suitability

Sample: *Standard solution A*

[NOTE—The relative retention times for acetaldehyde and ethylene oxide are 0.94 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 1.5 between acetaldehyde and ethylene oxide

Signal-to-noise: NLT 5 determined from the dioxane peak

Relative standard deviation: NMT 15%

Analysis

Samples: *Standard solution B* and *Sample solution*

Calculate the content of ethylene oxide, in ppm, in the portion of Ethylene Glycol and Vinyl Alcohol Graft Copolymer taken:

$$\text{Result} = A_E \times r_U/[(r_S \times W_U) - (r_U \times W_S)]$$

A_E = quantity of ethylene oxide added to *Standard solution B* (µg)

r_U = ethylene oxide peak response from the *Sample solution*

r_S = ethylene oxide peak response from *Standard solution B*

W_U = weight of Ethylene Glycol and Vinyl Alcohol Graft Copolymer taken to prepare the *Sample solution* (g)

W_S = weight of Ethylene Glycol and Vinyl Alcohol Graft Copolymer taken to prepare *Standard solution B* (g)

Calculate the content of dioxane, in ppm, in the portion of Ethylene Glycol and Vinyl Alcohol Graft Copolymer taken:

$$\text{Result} = A_D \times r_U/[(r_S \times W_U) - (r_U \times W_S)]$$

A_D = quantity of dioxane added to *Standard solution B* (µg)

r_U = dioxane peak response from the *Sample solution*

r_S = dioxane peak response from *Standard solution B*

W_U = weight of Ethylene Glycol and Vinyl Alcohol Graft Copolymer taken to prepare the *Sample solution* (g)

W_S = weight of Ethylene Glycol and Vinyl Alcohol Graft Copolymer taken to prepare *Standard solution B* (g)

Acceptance criteria: NMT 1 ppm of ethylene oxide; NMT 10 ppm of dioxane

• **Procedure 2: Vinyl Acetate**

Solution A: Acetonitrile, methanol, and water (5:5:90)

Solution B: Acetonitrile, methanol, and water (45:5:50)

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	100	0
2	100	0
40	85	15
42	0	100
48	0	100
51	100	0

System suitability solution: Transfer 50 mg of vinyl acetate and 50 mg of 1-vinylpyrrolidin-2-one to a 50-mL volumetric flask, add 10 mL of methanol, and sonicate or gently shake the flask to dissolve the materials. Dilute with *Solution A* to volume. Dilute 10 mL of this solution with *Solution A* to 100 mL. Dilute 5 mL of this solution with *Solution A* to 100 mL. The *System suitability solution* contains about 5 µg/mL each of vinyl acetate and 1-vinylpyrrolidin-2-one.

Standard solution: Transfer 50 mg of vinyl acetate to a 100-mL volumetric flask, dissolve in and dilute with methanol to volume, and mix well. Dilute 5.0 mL of the solution with *Solution A* to 100 mL. Dilute 10.0 mL of this solution with *Solution A* to 100 mL. The *Standard solution* contains about 2.5 µg/mL of vinyl acetate. [NOTE—This solution should be analyzed within 1 h when stored at room temperature.]

Sample solution: Transfer 250 mg of Ethylene Glycol and Vinyl Alcohol Graft Copolymer to a 10-mL volumetric flask, and add 1–2 mL of methanol, using an ultrasonic bath if necessary. After cooling to ambient temperature, dilute with water to volume, and mix. Pass through a ▲0.2-µm▲ (ERR 1-Jun-2018) membrane filter. [NOTE—This solution should be analyzed within 1 h when stored at room temperature.]

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 205 nm

Column: 4.0-mm × 25-cm analytical column; 5-µm packing L1. A 4.0-mm × 3-cm pre-column; 5-µm packing L1 may be used if a matrix effect is observed. [NOTE—The matrix effect may result in poor reproducibility of the retention times and of the peak shapes.]

Column temperature: 30°

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Flow rate: 1 mL/min
Injection size: 10 µL
System suitability
Sample: *System suitability solution*
[NOTE—The relative retention times for vinyl acetate and 1-vinylpyrrolidin-2-one are 1.0 and 1.4, respectively.]
Suitability requirements
Resolution: NLT 5.0 between vinyl acetate and 1-vinylpyrrolidin-2-one
Relative standard deviation: NMT 5.0% determined from the 1-vinylpyrrolidin-2-one peak
Analysis
Samples: *Standard solution* and *Sample solution*
Acceptance criteria: The response of the vinyl acetate peak from the *Sample solution* is NMT that of the vinyl acetate peak from the *Standard solution*, corresponding to NMT 100 ppm of vinyl acetate.

• **Procedure 3: Acetic Acid/Acetate**
Mobile phase: 5 mM sulfuric acid
Standard solution: 0.3 mg/mL for each of acetic acid and citric acid in *Mobile phase*
Sample solution: 20 mg/mL of Ethylene Glycol and Vinyl Alcohol Copolymer
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 205 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column clean: After each injection, rinse the column with a mixture of equal volumes of *Mobile phase* and acetonitrile.
Column temperature: Ambient
Flow rate: 1 mL/min
Injection size: 20 µL
System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for acetic acid and citric acid are 1.0 and 1.3, respectively.]
Suitability requirements
Resolution: NLT 2.0 between acetic acid and citric acid
Relative standard deviation: NMT 5.0% determined from the acetic acid peak
Analysis
Samples: *Standard solution* and *Sample solution*
Acceptance criteria: The response of the acetic acid peak from the *Sample solution* is NMT that of the acetic acid peak from the *Standard solution*, corresponding to NMT 1.5% of acetic acid.

SPECIFIC TESTS
• **FATS AND FIXED OILS,** *Ester Value* ⟨401⟩**:** 10–75
• **LOSS ON DRYING** ⟨731⟩**:** Dry 1.0 g of Ethylene Glycol and Vinyl Alcohol Graft Copolymer in a vacuum at 140° for 1 h: it loses NMT 5.0% of its weight.
• **pH** ⟨791⟩**:** 5.0–8.0, in a solution of 20% (w/w) in carbon dioxide-free water
• **VISCOSITY—ROTATIONAL METHODS** ⟨912⟩**:** After determining the *Loss on Drying*, weigh a quantity of undried Ethylene Glycol and Vinyl Alcohol Graft Copolymer, equivalent to 100.0 g on the dried basis. Transfer the sample to a short form, 600-mL beaker (internal diameter about 80 mm and height 120 mm), and add water to make the mixture weigh 500 g. Ensure a homogeneous solution by gently stirring at room temperature for 48 h. Afterwards allow the container to stand for 24 h to let the entrapped air dissipate. [NOTE—Ensure that the concentration of this solution is 20% (w/w).] Determine the viscosity of this solution at 23 ± 0.1° using a suitable rotational viscometer with a spindle having a cylinder 4.7 cm in diameter and 0.2 cm high attached to a shaft 0.3 cm in diameter, the distance from the top of the cylinder to the lower tip of the shaft being 2.7 cm, and the immersion depth being 4.9 cm.[2] Operate the viscometer at 100 rpm. Follow the instrument manufacturer's directions to measure the apparent viscosity.
Acceptance criteria: 25–250 mPa · s

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at a temperature below 25°.
• **LABELING:** Label it to indicate the viscosity, giving the viscosity measurement parameters, concentration of the solution, and the type of equipment used. The labeling also indicates the name and quantity of any added glidant.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Ethylene Glycol and Vinyl Alcohol Graft Copolymer RS

Ethylene Glycol Stearates

DEFINITION
Ethylene Glycol Stearates is a mixture of ethylene glycol monoesters and diesters of stearic and palmitic acids. It contains NLT 50.0% of monoesters produced from the condensation of ethylene glycol and stearic acid of vegetable or animal origin.

IDENTIFICATION
• **A.** It meets the requirements in *Specific Tests* for *Melting Range or Temperature, Class II* ⟨741⟩.
• **B.** It meets the requirements in *Specific Tests* for *Fats and Fixed Oils, Fatty Acid Composition* ⟨401⟩.

ASSAY
• **PROCEDURE**
Mobile phase: Tetrahydrofuran
Sample solution: 40 mg/mL of Ethylene Glycol Stearates in tetrahydrofuran
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Refractive index
Column: 7.5-mm × 60-cm; containing 5-µm 100Å packing L21. [NOTE—Two or three 7.5-mm × 30-cm L21 columns may be used in place of the one 60-cm column, provided that *System suitability* requirements are met.]
Temperatures
Column: 40°. [NOTE—The column temperature may be lowered to ambient temperature, although working at 40° provides stable separation conditions and ensures better sample solubility.]
Detector: 40°
Flow rate: 1 mL/min
Injection volume: 40 µL
System suitability
Sample: *Sample solution*
[NOTE—The relative retention times for diesters, monoesters, and ethylene glycol are 0.76, 0.83, and 1.0, respectively.]
Suitability requirements
Relative standard deviation: NMT 2.0% for the monoesters peak
Analysis
Sample: *Sample solution*
Calculate the percentage of free fatty acids, *E*, in the portion of Ethylene Glycol Stearates taken:

$$E = I_A \times 270/561.1$$

[2] A commercial instrument is available as an RV2 spindle from Brookfield.

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I_A = acid value, determined in *Specific Tests* for *Fats and Fixed Oils, Acid Value (Free Fatty Acids)* ⟨401⟩

Calculate the percentage of monoesters in the portion of *Sample* taken:

$$\text{Result} = [r_M/(r_M + r_D)](100 - D - E)$$

r_M = peak response for the monoesters
r_D = peak response for the diesters
D = percentage of free ethylene glycol in the portion of Ethylene Glycol Stearates taken, as determined in *Impurities* for *Limit of Free Ethylene Glycol*

Acceptance criteria: NLT 50.0% of monoesters

IMPURITIES

• **LIMIT OF FREE ETHYLENE GLYCOL**

Mobile phase, Sample solution, Chromatographic system, and **System suitability:** Proceed as directed in the *Assay*.

Standard solution A: 0.5 mg/mL of ethylene glycol in tetrahydrofuran

Standard solution B: 1.0 mg/mL of ethylene glycol in tetrahydrofuran

Standard solution C: 2.0 mg/mL of ethylene glycol in tetrahydrofuran

Standard solution D: 4.0 mg/mL of ethylene glycol in tetrahydrofuran

Analysis

Samples: *Sample solution* and *Standard solutions*

Plot the ethylene glycol peak responses obtained versus the concentration of ethylene glycol in the *Standard solutions*. From the standard curve so obtained, determine the ethylene glycol concentration in the *Sample solution*.

Calculate the percentage of free ethylene glycol in the portion of sample taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of ethylene glycol, determined from the standard curve (mg/mL)
C_U = concentration of the *Sample solution* (mg/mL)

Acceptance criteria: NMT 5.0%

SPECIFIC TESTS

• **MELTING RANGE OR TEMPERATURE,** *Class II* ⟨741⟩**:** 54°–60°

• **FATS AND FIXED OILS,** *Acid Value (Free Fatty Acids)* ⟨401⟩

Sample: 10.0 g

Acceptance criteria: NMT 3.0

• **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩**:** NMT 3.0

• **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩

Sample: 2.0 g

Acceptance criteria: 170–195

• **FATS AND FIXED OILS,** *Fatty Acid Composition* ⟨401⟩**:** 40.0%–60.0% of stearic acid, and the sum of palmitic and stearic acids is NLT 90.0%.

• **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩

Sample: 1.0 g

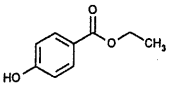
Acceptance criteria: NMT 0.1%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.

• **LABELING:** Label it to indicate that it is intended for topical use only.

Ethylparaben



$C_9H_{10}O_3$ 166.17

Benzoic acid, 4-hydroxy-, ethyl ester;
Ethyl *p*-hydroxybenzoate [120-47-8].

DEFINITION

Ethylparaben contains NLT 98.0% and NMT 102.0% of $C_9H_{10}O_3$.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197M**▲ (CN 1-May-2020)

• **B. MELTING RANGE OR TEMPERATURE** ⟨741⟩**:** 115°–118°

ASSAY

• **PROCEDURE**

Mobile phase, Sample solution, Standard solution B, and **Chromatographic system:** Proceed as described in the procedure for *Related Substances*.

System suitability

Sample: *Standard solution B*

Suitability requirements

Relative standard deviation: NMT 0.85% for 6 injections

Analysis

Samples: *Sample solution* and *Standard solution B*

Calculate the percentage of Ethylparaben in the *Sample solution*:

$$\text{Result} = P \times (r_U \times C_S)/(r_S \times C_U)$$

P = labeled purity of USP Ethylparaben RS expressed as a percentage
r_U = peak area of ethylparaben from the *Sample solution*
C_S = concentration of ethylparaben in *Standard solution B*
r_S = peak area of ethylparaben from *Standard solution B*
C_U = concentration of Ethylparaben in the *Sample solution*

Acceptance criteria: 98.0%–102.0%

IMPURITIES

INORGANIC IMPURITIES

• **Residue on Ignition** ⟨281⟩**:** NMT 0.1%, determined on 1.0 g

ORGANIC IMPURITIES

• **Procedure: Related Substances**

Mobile phase: Methanol and a 6.8-g/L solution of potassium dihydrogen phosphate (65:35 v/v)

Sample solution: Dissolve 50.0 mg of Ethylparaben in 2.5 mL of methanol, and dilute with *Mobile phase* to 50.0 mL. Dilute 10.0 mL of this solution with *Mobile phase* to 100.0 mL.

Standard solution A: 5.0 µg/mL each of *p*-hydroxy benzoic acid, USP Methylparaben RS, and USP Ethylparaben RS in *Mobile phase*

Standard solution B: Dissolve 50.0 mg of USP Ethylparaben RS in 2.5 mL of methanol, and dilute with *Mobile phase* to 50.0 mL. Dilute 10.0 mL of this solution with *Mobile phase* to 100.0 mL.

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Standard solution C: Dilute 1.0 mL of the *Sample solution* with *Mobile phase* to 20.0 mL. Dilute 1.0 mL of this solution with *Mobile phase* to 10.0 mL.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 272 nm
Column: 4.6-mm × 15-cm; 5-µm packing L1
Flow rate: 1.3 mL/min
Injection size: 10 µL
Run time: About 4 times the retention time of ethylparaben

System suitability
Sample: *Standard solution A*
[NOTE—The retention time of ethylparaben is about 3.0 min; the relative retention times for *p*-hydroxybenzoic acid, ethylparaben, and methylparaben are about 0.5, 1.0, and 0.8, respectively.]
Suitability requirements
Resolution: NLT 2.0 between the methylparaben and ethylparaben peaks

Analysis
Samples: *Sample solution* and *Standard solution C*
[NOTE—Disregard any limit that is 0.2 times the area of the principal peak in the chromatogram obtained with *Standard solution C* (0.1%).]

Acceptance criteria
***p*-Hydroxybenzoic acid:** The peak area in the *Sample solution*, multiplied by 1.4 to correct for the calculation of content, is NMT the area of the principal peak in *Standard solution C* (0.5%).
Unspecified impurities: The peak area of each impurity in the *Sample solution* is NMT the area of the principal peak in *Standard solution C* (0.5%).
Total impurities: The total peak area for all impurities in the *Sample solution* is NMT twice the area of the principal peak in *Standard solution C* (1.0%).

SPECIFIC TESTS

• **COLOR OF SOLUTION**
Sample solution: 100 mg/mL in alcohol
Comparison solution: Mix 2.4 mL of ferric chloride CS, 1.0 mL of cobaltous chloride CS, and 0.4 mL of cupric sulfate CS with 0.3 N hydrochloric acid to make 10 mL. Dilute 5 mL of this solution with 0.3 N hydrochloric acid to make 100 mL. [NOTE—Prepare and use this solution immediately.]
Analysis
Samples: Alcohol, *Sample solution*, and *Comparison solution*
Make the comparison by viewing the solutions downward in matched color-comparison tubes against a white surface (see *Color and Achromicity* ⟨631⟩).
Acceptance criteria: The *Sample solution* is clear and not more intensely colored than alcohol or the *Comparison solution*.

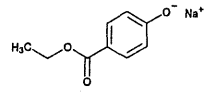
• **ACIDITY**
Sample solution: To 2 mL of *Sample solution* prepared in the test for *Color of Solution*, add 3 mL of alcohol, 5 mL of carbon dioxide-free water, and 0.1 mL of bromocresol green TS.
Analysis: Titrate with 0.10 N sodium hydroxide.
Acceptance criteria: NMT 0.1 mL is required to produce a blue color.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

• **USP REFERENCE STANDARDS** ⟨11⟩
USP Ethylparaben RS
USP Methylparaben RS

Ethylparaben Sodium



$C_9H_9NaO_3$ 188.2
Benzoic acid, 4-hydroxy-, methyl ester, sodium salt;
Ethyl *p*-hydroxybenzoate, sodium salt;
Sodium 4-ethoxycarbonylphenolate [35285-68-8].

DEFINITION

Ethylparaben Sodium contains NLT 95.0% and NMT 102.0% of ethylparaben sodium ($C_9H_9NaO_3$), calculated on the anhydrous basis.

IDENTIFICATION

• **A.**
Standard: 0.5 g of USP Ethylparaben RS
Sample: 0.5 g of Ethylparaben Sodium
Analysis: Dissolve the *Sample* in 5 mL of water, acidify with hydrochloric acid, and filter the resulting precipitate. Wash the precipitate with water, and dry under vacuum at 80° for 2 h.
Acceptance criteria: The IR absorption spectrum of a mineral oil dispersion of the *Sample* exhibits maxima only at the same wavelengths as those of a similar preparation of the *Standard*.

• **B.**
Sample solution: Ignite 0.3 g of Ethylparaben Sodium, cool, and dissolve the residue in about 3 mL of 3 N hydrochloric acid.
Acceptance criteria: A platinum wire dipped in the *Sample solution* imparts an intense, persistent yellow color to a nonluminous flame.

ASSAY

• **PROCEDURE**
Mobile phase: Methanol and a 6.8-g/L solution of potassium dihydrogen phosphate (65:35)
System suitability solution: 5.0 µg/mL each of *p*-hydroxybenzoic acid, USP Methylparaben RS, and USP Ethylparaben RS in *Mobile phase*
Standard solution: Dissolve 50.0 mg of USP Ethylparaben RS in 2.5 mL of methanol, and dilute with *Mobile phase* to 50.0 mL. Dilute 10.0 mL of this solution with *Mobile phase* to 100.0 mL.
Sample solution: Dissolve 50.0 mg of Ethylparaben Sodium in 2.5 mL of methanol, and dilute with *Mobile phase* to 50.0 mL. Dilute 10.0 mL of this solution with *Mobile phase* to 100.0 mL.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 272 nm
Column: 4.6-mm × 15-cm; 5-µm packing L1
Flow rate: 1.3 mL/min
Injection volume: 10 µL
Run time: About 4 times the retention time of the ethylparaben peak
System suitability
Samples: *System suitability solution* and *Standard solution*

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[NOTE—The retention time of ethylparaben is about 2.9 min; the relative retention times for *p*-hydroxybenzoic acid, methylparaben, and ethylparaben are about 0.5, 0.8, and 1.0, respectively.]

Suitability requirements

Resolution: NLT 2.0 between methylparaben and ethylparaben peaks, *System suitability solution*

Relative standard deviation: NMT 0.85% for six injections, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of ethylparaben sodium in the portion of Ethylparaben Sodium taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times P$$

r_U = peak area of ethylparaben from the *Sample solution*

r_S = peak area of ethylparaben from the *Standard solution*

C_S = concentration of USP Ethylparaben RS in the *Standard solution*

C_U = concentration of Ethylparaben Sodium in the *Sample solution*

M_{r1} = molecular weight of ethylparaben sodium, 188.2

M_{r2} = molecular weight of ethylparaben, 166.17

P = labeled purity of USP Ethylparaben RS expressed as a percentage

Acceptance criteria: 95.0%–102.0% on the anhydrous basis

IMPURITIES

• **RELATED COMPOUNDS**

Mobile phase, System suitability solution, Sample solution, and Chromatographic system: Proceed as directed in the *Assay*.

Standard solution: Dilute 1.0 mL of the *Sample solution* with *Mobile phase* to 20.0 mL. Dilute 1.0 mL of this solution with *Mobile phase* to 10.0 mL.

System suitability

Sample: *System suitability solution*

[NOTE—The retention time of ethylparaben is about 2.9 min; the relative retention times for *p*-hydroxybenzoic acid, methylparaben, and ethylparaben are about 0.5, 0.8, and 1.0, respectively.]

Suitability requirements

Resolution: NLT 2.0 between methylparaben and ethylparaben peaks

Analysis

Samples: *Standard solution* and *Sample solution*

Acceptance criteria

***p*-Hydroxybenzoic acid:** The peak area in the *Sample solution*, multiplied by 1.4 to correct for the calculation of content, is NMT 6 times the area of the principal peak in the *Standard solution*; NMT 3.0%.

Unspecified impurities: The peak area of each impurity in the *Sample solution* is NMT the area of the principal peak in the *Standard solution*; NMT 0.5%.

Total impurities: The total peak area for all unspecified impurities in the *Sample solution* is NMT twice the area of the principal peak in the *Standard solution*; NMT 1.0%.

• **CHLORIDE AND SULFATE,** *Chloride* ⟨221⟩

Sample: 0.2 g

Control solution: 0.10 mL of 0.020 N hydrochloric acid

Acceptance criteria: 0.035%; the *Sample* shows no more chloride than the *Control solution*.

• **CHLORIDE AND SULFATE,** *Sulfate* ⟨221⟩

Sample: 1.0 g

Control solution: 0.31 mL of 0.020 N sulfuric acid

Acceptance criteria: 0.030%; the *Sample* shows no more sulfate than the *Control solution*.

SPECIFIC TESTS

• **pH** ⟨791⟩

Sample solution: 1 mg/mL

Acceptance criteria: 9.5–10.5

• **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 5.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Ethylparaben RS

USP Methylparaben RS

Eucalyptus Oil

[8000-48-4].

DEFINITION

Eucalyptus Oil is obtained by steam distillation and rectification from the fresh leaves or the fresh terminal branchlets of various species of *Eucalyptus* rich in 1,8-cineole. The species mainly used are *Eucalyptus globulus* Labill., *Eucalyptus polybractea* R.T. Baker, and *Eucalyptus smithii* R.T. Baker. It contains NLT 70.0% and NMT 95.0% of eucalyptol (1,8-cineole, $C_{10}H_{18}O$).

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F** ▲(CN 1-May-2020)

• **B. IDENTITY BY AROMA SUBSTANCE PROFILE:** The retention times of the (+)-α-pinene, β-pinene, (*R*)-(–)-α-phellandrene, (*R*)-(+)-limonene, and eucalyptol peaks of the *Sample solution* correspond to those of the *Standard solution*, as obtained in the test for *Content of Aroma Substances* in the *Assay*.

ASSAY

• **CONTENT OF AROMA SUBSTANCES**

Standard solution A: 1.0 µL/mL of (+)-α-pinene, 0.5 µL/mL of β-pinene, 0.5 µL/mL of sabinene, 0.5 µL/mL of (*R*)-(–)-α-phellandrene, 1.0 µL/mL of (*R*)-(+)-limonene, 5 µL/mL of USP Eucalyptol RS, and 5 mg/mL of USP Camphor RS in heptane

Standard solution B: 0.01 µL/mL of (*R*)-(+)-limonene in heptane

Sample solution: 20 µL/mL of Eucalyptus Oil in heptane

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.25-mm × 60-m fused-silica capillary; 0.25-µm layer of phase G16

Temperatures

Injection port: 220°

Detector: 220°

Column: See *Table 1*.

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Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
60	—	60	5
60	5	200	5

Carrier gas: Helium
Flow rate: 1.5 mL/min
Injection type: Split ratio, 1:50
Injection volume: 1 µL
System suitability
Sample: *Standard solution A*
[NOTE—The relative retention times are listed in *Table 2*.]

Table 2

Name	Relative Retention Time
(+)-α-Pinene	0.64
β-Pinene	0.80
Sabinene	0.83
(*R*)-(–)-α-Phellandrene	0.91
(*R*)-(+)-Limonene	0.98
Eucalyptol	1.00
Camphor	1.58

Suitability requirements
Resolution: NLT 1.5 between (*R*)-(+)-limonene and eucalyptol
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Identify the peaks in the *Sample solution* based on those in *Standard solution A* as well as in *Table 3*.

Table 3

Name	Relative Retention Time
β-Myrcene	0.86–0.90
γ-Terpinene	1.05–1.07
p-Cymene	1.12–1.13
Terpinen-4-ol	1.68
α-Terpineol	1.82

β-Myrcene elutes before (*R*)-(–)-α-phellandrene and after sabinene; γ-terpinene and *p*-cymene elute between eucalyptol and camphor; and terpinen-4-ol and α-terpineol elute after camphor.
Calculate the percentage of (+)-α-pinene [β-pinene, sabinene, (*R*)-(–)-α-phellandrene, (*R*)-(+)-limonene, or eucalyptol] in the portion of Eucalyptus Oil taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of (+)-α-pinene [β-pinene, sabinene, (*R*)-(–)-α-phellandrene, (*R*)-(+)-limonene, or eucalyptol] from the *Sample solution*
r_S = peak response of (+)-α-pinene [β-pinene, sabinene, (*R*)-(–)-α-phellandrene, (*R*)-(+)-limonene, or eucalyptol] from the *Standard solution*
C_S = concentration of (+)-α-pinene [β-pinene, sabinene, (*R*)-(–)-α-phellandrene, (*R*)-(+)-limonene, or USP Eucalyptol RS] in the *Standard solution* (µL/mL)
C_U = concentration of Eucalyptus Oil in the *Sample solution* (µL/mL)

Calculate the percentage of camphor in the portion of Eucalyptus Oil taken:

$$\text{Result} = (r_U/r_S) \times [C_S/(C_U \times D)] \times 100$$

r_U = peak response of camphor from the *Sample solution*
r_S = peak response of camphor from the *Standard solution*
C_S = concentration of USP Camphor RS in the *Standard solution* (mg/mL)
C_U = concentration of Eucalyptus Oil in the *Sample solution* (µL/mL)
D = density of Eucalyptus Oil (mg/µL)

Calculate the percentage of β-myrcene (γ-terpinene, *p*-cymene, terpinen-4-ol, or α-terpineol) in the portion of Eucalyptus Oil taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of β-myrcene (γ-terpinene, *p*-cymene, terpinen-4-ol, or α-terpineol) from the *Sample solution*
r_T = sum of the total peak responses, except for the peaks due to solvent, from the *Sample solution*

Acceptance criteria: See *Table 4*. Disregard any peak with an area less than the major peak area from *Standard solution B*, corresponding to 0.05%.

Table 4

Name	Acceptance Criteria, NMT (%)
(+)-α-Pinene	0.2–10.0
β-Pinene	0.05–1.5
Sabinene	0.3
(*R*)-(–)-α-Phellandrene	0.05–1.5
(*R*)-(+)-Limonene	2.0–15.0
Eucalyptol	70.0–95.0
Camphor	0.1

Percentage of γ-terpinene: 0.1%–6.0%
Percentage of *p*-cymene: 0.5%–15.0%
Total percentage of all identified aroma substances [(+)-α-pinene, β-pinene, sabinene, (*R*)-(–)-α-phellandrene, (*R*)-(+)-limonene, eucalyptol, camphor, β-myrcene, γ-terpinene, *p*-cymene, terpinen-4-ol, and α-terpineol]: NLT 98.0%

IMPURITIES

• **TEST FOR ALDEHYDE**
Alcoholic hydroxylamine solution: Dissolve 3.5 g of hydroxylamine hydrochloride in 95 mL of 60% alcohol (v/v) and add 0.5 mL of a 2-mg/mL solution of methyl

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orange in 60% alcohol (v/v) and sufficient 0.5 M potassium hydroxide in 60% alcohol (v/v) to give a pure yellow color. Dilute with 60% alcohol (v/v) to 100 mL.

Analysis: In a ground-glass-stoppered tube, 25 mm in diameter and 150 mm long, add 10 mL of Eucalyptus Oil. Then add 5 mL of toluene and 4 mL of *Alcoholic hydroxylamine solution*. Shake vigorously, and titrate immediately with 0.5 M potassium hydroxide in 60% alcohol (v/v) until the red color changes to yellow. Continue the titration with shaking; the endpoint is reached when the pure yellow color of the indicator is permanent in the lower layer after shaking vigorously for 2 min and allowing separation to take place. The reaction is complete in about 15 min.

Repeat the titration using a further 10 mL of Eucalyptus Oil and, as a reference solution for the endpoint, the titrated liquid from the first determination, to which has been added 0.5 mL of 0.5 M potassium hydroxide in 60% alcohol (v/v).

Acceptance criteria: NMT 2.0 mL of 0.5 M potassium hydroxide in 60% alcohol (v/v) is required in the second titration.

SPECIFIC TESTS

- **SPECIFIC GRAVITY** ⟨841⟩: 0.906–0.927, at 20°
- **REFRACTIVE INDEX** ⟨831⟩: 1.450–1.470, at 20°
- **OPTICAL ROTATION** ⟨781⟩: 0°–10°, at 20°

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers. Do not store above 25°.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Camphor RS
  USP Eucalyptol RS
  USP Eucalyptus Oil RS

Hard Fat

DEFINITION

Hard Fat is a mixture of glycerides of saturated fatty acids.

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.05%
- **ALKALINE IMPURITIES**

Sample: 2.0 g

Analysis: Dissolve the *Sample* in a mixture of 1.5 mL of alcohol and 3.0 mL of ether. Add 0.05 mL of bromophenol blue TS, and titrate with 0.01 N hydrochloric acid to a yellow endpoint.

Acceptance criteria: NMT 0.15 mL of 0.01 N hydrochloric acid

SPECIFIC TESTS

- **MELTING RANGE OR TEMPERATURE,** *Class II* ⟨741⟩: The melting temperature does not differ by more than 2° from the nominal value given in *Labeling*.
- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩: NMT 1.0
- **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩: NMT 70
- **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩: NMT 7.0
- **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩: 215–255
- **FATS AND FIXED OILS,** *Unsaponifiable Matter* ⟨401⟩: NMT 3.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers at a temperature that is 5° or more below the melting range stated in the labeling.
- **LABELING:** The labeling includes the nominal melting temperature, which is 27°–44°.

Fennel Oil

DEFINITION

Fennel Oil is the volatile oil distilled with steam from the dried, ripe fruit of *Foeniculum vulgare* Mill. (Fam. Apiaceae).

[NOTE—If solid material has separated, carefully warm the Fennel Oil until it is completely liquefied, and mix before using.]

SPECIFIC TESTS

- **SPECIFIC GRAVITY** ⟨841⟩: 0.953–0.973
- **CONGEALING TEMPERATURE** ⟨651⟩: NLT 3°
- **OPTICAL ROTATION,** *Angular Rotation* ⟨781A⟩: +12° to +24°
- **REFRACTIVE INDEX** ⟨831⟩: 1.528–1.538 at 20°
- **SOLUBILITY IN 90% ALCOHOL:** 1 volume dissolves in 1 volume of 90% alcohol.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant source from which the article was derived. The label also states that if solid material has separated, carefully warm Oil until it is completely liquefied, and mix before using.

Ferric Oxide

DEFINITION

Ferric Oxide contains NLT 97.0% and NMT 100.5% of ferric oxide (Fe_2O_3), calculated on the ignited basis.

IDENTIFICATION

- **A. IDENTIFICATION TESTS—GENERAL,** *Iron* ⟨191⟩

Sample solution: Dissolve 0.5 g in 50 mL of hydrochloric acid, and dilute with water to 200 mL.

Acceptance criteria: Meets the requirements of the test for *Ferric Salts*

ASSAY

- **PROCEDURE**

To calculate the percentage of ferric oxide on the ignited basis, ignite about 2 g at 800 ± 25° to constant weight as directed in *Loss on Ignition* ⟨733⟩. [NOTE—Ignited Ferric Oxide is hygroscopic.]

Sample: 1.5 g

Blank: 25 mL of hydrochloric acid

Titrimetric system

Mode: Direct titration

Titrant: 0.1 N sodium thiosulfate VS

Endpoint detection: Visual

Analysis: Digest the *Sample* in 25 mL of hydrochloric acid on a water bath until dissolved. Add 10 mL of hydrogen peroxide TS, and evaporate on a water bath almost to dryness to volatilize all hydrogen peroxide. Dissolve the residue by warming with 5 mL of hydrochloric acid; add 25 mL of water; filter into a 250-mL volumetric flask, washing the filter with water; and add water to volume. Transfer a 50-mL aliquot to a glass-stoppered flask, add 3 g of potassium iodide and 5 mL of hydrochloric acid, and insert the stopper into the flask. Allow the mixture to stand for 15 min, add 50 mL of water, and titrate the liberated iodine with *Titrant*, using starch TS as the indicator. Perform a blank determination in the same manner.

Calculate the percentage of ferric oxide (Fe_2O_3) in the portion of the sample taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

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V_S = *Titrant* volume consumed by the *Sample* (mL)
V_B = *Titrant* volume consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 79.85 mg/mEq
W = *Sample* weight, calculated with a correction for loss on ignition (mg)

Acceptance criteria: 97.0%–100.5% on the ignited basis

IMPURITIES

• **MERCURY,** *Method IIa* ⟨261⟩
Test preparation: Combine 0.67 g of Ferric Oxide and 35 mL of 0.5 N hydrochloric acid. Heat to boiling, and allow to cool.
Analysis: Proceed as directed for *Test preparation* in the chapter, beginning with "Add 2 drops of phenolphthalein".
Acceptance criteria: NMT 3 µg/g

• **LIMIT OF ARSENIC**
Lead acetate cotton: Immerse absorbent cotton pledgets in a mixture of lead acetate TS and 2 N acetic acid (10:1). Free the cotton pledgets from excess liquid by expression, and allow to air-dry.
Sodium borohydride solution: 30 mg/mL of sodium borohydride in 0.25 N sodium hydroxide. Store in a loosely covered container protected from direct sunlight.
Mercuric bromide paper: Immerse several filter paper disks with a 15-mm diameter in alcoholic mercuric bromide TS, remove the disks from the solution, and allow to dry, protected from light. Store in a glass-stoppered container protected from light.
Arsenic trioxide stock solution: Dissolve 132.0 mg of arsenic trioxide in 2.0 mL of 2 N sodium hydroxide, and dilute with water to 100 mL.
Standard stock solution: On the day of use, dilute 1.0 mL of *Arsenic trioxide stock solution* with water to 1000 mL.
Standard solution: Dilute 1.5 mL of the *Standard stock solution* with hydrochloric acid to 10 mL. This solution contains 0.15 µg/mL of arsenic.
Sample solution: Dissolve 0.5 g of Ferric Oxide in several mL of hydrochloric acid with the aid of heat, and dilute with hydrochloric acid to 10.0 mL.
Apparatus: Prepare a 300-mL, side-arm conical flask containing a magnetic stirring bar. Attach to the conical flask a ground-glass stopper. Pass through the ground-glass stopper a glass tube 20 cm long with an internal diameter of 5 mm. The lower end of this tube is inside the conical flask, and it has been drawn to a tip with an internal diameter of 1 mm. There is an orifice, 2.5 mm in diameter, 15 mm from the tip, and at least 3 mm below the lower surface of the stopper. The upper end of the tube has a flat ground surface at a right angle to the axis of the tube.
A second glass tube, 30 mm long with an internal diameter of 5 mm and with a similar flat ground surface, is placed in contact with the ground surface of the first tube and is held in position by a clamp and springs.
Into the lower tube insert 55 mg of loosely packed *Lead acetate cotton*. Between the flat surfaces of the tubes place a disk of *Mercuric bromide paper*.
Analysis: Before placing the tube assembly into the flask, transfer the *Sample solution* to the flask, and add 5.0 mL of potassium iodide TS and 20 mL of water. Assemble the apparatus immediately, and stir while slowly adding, over a period of 20 min, 40 mL of *Sodium borohydride solution* through the side arm of the flask. Examine the stain produced on the *Mercuric bromide paper*. Perform the same *Analysis* using the *Standard solution*.
Acceptance criteria: NMT 3 µg/g; the stain produced on the *Mercuric bromide paper* from the *Sample solution* is not more intense than that from the *Standard solution*.

• **LIMIT OF LEAD**
Lead nitrate stock solution: 1.598 mg/mL of lead nitrate in 0.5 M nitric acid. Prepare and store this solution in glass containers free from soluble lead salts.
Standard stock solution: On the day of use, combine 5.0 mL of *Lead nitrate stock solution* and 10 mL of 1 N hydrochloric acid, and dilute with water to 100 mL.
Standard solution: Transfer 1.0 mL of the *Standard stock solution* to a 100-mL volumetric flask, add 10 mL of 1 N hydrochloric acid, and dilute with water to volume. This solution contains 0.5 µg/mL of lead.
Sample solution: Transfer 2.5 g of Ferric Oxide to a 100-mL, glass-stoppered conical flask. Add 35 mL of 0.1 N hydrochloric acid, and stir for 1 h. Filter, collecting the filtrate in a 50-mL volumetric flask, and dilute with 0.1 N hydrochloric acid to volume.
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometer equipped with a flow spoiler
Analytical wavelength: 217.0 nm (lead emission line)
Lamp: Lead hollow-cathode
Flame: Air–acetylene oxidizing
Analysis
Samples: *Standard solution* and *Sample solution*
Acceptance criteria: 0.001%; the absorbance of the *Sample solution* does not exceed that of the *Standard solution*.

SPECIFIC TESTS

• **WATER-SOLUBLE SUBSTANCES**
Sample: 2.0 g
Analysis: Digest the *Sample* in 100 mL of water on a boiling water bath for 2 h, filter, and wash the filter with water. Evaporate the filtrate and washings, and dry the residue at 105° for 1 h.
Acceptance criteria: 1.0%; NMT 20 mg of residue

• **ACID-INSOLUBLE SUBSTANCES**
Sample: 2.0 g
Analysis: Digest the *Sample* in 25 mL of hydrochloric acid by boiling for 20 min. Add 100 mL of hot water, and filter quantitatively through a tared filtering crucible, with the aid of hot wash water, until the filtrate tests negative for chloride. Dry the crucible and contents at 105° for 1 h.
Acceptance criteria: 0.3%; the residue weighs NMT 6 mg.

• **ORGANIC COLORS AND LAKES**
Sample: 3.0 g
Analysis: Place 1.0 g of the *Sample* in each of 3 beakers, and add 25 mL of each of the following reagents, one reagent to each beaker: 1-chloronaphthalene, alcohol, and chloroform. Heat the beakers containing alcohol and chloroform just to boiling. Heat the other beaker on a boiling water bath for 15 min, with occasional swirling. Pass the contents of the beakers through retentive, solvent-resistant filter paper. If any of the filtrates shows visible turbidity, centrifuge for 15 min. Record the spectra against respective solvent blanks in 1-cm cells from 350 to 750 nm.
Acceptance criteria: No peak above the noise level with a slope greater than +0.001 absorbance unit/nm is found.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Ferrosoferric Oxide

Fe_3O_4 ($FeO \cdot Fe_2O_3$) 231.54

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Magnetite;
Ferrous-ferric oxide;
Iron (II, III) oxide [1317-61-9].

DEFINITION
Ferrosoferric Oxide contains NLT 97.0% and NMT 100.5% of Fe_2O_3, calculated on the ignited basis.

IDENTIFICATION
• **A.**
Sample solution: Dissolve 0.1 g in 5 mL of hydrochloric acid, and dilute with water to 50 mL.
Analysis 1: Transfer 5 mL of the *Sample solution* to a test tube, and add a few drops of potassium ferrocyanide TS.
Analysis 2: Transfer 5 mL of the *Sample solution* to a test tube, and add a few drops of potassium ferricyanide TS.
Acceptance criteria: For both *Analysis 1* and *Analysis 2*, a blue precipitate (Prussian blue) is formed, which does not dissolve in dilute hydrochloric acid subsequently added. The precipitate dissolves in oxalic acid and sodium or potassium hydroxide.
• **B.** Ferrosoferric Oxide presents as black powder, which is distinguished from ferric oxide exhibiting two basic colors (red and yellow). It is attracted to a magnet.

ASSAY
• **PROCEDURE**
To enable the calculation of the percentage of Fe_2O_3 on the ignited basis, ignite about 2 g at 800 ± 25° to constant weight as directed in *Loss on Ignition* ⟨733⟩. [NOTE—Ignited Ferrosoferric Oxide is hygroscopic.]
Sample: 1.5 g
Blank: 25 mL of hydrochloric acid
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.1 N sodium thiosulfate VS
Endpoint detection: Visual
Analysis: Digest the *Sample* in 25 mL of hydrochloric acid on a water bath until dissolved. Add 10 mL of hydrogen peroxide TS, and evaporate on a water bath almost to dryness in order to volatilize all hydrogen peroxide. Dissolve the residue by warming with 5 mL of hydrochloric acid; add 25 mL of water; filter into a 250-mL volumetric flask, washing the filter with water; and add water to volume. Transfer a 50-mL aliquot to a glass-stoppered flask, add 3 g of potassium iodide and 5 mL of hydrochloric acid, and insert the stopper into the flask. Allow the mixture to stand for 15 min, add 50 mL of water, and titrate the liberated iodine with 0.1 N sodium thiosulfate VS, using starch TS as the indicator. Perform a blank determination in the same manner.
Calculate the percentage of the labeled amount as ferric oxide (Fe_2O_3) in the portion of the *Sample* taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

V_S = *Titrant* volume consumed by the *Sample* (mL)
V_B = *Titrant* volume consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 79.85 mg/mEq
W = weight of the *Sample*, calculated with a correction for loss on ignition (mg)

Acceptance criteria: 97.0%–100.5% on the ignited basis

IMPURITIES
• **LIMIT OF ARSENIC (AS)**
[NOTE—Select all reagents to have as low contents of elemental impurities as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse all glassware before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]
Solvent: 10% hydrochloric acid in water
Standard stock solution: Prepare a solution containing 2 ppm of arsenic from an Arsenic ICP/AA standard solution in *Solvent*. [NOTE—A commercially available certified standard solution can be used.]
Sample stock solution: In a 100-mL volumetric flask, dissolve 5.0 g of Ferrosoferric Oxide in 50 mL of hydrochloric acid by heating without boiling for about 1 h until the sample is dissolved. Cool down to room temperature. Pass through a filter, if necessary. Dilute with water to volume. Transfer 20.0 mL of the solution to a 100-mL volumetric flask, add 40 mL of ascorbic acid solution (200 mg/mL of ascorbic acid in water) and 20 mL of potassium iodide solution (400 mg/mL of potassium iodide in water), and dilute with water to volume. [NOTE—Reduce arsenic to the lowest possible oxidation state.] The resulting solution contains 10 mg/mL (g/L) of Ferrosoferric Oxide.
Sample solution A: Transfer 20 mL of the *Sample stock solution* to a 100-mL volumetric flask. Dilute with *Solvent* to volume. The resulting solution contains 2 mg/mL (g/L) of Ferrosoferric Oxide.
Sample solution B: Transfer 20 mL of the *Sample stock solution* to a 100-mL volumetric flask. Add 25 mL of the *Standard stock solution*, and dilute with *Solvent* to volume.
Sample solution C: Transfer 20 mL of the *Sample stock solution* to a 100-mL volumetric flask. Add 50 mL of the *Standard stock solution*, and dilute with *Solvent* to volume.
Blank: *Solvent*
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometer
Lamp: Arsenic hollow-cathode
Flame: Air–acetylene
Analytical wavelength: 193.696 nm
Analysis
Samples: *Sample solution A*, *Sample solution B*, and *Sample solution C*
[NOTE—To minimize matrix interference, it is recommended that the method of standard additions be used.]
Determine the absorbances of the *Samples* against the *Blank*, and plot the corrected absorbances versus their added content of arsenic (0, 0.5, and 1.0 ppm). Draw the straight line best fitting the three points, and extrapolate the line until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of arsenic, C_A, in ppm (µg/g) in the 2 mg/mL of Ferrosoferric Oxide in *Solvent*.
Calculate the content of arsenic, in µg/g, in the portion of Ferrosoferric Oxide taken:

$$\text{Result} = C_A \times D$$

D = dilution factor, 25

Acceptance criteria: NMT 2 ppm
• **LIMIT OF CADMIUM (CD) AND CHROMIUM (CR)**
[NOTE—Select all reagents to have as low contents of elemental impurities as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse all glassware before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]
Solvent: 10% hydrochloric acid in water

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Standard stock solution: Prepare a solution containing 2 ppm each of cadmium and chromium from an ICP standard solution containing cadmium and chromium in *Solvent*. [NOTE—A commercially available certified standard solution can be used.]
Sample stock solution: In a 100-mL volumetric flask, dissolve 5.0 g of Ferrosoferric Oxide in 50 mL of hydrochloric acid by heating without boiling for about 1 h until the sample is dissolved. Cool down to room temperature. Pass through a filter, if necessary. Dilute with water to volume. Transfer 20.0 mL of the solution to a 100-mL volumetric flask, and add water to volume. The resulting solution contains 10 mg/mL of Ferrosoferric Oxide.
Sample solution A: Transfer 20 mL of the *Sample stock solution* to a 100-mL volumetric flask. Dilute with *Solvent* to volume. The resulting solution contains 2 mg/mL of Ferrosoferric Oxide.
Sample solution B: Transfer 20 mL of the *Sample stock solution* to a 100-mL volumetric flask. Add 25 mL of the *Standard stock solution*, and dilute with *Solvent* to volume.
Sample solution C: Transfer 20 mL of the *Sample stock solution* to a 100-mL volumetric flask. Add 50 mL of the *Standard stock solution*, and dilute with *Solvent* to volume.
Blank: *Solvent*
Instrumental conditions
(See *Plasma Spectrochemistry* ⟨730⟩.)
Mode: Inductively coupled plasma–atomic emission spectrometer (ICP–AES)
Analytical wavelength
Cadmium: 228.802 nm
Chromium: 267.716 nm
Analysis
Samples: *Sample solution A*, *Sample solution B*, and *Sample solution C*
[NOTE—The operating conditions may be developed and optimized based on the manufacturer's recommendation.]
[NOTE—To minimize matrix interference, it is recommended that the method of standard additions be used.]
Instrument performance must be verified to conform to the manufacturer's specifications for resolution and sensitivity. Before analyzing samples, the instrument must pass a suitable performance check. Aspirate the *Samples*, at least in duplicate, and calculate the average absorbances for each against the *Blank*. Plot the average absorbances of the *Samples* versus their added content (0, 0.5, and 1.0 ppm) of cadmium (or chromium). Draw the straight line best fitting the three points, and extrapolate the line until it intercepts the concentration axis. The distance between this point and the intersection of the axes represents the concentration of cadmium (or chromium), C_C, in ppm (µg/g) in the 2 mg/mL of Ferrosoferric Oxide in *Solvent*.
Calculate the content of cadmium (or chromium), in µg/g, in the portion of Ferrosoferric Oxide taken:

$$\text{Result} = C_C \times D$$

D = dilution factor, 25

Acceptance criteria
Cadmium: NMT 1 ppm
Chromium: NMT 100 ppm

• **LIMIT OF LEAD (PB)**
[NOTE—Select all reagents to have as low contents of elemental impurities as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse all glassware before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]
Solvent: 4% hydrochloric acid and 8% nitric acid in water
Standard solution: Prepare a solution containing 25 ppb of lead in *Solvent*. [NOTE—A commercially available certified standard solution can be used.]
Sample stock solution: Dissolve 1.0 g of Ferrosoferric Oxide in 4 mL of hydrochloric acid and 8 mL of nitric acid in a 100-mL volumetric flask by heating without boiling until the sample is dissolved. Cool down to room temperature. Pass through a filter, if necessary. Dilute with water to volume. The resulting solution in the flask contains 10 mg/mL of Ferrosoferric Oxide.
Sample solution A: Transfer 10 mL of the *Sample stock solution* to a 100-mL volumetric flask, and dilute with *Solvent* to volume. The solution contains 1 mg/mL of Ferrosoferric Oxide.
Sample solution B: Transfer 10 mL of the *Sample stock solution* to a 100-mL volumetric flask. Add 4 mL of the *Standard solution*, and dilute with *Solvent* to volume.
Sample solution C: Transfer 10 mL of the *Sample stock solution* to a 100-mL volumetric flask. Add 12 mL of the *Standard solution*, and dilute with *Solvent* to volume.
Sample solution D: Transfer 10 mL of the *Sample stock solution* to a 100-mL volumetric flask. Add 20 mL of the *Standard solution*, and dilute with *Solvent* to volume.
Blank: *Solvent*
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometer equipped with graphite heating device
Analytical wavelength: 283.3 nm
Lamp: Lead hollow-cathode
Analysis
Samples: *Sample solution A*, *Sample solution B*, *Sample solution C*, and *Sample solution D*
[NOTE—To minimize matrix interference, it is recommended that the method of standard additions be used.]
Determine the absorbances of the *Samples* against the *Blank*, and plot the corrected absorbances versus their added content (0, 1, 3, and 5 ppb) of lead. Draw the straight line best fitting the four points, and extrapolate the line until it intercepts the concentration axis. The distance between this point and the intersection of the axes represents the concentration of lead, C_L, in ppm (µg/g) in the 1 mg/mL of Ferrosoferric Oxide.
Calculate the content of lead, in µg/g, in the portion of Ferrosoferric Oxide taken:

$$\text{Result} = C_L \times D$$

D = dilution factor, 10

Acceptance criteria: NMT 10 ppm

• **LIMIT OF MERCURY (HG) AND NICKEL (NI)**
[NOTE—Select all reagents to have as low contents of elemental impurities as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse all glassware before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]
Solvent: 10% hydrochloric acid in water
Standard solution: Prepare a solution containing 2 ppm of mercury (or nickel) from a Mercury (or Nickel) ICP/AA standard solution in *Solvent*. [NOTE—A commercially available certified standard solution can be used.]

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Sample stock solution: In a 100-mL volumetric flask, dissolve 5.0 g of Ferrosoferric Oxide in 50 mL of hydrochloric acid by heating without boiling for about 1 h until the sample is dissolved. Cool down to room temperature. Pass through a filter, if necessary. Dilute with water to volume. Transfer 20.0 mL of the solution to a 100-mL volumetric flask, and add water to volume. The resulting solution contains 10 mg/mL of Ferrosoferric Oxide.

Sample solution A: Transfer 20 mL of the *Sample stock solution* to a 100-mL volumetric flask. Dilute with *Solvent* to volume. The resulting solution contains 2 mg/mL of Ferrosoferric Oxide.

Sample solution B: Transfer 20 mL of the *Sample stock solution* to a 100-mL volumetric flask. Add 25 mL of the *Standard solution*, and dilute with *Solvent* to volume.

Sample solution C: Transfer 20 mL of the *Sample stock solution* to a 100-mL volumetric flask. Add 50 mL of the *Standard solution*, and dilute with *Solvent* to volume.

Blank: *Solvent*

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometer

Detection

Mercury: Flameless

Nickel: Air–acetylene flame

Lamp: Mercury hollow-cathode or nickel hollow-cathode

Analytical wavelength

Mercury: 253.7 nm

Nickel: 231.604 nm

Analysis

Samples: *Sample solution A*, *Sample solution B*, and *Sample solution C*

[NOTE—To minimize matrix interference, it is recommended that the method of standard additions be used.]

Determine the absorbances of the *Samples* against the *Blank*, and plot the corrected absorbances versus their content (0, 0.5, and 1.0 ppm) of mercury (or nickel). Draw the straight line best fitting the three points, and extrapolate the line until it intercepts the concentration axis. The distance between this point and the intersection of the axes represents the concentration of mercury (or nickel), C_S, in ppm (µg/g) in the 2 mg/mL of Ferrosoferric Oxide in *Solvent*.

Calculate the content of mercury (or nickel), in µg/g, in the portion of Ferrosoferric Oxide taken:

$$\text{Result} = C_S \times D$$

D = dilution factor, 25

Acceptance criteria

Mercury: NMT 1 ppm

Nickel: NMT 200 ppm

SPECIFIC TESTS

• **WATER-SOLUBLE SUBSTANCES**

Sample: 2.0 g

Analysis: Digest the *Sample* in 100 mL of water on a boiling water bath for 2 h, filter, and wash the filter with water. Evaporate the filtrate and washings, and dry the residue at 105° for 1 h.

Acceptance criteria: The weight of the dried residue is NMT 20 mg, corresponding to NMT 1.0%.

• **ACID-INSOLUBLE SUBSTANCES**

Sample: 2.0 g

Analysis: Digest the *Sample* in 25 mL of hydrochloric acid by boiling for 20 min. Add 100 mL of hot water, and filter quantitatively through a tared filtering[1] crucible, with the aid of hot wash water, until the filtrate tests negative for chloride. Dry the crucible and contents at 105° for 1 h.

Acceptance criteria: The weight of the residue is NMT 6 mg, corresponding to NMT 0.3%.

• **ORGANIC COLORS AND LAKES**

Analysis: Place 1.0 g in each of 3 beakers, and add 25 mL of each of the following reagents, respectively: 1-chloronaphthalene, alcohol, and chloroform. Heat the beakers containing alcohol and chloroform just to boiling. Heat the other beaker on a boiling water bath for 15 min, with occasional swirling. Pass the contents of the beakers through retentive, solvent-resistant filter paper.[2] If any of the filtrates show visible turbidity, centrifuge for 15 min. Record the spectra against respective solvent blanks in 1-cm cells from 350 to 750 nm.

Acceptance criteria: Absorbance in the range of 350–750 nm is NMT 0.01 AU.

• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.

• **pH** ⟨791⟩**:** 6–9 in a 10% suspension

• **LOSS ON DRYING** ⟨731⟩

Analysis: Dry a sample at 105° for 1 h.

Acceptance criteria: NMT 1.0% of its weight

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers. Store at room temperature.

Fructose—*see Fructose General Monographs*

Fumaric Acid

$C_4H_4O_4$ 116.07

2-Butenedioic acid, *E*-;
Fumaric acid [110-17-8].

DEFINITION

Fumaric Acid contains NLT 99.5% and NMT 100.5% of $C_4H_4O_4$, calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

▲• **SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197A**▲ (CN 1-May-2020)

ASSAY

• **PROCEDURE**

Sample solution: Dissolve 1 g of Fumaric Acid in 50 mL of methanol, and warm gently on a steam bath to effect solution. Cool, and add phenolphthalein TS.

Analysis: Titrate the *Sample solution* with 0.5 N sodium hydroxide VS to the first appearance of a pink color that persists for not less than 30 s. Perform a blank titration (see *Titrimetry* ⟨541⟩). Each mL of 0.5 N sodium hydroxide is equivalent to 29.02 mg of $C_4H_4O_4$.

[1] A glass-filter disc (P4; pore size diameter 9 to 15 µm) can be used, or equivalent.

[2] A PTFE filter (diameter 25 mm, 0.2-µm pore size), or equivalent.

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Acceptance criteria: 99.5%–100.5% on the anhydrous basis

IMPURITIES

INORGANIC IMPURITIES

• **Residue on Ignition** ⟨281⟩: NMT 0.1%

ORGANIC IMPURITIES

• **Procedure: Limit of Maleic Acid**

Mobile phase: 0.005 N sulfuric acid

Standard solution: 1 µg/mL of USP Maleic Acid RS in *Mobile phase*

Sample solution: 1 mg/mL of Fumaric Acid in *Mobile phase*

System suitability solution: 10 µg/mL of USP Fumaric Acid RS and 5 µg/mL of USP Maleic Acid RS in *Mobile phase*

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 210 nm

Column: 4.6-mm × 22-cm; packing L17

Flow rate: 0.3 mL/min

Injection size: 5 µL

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for maleic acid and fumaric acid are about 0.5 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 2.5 of the maleic acid and fumaric acid peaks

Relative standard deviation: NMT 2.0% of the maleic acid peak

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of maleic acid in the total weight of Fumaric Acid taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of maleic acid from the *Sample solution*

r_S = peak response of maleic acid from the *Standard solution*

C_S = concentration of USP Maleic Acid RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of the *Sample solution* (mg/mL)

Acceptance criteria: NMT 0.1%

SPECIFIC TESTS

• **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 0.5%

ADDITIONAL REQUIREMENTS

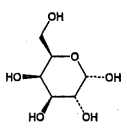
• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Fumaric Acid RS

USP Maleic Acid RS

Galactose



$C_6H_{12}O_6$ 180.16

α-D-Galactopyranose [3646-73-9].

DEFINITION

Galactose is one of the products of the metabolism of lactose, a naturally occurring sugar in dairy products, by the digestive enzyme lactase.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

• **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩

Solution A: Methanol and water (60:40)

Standard solution A: 500 µg/mL of USP Galactose RS in *Solution A*

Standard solution B: 500 µg/mL each of USP Galactose RS, USP Dextrose RS, and USP Lactose Monohydrate RS in *Solution A*

Sample solution: Dissolve 10 mg of Galactose in 20 mL of *Solution A*.

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Application volume: 2 µL

Developing solvent system: Propanol and water (85:15)

Spray reagent: 0.5 g of thymol in a mixture of alcohol and sulfuric acid (95:5)

System suitability

Sample: *Standard solution B*

Suitability requirements

Resolution: There must be three clearly resolved spots in the chromatogram for *Standard solution B*.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the plate in an unsaturated tank. After the solvent front has moved over 15 cm, remove the plate from the tank. Dry the plate with warm air, then spray the plate with *Spray reagent*. Heat for 10 min in an oven at 130°.

Acceptance criteria: The R_F of the principal spot of the *Sample solution* corresponds to that of *Standard solution A*.

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%

• **LIMIT OF LEAD**

Diluent: Dilute 12 mL of acetic acid with water to 100 mL. Mix equal parts of this solution and water to prepare the *Diluent*.

Lead standard solution: 16 µg/mL of lead nitrate

Standard solutions: To three identical flasks add 0.5, 1.0, and 1.5 mL of *Lead standard solution*, respectively, and then add to each flask 20.0 g of Galactose. Dilute with *Diluent* to 100 mL. To each flask add 2.0 mL of ammonium pyrrolidinedithiocarbamate solution (10 mg/mL) and 10.0 mL of methyl isobutyl ketone, then shake for 30 s. Protect from light. Allow the layers to separate, and use the methyl isobutyl ketone (upper) layer.

Sample solution: Dissolve 20.0 g of Galactose in 100 mL of *Diluent*. Add 2.0 mL of ammonium pyrrolidinedithiocarbamate solution (10 mg/mL) and 10.0 mL of methyl isobutyl ketone, and shake for 30 s. Protect from light. Allow the layers to separate, and use the methyl isobutyl ketone (upper) layer.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: 283.3 nm

Lamp: Lead hollow-cathode

Flame: Air–acetylene

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Analysis
Samples: *Standard solutions* and *Sample solution*
Concomitantly determine, at least in triplicate, the absorbances of the *Samples*. Record the average steady readings for each of the *Standard solutions* and the *Sample solution*. Plot the absorbances of the *Standard solutions* and the *Sample solution* versus the amount of lead added. Extrapolate the line joining the points on the graph until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of lead in the *Sample solution*.
Acceptance criteria: NMT 0.5 µg/g

• **BARIUM**
Standard solution: Add 6 mL of water to 5 mL of the *Sample solution* prepared for the *Acidity* test.
Sample solution: Add 5 mL of water and 1 mL of dilute sulfuric acid to 5 mL of the *Sample solution* prepared for the *Acidity* test. Allow to stand for 1 h.
Acceptance criteria: Any opalescence in the *Sample solution* is not more intense than that in the *Standard solution*.

SPECIFIC TESTS

• **APPEARANCE OF SOLUTION**
Sample solution: Dissolve, with heating at 50°, 10.0 g of Galactose in 50 mL of carbon dioxide-free water.
Control solution: Prepare immediately before use by mixing 3.0 mL of ferric chloride CS, 3.0 mL of cobaltous chloride CS, and 2.4 mL of cupric sulfate CS with dilute hydrochloric acid (10 mg/mL) to make 10 mL, and diluting 1.5 mL of this solution with the dilute hydrochloric acid to 100 mL.
Analysis: Compare by viewing the *Sample solution* and the *Control solution* downward in matched color-comparison tubes against a white surface (see *Color and Achromicity* ⟨631⟩).
Acceptance criteria: The *Sample solution* is not more intensely colored than the *Control solution*.

• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g. It meets the requirements of the test for absence of *Salmonella* species, *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*.

• **OPTICAL ROTATION,** *Specific Rotation* ⟨781S⟩
Sample solution: Transfer 10.0 g to a 100-mL volumetric flask, and dissolve in 80 mL of water. Add 0.2 mL of ammonia TS, allow to stand for 30 min, then dilute with water to volume.
Analysis: Perform at 20°.
Acceptance criteria: +78.0° to +81.5°

• **ACIDITY**
Sample solution: Dissolve 10.0 g of Galactose, with heating at 50°, in 40 mL of carbon dioxide-free water. Dilute with carbon dioxide-free water to 50 mL. [NOTE—Use this solution for the *Barium* test.]
Analysis: To 30 mL of the *Sample solution* add 0.3 mL of phenolphthalein TS, and titrate with 0.01 N sodium hydroxide to a pink color.
Acceptance criteria: NMT 1.5 mL of 0.01 N sodium hydroxide is required.

• **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 1.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in a tight container. No storage requirements specified.

• **USP REFERENCE STANDARDS** ⟨11⟩
USP Dextrose RS
USP Galactose RS
USP Lactose Monohydrate RS

Galageenan

DEFINITION

Galageenan is the hydrocolloid obtained by extraction with water or aqueous alkali from the red seaweed class Rhodophyceae species *Eucheuma gelatinae*. Galageenan consists chiefly of potassium, sodium, calcium, magnesium, and ammonium sulfate esters of galactose and 3,6-anhydrogalactose copolymers. These hexoses are alternately linked as α-1,3 and β-1,4 in the polymer. The ester sulfate content ranges from 8%–18%. In addition, it contains inorganic salts that originate from the seaweed and from the process of recovery from the extract.
Galageenan is recovered by alcohol precipitation or by freezing and pressing.

IDENTIFICATION

• **A. FILM FORMATION**
Solution A: A solution (1 in 50), prepared by heating a uniform dispersion in a hot water bath to 80°
Acceptance criteria: *Solution A* becomes more viscous upon cooling and may form a gel.

• **B.**
Analysis: Dilute a portion of *Solution A* (retained from *Identification* test *A*) with 4 parts water, and add 2–3 drops of methylene blue TS.
Acceptance criteria: A blue, stringy precipitate is formed.

Change to read:

• **C. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F** ▲ (CN 1-May-2020)
Sample solution: Disperse 2 g of Galageenan in 400 mL of a solution containing 5 g of edetate disodium in 1000 mL of 60% isopropyl alcohol, and stir for 2 h. Filter with the aid of vacuum, and wash the residue with a total of 200 mL of 65% isopropyl alcohol. Finish washing with a total of 100 mL of 80% isopropyl alcohol. Dry the residue for 30 min in a 60° oven, and overnight in a 70° vacuum oven. Break lumps by grinding with a mortar and pestle. Dissolve 15 mg of the alcohol-treated material in 5 mL of water. Heat for 10 min in a water bath. Pipet 2 mL onto a suitable nonsticking surface to produce a 5-µm-thick film (when dry).
Spectral range: 2000–600 cm^{-1}
Analysis: Subtract the baseline (drawn by connecting the minima in the range of 1500 and 800 cm^{-1}) from the raw spectrum. Record the absorbances for the bands at 1220–1260, 928–933, 840–850, and 800–805 cm^{-1} relative to the absorbance at 1050 cm^{-1}.
Acceptance criteria: The absorbance values so obtained are within the ranges specified in *Table 1*.

Table 1

Wave Number (cm^{-1})	Molecular Assignment	Absorbance Relative to 1050 cm^{-1} Galageenan
1220–1260	Ester sulfate	0.3–0.6
928–933	3,6-Anhydrogalactose	0.3–0.6
840–850	Galactose-4-sulfate	0.1–0.3

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Table 1 *(continued)*

Wave Number (cm^{-1})	Molecular Assignment	Absorbance Relative to 1050 cm^{-1} Galageenan
800–805	3,6-Anhydrogalactose-2-sulfate	0.0–0.1

ASSAY

- **CONTENT OF SULFATE**

Sample: 300 mg

Analysis: Weigh the *Sample* on an ashless filter paper. Fold the paper so as to enclose the *Sample*, and place it in a 500-mL Kjeldahl flask. Add 45 mL of nitric acid, and bring to a boil on a hot plate in a fume hood. Add nitric acid as necessary to keep the sample from evaporating to dryness. Continue boiling until digestion is complete and the volume of nitric acid remaining is about 10 mL. Cool the mixture, and reduce the excess nitric acid by adding formaldehyde TS until the evolution of nitrogen oxide vapors has ceased. Heat this mixture on a hot plate to reduce the volume to about 10 mL. Transfer the mixture to a 150-mL beaker with the aid of several portions of water until the total volume is approximately 100 mL. Add 0.5 mL of hydrochloric acid, and bring to a boil on a hot plate. Add carefully 10 mL of 0.25 M barium chloride, and allow to boil for 1 min. Cover with a watch glass, and allow to stand overnight. Filter the solution through a tared, fine-porosity filtering crucible previously ignited in a muffle furnace at 550° for 30 min and cooled in a desiccator for 30 min. Wash the barium sulfate precipitate so obtained on the crucible several times with boiling water. Place the crucible in an oven, and heat at 100° for 30 min. Transfer the crucible to a muffle furnace, and ignite for 30 min at 550°. Remove the crucible, and cool in a desiccator for 30 min.

Weigh and calculate the percentage of sulfate groups:

$$\text{Result} = (W_B/W_S) \times (M_{r1}/M_{r2}) \times 100$$

W_B = weight of barium sulfate obtained (mg)
W_S = weight of Galageenan taken (mg)
M_{r1} = molecular weight of the sulfate group, 96.02
M_{r2} = molecular weight of barium sulfate, 233.43

Acceptance criteria: 8%–18% sulfate

IMPURITIES

- **LEAD** ⟨251⟩**:** NMT 5 ppm
- **ACID-INSOLUBLE MATTER**

Sample: 2 g

Analysis: Transfer the *Sample* to a 250-mL beaker containing 150 mL of water and 1.5 mL of sulfuric acid. Cover with a watch glass, and heat on a steam bath for 6 h, rubbing down the wall of the beaker frequently with a rubber-tipped stirring rod, and replacing any water lost by evaporation. Transfer 500 mg of a suitable filter aid to the beaker, and pass through a tared filtering crucible equipped with a 2.4-cm glass fiber filter. Wash the residue several times with hot water, dry at 105° for 3 h, cool in a desiccator, and weigh. The difference between the total weight and the sum of the weights of the filter aid, crucible, and glass fiber filter is the weight of the acid-insoluble matter.

Acceptance criteria: NMT 2.0% of the weight of Galageenan taken

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic microbial count does not exceed 200 cfu/g, the total combined molds and yeasts count does not exceed 20 cfu/g, and it meets the requirements of the tests for absence of *Escherichia coli* and *Salmonella* species.
- **VISCOSITY—ROTATIONAL METHODS** ⟨912⟩

Sample solution: Transfer 7.5 g of Galageenan to a tared, tall-form, 600-mL beaker. Add 450 mL of water, and disperse with agitation for 15 min. Add water to bring the weight to 500 g, and heat in a water bath, with continuous agitation, until a temperature of 80° is reached. Add water to adjust for loss by evaporation, cool to between 76° and 77°, and place in a constant-temperature bath maintained at 75°.

Analysis: Use a suitable rotational viscometer equipped with a spindle having a cylinder 1.88 cm in diameter and 6.51 cm in height, and an immersion depth of 8.10 cm (No. 1 spindle). Allow the spindle to rotate in the solution at 30 rpm for six revolutions, then observe the scale reading. Convert the scale reading to centipoises by multiplying by the constant for the spindle and speed used.

Acceptance criteria: NLT 15 centipoises (at 75°)

- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 35.0%
- **LOSS ON DRYING** ⟨731⟩**:** Dry a sample at a pressure not exceeding 10 mm of mercury at 70° for 18 h, cool in a desiccator, and weigh: it loses NMT 12.5% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, preferably in a cool place.

Gelatin

Portions of this monograph that are national *USP* text, and are not part of the harmonized text, are marked with symbols (♦ ♦) to specify this fact.

Change to read:

DEFINITION

Purified protein obtained from collagen of animals ▲♦▲ (NF 1-May-2020) (including fish and poultry) ▲♦▲ (NF 1-May-2020) by partial alkaline and/or acid hydrolysis, ▲and/or▲ (NF 1-May-2020) enzymatic hydrolysis, or by thermal hydrolysis.

The hydrolysis leads to gelling or non-gelling grades. This monograph covers both the gelling ▲grades▲ (NF 1-May-2020) and non-gelling grades ▲(called also▲ (NF 1-May-2020) hydrolyzed gelatin▲).▲ (NF 1-May-2020)

IDENTIFICATION

Change to read:

- **♦A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: 197A▲ (CN 1-May-2020)[NOTE—Compare the spectra of the test specimen and USP Gelatin RS over the range from 800 to 3500 cm^{-1}. Disregard any peak at about 1403 cm^{-1}.]♦
- **B.**

Sample solution: Dissolve 1.00 g in carbon dioxide-free water at about 55°, dilute with the same solvent to 100 mL, and keep at 55°. Save the unused portion of this solution for use in the test for *pH*.

Analysis: To 2 mL of the *Sample solution* add 0.05 mL of a 125-g/L solution of copper sulfate pentahydrate. Mix, and add 0.5 mL of an 85-g/L solution of sodium hydroxide.

Acceptance criteria: A violet color is produced.

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Change to read:

• **C.**
Sample: 0.5 g
Analysis: Place the *Sample* in a test tube of about 15-mm internal diameter, and add 10 mL of water. Allow to stand for 10 min, heat at 60° for 15 min, and keep the tube upright at ▲2°–8°▲ (NF 1-May-2020) for 6 h. Invert the tube.
Acceptance criteria: The contents do not flow out immediately for gelling grades. The contents immediately flow out for non-gelling grades.

Change to read:

• **D. For Non-Gelling Grades**
Medium: pH 6.8 phosphate buffer. Mix 77.3 mL of a 71.5-g/L solution of disodium hydrogen phosphate dodecahydrate with 22.7 mL of a 21-g/L solution of citric acid.
Sample: 0.5 g
Color reagent: Prepare immediately before use. Dissolve 1.0 g of dimethylaminobenzaldehyde in 3.5 mL of perchloric acid [600 g/L of perchloric acid ($HClO_4$)].
Analysis: Place the *Sample* in a 250-mL bottle. Add 10 mL of water and 5 mL of sulfuric acid. Place the bottle, partly but not completely closed (for example, using a watch glass), in an oven at 105° for 4 h. Allow to cool, and add 200 mL of water. Adjust with a 200-g/L solution of sodium hydroxide to a pH of 6.0–8.0. Place 2 mL of the solution in a test tube, and add 2 mL of oxidizing reagent (14-g/L solution of chloramine T in the *Medium*; prepare immediately before use). Mix, and allow to stand for 20 min. Add 2 mL of *Color reagent*, and slowly add 6.5 mL of 2-propanol). Mix, and place in a water bath at 60° for about 15 min.
Acceptance criteria: A red ▲to violet▲ (NF 1-May-2020) color develops.

SPECIFIC TESTS
• **pH** ⟨791⟩
Sample: Use the *Sample solution* prepared in *Identification B*.
Acceptance criteria: 3.8–7.6 at 55°
• **Water Conductivity** ⟨645⟩
Sample: 1.0% solution at 30 ± 1.0°
Analysis: Determine without the use of the temperature compensation.
Acceptance criteria: NMT 1 $mS \cdot cm^{-1}$

Change to read:

• **Sulfur Dioxide**
Sample: 25.0 g
Titrimetric system
Mode: Direct titration
Titrant: 0.1 ▲N▲ (NF 1-May-2020) sodium hydroxide VS
Endpoint detection: Visual
Analysis: Introduce 150 mL of water into the flask (see *A* in *Figure 1*), and pass carbon dioxide through the whole system for 15 min at a rate of 100 mL/min. To 10 mL of hydrogen peroxide solution [30 g/L of hydrogen peroxide (H_2O_2)] add 0.15 mL of bromophenol blue TS. Add 0.1 M sodium hydroxide until a violet-blue color is obtained, without exceeding the endpoint. Place the solution in the test tube (*D*). Without interrupting the stream of carbon dioxide, remove the funnel (*B*), and introduce through the opening into the flask (*A*) the *Sample* with the aid of 100 mL of water. Add through the funnel 80 mL of diluted hydrochloric acid [73 g/L of hydrogen chloride (HCl)], and boil for 1 h. Open the tap of the funnel, stop the flow of carbon dioxide, and also stop the heating and the cooling water. Transfer the contents of the test tube with the aid of a little water to a 200-mL wide-necked, conical flask. Heat on a water bath for 15 min, and allow to cool. Add 0.1 mL of bromophenol blue TS, and titrate with *Titrant* until the color changes from yellow to violet-blue.

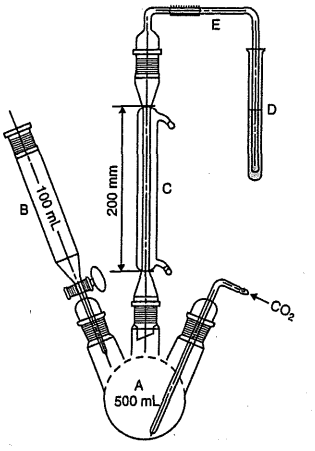


Figure 1. Apparatus for determination of sulfur dioxide.

Carry out a blank titration, and calculate the content of sulfur dioxide, in ppm:

$$\text{Result} = [(V_S - V_B) \times (M/W)] \times 32{,}030$$

V_S = volume of 0.1 M sodium hydroxide consumed by the *Sample* (mL)
V_B = volume of 0.1 M sodium hydroxide consumed by the blank (mL)
M = actual molarity of the *Titrant* (mol/L)
W = weight of the *Sample* (g)

Acceptance criteria: NMT 50 ppm
• **Peroxides**
Peroxidase transfers oxygen from peroxides to an organic redox indicator that is converted to a blue oxidation product. The intensity of the color obtained is proportional to the quantity of peroxide and can be compared with a color scale provided with the test strips to determine the peroxide concentration.
Peroxide test strips: Use commercial test strips with a suitable scale covering the range of 0–25 ppm of peroxide.
Sample: 20.0 ± 0.1 g
Suitability test
Hydrogen peroxide standard solution: 2 ppm of hydrogen peroxide (H_2O_2) prepared by dilution of hydrogen peroxide solution [30 g/L of hydrogen peroxide (H_2O_2)]
Procedure: Dip a test strip for 1 s into *Hydrogen peroxide standard solution*, such that the reaction zone is properly wetted. Remove the test strip, shake off excess liquid, and after 15 s, compare the reaction zone with the color scale provided. The test strips are suitable if the color matches that of the 2-ppm concentration.
Analysis: Weigh the *Sample* in a beaker, and add 80.0 ± 0.2 mL of water. Stir to moisten all of the gelatin, and allow the *Sample* to stand at room temperature for 1–3 h. Cover the beaker with a watch glass. If the *Sample* is not dissolved completely, place the beaker in a water bath at 65 ± 2° for 20 ± 5 min to dissolve the *Sample*. Stir the contents of the beaker with a glass rod to achieve a homogeneous solution. Dip a test strip for 1 s into the test

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solution, such that the reaction zone is properly wetted. Remove the test strip, shake off excess liquid, and after 15 s, compare the reaction zone with the color scale provided. Multiply the concentration read from the color scale by a factor of 5 to calculate the concentration, in ppm, of peroxide in the test substance.

Acceptance criteria: NMT 10 ppm

• **GEL STRENGTH (BLOOM VALUE)**

For gelling grades

The gel strength is expressed as the mass in grams necessary to produce the force that, applied to a plunger 12.7 mm in diameter, makes a depression 4-mm deep in a gel with a concentration of 6.67% m/m and matured at 10°.

Sample: 7.5 g

Apparatus: Texture analyzer or gelometer with a cylindrical piston 12.7 ± 0.1 mm in diameter with a plane pressure surface and a sharp bottom edge, and a bottle 59 ± 1 mm in internal diameter and 85 mm high. Adjust the apparatus according to the manufacturer's manual.

Settings

Distance: 4 mm

Test speed: 0.5 mm/s

Analysis: Place the *Sample* in a bottle. Add 105 mL of water, place a watch glass over the bottle, and allow to stand for 1–4 h. Heat in a water bath at 65 ± 2° for 15 min. While heating, stir gently with a glass rod. Ensure that the solution is uniform and that any condensed water on the inner walls of the bottle is incorporated. Allow to cool at room temperature for 15 min, and transfer the bottle to a thermostatically controlled bath at 10.0 ± 0.1°, fitted with a device to ensure that the platform on which the bottle stands is perfectly horizontal. Close the bottle with a rubber stopper, and allow to stand for 17 ± 1 h. Remove the *Sample* bottle from the bath, and quickly wipe the water from the exterior of the bottle. Center the bottle on the platform of the *Apparatus* so that the plunger contacts the *Sample* as nearly at its midpoint as possible, and start the measurement.

Acceptance criteria: 80%–120% of the labeled nominal value

• **IRON**

Atomic absorption spectrometry, standard additions method

Iron standard solution (8 ppm): Dissolve 80 mg of iron in 50 mL of hydrochloric acid [220 g/L of hydrogen chloride (HCl)], and dilute with water to 1000.0 mL. Immediately before use, prepare a 1:10 dilution with water.

Standard solution: Prepare using the *Iron standard solution*, diluted with water as necessary.

Sample solution: In a conical flask, add 10 mL of hydrochloric acid [37% m/m of hydrogen chloride (HCl)] to 5.00 g of the substance to be examined. Close the flask, and place in a water bath at 75°–80° for 2 h. (If necessary for proper solubilization, the gelatin may be allowed to swell after addition of the acid and before heating, the heating time may be prolonged, and a higher temperature may be used.) Allow to cool, and adjust the contents of the flask with water to 100.0 g.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Analytical wavelength: 248.3 nm

Acceptance criteria: NMT 30 ppm

• **CHROMIUM**

Atomic absorption spectrometry, standard additions method

Chromium standard solution (100 ppm): 0.283 mg/mL of potassium dichromate ($K_2Cr_2O_7$) in water

Standard solution: Prepare using the *Chromium standard solution*, diluted with water if necessary.

Sample solution: Use the *Sample solution* described in the test for *Iron*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Analytical wavelength: 357.9 nm

Acceptance criteria: NMT 10 ppm

• **ZINC**

Atomic absorption spectrometry, standard additions method

Zinc standard solution (10 ppm): Dissolve 0.440 g of zinc sulfate heptahydrate and 1 mL of acetic acid [300 g/L of acetic acid ($C_2H_4O_2$)] in water, and dilute to 100.0 mL. Immediately before use, prepare a 1:100 dilution in water.

Standard solution: Prepare using the *Zinc standard solution*, diluted with water if necessary.

Sample solution: Use the *Sample solution* described in the test for *Iron*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Analytical wavelength: 213.9 nm

Acceptance criteria: NMT 30 ppm

• **LOSS ON DRYING** ⟨731⟩

Sample: 5.0 g

Analysis: Dry the *Sample* in an oven at 105° for 16 h.

Acceptance criteria: NMT 15%

• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩: The total bacterial count does not exceed 10^3 cfu/g, the total yeasts and molds count does not exceed 10^2 cfu/g, and the tests for *Salmonella* species and *Escherichia coli* are negative.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Protect from heat and moisture.

• **LABELING:** The label states the *Gel Strength (Bloom Value)* or that it is a non-gelling grade.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Gelatin RS

Gellan Gum

[71010-52-1].

DEFINITION

Gellan Gum is a high-molecular-weight polysaccharide gum produced by a pure-culture fermentation of a carbohydrate with *Pseudomonas elodea*, purified by recovery with isopropyl alcohol, and then dried and milled. It is a heteropolysaccharide comprising a tetrasaccharide repeating unit of one rhamnose, one glucuronic acid, and two glucose units. The glucuronic acid is neutralized to mixed potassium, sodium, calcium, and magnesium salts. It may contain acyl (glyceryl and acetyl) groups as the *O*-glycosidically linked esters. It yields NLT 3.3% and NMT 6.8% of carbon dioxide, calculated on the dried basis.

IDENTIFICATION

• **A.**

Sample solution: Hydrate 1 g of Gellan Gum in 99 mL of deionized water. Stir the mixture for 2 h, using a motorized stirrer and a propeller-type stirring blade.

Analysis: Draw a small amount of the solution obtained into a wide-bore pipet, and transfer it to a 10% calcium chloride solution. [NOTE—Reserve the remaining portion of this solution for *Identification* test *B*.]

Acceptance criteria: A tough, wormlike gel will form instantly.

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• **B.**
Sample solution: The remaining *Sample solution* from *Identification* test *A*
Analysis: Add 0.5 g of sodium chloride, heat the solution to 80°, stirring constantly, and hold at 80° for 1 min. Stop heating and stirring the solution, and allow it to cool to room temperature.
Acceptance criteria: A firm gel will form.

ASSAY
• **ALGINATES ASSAY** ⟨311⟩
Sample: 1.2 g of undried Gellan Gum
Analysis: Proceed as directed for the *Procedure* in the chapter.
Acceptance criteria: 3.3%–6.8% of carbon dioxide on the dried basis

IMPURITIES
• **LEAD** ⟨251⟩
Test preparation: Prepare as directed, using a 2.0-g portion of Gellan Gum. Use 4 mL of *Diluted Standard Lead Solution* (4 µg of Pb) for the test.
Acceptance criteria: NMT 2.0 µg/g
• **ARSENIC,** *Method II* ⟨211⟩**:** NMT 3.0 µg/g
• **LIMIT OF ISOPROPYL ALCOHOL**
Internal standard solution: 1 mg/mL of tertiary butyl alcohol
Standard stock solution: 1 mg/mL of isopropyl alcohol
Standard solution: Pipet 4 mL of the *Standard stock solution* and 4 mL of the *Internal standard solution* into a 100-mL volumetric flask, dilute with water to volume, and mix.
Sample solution: Disperse 1 mL of a suitable antifoam emulsion in 200 mL of water contained in a 1000-mL, round-bottom distilling flask having a 24/40 standard taper ground joint. Add 5 g of Gellan Gum, and shake for 1 h on a wrist-action mechanical shaker. Connect the flask to a fractionating column, and distill about 100 mL, adjusting the heat so that foam does not enter the column. Add by pipet 4 mL of the *Internal standard solution*, and mix.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 3.2-mm × 1.8-m stainless steel; packed with 80- to 100-mesh surface silanized packing S3, or equivalent
Temperatures
Column: 165°
Detector: 200°
Injection port: 200°
Carrier gas: Helium
Injection volume: 4–5 µL
Analysis
Samples: *Standard solution* and *Sample solution*
[NOTE—The retention time of tertiary butyl alcohol is 1.5 relative to that of isopropyl alcohol.]
Calculate the amount of isopropyl alcohol in the portion of Gellan Gum taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of isopropyl alcohol to the internal standard from the *Sample solution*
R_S = peak response ratio of isopropyl alcohol to the internal standard from the *Standard solution*
C_S = concentration of isopropyl alcohol in the *Standard solution* (mg/mL)
C_U = concentration of the *Sample solution* (mg/mL)

Acceptance criteria: NMT 0.075%

SPECIFIC TESTS
• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*. The total aerobic microbial count is NMT 10^3 cfu/g, and the total combined molds and yeasts count is NMT 10^2 cfu/g.
• **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** 4.0%–14.0% on the dried basis
• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry at 105° for 2.5 h.
Acceptance criteria: NMT 15.0%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at room temperature.

Pharmaceutical Glaze

DEFINITION
Pharmaceutical Glaze is a specially denatured alcoholic solution of Shellac containing between 20.0% and 57.0% of anhydrous shellac and is made with either dehydrated alcohol or alcohol containing 5% water by volume. The solvent is a specially denatured alcohol approved for glaze manufacturing by the Internal Revenue Service. It contains NLT 90.0% and NMT 110.0% of the labeled amount of shellac. It may contain waxes and titanium dioxide as an opaquing agent.

IDENTIFICATION
• **A.**
Sample: Remaining sample solution retained from the *Assay*
Analysis: Pour the *Sample* from a volumetric flask onto a clean glass plate, and place the plate in a nearly vertical position. After drainage is complete, allow the resulting film to dry in a well-ventilated place at 20° for 1 h, then place the plate in an oven at a temperature of 43° for 16–24 h. Cool, and scrape the film from the plate with a sharp blade, discarding the thick edges. To 50 mg of the solid shellac from the film add a few drops of a mixture of 1 g of ammonium molybdate and 3 mL of sulfuric acid. Retain the remaining film for the tests for *Identification of Aleuritic Acid and Shellolic Acid by Thin-Layer Chromatography* and *Rosin*.
Acceptance criteria: A green color is produced, and it becomes lilac on standing for 5 min.
• **B. IDENTIFICATION OF ALEURITIC ACID AND SHELLOLIC ACID BY THIN-LAYER CHROMATOGRAPHY**
Standard solution: 6 mg/mL of USP Aleuritic Acid RS in methanol, heating slightly if necessary
Solid shellac sample: 500 mg of solid shellac from the film prepared in *Identification* test *A*
Sample solution: Weigh and finely powder the *Solid shellac sample*. Transfer 500 mg of the finely powdered shellac to a test tube and heat with 4 mL of 85-mg/mL sodium hydroxide solution in a vigorously boiling water bath for 5 min. Cool, add 10 mL of ethyl acetate, and transfer the content to a separatory funnel. With stirring, add slowly 4 mL of a 120-mg/mL solution of glacial acetic acid to the funnel. Shake the solution thoroughly and withdraw the lower layer. Transfer the upper layer to a small flask, add anhydrous sodium sulfate, and pass the content through a 0.45-µm syringe filter.[1] Collect the filtrate and use it as the sample.

[1] A 0.45-µm GHP membrane syringe filter is suitable.

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Chromatographic system
(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
Mode: TLC
Plate: 10-cm × 20-cm, or 20-cm × 20-cm, silica gel 60 F_{254}
Application volume: 10 µL, as 8-mm bands. [NOTE—An automated apparatus may be used.]
Developing solvent system: Ethyl acetate, methylene chloride, methyl alcohol, and acetic acid (60:32:8:1)
Spray reagent: Prepare the anisaldehyde solution by mixing in the following order. In 0.5 mL of anisaldehyde add 10 mL of glacial acetic acid, 85 mL of methyl alcohol, and 5 mL of sulfuric acid.

Analysis
Samples: *Standard solution* and *Sample solution*
Development: Apply the *Samples* in different bands to the previously marked starting point on a TLC plate, and develop the plate two times over a path of 15 cm. Dry the plate in air.
Detection: Spray with *Spray reagent*. Heat the plate at 100°–105° for 10 min, and examine in daylight (or white light).
[NOTE—The principal band of aleuritic acid shows strong intensity and purple color. The retardation factor (R_F) for the principal band of aleuritic acid is 0.41. A blue-gray band with medium intensity at R_F 0.22 could be assigned to shellolic acid.]

Acceptance criteria: The chromatogram from the *Sample solution* shows several colored bands. One of the colored bands is similar in position and color to the band in the chromatogram from the *Standard solution*, and it is assigned to aleuritic acid. Below the aleuritic acid band, a blue-gray band is assigned to shellolic acid.

ASSAY

• **PROCEDURE**

Sample solution A: When testing Glaze that does not contain titanium dioxide, transfer a quantity of Glaze containing 17 g of shellac to a 100-mL volumetric flask, and add alcohol to volume. Pipet 3 mL into a tared dish containing 10 g of washed sand and a small glass rod. Retain the remaining solution in the volumetric flask for *Identification* test *A*. The tare weight includes the combined weights of the dish, the washed sand, and the glass rod. Stir until a uniform mixture is obtained, allow the glass rod to remain in the dish, dry at 105° for 1 h in an explosion-proof oven, cool, and weigh.

Sample solution B: When testing Glaze that contains titanium dioxide, transfer a quantity containing 10 g of solids to a beaker, and add 10 mL of alcohol. Filter off the pigment with the aid of a vacuum. Wash the filter with alcohol; transfer the combined filtrate and washing, with the aid of alcohol, to a 200-mL volumetric flask; add alcohol to volume; and mix. Pipet 6 mL into a tared dish containing 10 g of washed sand and a small glass rod. Proceed as directed in *Sample solution A*, beginning with "The tare weight...".

Analysis: The weight of shellac, W_F, in g, in the quantity of Glaze taken is obtained by subtracting the tare weight from the final weight of the dried dish and contents.

For Glaze not containing titanium dioxide calculate the percentage of shellac in the quantity of Glaze taken:

$$\text{Result} = [(W_F/V_F) \times V_I]/W \times 100$$

V_F = final volume used, 3 mL
V_I = initial volume prepared, 100 mL
W = weight of Glaze (g) for preparation of 100-mL solution

For Glaze containing titanium dioxide calculate the percentage of shellac in the quantity of Glaze taken:

$$\text{Result} = [(W_F/V_F) \times V_I]/W \times 100$$

V_F = final volume used, 6 mL
V_I = initial volume prepared, 200 mL
W = weight of Glaze (g) for preparation of 200-mL solution

Acceptance criteria: 90.0%–110.0% of the labeled amount of shellac

OTHER COMPONENTS

• **WAX**

Sample: Weigh, by difference, a quantity of Glaze containing 10 g of shellac into a 200-mL tall-form beaker.
Analysis: To the *Sample* add with stirring 150 mL of hot water containing 2.5 g of sodium carbonate, immerse the beaker in a boiling water bath, and stir until the solid is dissolved. Cover the beaker with a watch glass, and maintain the heat for 3 h more without agitation. Remove the beaker to a cold water bath. When the wax has floated to the surface, pass the solution through medium-speed quantitative ashless filter paper, transferring the wax to the paper, and wash the filter with water. Pour 5–10 mL of alcohol onto the filter to facilitate drying. Wrap the paper loosely in a larger piece of filter paper, bind with a piece of fine wire, and dry with the aid of gentle heat. Extract with chloroform in a suitable continuous extraction apparatus for 2 h, using a weighed flask to receive the extracted wax and solvent. Evaporate the solvent, and dry the wax at 105° to constant weight.
Acceptance criteria: It meets the requirements in *Table 1*.

Table 1

Type of Shellac	Wax
Orange shellac	NMT 5.5%
Refined orange shellac	NMT 0.2%
Regular bleached shellac	NMT 5.5%
Refined bleached shellac	NMT 0.2%

IMPURITIES

• **ROSIN**

Sample: 2 g of the solid shellac from the film prepared in *Identification* test *A*
Analysis: Dissolve the *Sample* by shaking with 10 mL of dehydrated alcohol. Add slowly, with shaking, 50 mL of solvent hexane, wash with two successive 50-mL portions of water, filter the washed alcohol–solvent hexane solution, and evaporate to dryness. To the residue add 2 mL of a mixture of liquefied phenol, dehydrated alcohol, and solvent hexane (1: 0.5: 2). Stir, and transfer a portion of the solution to the cavity of a color-reaction plate. Fill an adjacent cavity with a mixture of bromine and solvent hexane (1:4), and cover both cavities with an inverted watch glass.
Acceptance criteria: No purple or deep indigo-blue color is produced in or above the liquid containing the residue.

SPECIFIC TESTS

• **ACID VALUE**

Sample: Weigh, by difference, a quantity of Glaze containing 2 g of shellac.
Analysis: Dissolve the *Sample* in 50 mL of alcohol that has been neutralized to phenolphthalein with 0.1 N sodium hydroxide, add additional phenolphthalein TS if necessary, and titrate with 0.1 N sodium hydroxide VS to a pink

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endpoint, or determine the endpoint potentiometrically (see *Titrimetry* ⟨541⟩). [NOTE—For Glaze containing orange shellac, titrate slowly, stirring vigorously, until a glass rod dipped into the titrated solution produces a color change when touched to a drop of thymol blue TS on a spot plate.] Express the acid value in terms of the number of mg of potassium hydroxide required per g of dried shellac. Calculate the acid value as directed in *Fats and Fixed Oils* ⟨401⟩, *Acid Value*.

Acceptance criteria: It meets the requirements in *Table 2*.

Table 2

Type of Shellac	Acid Value (on Dried Basis)
Orange shellac	68–76
Refined orange shellac	68–79
Regular bleached shellac	73–89
Refined bleached shellac	75–91

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, lined metal or plastic containers, protected from excessive heat, preferably at a temperature below 25°.
- **LABELING:** Label it to indicate the type of shellac. Label it also to indicate the shellac concentration, the composition of the solvent, and the quantity of titanium dioxide, if present. Where titanium dioxide or waxes are present, the label must state that the Glaze requires mixing before use.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Aleuritic Acid RS

Gluconolactone—*see Gluconolactone General Monographs*

Liquid Glucose

[8027-56-3].

DEFINITION

Liquid Glucose is a product obtained by the incomplete hydrolysis of starch. It consists chiefly of dextrose, dextrins, maltose, and water.

IDENTIFICATION

- **A.** It meets the requirements in the *Assay* for *Reducing Sugars (Dextrose Equivalent)*.

ASSAY

Change to read:

- **REDUCING SUGARS (DEXTROSE EQUIVALENT)**
  Methylene blue solution: 10 mg/mL
  Standard solution: 6 mg/mL of USP Dextrose RS
  Sample solution: Transfer a quantity of Liquid Glucose, equivalent to about 3.0 g of reducing sugars (dextrose equivalent), to a 500-mL volumetric flask, and dilute with water to volume.
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Transfer 25.0-mL portions of alkaline cupric tartrate TS to each of two boiling flasks. Bring the contents of one flask to boiling within about 2 min while titrating with *Standard solution* to within 0.5 mL of the anticipated endpoint. Boil gently for 2 min. Continue to boil gently, add 2 drops of *Methylene blue solution*, and complete the titration within 1 min by adding the *Standard solution* dropwise or in small increments until the blue color disappears, determined by viewing against a white background in daylight or under equivalent illumination. If more than 0.5 mL of the titrant was required after the addition of the indicator, repeat the titration, adding the necessary volume of titrant before adding the indicator. Bring the contents of the second flask to boiling, and similarly titrate with the *Sample solution*.
  Calculate the dextrose equivalent, on the anhydrous basis, taken:

$$\text{Result} = (C_S/C_U) \times (V_S/V_U) \times [1/(1 - 0.01 \times A)] \times 100$$

C_S = concentration of USP Dextrose RS in the *Standard solution* (mg/mL)
C_U = concentration of ▲Liquid Glucose▲ (ERR 1-Jun-2019) in the *Sample solution* (mg/mL)
V_S = *Titrant* volume consumed by the *Standard solution* (mL)
V_U = *Titrant* volume consumed by the *Sample solution* (mL)
A = percentage of water in the Liquid Glucose taken

Acceptance criteria: 90%–110% of the labeled value on the anhydrous basis

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.5%
- **SULFITE**
  Sample solution: 5 g in 50 mL of water
  Analysis: To the *Sample solution* add 0.2 mL of 0.1 N iodine, then add 0.5 mL of starch TS.
  Acceptance criteria: A blue color is produced.
- **STARCH**
  Sample solution: 5 g in 50 mL of water
  Analysis: Boil the *Sample solution* for 1 min, cool, and add 0.2 mL of 0.1 N iodine.
  Acceptance criteria: No blue color is produced.

SPECIFIC TESTS

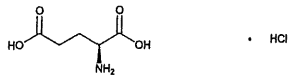
- **ACIDITY**
  Sample solution: 5.0 g in 15 mL of water
  Analysis: To the *Sample solution* add 5 drops of phenolphthalein TS, and titrate with 0.10 N sodium hydroxide.
  Acceptance criteria: NMT 0.60 mL of 0.10 N sodium hydroxide is required to produce a pink color.
- **WATER DETERMINATION,** *Method Ia* ⟨921⟩
  Sample: 100 mg
  Acceptance criteria: NMT 21.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tightly closed containers. No storage requirements specified.
- **LABELING:** Label it to indicate the natural source of starch. Label it to indicate its nominal dextrose equivalent.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Dextrose RS

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L-Glutamic Acid, Hydrochloride



$C_5H_9NO_4 \cdot HCl$ 183.59
L-2-Aminoglutaric acid, hydrochloride;
2-Aminopentanedioic acid, hydrochloride [138-15-8].

DEFINITION
L-Glutamic Acid, Hydrochloride, contains NLT 98.5% and NMT 101.5% of $C_5H_9NO_4 \cdot HCl$, calculated on the dried basis.

IDENTIFICATION

Change to read:

▲• **SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

ASSAY
• **PROCEDURE**
Sample: 100 mg of L-Glutamic Acid, Hydrochloride, previously dried
Analysis: Dissolve the *Sample* in 0.5 mL of water, add 15.0 mL of 0.1 N perchloric acid VS, and heat on a water bath for 30 min. After cooling, add 45 mL of glacial acetic acid, and titrate the excess perchloric acid with 0.1 N sodium acetate, determining the endpoint potentiometrically. Perform a blank determination (see *Titrimetry* ⟨541⟩). Each mL of 0.1 N perchloric acid is equivalent to 18.36 mg of $C_5H_9NO_4 \cdot HCl$.
Acceptance criteria: 98.5%–101.5% on the dried basis

IMPURITIES
INORGANIC IMPURITIES
• **Residue on Ignition** ⟨281⟩: NMT 0.25%

SPECIFIC TESTS
• **OPTICAL ROTATION,** *Specific Rotation* ⟨781S⟩: +25.2° to +25.8°, determined at 20°
Sample solution: 100 mg/mL, in 2 N hydrochloric acid
• **LOSS ON DRYING** ⟨731⟩: Dry a sample at 80° for 4 h: it loses NMT 0.5% of its weight.

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Store in well-closed, light-resistant containers.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP l-Glutamic Acid, Hydrochloride RS

Glutaral Disinfectant Solution

DEFINITION
Glutaral Disinfectant Solution contains NLT 100.0% and NMT 110.0%, by weight, of the labeled amount of glutaraldehyde ($C_5H_8O_2$).

IDENTIFICATION
• **A. MELTING RANGE OR TEMPERATURE** ⟨741⟩
2,4-Dinitrophenylhydrazine reagent: To 0.8 g of 2,4-dinitrophenylhydrazine add 4 mL of sulfuric acid, then add 6 mL of water, dropwise, with swirling. When dissolution is essentially complete, add 20 mL of alcohol, and filter. Use the filtrate.
Sample: 5 mL
Analysis: To 20 mL of *2,4-Dinitrophenylhydrazine reagent* add the *Sample*, mix by swirling, and allow to stand for 5 min. Collect the precipitate on a filter, and rinse thoroughly with alcohol. Dissolve the precipitate in 20 mL of hot ethylene dichloride, filter, and cool the filtrate in an ice bath until crystallization occurs. Collect the precipitate on a filter. Redissolve the precipitate by refluxing with 30 mL of acetone, filter, and cool the filtrate in an ice bath until crystallization occurs. Collect the precipitate on a filter.
Acceptance criteria: The 2,4-dinitrophenylhydrazone so obtained melts between 185° and 195°, within a 3° range.

ASSAY
• **PROCEDURE**
Buffer: Dissolve 2.59 g of monobasic potassium phosphate and 6.77 g of anhydrous dibasic sodium phosphate in 500 mL of water in a 1000-mL volumetric flask. Dilute to volume.
Hydroxylamine hydrochloride solution: 70 µg/mL of hydroxylamine hydrochloride in *Buffer*
Standard solution: 50 µg/mL of glutaraldehyde in water from Glutaral Concentrate
Standard blank solution: Add 10.0 mL of *Standard solution* and 10.0 mL of *Buffer* to a 50-mL volumetric flask, and dilute with water to volume.
Sample solution: 50 µg/mL of glutaraldehyde in water from Disinfectant Solution
Sample blank solution: Add 10.0 mL of *Sample solution* and 10.0 mL of *Buffer* to a 50-mL volumetric flask, and dilute with water to volume.
Reagent blank solution: Add 10.0 mL of *Buffer* and 10.0 mL of *Hydroxylamine hydrochloride solution* to a 50-mL volumetric flask, and dilute with water to volume.
Instrumental conditions
Mode: UV
Analytical wavelength: 238 nm
Blank: *Reagent blank solution*
Analysis
Samples: *Standard solution, Standard blank solution, Sample solution,* and *Sample blank solution*
Transfer 10.0 mL each of the *Standard solution* and the *Sample solution* to separate 50-mL volumetric flasks. To each flask add 10.0 mL of *Hydroxylamine hydrochloride solution*, dilute with water to volume, mix, and allow each flask to stand for 25 min.
Concomitantly determine the absorbances of the *Standard solution, Sample solution, Standard blank solution,* and *Sample blank solution.*
Calculate the percentage of the labeled amount of glutaraldehyde ($C_5H_8O_2$) in the portion of Disinfectant Solution taken:

$$\text{Result} = [(A_U - A_{Ub})/(A_S - A_{Sb})] \times (C_S/C_U) \times 100$$

A_U = absorbance of the *Sample solution*
A_{Ub} = absorbance of the *Sample blank solution*
A_S = absorbance of the *Standard solution*
A_{Sb} = absorbance of the *Standard blank solution*
C_S = concentration of glutaraldehyde in the *Standard solution* (µg/mL)
C_U = concentration of the *Sample solution* (µg/mL)

Acceptance criteria: 100.0%–110.0%

SPECIFIC TESTS
• **pH** ⟨791⟩: 2.7–3.7

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and avoid exposure to excessive heat.

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Glycerin—*see Glycerin General Monographs*

Glyceryl Behenate

(Title for this monograph—not to change until December 1, 2019)
(Prior to December 1, 2019, the current practice of labeling the article of commerce with the name Glyceryl Behenate may be continued. Use of the name Glyceryl Dibehenate will be permitted as of December 1, 2014; however, the use of this name will not be mandatory until December 1, 2019. The 60-month extension will provide the time needed by manufacturers and users to make necessary changes.)

DEFINITION

Glyceryl Behenate is a mixture of diglycerides, mainly glyceryl dibehenate, together with variable quantities of monoglycerides and triglycerides. It contains NLT 15.0% and NMT 23.0% of monoglycerides, NLT 40.0% and NMT 60.0% of diglycerides, and NLT 21.0% and NMT 35.0% of triglycerides. It is obtained by esterification of glycerin with behenic (docosanoic) acid. The fatty acid may be of vegetable or synthetic origin.

IDENTIFICATION

- **A.** It meets the requirements for the content of diglycerides in the *Assay*.
- **B.** It meets the requirements for *Fats and Fixed Oils* ⟨401⟩, *Fatty Acid Composition* in *Specific Tests*.
- **C. MELTING RANGE OR TEMPERATURE,** *Class II* ⟨741⟩: 65°–77°

ASSAY

- **PROCEDURE**

Mobile phase: Tetrahydrofuran

System suitability solution: 40.0 mg/mL of USP Glyceryl Dibehenate RS in tetrahydrofuran. Heat gently at about 35°.

Sample solution: 40.0 mg/mL of Glyceryl Behenate in tetrahydrofuran. Heat gently at about 35°. Determine the total mass of solvent and substance.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 7.5-mm × 60-cm; 5-µm 100-Å packing L21

[NOTE—Two 7.5-mm × 30-cm L21 columns may be used in place of one 60-cm column, provided system suitability requirements are met.]

Temperatures

Detector: 35°

Column: 35°

Flow rate: 1.0 mL/min

Injection volume: 40 µL

[NOTE—Maintain the temperature of the sample at 35° to avoid precipitation.]

System suitability

Samples: *System suitability solution* and *Sample solution*

Suitability requirements: The retention times of the major peaks of the *Sample solution* correspond to those of the *System suitability solution*.

[NOTE—The relative retention times for triglycerides, diglycerides, monoglycerides, and glycerin are about 0.73, 0.76, 0.82, and 1.0, respectively.]

Analysis

Sample: *Sample solution*

Calculate the percentage of monoglycerides in the portion of Glyceryl Behenate taken:

$$\text{Result} = [(r_{U1}/r_S) \times (100 - A - B)] - D$$

r_{U1} = peak area of monoglycerides
r_S = sum of the peak areas of monoglycerides, diglycerides, and triglycerides
A = content of free glycerin from *Limit of Free Glycerin* (%)
B = content of water from the *Water Determination* test in *Specific Tests* (%)
D = content of free fatty acids (%) using the following formula:

$$D = AV \times [M_{r1}/(M_{r2} \times 1000)] \times 100$$

AV = acid value, determined in the *Acid Value* test in *Specific Tests*
M_{r1} = molecular weight of behenic acid, 340.58
M_{r2} = molecular weight of potassium hydroxide, 56.11
1000 = conversion of mg to g

Calculate the percentage of diglycerides in the portion of Glyceryl Behenate taken:

$$\text{Result} = (r_{U2}/r_S) \times (100 - A - B)$$

r_{U2} = peak area of diglycerides
r_S = sum of the peak areas of monoglycerides, diglycerides, and triglycerides
A = content of free glycerin from *Limit of Free Glycerin* (%)
B = content of water from the *Water Determination* test in *Specific Tests* (%)

Calculate the percentage of triglycerides in the portion of Glyceryl Behenate taken:

$$\text{Result} = (r_{U3}/r_S) \times (100 - A - B)$$

r_{U3} = peak area of triglycerides
r_S = sum of the peak areas of monoglycerides, diglycerides, and triglycerides
A = content of free glycerin from *Limit of Free Glycerin* (%)
B = content of water from the *Water Determination* test in *Specific Tests* (%)

Acceptance criteria

Monoglycerides: 15.0%–23.0%
Diglycerides: 40.0%–60.0%
Triglycerides: 21.0%–35.0%

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%
- **LIMIT OF FREE GLYCERIN**

Mobile phase, System suitability solution, and **Chromatographic system:** Proceed as directed in the *Assay*.

Standard stock solution: 4.0 mg/mL of USP Glycerin RS in tetrahydrofuran

Standard solutions: To four 15-mL flasks transfer 0.25, 0.5, 1.0, and 2.0 mL of the *Standard stock solution*, respectively, and add 5.0 mL of tetrahydrofuran. Determine the concentration for each (mg/g).

Sample solution: Transfer 0.2 g of Glyceryl Behenate to a 15-mL flask, and add 5.0 mL of tetrahydrofuran. Heat gently at about 35°. Determine the total mass of solvent and substance.

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System suitability
Samples: *System suitability solution* and *Sample solution*
Suitability requirements: The retention times of the major peaks of the *Sample solution* correspond to those of the *System suitability solution.*
[NOTE—The relative retention times for triglycerides, diglycerides, monoglycerides, and glycerin are about 0.73, 0.76, 0.82, and 1.0, respectively.]
Analysis
Samples: *Standard solutions* and *Sample solution*
Using the chromatograms from the *Standard solutions*, measure the responses for the glycerin peaks. Plot the concentration, in mg/g, of USP Glycerin RS in the *Standard solutions* versus the glycerin peak responses obtained. From the standard curve so obtained, determine the glycerin concentration, *C*, in mg/g, in the *Sample solution.*
Calculate the percentage of free glycerin in the portion of Glyceryl Behenate taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of glycerin determined from the calibration curve in the *Sample solution* (mg/g)
C_U = concentration of Glyceryl Behenate in the *Sample solution* (mg/g)

Acceptance criteria: NMT 1.0%

SPECIFIC TESTS
- **FATS AND FIXED OILS,** *Acid Value, Method I* ⟨401⟩
Sample: 1 g
Analysis: Suspend the *Sample* in 50 mL of a mixture of alcohol and toluene (1:1) with gentle heating. Continue as directed in *Fats and Fixed Oils* ⟨401⟩, *Acid Value,* beginning with "Add 1 mL of phenolphthalein TS".
Acceptance criteria: NMT 4
- **FATS AND FIXED OILS,** *Fatty Acid Composition* ⟨401⟩**:** Glyceryl Behenate exhibits the composition profile of fatty acids in *Table 1*, as determined in the chapter.

Table 1

Carbon-Chain Length	No. of Double Bonds	Percentage (%)
16	0	≤3.0
18	0	≤5.0
20	0	≤10.0
22	0	≥83.0
22	1	≤3.0
24	0	≤3.0

- **WATER DETERMINATION,** *Method Ia* ⟨921⟩**:** NMT 1.0% on 1.0-g sample. Use pyridine as the solvent.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers at a temperature not higher than 35°.
- **LABELING:** Label to indicate the origin of behenic acid and glycerin.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Glycerin RS
USP Glyceryl Dibehenate RS

Glyceryl Dibehenate

(Title for this monograph—not to become official until December 1, 2019)
(Prior to December 1, 2019, the current practice of labeling the article of commerce with the name Glyceryl Behenate may be continued. Use of the name Glyceryl Dibehenate will be permitted as of December 1, 2014; however, the use of this name will not be mandatory until December 1, 2019. The 60-month extension will provide the time needed by manufacturers and users to make necessary changes.)

DEFINITION
Glyceryl Dibehenate is a mixture of diglycerides, mainly glyceryl dibehenate, together with variable quantities of monoglycerides and triglycerides. It contains NLT 15.0% and NMT 23.0% of monoglycerides, NLT 40.0% and NMT 60.0% of diglycerides, and NLT 21.0% and NMT 35.0% of triglycerides. It is obtained by esterification of glycerin with behenic (docosanoic) acid. The fatty acid may be of vegetable or synthetic origin.

IDENTIFICATION
- **A.** It meets the requirements for the content of diglycerides in the *Assay.*
- **B.** It meets the requirements for *Fats and Fixed Oils* ⟨401⟩, *Fatty Acid Composition* in *Specific Tests.*
- **C. MELTING RANGE OR TEMPERATURE,** *Class II* ⟨741⟩**:** 65°–77°

ASSAY
- **PROCEDURE**
Mobile phase: Tetrahydrofuran
System suitability solution: 40.0 mg/mL of USP Glyceryl Dibehenate RS in tetrahydrofuran. Heat gently at about 35°.
Sample solution: 40.0 mg/mL of Glyceryl Dibehenate in tetrahydrofuran. Heat gently at about 35°. Determine the total mass of solvent and substance.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability.*)
Mode: LC
Detector: Refractive index
Column: 7.5-mm × 60-cm; 5-µm 100-Å packing L21
[NOTE—Two 7.5-mm × 30-cm L21 columns may be used in place of one 60-cm column, provided system suitability requirements are met.]
Temperatures
Detector: 35°
Column: 35°
Flow rate: 1.0 mL/min
Injection volume: 40 µL
[NOTE—Maintain the temperature of the sample at 35° to avoid precipitation.]
System suitability
Samples: *System suitability solution* and *Sample solution*
Suitability requirements: The retention times of the major peaks of the *Sample solution* correspond to those of the *System suitability solution.*
[NOTE—The relative retention times for triglycerides, diglycerides, monoglycerides, and glycerin are about 0.73, 0.76, 0.82, and 1.0, respectively.]
Analysis
Sample: *Sample solution*
Calculate the percentage of monoglycerides in the portion of Glyceryl Dibehenate taken:

$$\text{Result} = [(r_{U1}/r_S) \times (100 - A - B)] - D$$

r_{U1} = peak area of monoglycerides

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r_S = sum of the peak areas of monoglycerides, diglycerides, and triglycerides
A = content of free glycerin from *Limit of Free Glycerin* (%)
B = content of water from the *Water Determination* test in *Specific Tests* (%)
D = content of free fatty acids (%) using the following formula:

$$D = AV \times [M_{r1}/(M_{r2} \times 1000)] \times 100$$

AV = acid value, determined in the *Acid Value* test in *Specific Tests*
M_{r1} = molecular weight of behenic acid, 340.58
M_{r2} = molecular weight of potassium hydroxide, 56.11
1000 = conversion of mg to g

Calculate the percentage of diglycerides in the portion of Glyceryl Dibehenate taken:

$$\text{Result} = (r_{U2}/r_S) \times (100 - A - B)$$

r_{U2} = peak area of diglycerides
r_S = sum of the peak areas of monoglycerides, diglycerides, and triglycerides
A = content of free glycerin from *Limit of Free Glycerin* (%)
B = content of water from the *Water Determination* test in *Specific Tests* (%)

Calculate the percentage of triglycerides in the portion of Glyceryl Dibehenate taken:

$$\text{Result} = (r_{U3}/r_S) \times (100 - A - B)$$

r_{U3} = peak area of triglycerides
r_S = sum of the peak areas of monoglycerides, diglycerides, and triglycerides
A = content of free glycerin from *Limit of Free Glycerin* (%)
B = content of water from the *Water Determination* test in *Specific Tests* (%)

Acceptance criteria
Monoglycerides: 15.0%–23.0%
Diglycerides: 40.0%–60.0%
Triglycerides: 21.0%–35.0%

IMPURITIES

- **Residue on Ignition** ⟨281⟩: NMT 0.1%
- **Limit of Free Glycerin**

Mobile phase, System suitability solution, and **Chromatographic system:** Proceed as directed in the *Assay*.
Standard stock solution: 4.0 mg/mL of USP Glycerin RS in tetrahydrofuran
Standard solutions: To four 15-mL flasks transfer 0.25, 0.5, 1.0, and 2.0 mL of the *Standard stock solution*, respectively, and add 5.0 mL of tetrahydrofuran. Determine the concentration for each (mg/g).
Sample solution: Transfer 0.2 g of Glyceryl Dibehenate to a 15-mL flask, and add 5.0 mL of tetrahydrofuran. Heat gently at about 35°. Determine the total mass of solvent and substance.
System suitability
Samples: *System suitability solution* and *Sample solution*
Suitability requirements: The retention times of the major peaks of the *Sample solution* correspond to those of the *System suitability solution*.
[NOTE—The relative retention times for triglycerides, diglycerides, monoglycerides, and glycerin are about 0.73, 0.76, 0.82, and 1.0, respectively.]
Analysis
Samples: *Standard solutions* and *Sample solution*
Using the chromatograms from the *Standard solutions*, measure the responses for the glycerin peaks. Plot the concentration, in mg/g, of USP Glycerin RS in the *Standard solutions* versus the glycerin peak responses obtained. From the standard curve so obtained, determine the glycerin concentration, C, in mg/g, in the *Sample solution*.
Calculate the percentage of free glycerin in the portion of Glyceryl Dibehenate taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of glycerin determined from the calibration curve in the *Sample solution* (mg/g)
C_U = concentration of Glyceryl Dibehenate in the *Sample solution* (mg/g)

Acceptance criteria: NMT 1.0%

SPECIFIC TESTS

- **Fats and Fixed Oils,** *Acid Value, Method I* ⟨401⟩
Sample: 1 g
Analysis: Suspend the *Sample* in 50 mL of a mixture of alcohol and toluene (1:1) with gentle heating. Continue as directed in *Fats and Fixed Oils* ⟨401⟩, *Acid Value*, beginning with "Add 1 mL of phenolphthalein TS".
Acceptance criteria: NMT 4
- **Fats and Fixed Oils,** *Fatty Acid Composition* ⟨401⟩**:** Glyceryl Dibehenate exhibits the composition profile of fatty acids in *Table 1*, as determined in the chapter.

Table 1

Carbon-Chain Length	No. of Double Bonds	Percentage (%)
16	0	≤3.0
18	0	≤5.0
20	0	≤10.0
22	0	≥83.0
22	1	≤3.0
24	0	≤3.0

- **Water Determination,** *Method Ia* ⟨921⟩**:** NMT 1.0% on 1.0-g sample. Use pyridine as the solvent.

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in tight containers at a temperature not higher than 35°.
- **Labeling:** Label to indicate the origin of behenic acid and glycerin.
- **USP Reference Standards** ⟨11⟩
USP Glycerin RS
USP Glyceryl Dibehenate RS

Glyceryl Distearate

625.01

[1323-83-7].

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DEFINITION

Glyceryl Distearate is a mixture of diglycerides, mainly glyceryl distearate, together with variable quantities of monoglycerides and triglycerides. It contains NLT 8.0% and NMT 22.0% of monoglycerides, NLT 40.0% and NMT 60.0% of diglycerides, and NLT 25.0% and NMT 35.0% of triglycerides. It is obtained by partial glycerolysis of vegetable oil that consists mainly of triglycerides of palmitic or stearic acid or by esterification of glycerol with stearic acid. The fatty acids may be of vegetable or animal origin.

IDENTIFICATION

• **A.** It meets the requirements in *Specific Tests* for *Melting Range or Temperature, Class II* ⟨741⟩.

• **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩

Standard solution: 50 mg/mL of USP Glyceryl Distearate RS in methylene chloride

Sample solution: 50 mg/mL in methylene chloride

Chromatographic system

Developing solvent system: Ether and hexane (70:30)

Spray reagent: 0.1 mg/mL of rhodamine B in alcohol

Analysis: Proceed as directed in the chapter. Spray with the *Spray reagent*, and locate the spots on the plate by examination under UV light at a wavelength of 365 nm.

Acceptance criteria: The principal spot of the *Sample solution* corresponds in color, size, and R_F value to that of the *Standard solution*.

ASSAY

• **PROCEDURE**

Mobile phase: Tetrahydrofuran

Sample solution: 40 mg/mL of Glyceryl Distearate in tetrahydrofuran

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 7.5-mm × 60-cm; 5-µm 100-Å packing L21.

[NOTE—Two or three 7.5-mm × 30-cm L21 columns may be used in place of the one 60-cm column, provided that *System suitability* requirements are met. The column temperature may be lowered to ambient temperature, although working at 40° provides stable separation conditions and ensures better sample solubility.]

Temperatures

Detector: 40°

Column: 40°

Flow rate: 1 mL/min

Injection volume: 40 µL

System suitability

Sample: *Sample solution*

[NOTE—The relative retention times for triglycerides, diglycerides, monoglycerides, and glycerin are 0.75, 0.78, 0.84, and 1.0, respectively.]

Suitability requirements

Resolution: NLT 1.0 between the diglycerides and monoglycerides peaks

Relative standard deviation: NMT 2.0%, determined from the monoglycerides peak

Analysis

Sample: *Sample solution*

Calculate the percentage of monoglycerides, diglycerides, and triglycerides in the portion of Glyceryl Distearate taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = individual peak response for the monoglycerides, diglycerides, and triglycerides, as appropriate

r_T = sum of the responses for all of the glyceride peaks

Acceptance criteria

Monoglycerides: 8.0%–22.0%

Diglycerides: 40.0%–60.0%

Triglycerides: 25.0%–35.0%

IMPURITIES

• **LIMIT OF NICKEL**

[CAUTION—When using closed high-pressure digestion vessels and microwave laboratory equipment, be familiar with the safety and operating instructions given by the manufacturer.]

Magnesium nitrate solution: 5 mg/mL of magnesium nitrate

Standard stock solution: Transfer 5.0 mL of nickel standard solution TS to a 10-mL volumetric flask. Add 0.5 mL of nitric acid and 1.0 mL of 30% hydrogen peroxide, and dilute with water to volume.

Standard solutions: Into four identical 25-mL volumetric flasks, each containing 6 mL of nitric acid, transfer 20, 50, 100, and 150 µL, respectively, of the *Standard stock solution*, and dilute with water to volume. These solutions contain 4, 10, 20, and 30 ng/mL of nickel, respectively.

Sample solution: Transfer 0.1 g of Glyceryl Distearate into a suitable high-pressure resistant digestion vessel (fluoropolymer or quartz), and add 6.0 mL of nitric acid and 2.0 mL of 30% hydrogen peroxide. Place the closed vessel in a laboratory microwave oven, and digest using an appropriate program (for example, 250 W for 10 min; 600 W for 5 min; 400 W for 5 min; and 250 W for 7 min). Allow the digestion vessel to cool before opening. Quantitatively transfer the contents to a 25-mL volumetric flask, and dilute with water to volume.

Blank: Add 6.0 mL of nitric acid and 2.0 mL of 30% hydrogen peroxide to a high-pressure-resistant digestion vessel, and proceed as directed for the *Sample solution*.

Zero solution: Transfer 6.0 mL of nitric acid into a 25-mL volumetric flask. Dilute with water to volume.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry equipped with a pyrolytically coated tube with a platform

Analytical wavelength: 232 nm

Lamp: Nickel hollow-cathode

Graphite furnace parameters: Maintain the drying temperature of the furnace at 100° for 10 s after a 10-s ramp, the ashing temperature at 1400° for 10 s after a 20-s ramp, and the atomization temperature at 2500° for 5 s. Use the *Zero solution* to set the instrument to zero.

Analysis

Samples: Into seven separate 25-mL flasks, each containing 5.0 mL of *Magnesium nitrate solution*, transfer respectively 10.0 mL of each of the following: the *Sample solution*, the *Blank*, the four *Standard solutions*, and the *Zero solution*.

Concomitantly determine the absorbances of the *Samples* at least three times each. Record the average of the steady readings for each of the solutions. [NOTE—If necessary, dilute the *Sample solution* with the *Zero solution* to obtain a reading within the calibrated absorbance range.]

Acceptance criteria: NMT 1 µg/g

• **LIMIT OF FREE GLYCERIN**

Mobile phase, Sample solution, Chromatographic system, and System suitability: Proceed as directed in the *Assay*.

Standard solutions: 0.2, 0.4, 1.0, and 2.0 mg/mL of glycerin in tetrahydrofuran

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Analysis
Samples: *Standard solutions* and *Sample solution*
Plot the glycerin peak responses versus the concentration of glycerin in the *Standard solutions*. From the standard curve so obtained, determine the glycerin concentration in the *Sample solution*.
Calculate the percentage of free glycerin in the portion of Glyceryl Distearate taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of glycerin in the *Sample solution* from the standard curve (mg/mL)
C_U = concentration of Glyceryl Distearate in the *Sample solution* (mg/mL)

Acceptance criteria: NMT 1.0%

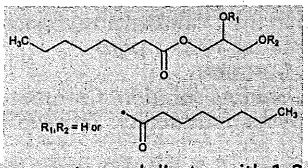
SPECIFIC TESTS
- **MELTING RANGE OR TEMPERATURE,** *Class II* ⟨741⟩**:** 50°–70°
- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩
Sample: 1.0 g
Analysis: Use a mixture of alcohol and toluene (1:1, v/v) as the solvent and with gentle heating.
Acceptance criteria: NMT 6.0
- **FATS AND FIXED OILS,** *Fatty Acid Composition* ⟨401⟩**:** The fatty acid fraction of it contains NLT 40.0% of stearic acid, and the sum of the contents of palmitic and stearic acids is NLT 90.0%.
- **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩**:** NMT 3.0
- **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩
Sample: 2.0 g
Analysis: Carry out the titration with heating.
Acceptance criteria: 165–195
- **WATER DETERMINATION,** *Method I* ⟨921⟩
Analysis: Use pyridine in place of methanol in the titration vessel.
Acceptance criteria: NMT 1.0%
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 0.1%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements are specified.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Glyceryl Distearate RS

Add the following:

▲Glyceryl Mono and Dicaprylate

(Title for this monograph—not to become official until May 1, 2025.) (Prior to May 1, 2025, the current practice of labeling the article of commerce with the name Glyceryl Monocaprylate Type I may be continued. Use of the name Glyceryl Mono and Dicaprylate will be permitted as of May 1, 2020; however, the use of this name will not be mandatory until May 1, 2025. The 60-month extension will provide the time needed by manufacturers and users to make necessary changes.)



Octanoic acid, monoester and diester with 1,2,3-propanetriol; Caprylic acid, monoglyceride and diglyceride; Glyceryl mono and dioctanoate; Mono and dicapryl glycerides [11140-04-8].

DEFINITION
Glyceryl Mono and Dicaprylate is a mixture of glyceryl monoester and diesters, mainly mono- and di-*O*-octanoylglycerol, containing variable quantities of triesters of fatty acids composed predominately of caprylic acid. The requirements for monoester, diester, and triester content are: Monoesters: 40.0%–75.0%; Diesters: 20.0%–50.0%; and Triesters: NMT 15.0%.

IDENTIFICATION
- **A. FATTY ACID COMPOSITION**
Boron trifluoride methanol solution: 140 mg/mL of boron trifluoride in methanol
Saturated sodium chloride solution: Mix 1 part sodium chloride with 2 parts water, shake from time to time, and allow to stand. Before use, decant the solution from any undissolved substance and filter, if necessary.
Standard solution 1: 1.0 mg/mL of USP Methyl Caproate RS, 1.0 mg/mL of USP Methyl Caprylate RS, and 2.0 mg/mL of USP Methyl Caprate RS in *n*-heptane
Standard solution 2: 0.1 mg/mL of USP Methyl Caproate RS, 0.1 mg/mL of USP Methyl Caprylate RS, and 0.2 mg/mL of USP Methyl Caprate RS in *n*-heptane, diluted from *Standard solution 1*
Standard solution 3: Prepare an ester mixture either by mixing a commercially available ester mixture with methyl caprylate and methyl caprate or by using USP Methyl Caproate RS, USP Methyl Caprylate RS, USP Methyl Caprate RS, USP Methyl Laurate RS, and USP Methyl Laurate RS. Dissolve a quantity of the prepared ester mixture containing methyl caproate, methyl caprylate, methyl caprate, methyl laurate, and methyl myristate in *n*-heptane to make a solution of about 9.0 mg/mL for methyl caprylate, 1.0 mg/mL for methyl caprate, and 0.05–0.1 mg/mL for each of the other methyl esters.
Sample solution: Transfer 100 mg of Glyceryl Mono and Dicaprylate to a 25-mL conical flask fitted with a suitable water-cooled reflux condenser and a magnetic stir bar. Add 2 mL of a 20-mg/mL solution of sodium hydroxide in methanol, mix, and reflux for about 30 min. Add 2 mL of *Boron trifluoride methanol solution* through the condenser, and reflux for about 30 min. Add 4 mL of *n*-heptane through the condenser and reflux for 5 min. Cool, remove the condenser, add about 10 mL of *Saturated sodium chloride solution*, shake, add a quantity of *Saturated sodium chloride solution* to bring the upper layer up to the neck of the flask, and allow the layers to separate. Collect 2 mL of the *n*-heptane layer (upper layer), wash with three quantities each of 2 mL of water, and dry the *n*-heptane phase over anhydrous sodium sulfate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame Ionization
Column: 0.53-mm × 30-m capillary; bonded with a 1.0-µm layer of phase G16
Temperatures
Injection port: 250°
Detector: 250°
Column: See *Table 1*.

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Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
50	20	180	—
180	9	240	12

Carrier gas: Helium
Flow rate: 10 mL/min
Injection volume: 2 µL
Injection type: Split injection; split ratio, 4:1
System suitability
Samples: *Standard solution 1* and *Standard solution 2*
[NOTE—The relative retention times for methyl caproate, methyl caprylate, and methyl caprate are about 0.7, 1.0, and 1.3, respectively.]
Suitability requirements
Resolution: NLT 4.0 between the methyl caprylate and methyl caprate peaks, *Standard solution 1*
Signal-to-noise ratio: NLT 10 for the methyl caproate peak, *Standard solution 2*
Analysis
Samples: *Standard solution 3* and *Sample solution*
Measure the peak areas for the methyl esters of the fatty acids. Disregard any peaks with an area less than 0.05% of the total area and any peak due to the solvent.
[NOTE—The relative retention times for several methyl esters are summarized in *Table 2*.]

Table 2

Carbon-Chain Length	Number of Double Bonds	Relative Retention Times
6	0	0.7
8	0	1.0
10	0	1.3
12	0	1.6
14	0	1.9

Take the main component in *Standard solution 3* as a reference component and calculate the calibration factor, F_{FA}, for each fatty acid methyl ester:

$$F_{FA} = (F_{MC} \times P_{FA1} \times A_{MC})/(P_{MC} \times A_{FA1})$$

F_{MC} = factor for the main component, 1
P_{FA1} = percentage by weight of the fatty acid methyl ester in *Standard solution 3*
A_{MC} = peak area of the main component from *Standard solution 3*
P_{MC} = percentage by weight of the main component in *Standard solution 3*
A_{FA1} = peak area of the fatty acid methyl ester from *Standard solution 3*

Calculate the percentage of the fatty acid methyl ester by weight in the portion of Glyceryl Mono and Dicaprylate taken:

$$\text{Result} = [(A_{FA2} \times F_{FA})/A_T] \times 100$$

A_{FA2} = peak area of the fatty acid methyl ester from the *Sample solution*
A_T = sum of the peak areas of the fatty acid methyl esters from the *Sample solution*

Acceptance criteria: Glyceryl Mono and Dicaprylate exhibits the composition profile of fatty acids shown in *Table 3*.

Table 3

Carbon-Chain Length	Number of Double Bonds	Percentage (%, w/w)
6	0	≤1.0
8	0	≥90.0
10	0	≤10.0
12	0	≤1.0
14	0	≤0.5

• **B. GLYCERIDE CONTENT:** It meets the requirements of the test for *Content of Monoglycerides, Diglycerides, and Triglycerides* in the *Assay*.
• **C. FATS AND FIXED OILS** ⟨401⟩, *Procedures, Saponification Value*: 266–300

ASSAY

• **CONTENT OF MONOGLYCERIDES, DIGLYCERIDES, AND TRIGLYCERIDES**
System suitability solution: 20 mg/mL each of 1-monooctanoyl-rac-glycerol and 1-monodecanoyl-rac-glycerol in tetrahydrofuran
Standard solution: 50 mg/mL of USP Glyceryl Monocaprylate RS in tetrahydrofuran
Sample solution: 50 mg/mL of Glyceryl Mono and Dicaprylate in tetrahydrofuran
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.32-mm × 10-m; bonded with a 0.1-µm layer of phase G27
Temperatures
Injection port: 350°
Detector: 370°
Column: See *Table 4*.

Table 4

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
60	—	60	3
60	8	340	12

Carrier gas: Helium
Flow rate: 2.3 mL/min
Injection volume: 1 µL
Injection type: Split injection; split ratio, 50:1
System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The typical relative retention times for monoglycerides, diglycerides, and triglycerides are 1.0–1.2, 1.5–1.9, and 2.0–2.3, respectively, *Standard solution*.]
Suitability requirements
Resolution: NLT 5.0 between the 1-monooctanoyl-rac-glycerol and

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1-monodecanoyl-rac-glycerol peaks, *System suitability solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Based on the chromatogram from the *Standard solution*, identify the peaks due to monoglycerides, diglycerides, and triglycerides in the *Sample solution*.

For the calculation of the contents of monoglycerides, diglycerides, and triglycerides, disregard the peaks with retention times less than those of the monoglycerides, which are due to impurities of the solvent and to the free fatty acids.

Calculate the percentage content of free fatty acids (C_A):

$$C_A = [(I_A \times F \times M_{r1})/M_{r2}] \times 100$$

I_A = acid value for Glyceryl Mono and Dicaprylate, determined from the test for *Acid Value*
F = conversion factor, 10^{-3} g/mg
M_{r1} = molecular weight of caprylic acid, 144.21 g/mol
M_{r2} = molecular weight of potassium hydroxide, 56.11 g/mol

Calculate the percentage of monoglycerides, diglycerides, or triglycerides in the portion of Glyceryl Mono and Dicaprylate taken:

$$\text{Result} = (r_U/r_T) \times [(100 - C_A - C_W - C_G)/100] \times 100$$

r_U = peak response of the monoglycerides, diglycerides, or triglycerides from the *Sample solution*
r_T = sum of all the glyceride peak responses from the *Sample solution*
C_A = percentage of free fatty acids, determined above
C_W = percentage of water, determined from the test for *Water Determination*
C_G = percentage of free glycerin, determined from the test for *Limit of Free Glycerol*

Acceptance criteria

Monoesters: 40.0%–75.0%
Diesters: 20.0%–50.0%
Triesters: NMT 15.0%

IMPURITIES

• **TOTAL ASH**

Sample: 1.0 g

Analysis: Heat a silica or platinum crucible to redness for 30 min, allow to cool in a desiccator, and weigh. Evenly distribute the *Sample* in the crucible. Dry at 100°–105° for 1 h and ignite to constant weight in a muffle furnace at 600 ± 25°, allowing the crucible to cool in a desiccator after each ignition. Flames should not be produced at any time during the procedure. If after prolonged ignition the ash still contains black particles, take up with hot water, pass through an ashless filter paper, and ignite the residue and the filter paper. Combine the filtrate with the ash, carefully evaporate to dryness, and ignite to constant mass.

Acceptance criteria: NMT 0.5%

• **LIMIT OF FREE GLYCEROL**

Sample: 1.2 g

Periodic acetic acid solution: Dissolve 0.446 g of sodium periodate in 2.5 mL of a 25% (v/v) solution of sulfuric acid, and dilute with glacial acetic acid to 100.0 mL.

Potassium iodide solution: 75 mg/mL of potassium iodide

Blank: 25 mL of methylene chloride

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Residual titration

Titrant: 0.1 M sodium thiosulfate VS

Endpoint detection: Visual

Analysis: Dissolve the *Sample* in 25 mL of methylene chloride. Heat to about 50° and allow to cool. Add 100 mL of water. Shake and add 25 mL of *Periodic acetic acid solution*. Shake again and allow to stand for 30 min. Add 40 mL of *Potassium iodide solution*, and allow to stand for 1 min. Add 1 mL of starch TS and titrate the liberated iodine with *Titrant* until the aqueous phase becomes colorless. Perform a blank determination.

Calculate the percentage of free glycerol in the portion of Glyceryl Mono and Dicaprylate taken:

$$\text{Result} = \{[(V_B - V_S) \times N \times E \times F]/W\} \times 100$$

V_B = volume of *Titrant* consumed in the *Blank* titration (mL)
V_S = volume of *Titrant* consumed in the *Sample* titration (mL)
N = actual normality of the *Titrant* (mEq/mL)
E = equivalent factor for glycerol, 23 mg/mEq
F = conversion factor, 10^{-3} g/mg
W = weight of Glyceryl Mono and Dicaprylate taken for the titration (g)

Acceptance criteria: NMT 3.0%

SPECIFIC TESTS

• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Acid Value*: NMT 3.0
• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Peroxide Value*: NMT 1
• **WATER DETERMINATION** ⟨921⟩, *Method I, Method Ia*: NMT 1.0%

ADDITIONAL REQUIREMENTS

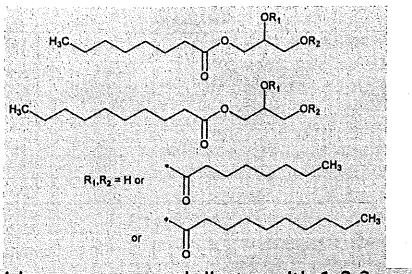
• **PACKAGING AND STORAGE:** Preserve in tight containers, and store at room temperature. Protect from moisture.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Glyceryl Monocaprylate RS
USP Methyl Caprate RS
USP Methyl Caproate RS
USP Methyl Caprylate RS
USP Methyl Laurate RS
USP Methyl Myristate RS ▲(NF 1-May-2020)

Add the following:

▲Glyceryl Mono and Dicaprylocaprate

(Title for this monograph—not to become official until May 1, 2025) (Prior to May 1, 2025, the current practice of labeling the article of commerce with the name Glyceryl Monocaprylocaprate Type I may be continued. Use of the name Glyceryl Mono and Dicaprylocaprate will be permitted as of May 1, 2020; however, the use of this name will not be mandatory until May 1, 2025. The 60-month extension will provide the time needed by manufacturers and users to make necessary changes.)

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Octanoic acid, monoester and diester with 1,2,3-propanetriol and decanoic acid, monoester and diester with 1,2,3-propanetriol;
Caprylic acid monoglyceride and diglyceride and capric acid monoglyceride and diglyceride;
Glyceryl mono and dioctanoate and glyceryl mono and didecanoate;
Glycerides, $C_{8\text{-}10}$ monoglycerides and diglycerides;
$C_{8\text{-}10}$ mono- and diglycerides [85536-07-8].

DEFINITION

Glyceryl Mono and Dicaprylocaprate is a mixture of glyceryl monoesters and diesters, mainly mono- and di-*O*-octanoylglycerol and mono- and di-*O*-decanoylglycerol, containing variable quantities of triesters of fatty acids composed predominately of caprylic acid and capric acid. The requirements for monoester, diester, and triester content are: Monoesters: 40.0%–75.0%; Diesters: 20.0%–50.0%; and Triesters: NMT 15.0%.

IDENTIFICATION

• A. Fatty Acid Composition

Boron trifluoride methanol solution: 140 mg/mL of boron trifluoride in methanol

Saturated sodium chloride solution: Mix 1 part sodium chloride with 2 parts water, shake from time to time, and allow to stand. Before use, decant the solution from any undissolved substance and filter, if necessary.

Standard solution 1: 1.0 mg/mL of USP Methyl Caproate RS, 1.0 mg/mL of USP Methyl Caprylate RS, and 2.0 mg/mL of USP Methyl Caprate RS in *n*-heptane

Standard solution 2: 0.1 mg/mL of USP Methyl Caproate RS, 0.1 mg/mL of USP Methyl Caprylate RS, and 0.2 mg/mL of USP Methyl Caprate RS in *n*-heptane, diluted from *Standard solution 1*

Standard solution 3: Prepare an ester mixture either by mixing a commercially available ester mixture with methyl caprylate and methyl caprate, or by using USP Methyl Caproate RS, USP Methyl Caprylate RS, USP Methyl Caprate RS, USP Methyl Laurate RS, and USP Methyl Myristate RS. Dissolve a quantity of the prepared ester mixture containing methyl caproate, methyl caprylate, methyl caprate, methyl laurate, and methyl myristate in *n*-heptane to make a solution of about 5.0–9.0 mg/mL for methyl caprylate, 1.0–5.0 mg/mL for methyl caprate, and 0.1–0.3 mg/mL for each of the other methyl esters.

Sample solution: Transfer 100 mg of Glyceryl Mono and Dicaprylocaprate to a 25-mL conical flask fitted with a suitable water-cooled reflux condenser and a magnetic stir bar. Add 2 mL of a 20-mg/mL solution of sodium hydroxide in methanol, mix, and reflux for about 30 min. Add 2 mL of *Boron trifluoride methanol solution* through the condenser and reflux for about 30 min. Add 4 mL of *n*-heptane through the condenser and reflux for 5 min. Cool, remove the condenser, add about 10 mL of *Saturated sodium chloride solution*, shake, add a quantity of *Saturated sodium chloride solution* to bring the upper layer up to the neck of the flask, and allow the layers to separate. Collect 2 mL of the *n*-heptane layer (upper layer), wash with three quantities each of 2 mL of water, and dry the *n*-heptane phase over anhydrous sodium sulfate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.53-mm × 30-m capillary; bonded with a 1.0-µm layer of phase G16
Temperatures
Injection port: 250°
Detector: 250°
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
50	20	180	—
180	9	240	12

Carrier gas: Helium
Flow rate: 10 mL/min
Injection volume: 2 µL
Injection type: Split injection; split ratio, 4:1

System suitability
Samples: *Standard solution 1* and *Standard solution 2*
[Note—The relative retention times for methyl caproate, methyl caprylate, and methyl caprate are about 0.7, 1.0, and 1.3, respectively.]
Suitability requirements
Resolution: NLT 4.0 between methyl caprylate and methyl caprate peaks, *Standard solution 1*
Signal-to-noise ratio: NLT 10 for the methyl caproate peak, *Standard solution 2*

Analysis
Samples: *Standard solution 3* and *Sample solution*
Measure the peak areas for the methyl esters of the fatty acids. Disregard any peaks with an area less than 0.05% of the total area and any peak due to the solvent.
[Note—The relative retention times for several methyl esters are summarized in *Table 2*.]

Table 2

Carbon-Chain Length	Number of Double Bonds	Relative Retention Times
6	0	0.7
8	0	1.0
10	0	1.3
12	0	1.6
14	0	1.9

Take the main component in *Standard solution 3* as a reference component and calculate the calibration factor, F_{FA}, for each fatty acid methyl ester:

$$F_{FA} = (F_{MC} \times P_{FA1} \times A_{MC})/(P_{MC} \times A_{FA1})$$

F_{MC} = factor for the main component, 1

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P_{FAT} = percentage by weight of the fatty acid methyl ester in *Standard solution 3*
A_{MC} = peak area of the main component from *Standard solution 3*
P_{MC} = percentage by weight of the main component in *Standard solution 3*
A_{FAT} = peak area of the fatty acid methyl ester from *Standard solution 3*

Calculate the percentage of the fatty acid methyl ester by weight in the portion of Glyceryl Mono and Dicaprylocaprate taken:

$$\text{Result} = [(A_{FA2} \times F_{FA})/A_T] \times 100$$

A_{FA2} = peak area of the fatty acid methyl ester from the *Sample solution*
A_T = sum of the peak areas of the fatty acid methyl esters from the *Sample solution*

Acceptance criteria: Glyceryl Mono and Dicaprylocaprate exhibits the composition profile of fatty acids shown in *Table 3*.

Table 3

Carbon-Chain Length	Number of Double Bonds	Percentage (%, w/w)
6	0	≤3.0
8	0	50.0–90.0
10	0	10.0–50.0
12	0	≤3.0
14	0	≤1.0

• **B. Glyceride Content:** It meets the requirements of the test for *Content of Monoglycerides, Diglycerides, and Triglycerides* in the *Assay*.
• **C. Fats and Fixed Oils** ⟨401⟩, *Procedures, Saponification Value*: 250–280

ASSAY

• **Content of Monoglycerides, Diglycerides, and Triglycerides**

System suitability solution: 20 mg/mL each of 1-monooctanoyl-rac-glycerol and 1-monodecanoyl-rac-glycerol in tetrahydrofuran
Standard solution: 50 mg/mL of USP Glyceryl Mono and Dicaprylocaprate RS in tetrahydrofuran
Sample solution: 50 mg/mL of Glyceryl Mono and Dicaprylocaprate in tetrahydrofuran
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.32-mm × 10-m; bonded with a 0.1-µm layer of phase G27
Temperatures
Injection port: 350°
Detector: 370°
Column: See *Table 4*.

Table 4

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
60	—	60	3
60	8	340	12

Carrier gas: Helium
Flow rate: 2.3 mL/min
Injection volume: 1 µL
Injection type: Split injection; split ratio, 50:1
System suitability
Samples: *System suitability solution* and *Standard solution*
[Note—The typical relative retention times for monoglycerides, diglycerides, and triglycerides are 1.0–1.2, 1.5–1.9, and 2.0–2.3, respectively, *Standard solution*.]
Suitability requirements
Resolution: NLT 5.0 between the 1-monooctanoyl-rac-glycerol and 1-monodecanoyl-rac-glycerol peaks, *System suitability solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Based on the chromatogram from the *Standard solution*, identify the peaks due to monoglycerides, diglycerides, and triglycerides in the *Sample solution*.
For the calculation of the contents of monoglycerides, diglycerides, and triglycerides, disregard the peaks with retention times less than those of the monoglycerides, which are due to impurities of the solvent and to the free fatty acids.
Calculate the percentage content of free fatty acids (C_A):

$$C_A = [(I_A \times F \times M_{r1})/M_{r2}] \times 100$$

I_A = acid value for Glyceryl Mono and Dicaprylocaprate, determined from the test for *Acid Value*
F = conversion factor, 10^{-3} g/mg
M_{r1} = molecular weight of caprylic acid, 144.21 g/mol
M_{r2} = molecular weight of potassium hydroxide, 56.11 g/mol

Calculate the percentage of monoglycerides, diglycerides, or triglycerides in the portion of Glyceryl Mono and Dicaprylocaprate taken:

$$\text{Result} = (r_U/r_T) \times [(100 - C_A - C_W - C_G)/100] \times 100$$

r_U = peak response of the monoglycerides, diglycerides, or triglycerides from the *Sample solution*
r_T = sum of all the glyceride peak responses from the *Sample solution*
C_A = percentage of free fatty acids, determined above
C_W = percentage of water, determined from the test for *Water Determination*
C_G = percentage of free glycerin, determined from the test for *Limit of Free Glycerol*

Acceptance criteria
Monoesters: 40.0%–75.0%
Diesters: 20.0%–50.0%
Triesters: NMT 15.0%

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IMPURITIES

• **TOTAL ASH**

Sample: 1.0 g

Analysis: Heat a silica or platinum crucible to redness for 30 min, allow to cool in a desiccator, and weigh. Evenly distribute the *Sample* in the crucible. Dry at 100°–105° for 1 h and ignite to constant weight in a muffle furnace at 600 ± 25°, allowing the crucible to cool in a desiccator after each ignition. Flames should not be produced at any time during the procedure. If after prolonged ignition the ash still contains black particles, take up with hot water, pass through an ashless filter paper, and ignite the residue and the filter paper. Combine the filtrate with the ash, carefully evaporate to dryness, and ignite to constant mass.

Acceptance criteria: NMT 0.5%

• **LIMIT OF FREE GLYCEROL**

Sample: 1.2 g

Periodic acetic acid solution: Dissolve 0.446 g of sodium periodate in 2.5 mL of a 25% (v/v) solution of sulfuric acid, and dilute with glacial acetic acid to 100.0 mL.

Potassium iodide solution: 75 mg/mL of potassium iodide

Blank: 25 mL of methylene chloride

Titrimetric system

(See *Titrimetry* ⟨541⟩)

Mode: Residual titration

Titrant: 0.1 M sodium thiosulfate VS

Endpoint detection: Visual

Analysis: Dissolve the *Sample* in 25 mL of methylene chloride. Heat to about 50° and allow to cool. Add 100 mL of water. Shake and add 25 mL of *Periodic acetic acid solution*. Shake again and allow to stand for 30 min. Add 40 mL of *Potassium iodide solution* and allow to stand for 1 min. Add 1 mL of starch TS and titrate the liberated iodine with *Titrant* until the aqueous phase becomes colorless. Perform a *Blank* determination.

Calculate the percentage of free glycerol in the portion of Glyceryl Mono and Dicaprylocaprate taken:

$$\text{Result} = \{[(V_B - V_S) \times N \times E \times F]/W\} \times 100$$

V_B = volume of *Titrant* consumed in the *Blank* titration (mL)

V_S = volume of *Titrant* consumed in the *Sample* titration (mL)

N = actual normality of the *Titrant* (mEq/mL)

E = equivalent factor for glycerol, 23 mg/mEq

F = conversion factor, 10^{-3} g/mg

W = weight of Glyceryl Mono and Dicaprylocaprate taken for the titration (g)

Acceptance criteria: NMT 3.0%

SPECIFIC TESTS

• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Acid Value*: NMT 3.0

• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Peroxide Value*: NMT 1

• **WATER DETERMINATION** ⟨921⟩, *Method I, Method Ia*: NMT 1.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers, and store at room temperature. Protect from moisture.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Glyceryl Mono and Dicaprylocaprate RS

[NOTE—May also be labeled as USP Glyceryl Monocaprylocaprate RS (Type I) until May 1, 2025.]

USP Methyl Caprate RS

USP Methyl Caproate RS

USP Methyl Caprylate RS

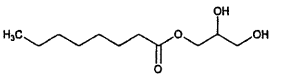
USP Methyl Laurate RS

USP Methyl Myristate RS ▲(NF 1-May-2020)

Glyceryl Monocaprylate

Add the following:

▲Any article currently titled Glyceryl Monocaprylate Type I will be officially titled Glyceryl Mono and Dicaprylate after May 1, 2025. Any article currently titled Glyceryl Monocaprylate Type II will be officially titled Glyceryl Monocaprylate after May 1, 2025.▲ (NF 1-May-2020)



$C_{11}H_{22}O_4$ 218.29

Octanoic acid, monoester with 1,2,3-propanetriol;
Caprylic acid monoglyceride;
Glyceryl monooctanoate;
Monocapryl glyceride [26402-26-6].

DEFINITION

Change to read:

Glyceryl Monocaprylate is a mixture of glyceryl monoesters, mainly mono-*O*-octanoylglycerol, containing variable quantities of diesters and triesters of fatty acids composed predominately of caprylic acid. ▲The requirements for monoester, diester, and triester content are: Monoesters: NLT 75.1%; Diesters: NMT 24.9%; and Triesters: NMT 5.0%.▲ (NF 1-May-2020)

IDENTIFICATION

• **A. FATTY ACID COMPOSITION**

Boron trifluoride methanol solution: 140 mg/mL of boron trifluoride in methanol

Saturated sodium chloride solution: Mix 1 part sodium chloride with 2 parts water, shake from time to time, and allow to stand. Before use, decant the solution from any undissolved substance and filter, if necessary.

Standard solution 1: 1.0 mg/mL of USP Methyl Caproate RS, 1.0 mg/mL of USP Methyl Caprylate RS, and 2.0 mg/mL of USP Methyl Caprate RS in *n*-heptane

Standard solution 2: 0.1 mg/mL of USP Methyl Caproate RS, 0.1 mg/mL of USP Methyl Caprylate RS, and 0.2 mg/mL of USP Methyl Caprate RS in *n*-heptane, diluted from *Standard solution 1*

Standard solution 3: Prepare an ester mixture either by mixing a commercially available ester mixture with methyl caprylate and methyl caprate or by using USP Methyl Caproate RS, USP Methyl Caprylate RS, USP Methyl Caprate RS, USP Methyl Laurate RS, and USP Methyl Myristate RS. Dissolve a quantity of the prepared ester mixture containing methyl caproate, methyl caprylate, methyl caprate, methyl laurate, and methyl myristate in *n*-heptane to make a solution of about 9.0 mg/mL for methyl caprylate, 1.0 mg/mL for methyl caprate, and 0.05–0.1 mg/mL for each of the other methyl esters.

Sample solution: Transfer 100 mg of Glyceryl Monocaprylate to a 25-mL conical flask fitted with a suitable water-cooled reflux condenser and a magnetic stir bar. Add 2 mL of a 20-mg/mL solution of sodium hydroxide in methanol, mix, and reflux for about 30 min. Add 2 mL

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of *Boron trifluoride methanol solution* through the condenser, and reflux for about 30 min. Add 4 mL of *n*-heptane through the condenser and reflux for 5 min. Cool, remove the condenser, add about 10 mL of *Saturated sodium chloride solution*, shake, add a quantity of *Saturated sodium chloride solution* to bring the upper layer up to the neck of the flask, and allow the layers to separate. Collect 2 mL of the *n*-heptane layer (upper layer), wash with three quantities each of 2 mL of water, and dry the *n*-heptane phase over anhydrous sodium sulfate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.53-mm × 30-m capillary; bonded with a 1.0-µm layer of phase G16
Temperatures
Injection port: 250°
Detector: 250°
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
50	20	180	—
180	9	240	12

Carrier gas: Helium
Flow rate: 10 mL/min
Injection volume: 2 µL
Injection type: Split injection; split ratio, 4:1
System suitability
Samples: *Standard solution 1* and *Standard solution 2*
[NOTE—The relative retention times for methyl caproate, methyl caprylate, and methyl caprate are about 0.7, 1.0, and 1.3, respectively.]
Suitability requirements
Resolution: NLT 4.0 between the methyl caprylate and methyl caprate peaks, *Standard solution 1*
Signal-to-noise ratio: NLT 10 for the methyl caproate peak, *Standard solution 2*
Analysis
Samples: *Standard solution 3* and *Sample solution*
Measure the peak areas for the methyl esters of the fatty acids. Disregard any peaks with an area less than 0.05% of the total area and any peak due to the solvent.
[NOTE—The relative retention times for several methyl esters are summarized in *Table 2*.]

Table 2

Carbon-Chain Length	Number of Double Bonds	Relative Retention Time
6	0	0.7
8	0	1.0
10	0	1.3
12	0	1.6
14	0	1.9

Take the main component in *Standard solution 3* as a reference component and calculate the calibration factor, F_{FA}, for each fatty acid methyl ester:

$$F_{FA} = (F_{MC} \times P_{FA1} \times A_{MC})/(P_{MC} \times A_{FA1})$$

F_{MC} = factor for the main component, 1
P_{FA1} = percentage by weight of the fatty acid methyl ester in *Standard solution 3*
A_{MC} = peak area of the main component from *Standard solution 3*
P_{MC} = percentage by weight of the main component in *Standard solution 3*
A_{FA1} = peak area of the fatty acid methyl ester from *Standard solution 3*

Calculate the percentage of the fatty acid methyl ester by weight in the portion of Glyceryl Monocaprylate taken:

$$\text{Result} = [(A_{FA2} \times F_{FA})/A_T] \times 100$$

A_{FA2} = peak area of the fatty acid methyl ester from the *Sample solution*
A_T = sum of the peak areas of the fatty acid methyl esters from the *Sample solution*

Acceptance criteria: Glyceryl Monocaprylate exhibits the composition profile of fatty acids shown in *Table 3*.

Table 3

Carbon-Chain Length	Number of Double Bonds	Percentage (%, w/w)
6	0	≤1.0
8	0	≥90.0
10	0	≤10.0
12	0	≤1.0
14	0	≤0.5

• **B. GLYCERIDE CONTENT:** It meets the requirements of the test for *Content of Monoglycerides, Diglycerides, and Triglycerides* in the *Assay*.

Change to read:

• **C. FATS AND FIXED OILS** ⟨401⟩, *Procedures, Saponification Value*: ▲245–272▲ (NF 1-May-2020)

ASSAY

Change to read:

• **CONTENT OF MONOGLYCERIDES, DIGLYCERIDES, AND TRIGLYCERIDES**
System suitability solution: 20 mg/mL each of 1-monooctanoyl-rac-glycerol and 1-monodecanoyl-rac-glycerol in tetrahydrofuran
Standard solution: 50 mg/mL of USP Glyceryl Monocaprylate RS in tetrahydrofuran
Sample solution: 50 mg/mL of Glyceryl Monocaprylate in tetrahydrofuran
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.32-mm × 10-m; bonded with a 0.1-µm layer of phase G27
Temperatures
Injection port: 350°
Detector: 370°
Column: See *Table 4*.

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Table 4

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
60	—	60	3
60	8	340	12

Carrier gas: Helium
Flow rate: 2.3 mL/min
Injection volume: 1 µL
Injection type: Split injection; split ratio, 50:1

System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The typical relative retention times for monoglycerides, diglycerides, and triglycerides are 1.0–1.2, 1.5–1.9, and 2.0–2.3, respectively, *Standard solution*.]
Suitability requirements
Resolution: NLT 5.0 between the 1-monooctanoyl-rac-glycerol and 1-monodecanoyl-rac-glycerol peaks, *System suitability solution*

Analysis
Samples: *Standard solution* and *Sample solution*
Based on the chromatogram from the *Standard solution*, identify the peaks due to monoglycerides, diglycerides, and triglycerides in the *Sample solution*.
For the calculation of the contents of monoglycerides, diglycerides, and triglycerides, disregard the peaks with retention times less than those of the monoglycerides, which are due to impurities of the solvent and to the free fatty acids.
Calculate the percentage content of free fatty acids (C_A):

$$C_A = [(I_A \times F \times M_{r1})/M_{r2}] \times 100$$

I_A = acid value for Glyceryl Monocaprylate, determined from the test for *Acid Value*
F = conversion factor, 10^{-3} g/mg
M_{r1} = molecular weight of caprylic acid, 144.21 g/mol
M_{r2} = molecular weight of potassium hydroxide, 56.11 g/mol

Calculate the percentage of monoglycerides, diglycerides, or triglycerides in the portion of Glyceryl Monocaprylate taken:

$$\text{Result} = (r_U/r_T) \times [(100 - C_A - C_W - C_G)/100] \times 100$$

r_U = peak response of the monoglycerides, diglycerides, or triglycerides from the *Sample solution*
r_T = sum of all the glyceride peak responses from the *Sample solution*
C_A = percentage of free fatty acids, determined above
C_W = percentage of water, determined from the test for *Water Determination*
C_G = percentage of free glycerin, determined from the test for *Limit of Free Glycerol*

Acceptance criteria
▲**Monoesters:** NLT 75.1%
Diesters: NMT 24.9%
Triesters: NMT 5.0%▲ (NF 1-May-2020)

IMPURITIES

• **TOTAL ASH**
Sample: 1.0 g
Analysis: Heat a silica or platinum crucible to redness for 30 min, allow to cool in a desiccator, and weigh. Evenly distribute the *Sample* in the crucible. Dry at 100°–105° for 1 h and ignite to constant weight in a muffle furnace at 600 ± 25°, allowing the crucible to cool in a desiccator after each ignition. Flames should not be produced at any time during the procedure. If after prolonged ignition the ash still contains black particles, take up with hot water, pass through an ashless filter paper, and ignite the residue and the filter paper. Combine the filtrate with the ash, carefully evaporate to dryness, and ignite to constant mass.
Acceptance criteria: NMT 0.5%

• **LIMIT OF FREE GLYCEROL**
Sample: 1.2 g
Periodic acetic acid solution: Dissolve 0.446 g of sodium periodate in 2.5 mL of a 25% (v/v) solution of sulfuric acid, and dilute with glacial acetic acid to 100.0 mL.
Potassium iodide solution: 75 mg/mL of potassium iodide
Blank: 25 mL of methylene chloride
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Residual titration
Titrant: 0.1 M sodium thiosulfate VS
Endpoint detection: Visual
Analysis: Dissolve the *Sample* in 25 mL of methylene chloride. Heat to about 50° and allow to cool. Add 100 mL of water. Shake and add 25 mL of *Periodic acetic acid solution*. Shake again and allow to stand for 30 min. Add 40 mL of *Potassium iodide solution*, and allow to stand for 1 min. Add 1 mL of starch TS and titrate the liberated iodine with *Titrant* until the aqueous phase becomes colorless. Perform a blank determination.
Calculate the percentage of free glycerol in the portion of Glyceryl Monocaprylate taken:

$$\text{Result} = \{[(V_B - V_S) \times N \times E \times F]/W\} \times 100$$

V_B = volume of *Titrant* consumed in the *Blank* titration (mL)
V_S = volume of *Titrant* consumed in the *Sample* titration (mL)
N = actual normality of the *Titrant* (mEq/mL)
E = equivalent factor for glycerol, 23 mg/mEq
F = conversion factor, 10^{-3} g/mg
W = weight of Glyceryl Monocaprylate taken for the titration (g)

Acceptance criteria: NMT 3.0%

SPECIFIC TESTS

• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Acid Value*: NMT 3.0
• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Peroxide Value*: NMT 1
• **WATER DETERMINATION** ⟨921⟩, *Method I, Method Ia*: NMT 1.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers, and store at room temperature. Protect from moisture.

Delete the following:

▲• **LABELING:** Label it to indicate the type (Type I or Type II).▲ (NF 1-May-2020)
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Glyceryl Monocaprylate RS
USP Methyl Caprate RS
USP Methyl Caproate RS
USP Methyl Caprylate RS

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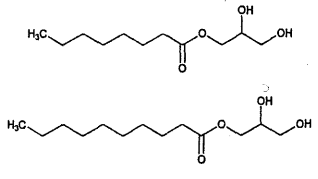
USP Methyl Laurate RS
USP Methyl Myristate RS

Glyceryl Monocaprylocaprate

Add the following:

▲Any article currently titled Glyceryl Monocaprylocaprate Type I will be officially titled Glyceryl Mono and Dicaprylocaprate after May 1, 2025. Any article currently titled Glyceryl Monocaprylocaprate Type II will be officially titled Glyceryl Monocaprylocaprate after May 1, 2025.▲ (NF 1-May-2020)

Change to read:



$C_{11}H_{22}O_4$

$C_{13}H_{26}O_4$

Octanoic acid, monoester with 1,2,3-propanetriol and decanoic acid, monoester with 1,2,3-propanetriol;
Caprylic acid monoglyceride and capric acid monoglyceride;
Glyceryl monooctanoate and glyceryl monodecanoate;
▲Monoglycerides, $C_{8\text{-}10}$
$C_{8\text{-}10}$ monoglycerides [91052-46-9▲ (NF 1-May-2020)].

DEFINITION

Change to read:

Glyceryl Monocaprylocaprate is a mixture of glyceryl monoesters, mainly mono-*O*-octanoylglycerol and mono-*O*-decanoylglycerol, containing variable quantities of diesters and triesters of fatty acids composed predominately of caprylic acid and capric acid. ▲The requirements for monoester, diester, and triester content are: Monoesters: NLT 75.1%; Diesters: NMT 24.9%; and Triesters: NMT 5.0%.▲ (NF 1-May-2020)

IDENTIFICATION

• **A. Fatty Acid Composition**

Boron trifluoride methanol solution: 140 mg/mL of boron trifluoride in methanol

Saturated sodium chloride solution: Mix 1 part sodium chloride with 2 parts water, shake from time to time, and allow to stand. Before use, decant the solution from any undissolved substance and filter, if necessary.

Standard solution 1: 1.0 mg/mL of USP Methyl Caproate RS, 1.0 mg/mL of USP Methyl Caprylate RS, and 2.0 mg/mL of USP Methyl Caprate RS in *n*-heptane

Standard solution 2: 0.1 mg/mL of USP Methyl Caproate RS, 0.1 mg/mL of USP Methyl Caprylate RS, and 0.2 mg/mL of USP Methyl Caprate RS in *n*-heptane, diluted from *Standard solution 1*

Standard solution 3: Prepare an ester mixture either by mixing a commercially available ester mixture with methyl caprylate and methyl caprate, or by using USP Methyl Caproate RS, USP Methyl Caprylate RS, USP Methyl Caprate RS, USP Methyl Laurate RS, and USP Methyl Myristate RS. Dissolve a quantity of the prepared ester mixture containing methyl caproate, methyl caprylate, methyl caprate, methyl laurate, and methyl myristate in *n*-heptane to make a solution of about 5.0–9.0 mg/mL for methyl caprylate, 1.0–5.0 mg/mL for methyl caprate, and 0.1–0.3 mg/mL for each of the other methyl esters.

Sample solution: Transfer 100 mg of Glyceryl Monocaprylocaprate to a 25-mL conical flask fitted with a suitable water-cooled reflux condenser and a magnetic stir bar. Add 2 mL of a 20-mg/mL solution of sodium hydroxide in methanol, mix, and reflux for about 30 min. Add 2 mL of *Boron trifluoride methanol solution* through the condenser and reflux for about 30 min. Add 4 mL of *n*-heptane through the condenser and reflux for 5 min. Cool, remove the condenser, add about 10 mL of *Saturated sodium chloride solution*, shake, add a quantity of *Saturated sodium chloride solution* to bring the upper layer up to the neck of the flask, and allow the layers to separate. Collect 2 mL of the *n*-heptane layer (upper layer), wash with three quantities each of 2 mL of water, and dry the *n*-heptane phase over anhydrous sodium sulfate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.53-mm × 30-m capillary; bonded with a 1.0-µm layer of phase G16
Temperatures
Injection port: 250°
Detector: 250°
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
50	20	180	—
180	9	240	12

Carrier gas: Helium
Flow rate: 10 mL/min
Injection volume: 2 µL
Injection type: Split injection; split ratio, 4:1

System suitability
Samples: *Standard solution 1* and *Standard solution 2*
[NOTE—The relative retention times for methyl caproate, methyl caprylate, and methyl caprate are about 0.7, 1.0, and 1.3, respectively.]
Suitability requirements
Resolution: NLT 4.0 between the methyl caprylate and methyl caprate peaks, *Standard solution 1*
Signal-to-noise ratio: NLT 10 for the methyl caproate peak, *Standard solution 2*

Analysis
Samples: *Standard solution 3* and *Sample solution*
Measure the peak areas for the methyl esters of the fatty acids. Disregard any peaks with an area less than 0.05% of the total area and any peak due to the solvent.
[NOTE—The relative retention times for several methyl esters are summarized in *Table 2*.]

Table 2

Carbon-Chain Length	Number of Double Bonds	Relative Retention Times
6	0	0.7
8	0	1.0

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Table 2 *(continued)*

Carbon-Chain Length	Number of Double Bonds	Relative Retention Times
10	0	1.3
12	0	1.6
14	0	1.9

Take the main component in *Standard solution 3* as a reference component and calculate the calibration factor, F_{FA}, for each fatty acid methyl ester:

$$F_{FA} = (F_{MC} \times P_{FA1} \times A_{MC})/(P_{MC} \times A_{FA1})$$

F_{MC} = factor for the main component, 1
P_{FA1} = percentage by weight of the fatty acid methyl ester in *Standard solution 3*
A_{MC} = peak area of the main component from *Standard solution 3*
P_{MC} = percentage by weight of the main component in *Standard solution 3*
A_{FA1} = peak area of the fatty acid methyl ester from *Standard solution 3*

Calculate the percentage of the fatty acid methyl ester by weight in the portion of Glyceryl Monocaprylocaprate taken:

$$\text{Result} = [(A_{FA2} \times F_{FA})/A_T] \times 100$$

A_{FA2} = peak area of the fatty acid methyl ester from the *Sample solution*
A_T = sum of the peak areas of the fatty acid methyl esters from the *Sample solution*

Acceptance criteria: Glyceryl Monocaprylocaprate exhibits the composition profile of fatty acids shown in *Table 3*.

Table 3

Carbon-Chain Length	Number of Double Bonds	Percentage (%, w/w)
6	0	≤3.0
8	0	50.0–90.0
10	0	10.0–50.0
12	0	≤3.0
14	0	≤1.0

- **B. GLYCERIDE CONTENT:** It meets the requirements of the test for *Content of Monoglycerides, Diglycerides, and Triglycerides* in the *Assay*.

Change to read:

- **C. FATS AND FIXED OILS** ⟨401⟩, *Procedures, Saponification Value*:
▲225–255▲ (NF 1-May-2020)

ASSAY

Change to read:

- **CONTENT OF MONOGLYCERIDES, DIGLYCERIDES, AND TRIGLYCERIDES**

System suitability solution: 20 mg/mL each of 1-monooctanoyl-rac-glycerol and 1-monodecanoyl-rac-glycerol in tetrahydrofuran

Standard solution: 50 mg/mL of ▲USP Glyceryl Mono and Dicaprylocaprate RS▲ (NF 1-May-2020) in tetrahydrofuran

Sample solution: 50 mg/mL of Glyceryl Monocaprylocaprate in tetrahydrofuran

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.32-mm × 10-m; bonded with a 0.1-µm layer of phase G27
Temperatures
Injection port: 350°
Detector: 370°
Column: See *Table 4*.

Table 4

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
60	—	60	3
60	8	340	12

Carrier gas: Helium
Flow rate: 2.3 mL/min
Injection volume: 1 µL
Injection type: Split injection; split ratio, 50:1

System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The typical relative retention times for monoglycerides, diglycerides, and triglycerides are 1.0–1.2, 1.5–1.9, and 2.0–2.3, respectively, *Standard solution*.]
Suitability requirements
Resolution: NLT 5.0 between the 1-monooctanoyl-rac-glycerol and 1-monodecanoyl-rac-glycerol peaks, *System suitability solution*

Analysis
Samples: *Standard solution* and *Sample solution*
Based on the chromatogram from the *Standard solution*, identify the peaks due to monoglycerides, diglycerides, and triglycerides in the *Sample solution*.
For the calculation of the contents of monoglycerides, diglycerides, and triglycerides, disregard the peaks with retention times less than those of the monoglycerides, which are due to impurities of the solvent and to the free fatty acids.
Calculate the percentage content of free fatty acids (C_A):

$$C_A = [(I_A \times F \times M_{r1})/M_{r2}] \times 100$$

I_A = acid value for Glyceryl Monocaprylocaprate, determined from the test for *Acid Value*
F = conversion factor, 10^{-3} g/mg
M_{r1} = molecular weight of caprylic acid, 144.21 g/mol
M_{r2} = molecular weight of potassium hydroxide, 56.11 g/mol

Calculate the percentage of monoglycerides, diglycerides, or triglycerides in the portion of Glyceryl Monocaprylocaprate taken:

$$\text{Result} = (r_U/r_T) \times [(100 - C_A - C_W - C_G)/100] \times 100$$

r_U = peak responses of the monoglycerides, diglycerides, or triglycerides from the *Sample solution*

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r_T = sum of all the glyceride peak responses from the *Sample solution*
C_A = percentage of free fatty acids, determined above
C_W = percentage of water, determined from the test for *Water Determination*
C_G = percentage of free glycerin, determined from the test for *Limit of Free Glycerol*

Acceptance criteria
▲**Monoesters:** NLT 75.1%
Diesters: NMT 24.9%
Triesters: NMT 5.0%▲ (NF 1-May-2020)

IMPURITIES
• **TOTAL ASH**
Sample: 1.0 g
Analysis: Heat a silica or platinum crucible to redness for 30 min, allow to cool in a desiccator, and weigh. Evenly distribute the *Sample* in the crucible. Dry at 100°–105° for 1 h and ignite to constant weight in a muffle furnace at 600 ± 25°, allowing the crucible to cool in a desiccator after each ignition. Flames should not be produced at any time during the procedure. If after prolonged ignition the ash still contains black particles, take up with hot water, pass through an ashless filter paper, and ignite the residue and the filter paper. Combine the filtrate with the ash, carefully evaporate to dryness, and ignite to constant mass.
Acceptance criteria: NMT 0.5%
• **LIMIT OF FREE GLYCEROL**
Sample: 1.2 g
Periodic acetic acid solution: Dissolve 0.446 g of sodium periodate in 2.5 mL of a 25% (v/v) solution of sulfuric acid, and dilute with glacial acetic acid to 100.0 mL.
Potassium iodide solution: 75 mg/mL of potassium iodide
Blank: 25 mL of methylene chloride
Titrimetric system
(See *Titrimetry* ⟨541⟩)
Mode: Residual titration
Titrant: 0.1 M sodium thiosulfate VS
Endpoint detection: Visual
Analysis: Dissolve the *Sample* in 25 mL of methylene chloride. Heat to about 50° and allow to cool. Add 100 mL of water. Shake and add 25 mL of *Periodic acetic acid solution*. Shake again and allow to stand for 30 min. Add 40 mL of *Potassium iodide solution* and allow to stand for 1 min. Add 1 mL of starch TS and titrate the liberated iodine with *Titrant* until the aqueous phase becomes colorless. Perform a blank determination.
Calculate the percentage of free glycerol in the portion of Glyceryl Monocaprylocaprate taken:

$$\text{Result} = \{[(V_B - V_S) \times N \times E \times F]/W\} \times 100$$

V_B = *Titrant* volume consumed in the *Blank* titration (mL)
V_S = *Titrant* volume consumed in the *Sample* titration (mL)
N = actual normality of the *Titrant* (mEq/mL)
E = equivalent factor for glycerol, 23 mg/mEq
F = conversion factor, 10^{-3} g/mg
W = weight of Glyceryl Monocaprylocaprate taken for the titration (g)

Acceptance criteria: NMT 3.0%

SPECIFIC TESTS
• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Acid Value*: NMT 3.0
• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Peroxide Value*: NMT 1
• **WATER DETERMINATION** ⟨921⟩, *Method I, Method Ia*: NMT 1.0%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight containers, and store at room temperature. Protect from moisture.

Delete the following:

▲• **LABELING:** Label it to indicate the type (Type I or Type II).▲ (NF 1-May-2020)

Change to read:

• **USP REFERENCE STANDARDS** ⟨11⟩
▲USP Glyceryl Mono and Dicaprylocaprate RS
[NOTE—May also be labeled as USP Glyceryl Monocaprylocaprate RS (Type I) until May 1, 2025.▲ (NF 1-May-2020)]
USP Methyl Caprate RS
USP Methyl Caproate RS
USP Methyl Caprylate RS
USP Methyl Laurate RS
USP Methyl Myristate RS

Glyceryl Monolinoleate

354.5

[26545-74-4].

DEFINITION
Glyceryl Monolinoleate is a mixture of monoglycerides, mainly glyceryl monooleate and glyceryl monolinoleate, together with variable quantities of diglycerides and triglycerides. It is obtained by partial glycerolysis of vegetable oil that consists mainly of triglycerides of linoleic acid. It contains NLT 32.0% and NMT 52.0% of monoglycerides, NLT 40.0% and NMT 55.0% of diglycerides, and NLT 5.0% and NMT 20.0% of triglycerides. A suitable antioxidant may be added.

IDENTIFICATION
• **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩
Standard solution: 50 mg/mL of USP Glyceryl Monolinoleate RS in methylene chloride
Sample solution: 50 mg/mL in methylene chloride
Chromatographic system
(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
Developing solvent system: Ether and hexane (70:30)
Spray reagent: 0.1 mg/mL of rhodamine B in alcohol
Analysis: Proceed as directed in the chapter. Spray with the *Spray reagent*, and locate the spots on the plate by examination under UV light at a wavelength of 365 nm.
Acceptance criteria: The principal spot from the *Sample solution* corresponds in color, size, and R_F value to that of the *Standard solution*.
• **B.** It meets the requirements in *Specific Tests* for *Fats and Fixed Oils, Iodine Value* ⟨401⟩.
• **C.** It meets the requirements in *Specific Tests* for *Fats and Fixed Oils, Fatty Acid Composition* ⟨401⟩.

ASSAY
• **PROCEDURE**
Mobile phase: Tetrahydrofuran
Sample solution: 40 mg/mL of Glyceryl Monolinoleate in tetrahydrofuran
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)

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Mode: LC
Detector: Refractive index
Column: 7.5-mm × 60-cm; 5-µm 100-Å packing L21
[NOTE—Two or three 7.5-mm × 30-cm L21 columns may be used in place of the one 60-cm column, provided that system suitability requirements are met. The column temperature may be lowered to ambient temperature, although working at 40° provides stable separation conditions and ensures better sample solubility.]
Temperatures
Detector: 40°
Column: 40°
Flow rate: 1 mL/min
Injection volume: 40 µL
System suitability
Sample: *Sample solution*
[NOTE—The relative retention times for triglycerides, diglycerides, monoglycerides, and glycerin are about 0.76, 0.80, 0.86, and 1.0, respectively.]
Suitability requirements
Resolution: NLT 1.0 between the diglycerides and monoglycerides peaks
Relative standard deviation: NMT 2.0%, determined from the monoglycerides peak
Analysis
Sample: *Sample solution*
Calculate the percentage of monoglycerides, diglycerides, and triglycerides in the portion of Glyceryl Monolinoleate taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = individual peak response for the monoglycerides, diglycerides, and triglycerides, as appropriate
r_T = sum of the responses for all of the glyceride peaks

Acceptance criteria
Monoglycerides: 32.0%–52.0%
Diglycerides: 40.0%–55.0%
Triglycerides: 5.0%–20.0%

IMPURITIES

• **LIMIT OF FREE GLYCERIN**
Mobile phase, Sample solution, Chromatographic system, and **System suitability:** Proceed as directed in the *Assay*.
Standard solutions: 0.4, 1.0, 2.0, and 4.0 mg/mL of glycerin in tetrahydrofuran
Analysis
Samples: *Sample solution* and *Standard solutions*
Plot the glycerin peak responses obtained versus the concentration of glycerin in the *Standard solutions*. From the standard curve so obtained, determine the glycerin concentration in the *Sample solution*.
Calculate the percentage of free glycerin in the portion of Glyceryl Monolinoleate taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of glycerin in the *Sample solution* from the standard curve (mg/mL)
C_U = concentration of Glyceryl Monolinoleate in the *Sample solution* (mg/mL)

Acceptance criteria: NMT 6.0%

SPECIFIC TESTS

• **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩
Sample: 1.0 g
Acceptance criteria: NMT 6.0
• **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩**:** 100–140
• **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩
Sample: 2.0 g
Acceptance criteria: NMT 12.0
• **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩
Sample: 2.0 g
Acceptance criteria: 160–180
• **FATS AND FIXED OILS,** *Fatty Acid Composition* ⟨401⟩**:** See *Table 1*.

Table 1

Carbon-Chain Length	No. of Double Bonds	Percentage (%)
16	0	4.0–20.0
18	0	NMT 6.0
18	1	10.0–35.0
18	2	NLT 50.0
18	3	NMT 2.0
20	0	NMT 1.0
20	1	NMT 1.0

• **WATER DETERMINATION,** *Method I* ⟨921⟩
Analysis: Use a mixture of methanol and methylene chloride (1:1) in place of methanol in the titration vessel.
Acceptance criteria: NMT 1.0%
• **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 0.1%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.
• **LABELING:** The labeling indicates the name and the concentration of any added antioxidant.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Glyceryl Monolinoleate RS

Glyceryl Monooleate

Oleic acid, 2,3-dihydroxypropyl ester, (±); (*RS*)-1-Glyceryl oleate 356.54
[25496-72-4].

DEFINITION

Glyceryl Monooleate is a mixture of monoglycerides, mainly glyceryl monooleate, together with variable quantities of diglycerides and triglycerides. It is obtained by partial glycerolysis of vegetable oil that consists mainly of triglycerides of oleic acid, or by esterification of glycerol with oleic acid of vegetable or animal origin. It is defined by the nominal content of monoglycerides. The assay requirements differ as set forth in the accompanying table. A suitable antioxidant may be added.

	Nominal Content of Monoglycerides (%)		
	40	60	90
Monoglycerides	32.0–52.0	55.0–65.0	90.0–101.0
Diglycerides	30.0–50.0	15.0–35.0	<10.0
Triglycerides	5.0–20.0	2.0–10.0	<2.0

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IDENTIFICATION

• **A. Thin-Layer Chromatographic Identification Test** ⟨201⟩

Standard solution: 50 mg/mL of USP Glyceryl Monooleate 40% RS or USP Glyceryl Monooleate 90% RS in methylene chloride

Sample solution: 50 mg/mL of Glyceryl Monooleate in methylene chloride

Application volume: 10 µL

Developing solvent system: Ether and hexane (7:3)

Spray reagent: 0.1 mg/mL of rhodamine B in alcohol

Analysis: Proceed as directed in the chapter. Spray with the *Spray reagent*, and locate the spots on the plate by examination under UV light at a wavelength of 365 nm.

Acceptance criteria: The principal spot of the *Sample solution* corresponds in color, size, and R_F value to that of the *Standard solution*.

• **B.** It meets the requirements in *Specific Tests* for *Fats and Fixed Oils, Iodine Value* ⟨401⟩.

ASSAY

• **Procedure**

Mobile phase: Tetrahydrofuran

Sample solution: 40 mg/mL of Glyceryl Monooleate in tetrahydrofuran

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 7.5-mm × 60-cm; 5-µm 100-Å packing L21

[Note—Two or three 7.5-mm × 30-cm L21 columns may be used in place of the one 60-cm column, provided that the system suitability requirements are met. The column temperature may be lowered to ambient temperature, although working at 40° provides stable separation conditions and ensures better sample solubility.]

Temperature

Column: 40°

Detector: 40°

Flow rate: 1 mL/min

Injection size: 40 µL

System suitability

Sample: *Sample solution*

[Note—The relative retention times for triglycerides, diglycerides, monoglycerides, and glycerin are about 0.76, 0.79, 0.85, and 1.0, respectively.]

Suitability requirements

Resolution: NLT 1.0 between the diglycerides and monoglycerides

Relative standard deviation: NMT 2.0%, determined from the monoglycerides peak

Analysis

Sample: *Sample solution*

Calculate the percentage of monoglycerides, diglycerides, and triglycerides in the portion of Glyceryl Monooleate taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = individual peak responses for the monoglycerides, diglycerides, and triglycerides, as appropriate

r_T = sum of all the glyceride peak responses

Acceptance criteria: See the table in the *Definition*.

IMPURITIES

• **Limit of Free Glycerin**

Mobile phase, Sample solution, and **Chromatographic system:** Proceed as directed in the *Assay*.

Standard solutions: Prepare four solutions by dissolving glycerin in tetrahydrofuran, and diluting each with tetrahydrofuran as necessary, to obtain solutions having known concentrations of 0.4, 1.0, 2.0, and 4.0 mg/mL.

Standard curve

Samples: *Standard solutions*

Plot: Record the chromatograms, and measure the responses for the glycerin peaks. Plot the glycerin peak responses obtained versus the concentration, in mg/mL, of glycerin in the *Standard solutions*.

Analysis

Sample: *Sample solution*

From the *Standard curve*, determine the glycerin concentration, in mg/mL, in the *Sample solution*.

Calculate the percentage of free glycerin in the portion of Glyceryl Monooleate taken:

$$\text{Result} = (C_S/C_U) \times 100$$

C_S = concentration of glycerin in the *Sample solution* from the *Standard curve* (mg/mL)

C_U = concentration of Glyceryl Monooleate in the *Sample solution* (mg/mL)

Acceptance criteria: NMT 6.0%

SPECIFIC TESTS

• **Fats and Fixed Oils,** *Acid Value* ⟨401⟩: NMT 6.0, determined on 1.0 g

• **Fats and Fixed Oils,** *Iodine Value* ⟨401⟩: 65.0–95.0

• **Fats and Fixed Oils,** *Peroxide Value* ⟨401⟩: NMT 12.0, determined on 2.0 g

• **Fats and Fixed Oils,** *Saponification Value* ⟨401⟩: 150–175, determined on 2.0 g

• **Fats and Fixed Oils,** *Fatty Acid Composition* ⟨401⟩: Glyceryl Monooleate exhibits the following composition profile of fatty acids (see *Table 1*), determined as directed in the chapter.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
16	0	NMT 12.0
18	0	NMT 6.0
18	1	NLT 60.0
18	2	NMT 35.0
18	3	NMT 2.0
20	0	NMT 2.0
20	1	NMT 2.0

• **Water Determination,** *Method I* ⟨921⟩: NMT 1.0%, using a mixture of methanol and methylene chloride (1:1) in place of methanol in the titration vessel

• **Articles of Botanical Origin,** *Total Ash* ⟨561⟩: NMT 0.1%

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Preserve in tight containers. No storage requirements specified.

• **Labeling:** The labeling indicates the nominal content of monoglycerides and the name and the concentration of any added antioxidant.

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• **USP REFERENCE STANDARDS** ⟨11⟩
USP Glyceryl Monooleate 40% RS
USP Glyceryl Monooleate 90% RS

Glyceryl Monostearate

Octadecanoic acid, monoester with 1,2,3-propanetriol; Monostearin [31566-31-1].

DEFINITION
Glyceryl Monostearate contains NLT 90.0% of monoglycerides of saturated fatty acids, chiefly glyceryl monostearate ($C_{21}H_{42}O_4$) and glyceryl monopalmitate ($C_{19}H_{38}O_4$). It may contain a suitable antioxidant.

ASSAY
• **PROCEDURE**
Mobile phase: Tetrahydrofuran
Sample solution: 8 mg/mL of Glyceryl Monostearate in tetrahydrofuran
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Refractive index
Column: 7.5-mm × 60-cm; 5-µm 100-Å packing L21
Temperature: Column and detector temperatures are maintained at 40°.
[NOTE—Two or three 7.5-mm × 30-cm L21 columns may be used in place of the one 60-cm column, provided that system suitability requirements are met. The column temperature may be lowered to ambient temperature, although working at 40° provides stable separation conditions and ensures better sample solubility.]
Flow rate: 1 mL/min
Injection size: 40 µL
System suitability
Sample: *Sample solution*
[NOTE—The relative retention times for triglycerides, diglycerides, monoglycerides, and glycerin are 0.77, 0.81, 0.86, and 1.0, respectively.]
Suitability requirements
Relative standard deviation: NMT 2.0%, determined from the monoglycerides peak
Analysis
Sample: *Sample solution*
Calculate the percentage of monoglycerides in the portion of Glyceryl Monostearate taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of the monoglycerides
r_T = sum of all the glyceride peak responses

Acceptance criteria: NLT 90.0% of monoglycerides of saturated fatty acids, chiefly $C_{21}H_{42}O_4$ and $C_{19}H_{38}O_4$

IMPURITIES
INORGANIC IMPURITIES
• **Residue on Ignition** ⟨281⟩: NMT 0.5%
ORGANIC IMPURITIES
• **Procedure: Limit of Free Glycerin**
Propionating reagent: Pyridine and propionic anhydride (1:2)
Internal standard solution: 0.2 mg/mL of tributyrin in chloroform
Standard solution: Transfer 15 mg of glycerin and 50 mg of tributyrin to a glass-stoppered, 25-mL conical flask. Add 3 mL of *Propionating reagent*, and heat at 75° for 30 min. Volatilize the reagents with the aid of a stream of nitrogen at room temperature, and add 12 mL of chloroform. Dilute 1 mL of this mixture with chloroform to 20 mL.
Sample solution: Transfer 50 mg of Glyceryl Monostearate to a glass-stoppered, 25-mL conical flask. Add 5 mL of *Internal standard solution* by pipet, and mix to dissolve. Immerse the flask in a water bath, maintained at a temperature between 45° and 50°, and volatilize the chloroform with the aid of a stream of nitrogen. Add 3 mL of *Propionating reagent*, and heat at 75° for 30 min. Volatilize the reagents with the aid of a stream of nitrogen at room temperature, and add 5 mL of chloroform.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization (under typical conditions)
Column: 4-mm × 2.4-m borosilicate glass, packed with 2% liquid phase G16, on 80- to 100-mesh support S1A
Temperature
Injector: 300°
Detector: 310°
Column: The column is maintained isothermally at a temperature between 190° and 200°.
Carrier gas: Helium
Flow rate: 70 mL/min
System suitability
Sample: *Standard solution* (6–10 injections)
Suitability requirements
Resolution: NLT 4.0 between derivatized glycerin and tributyrin
Relative standard deviation: NMT 2.0% of the ratio of their peak areas
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the response factor, F:

$$F = (A_D/A_S) \times (W_S/W_D)$$

A_D = peak area of tributyrin from the *Standard solution*
A_S = peak area of tripropionin from the *Standard solution*
W_S = weight of glycerin in the *Standard solution* (mg)
W_D = weight of tributyrin in the *Standard solution* (mg)

Calculate the percentage of glycerin in the portion of Glyceryl Monostearate taken:

$$\text{Result} = (A_U/A_S) \times (W_D/W_U) \times F \times 100$$

A_U = peak area of tripropionin in the *Sample solution*
A_S = peak area of tributyrin in the *Sample solution*
W_D = weight of tributyrin in 5 mL of *Internal standard solution* (mg)
W_U = weight of Glyceryl Monostearate in the *Sample solution* (mg)

Acceptance criteria: NMT 1.2%

SPECIFIC TESTS
• **MELTING RANGE OR TEMPERATURE,** *Class I* ⟨741⟩: Does not melt below 55°
• **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩: NMT 6
• **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩: 290–330
• **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩: NMT 3
• **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩: 150–165

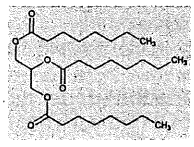
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ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** Label it to indicate the name and quantity of any added antioxidant.

Add the following:

▲Glyceryl Tricaprylate



$C_{27}H_{50}O_6$ 470.68

Octanoic acid, 1,1′,1″-(1,2,3-propanetriyl) ester;
Octanoic acid, 1,2,3-propanetriyl ester;
1,3-Di(octanoyloxy)propan-2-yl octanoate;
Glyceryl trioctanoate;
Tricapryloylglycerol [538-23-8].

DEFINITION

Glyceryl Tricaprylate contains NLT 90.0% of triglycerides of saturated fatty acids, chiefly glyceryl tricaprylate ($C_{27}H_{50}O_6$).

IDENTIFICATION

- **A. FATTY ACID COMPOSITION**

Boron trifluoride methanol solution: 140 mg/mL of boron trifluoride in methanol

Saturated sodium chloride solution: Mix 1 part of sodium chloride with 2 parts of water, shake intermittently, and allow to stand. Before use, decant the solution from any undissolved substance and filter, if necessary.

Standard solution 1: 1.0 mg/mL of USP Methyl Caproate RS, 1.0 mg/mL of USP Methyl Caprylate RS, and 2.0 mg/mL of USP Methyl Caprate RS in *n*-heptane

Standard solution 2: 0.1 mg/mL of USP Methyl Caproate RS, 0.1 mg/mL of USP Methyl Caprylate RS, and 0.2 mg/mL of USP Methyl Caprate RS in *n*-heptane, diluted from *Standard solution 1*

Standard solution 3: Prepare an ester mixture by mixing a commercially available ester mixture with methyl caprylate and methyl caprate, or prepare an ester mixture by using USP Methyl Caproate RS, USP Methyl Caprylate RS, USP Methyl Caprate RS, and USP Methyl Laurate RS. Dissolve a quantity of the prepared ester mixture containing methyl caproate, methyl caprylate, methyl caprate, and methyl laurate in *n*-heptane to make a solution of about 9.0 mg/mL for methyl caprylate, 0.5 mg/mL for methyl caprate, and 0.1 mg/mL for each of methyl caproate and methyl laurate.

Sample solution: Transfer 100 mg of Glyceryl Tricaprylate to a 25-mL conical flask fitted with a suitable water-cooled reflux condenser and a magnetic stir bar. Add 2 mL of a 20-mg/mL solution of sodium hydroxide in methanol, mix, and reflux for about 30 min. Add 2 mL of *Boron trifluoride methanol solution* through the condenser and reflux for about 30 min. Add 4 mL of *n*-heptane through the condenser and reflux for 5 min. Cool, remove the condenser, add about 10 mL of *Saturated sodium chloride solution*, shake, add a quantity of *Saturated sodium chloride solution* to bring the upper layer up to the neck of the flask, and allow the layers to separate. Collect 2 mL of the *n*-heptane layer (upper layer); wash with three quantities of water, 2 mL each; and dry the *n*-heptane phase over anhydrous sodium sulfate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.53-mm × 30-m capillary bonded with a 1.0-µm layer of phase *G16*
Temperatures
Injection port: 250°
Detector: 250°
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
50	20	180	—
180	9	240	12

Carrier gas: Helium
Flow rate: 10 mL/min
Injection volume: 2 µL
Injection type: Split ratio, 4:1
System suitability
Samples: *Standard solution 1* and *Standard solution 2*
[NOTE—The relative retention times for methyl caproate, methyl caprylate, and methyl caprate are about 0.7, 1.0, and 1.4, respectively.]
Suitability requirements
Resolution: NLT 4.0 between the methyl caprylate and methyl caprate peaks, *Standard solution 1*
Signal-to-noise ratio: NLT 10 for the methyl caproate peak, *Standard solution 2*
Analysis
Samples: *Standard solution 3* and *Sample solution*
Measure the peak areas of the methyl esters of the fatty acids. Disregard any peaks with an area less than 0.05% of the total area and any peak due to the solvent.
[NOTE—Relative retention times for several methyl esters are summarized in *Table 2*.]

Table 2

Carbon-Chain Length	Number of Double Bonds	Relative Retention Times
6	0	0.7
8	0	1.0
10	0	1.4
12	0	1.7

Take the main component in *Standard solution 3* as a reference component, and calculate the calibration factor (F_{FA}) for each fatty acid methyl ester:

$$F_{FA} = [(F_{MC} \times P_{FA1} \times A_{MC})/(P_{MC} \times A_{FA1})]$$

F_{MC} = factor for the main component, 1
P_{FA1} = percentage by weight of the fatty acid methyl ester in *Standard solution 3*
A_{MC} = peak area for the main component in *Standard solution 3*

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P_{MC} = percentage by weight of the main component in *Standard solution 3*
A_{FA1} = peak area for the fatty acid methyl ester in *Standard solution 3*

Calculate the percentage of each fatty acid methyl ester by weight in the portion of Glyceryl Tricaprylate taken:

$$\text{Result} = [(A_{FA2} \times F_{FA})/A_T] \times 100$$

A_{FA2} = peak area for the fatty acid methyl ester in the *Sample solution*
F_{FA} = calibration factor, determined previously
A_T = sum of the peak areas for the fatty acid methyl esters in the *Sample solution*

Acceptance criteria: Glyceryl Tricaprylate exhibits the following composition profile of fatty acids shown in *Table 3*.

Table 3

Carbon-Chain Length	Number of Double Bonds	Percentage (w/w)
6	0	≤1.0
8	0	≥90.0
10	0	≤5.0
12	0	≤1.0

• **B. Content of Triglycerides**
Analysis: Proceed as directed in the test for *Content of Triglycerides* in the *Assay*.
Acceptance criteria: NLT 90.0% of triglycerides
• **C. Fats and Fixed Oils** ⟨401⟩, *Procedures, Saponification Value*: 340–370

ASSAY

Change to read:

• **Content of Triglycerides**
System suitability solution: 20 ▲mg/mL▲ (ERR 1-Oct-2018) each of 1-monooctanoyl-rac-glycerol and 1-monodecanoyl-rac-glycerol in tetrahydrofuran
Standard solution 1: 50 mg/mL of USP Glyceryl Monocaprylate RS in tetrahydrofuran
Standard solution 2: 50 mg/mL of USP Glyceryl Tricaprylate RS in tetrahydrofuran
Sample solution: 50 mg/mL of Glyceryl Tricaprylate in tetrahydrofuran
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.32-mm × 10-m bonded with a 0.1-µm layer of phase G27
Temperatures
Injection port: 350°
Detector: 370°
Column: See *Table 4*.

Table 4

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
60	—	60	3

Table 4 *(continued)*

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
60	8	340	12

Carrier gas: Helium
Flow rate: 2.3 mL/min
Injection volume: 1 µL
Injection type: Split ratio, 50:1
System suitability
Samples: *System suitability solution*, *Standard solution 1*, and *Standard solution 2*
[Note—The relative retention times for monoglycerides, diglycerides, and triglycerides are 1.0–1.2, 1.5–1.9, and 1.9–2.3, respectively, *Standard solution 1* and *Standard solution 2*.]
Suitability requirements
Resolution: NLT 5.0 between the peaks due to 1-monooctanoyl-rac-glycerol and 1-monodecanoyl-rac-glycerol, *System suitability solution*
Analysis
Samples: *Standard solution 1*, *Standard solution 2*, and *Sample solution*
Based on the chromatograms from *Standard solution 1* and *Standard solution 2*, identify the peaks due to mono-, di-, and triglycerides in the *Sample solution*.
For the calculation of the contents of mono-, di-, and triglycerides, disregard the peaks with a retention time less than that of the monoglycerides, which are due to impurities of the solvent and to the free fatty acids.
Calculate the percentage of free fatty acids (C_A) in the portion of Glyceryl Tricaprylate taken:

$$C_A = [(I_A \times F \times M_{r1})/M_{r2}] \times 100$$

I_A = acid value for Glyceryl Tricaprylate, determined in the test for *Fats and Fixed Oils, Acid Value*
F = unit conversion factor, 10^{-3} g/mg
M_{r1} = molecular weight of caprylic acid, 144.21 g/mol
M_{r2} = molecular weight of potassium hydroxide, 56.11 g/mol

Calculate the percentage of triglycerides in the portion of Glyceryl Tricaprylate taken:

$$\text{Result} = (r_U/r_T) \times [(100 - C_A - C_W - C_G)/100] \times 100$$

r_U = peak response of the triglycerides from the *Sample solution*
r_T = sum of all the glyceride peak responses from the *Sample solution*
C_A = percentage of free fatty acids, determined previously
C_W = percentage of water, determined in the test for *Water Determination*
C_G = percentage of free glycerin, determined in the test for *Limit of Free Glycerin*

Acceptance criteria: NLT 90.0% of triglycerides

IMPURITIES

• **Residue on Ignition** ⟨281⟩: NMT 0.1%
• **Alkaline Impurities**
Analysis: Prepare a mixture of 2.0 g of Glyceryl Tricaprylate, 15 mL of alcohol, and 30 mL of ether. Dissolve by gently heating. Add 0.05 mL of bromophenol blue TS,

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and titrate with 0.01 N hydrochloric acid VS until the mixture turns yellow.
Acceptance criteria: NMT 0.4 mL of 0.01 N hydrochloric acid is required.

• **LIMIT OF FREE GLYCERIN**
Sample: 1.2 g
Periodic acetic acid solution: Dissolve 0.446 g of sodium periodate in 2.5 mL of a 25% (v/v) solution of sulfuric acid, and dilute with glacial acetic acid to 100.0 mL.
Potassium iodide solution: 75 mg/mL of potassium iodide
Blank: 25 mL of methylene chloride
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Residual titration
Titrant: 0.1 N sodium thiosulfate VS
Endpoint detection: Visual
Analysis: Dissolve the *Sample* in 25 mL of methylene chloride. Heat to about 50°, and allow to cool. Add 100 mL of water. Shake, and add 25 mL of *Periodic acetic acid solution*. Shake again, and allow to stand for 30 min. Add 40 mL of *Potassium iodide solution*, and allow to stand for 1 min. Add 1 mL of starch TS, and titrate the liberated iodine with *Titrant* until the aqueous phase becomes colorless. Perform a blank determination.
Calculate the percentage of free glycerol in the portion of Glyceryl Tricaprylate taken:

$$\text{Result} = \{[(V_B - V_S) \times N_A \times E \times F]/W\} \times 100$$

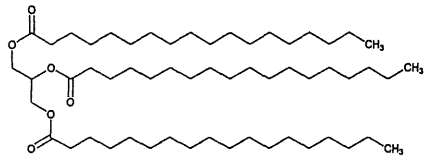
V_B = volume of *Titrant* consumed in the blank titration (mL)
V_S = volume of *Titrant* consumed in the *Sample* titration (mL)
N_A = actual normality of the *Titrant* (mEq/mL)
E = equivalent factor for glycerol, 23 mg/mEq
F = conversion factor, 10^{-3} g/mg
W = weight of Glyceryl Tricaprylate taken for the titration (g)

Acceptance criteria: NMT 0.5%

SPECIFIC TESTS
• **BACTERIAL ENDOTOXINS TEST ⟨85⟩:** Where the label states that Glyceryl Tricaprylate must be subjected to further processing during the preparation of injectable dosage forms, the level of bacterial endotoxins is such that the requirement in the relevant dosage form monograph(s) in which Glyceryl Tricaprylate is used can be met.
• **FATS AND FIXED OILS ⟨401⟩, *Procedures, Acid Value*:** NMT 0.2
• **FATS AND FIXED OILS ⟨401⟩, *Procedures, Hydroxyl Value*:** NMT 10.0
• **FATS AND FIXED OILS ⟨401⟩, *Procedures, Peroxide Value*:** NMT 1
• **WATER DETERMINATION ⟨921⟩, *Method I, Method Ia*:** NMT 0.2%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at room temperature. Protect from light and moisture.
• **LABELING:** Where Glyceryl Tricaprylate must be subjected to further processing during the preparation of injectable dosage forms to ensure acceptable levels of bacterial endotoxins, it is so labeled.
• **USP REFERENCE STANDARDS ⟨11⟩**
USP Glyceryl Monocaprylate RS
USP Glyceryl Tricaprylate RS
USP Methyl Caprate RS
USP Methyl Caproate RS
USP Methyl Caprylate RS
USP Methyl Laurate RS ▲1S (NF36)

Glyceryl Tristearate



$C_{57}H_{110}O_6$ 891.48
Octadecanoic acid, 1,1′,1″-(1,2,3-propanetriyl) ester;
Octadecanoic acid, 1,2,3-propanetriyl ester;
Glycerol trioctadecanoate;
Tristearoylglycerol [555-43-1].

DEFINITION
Glyceryl Tristearate contains NLT 90.0% of triglycerides of saturated fatty acids, chiefly glyceryl tristearate ($C_{57}H_{110}O_6$).

IDENTIFICATION
• **FATTY ACID COMPOSITION**
Boron trifluoride methanol solution: 140 mg/mL of boron trifluoride in methanol
Saturated sodium chloride solution: Mix 1 part of sodium chloride with 2 parts of water, shake from time to time, and allow to stand. Before use, decant the solution from any undissolved substance and filter, if necessary.
Standard solution 1: 0.5 mg/mL of USP Methyl Myristate RS, 2.0 mg/mL of USP Methyl Stearate RS, and 2.0 mg/mL of USP Methyl Oleate RS in *n*-heptane
Standard solution 2: 0.05 mg/mL of USP Methyl Myristate RS, 0.2 mg/mL of USP Methyl Stearate RS, and 0.2 mg/mL of USP Methyl Oleate RS in *n*-heptane, diluted from *Standard solution 1*
Standard solution 3: Dissolve a quantity of an ester mixture[1] containing methyl hexanoate, methyl caprylate, methyl caprate, methyl laurate, methyl myristate, methyl palmitate, methyl palmitoleate, methyl stearate, methyl oleate, methyl linoleate, and methyl arachidate in *n*-heptane to make a solution of about 9.0 mg/mL for methyl stearate and 0.1–0.2 mg/mL for each other methyl ester.
Sample solution: Transfer 100 mg of Glyceryl Tristearate to a 25-mL conical flask fitted with a suitable water-cooled reflux condenser and a magnetic stir bar. Add 2 mL of a 20-mg/mL solution of sodium hydroxide in methanol, mix, and reflux for about 30 min. Add 2 mL of *Boron trifluoride methanol solution* through the condenser, and reflux for about 30 min. Add 4 mL of *n*-heptane through the condenser, and reflux for 5 min. Cool, remove the condenser, add about 10 mL of *Saturated sodium chloride solution*, and shake. Add a quantity of *Saturated sodium chloride solution* to bring the upper layer up to the neck of the flask, and allow the layers to separate. Collect 2 mL of the *n*-heptane layer (the upper layer); wash with three quantities of water (2 mL each), and dry the *n*-heptane phase over anhydrous sodium sulfate.

[1] Ester mixture can be obtained commercially from Nu-Chek-Prep, Inc., P.O. Box 295, Elysian, MN 56028, or from Sigma-Aldrich; or it can be prepared by mixing a commercially-available ester mixture with methyl stearate.

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Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.53-mm × 30-m capillary, bonded with a 1.0-µm layer of phase G16
Temperatures
Injection port: 250°
Detector: 250°
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
50	20	180	—
180	9	240	12

Carrier gas: Helium
Flow rate: 10 mL/min
Injection volume: 2 µL
Injection type: Split ratio, 4:1
System suitability
Samples: *Standard solution 1* and *Standard solution 2*
[NOTE—The relative retention times for methyl myristate, methyl stearate, and methyl oleate are about 0.70, 1.00, and 1.01, respectively.]
Suitability requirements
Resolution: NLT 1.5 between methyl stearate and methyl oleate, *Standard solution 1*
Signal-to-noise ratio: NLT 5 for methyl myristate, *Standard solution 2*
Analysis
Samples: *Standard solution 3* and *Sample solution*
Measure the areas for the peaks of the methyl esters of the fatty acids. Disregard any peak with an area less than 0.05% of the total area and any peak due to the solvent. [NOTE—Relative retention times for several methyl esters are summarized in *Table 2*.]

Table 2

Carbon-Chain Length	Number of Double Bonds	Relative Retention Times
12	0	0.58
14	0	0.70
16	0	0.83
18	0	1.00
18	1	1.01
20	0	1.14

Take the main component of *Standard solution 3* as a reference component, and calculate the calibration factor, F_{FA}, for each fatty acid methyl ester:

$$\text{Result} = (F_{MC} \times P_{FA1} \times A_{MC})/(P_{MC} \times A_{FA1})$$

F_{MC} = factor for the main component, 1
P_{FA1} = percentage by weight of the fatty acid methyl ester in *Standard solution 3*
A_{MC} = peak area of the main component in *Standard solution 3*
P_{MC} = percentage by weight of the main component of *Standard solution 3*
A_{FA1} = peak area of the fatty acid methyl ester in *Standard solution 3*

Calculate the percentage of the fatty acid methyl ester by weight in the portion of Glyceryl Tristearate taken:

$$\text{Result} = [(A_{FA2} \times F_{FA})/A_T] \times 100$$

A_{FA2} = peak area of the fatty acid methyl ester in the *Sample solution*
F_{FA} = calibration factor
A_T = sum of the peak areas of the fatty acid methyl esters in the *Sample solution*

Acceptance criteria: Glyceryl Tristearate exhibits the composition profile of fatty acids shown in *Table 3*.

Table 3

Carbon-Chain Length	Number of Double Bonds	Percentage (w/w)
6, 8, 10	0	0.0–0.3
12	0	0.0–0.5
14	0	0.0–0.5
16	0	0.0–2.0
16	1	0.0–0.1
18	0	≥97
18	1	0.0–0.5
18	2	0.0–0.5
20	0	0.0–1.0

ASSAY

• **CONTENT OF TRIGLYCERIDES**
Mobile phase: Tetrahydrofuran
System suitability solution: 40 mg/mL of USP Glyceryl Distearate RS in *Mobile phase*
Sample solution: 8 mg/mL of Glyceryl Tristearate in *Mobile phase*
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Refractive index
Column: 7.5-mm × 60-cm; 3-µm or 5-µm 100-Å packing L21
Temperatures
Column: 40°
Detector: 40°
[NOTE—Two or three 7.5-mm × 30-cm L21 columns may be used in place of the one 60-cm column, provided that system suitability requirements are met. The column temperature may be lowered to ambient temperature, although working at 40° provides stable separation conditions and ensures better sample solubility.]
Flow rate: 1 mL/min
Injection volume: 40 µL
System suitability
Sample: *System suitability solution*
[NOTE—The relative retention times for triglycerides, diglycerides, monoglycerides, and glycerin are 0.78, 0.81, 0.86, and 1.00, respectively.]
Suitability requirements
Resolution: NLT 1.0 between the diglycerides and the monoglycerides
Relative standard deviation: NMT 2.0%, determined for the monoglycerides peak

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Analysis
Samples: *System suitability solution* and *Sample solution*
Calculate the percentage of triglycerides in the portion of Glyceryl Tristearate taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of the triglycerides from the *Sample solution*
r_T = sum of all the glyceride peak responses from the *Sample solution*

Acceptance criteria: NLT 90.0% of triglycerides

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%
- **ALKALINE IMPURITIES**

Analysis: Prepare a mixture of 2.0 g of Glyceryl Tristearate, 15 mL of alcohol, and 30 mL of ether. Dissolve by gentle heating. Add 0.05 mL of bromophenol blue TS, and titrate with 0.01 N hydrochloric acid VS until the mixture turns yellow.
Acceptance criteria: NMT 0.4 mL of 0.01 N hydrochloric acid is required.

- **LIMIT OF NICKEL**

[CAUTION—When using closed high-pressure digestion vessels and laboratory microwave equipment, be familiar with the safety and operating instructions given by the manufacturer.]
Magnesium nitrate solution: 10 mg/mL of magnesium nitrate in water
Ammonium dihydrogen phosphate solution: 100 mg/mL of ammonium dihydrogen phosphate in water
Standard stock solution: Transfer 5.0 mL of nickel standard solution TS to a 10-mL volumetric flask, and dilute with water to make a solution containing 5 µg/mL of nickel.
Standard solutions: To four identical 25-mL volumetric flasks, each containing 6 mL of nitric acid, transfer 25, 50, 75, and 100 µL, respectively, of the *Standard stock solution*. To each flask add 0.5 mL of *Magnesium nitrate solution* and 0.5 mL of *Ammonium dihydrogen phosphate solution*, and dilute with water to volume. These solutions contain 0.005, 0.01, 0.015, and 0.02 µg/mL, respectively, of nickel.
Sample solution: Transfer 0.25 g of Glyceryl Tristearate to a suitable high-pressure-resistant digestion vessel (fluoropolymer or quartz), and add 6.0 mL of nitric acid and 2.0 mL of 30% hydrogen peroxide. Place the closed vessel in a laboratory microwave oven, and digest using an appropriate program (e.g., 1000 W for 40 min). Allow the digestion vessel to cool before opening. Add 2.0 mL of 30% hydrogen peroxide, and repeat the digestion step. Allow the digestion vessel to cool before opening. Quantitatively transfer the contents to a 25-mL volumetric flask, add 0.5 mL of *Magnesium nitrate solution* and 0.5 mL of *Ammonium dihydrogen phosphate solution*, dilute with water to volume, and mix.
Blank solution: Add 6.0 mL of nitric acid and 2.0 mL of 30% hydrogen peroxide to a high-pressure-resistant digestion vessel, and proceed as directed for the *Sample solution*.
Zero solution: Into a 50.0-mL volumetric flask, introduce 1.0 mL of *Magnesium nitrate solution*, 1.0 mL of *Ammonium dihydrogen phosphate solution*, and 12.0 mL of nitric acid. Dilute with water to volume, and mix.
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Analytical wavelength: Maximum absorbance at 232.0 nm
Detection: Graphite furnace
Lamp: Nickel hollow-cathode
Tube: Pyrolytically coated
Other: Background compensation system
Furnace program: See *Table 4*.
[NOTE—The temperature program may be modified to obtain optimum furnace temperatures.]

Table 4

Step	Dry	Ash	Cool	Atomize
Temperature (°)	120	1100	800	2600
Ramp time (s)	5	30	5	—
Hold time (s)	35	10	5 (decrease)	7

Analysis
Samples: *Standard solutions*, *Sample solution*, and *Blank solution*
Use the *Zero solution* to set the instrument to zero. Concomitantly determine the absorbances of the *Samples* at least three times each. Record the average of the steady readings for each of the solutions. If necessary, dilute the *Sample solution* with the *Zero solution* to obtain a reading within the calibrated absorbance range.
Plot the absorbances versus the concentrations of nickel, in µg/mL, in the *Standard solutions*. On the basis of the calibration curve, determine the concentrations of nickel, in µg/mL, in the *Sample solution* and the *Blank solution* from the corresponding absorptions. Obtain the correct concentration of nickel, C_C, in µg/mL, in the *Sample solution*.
Calculate the content of nickel, in µg/g (ppm), in the portion of Glyceryl Tristearate taken:
[NOTE—If the *Sample solution* is diluted with the *Zero solution*, apply an appropriate correction factor for dilution.]

$$\text{Result} = (C_C \times V)/W$$

C_C = concentration of nickel in the *Sample solution* (µg/mL)
V = volume of the *Sample solution*, 25 mL
W = weight of Glyceryl Tristearate taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 1 µg/g (ppm)

SPECIFIC TESTS

- **MELTING RANGE OR TEMPERATURE** ⟨741⟩, *Procedures, Procedure for Class II*: 69°–73°
- **FATS AND FIXED OILS** ⟨401⟩, *Acid Value*: NMT 1.0
- **FATS AND FIXED OILS** ⟨401⟩, *Hydroxyl Value*: NMT 5.0
- **FATS AND FIXED OILS** ⟨401⟩, *Saponification Value*: 186–192
- **FATS AND FIXED OILS** ⟨401⟩, *Peroxide Value*: NMT 3
- **FATS AND FIXED OILS** ⟨401⟩, *Unsaponifiable Matter*: NMT 0.5%
- **WATER DETERMINATION** ⟨921⟩, *Method I, Method Ia*: NMT 0.1%

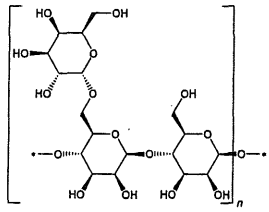
ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and store at room temperature. Protect from moisture.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Glyceryl Distearate RS
  USP Methyl Myristate RS
  USP Methyl Oleate RS
  USP Methyl Stearate RS

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Glycine— *see Glycine General Monographs*

Guar Gum



[9000-30-0].

DEFINITION

Guar Gum is the flour obtained by grinding the endosperms of seeds of *Cyamopsis tetragonolobus* (L.) Taub. (Fam. Leguminosae). It consists chiefly of high molecular weight hydrocolloidal polysaccharides composed of galactomannan. The content of galactomannan is NLT 66.0%. Galactomannan consists of a linear main chain of β-(1→4)-glycosidically linked mannopyranoses and single α-(1→6)-glycosidically linked galactopyranoses, and the ratio of the mannose and galactose is from 1.4:1 to 2.2:1.

IDENTIFICATION

• **A. Indication for a Polymeric Compound and Distinction from Locust Bean Gum**

Sample: 2 g

Analysis 1: Place the *Sample* in a 400-mL beaker, and moisten it with 4 mL of isopropyl alcohol. Add 200 mL of cold water with vigorous stirring, and continue stirring until the *Sample* is completely and uniformly dispersed.

Acceptance criteria 1: An opalescent, viscous dispersion results.

Analysis 2: Transfer 100 mL of the sample dispersion prepared above to a 400-mL beaker, heat in a boiling water bath for about 10 min, and then cool to room temperature.

Acceptance criteria 2: No appreciable increase in viscosity is produced (distinction from locust bean gum: see *Reagents, Indicators, and Solutions—Reagent Specifications*).

• **B. Identification of Constituting Mannose and Galactose by Thin-Layer Chromatography**

Mobile phase: Acetonitrile and water (85:15)

Standard solution: Dissolve 10 mg of USP Galactose RS and 10 mg of USP Mannose RS in 2 mL of water, and dilute with methanol to 20 mL.

Sample solution: Transfer 20 mg of Guar Gum to a test tube, add 4 mL of a 100 mg/mL solution of trifluoroacetic acid, and shake vigorously to dissolve the forming gel. Stopper the tube, and heat the mixture at 115° for 1 h 20 min in a dry bath (heating block) or oil bath. Cool, transfer the hydrolysate to a centrifuge tube, and centrifuge. Some suspended particles/gel are formed. Pass the supernatant solution through a 0.45-µm disc filter. Wash the test tube and the centrifuge tube with two 5-mL portions of water, and filter. Combine the washing filtrate with the filtered supernatant of the hydrolysate. Transfer the combined clear filtrate to a 50-mL flask, and evaporate the solution to dryness under reduced pressure. To the resulting residue add 0.2 mL of water and 1.8 mL of methanol.

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Mode: TLC

Absorbent layer: 0.25-mm silica gel 60 F_{254}

Application volume: 5 µL, as 9-mm bands, using an automated apparatus

Spray reagent: Dissolve 3 g of phthalic acid and 0.3 g of aminohippuric acid in ethyl alcohol, and dilute with ethyl alcohol to 100 mL.

Analysis

Samples: *Standard solution* and *Sample solution*

Develop over a path of 15 cm. Spray with *Spray reagent*, and dry at 120° for 5 min.

Acceptance criteria: The chromatogram from the *Standard solution* shows, in the lower region, two clearly separated brownish or yellowish zones due to galactose and mannose in order of increasing R_F value. The chromatogram from the *Sample solution* shows two zones due to galactose and mannose.

ASSAY

• **Content of Galactomannan and Ratio of Constituting Mannose and Galactose**

Mobile phase: Water

System suitability solution: 5 mg/mL of USP Galactose RS, 5 mg/mL of USP Mannose RS, 5 mg/mL of USP Xylose RS, and 5 mg/mL of USP Dextrose RS in *Mobile phase*

Standard solution: 10 mg/mL of USP Galactose RS and 10 mg/mL of USP Mannose RS in *Mobile phase*

Sample solution A: Transfer 100 mg of Guar Gum to a glass test tube. Add 2.0 mL of water and 2.0 mL of 1 M trifluoroacetic acid to the tube, and mix on a vortex mixer for 30 s. Incubate the solution at 105° in an oil-bath heating module for 6 h. After the first 15 min of incubation, mix on a vortex mixer for 30 s. After the 30 min of incubation, mix on a vortex mixer for 30 s. [Note—This ensures that Guar Gum does not stick to the bottom of the test tube and burn.] Before HPLC analysis, mix on a vortex mixer for 30 s, and pass the solution through a 0.45-µm PES (polyethersulfone) membrane syringe filter.

Sample solution B: 5 mg/mL of Guar Gum in *Mobile phase*

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 8.0-mm × 30-cm; 7-µm packing L22

Temperatures

Detector: 55°

Column: 80°

Flow rate: 0.75 mL/min

Injection volume: 10 µL

Detector purge time: 1 min

Run time: 17 min

System suitability

Samples: *System suitability solution* and *Standard solution*

[Note—The relative retention times for glucose, xylose, galactose, and mannose are 0.88, 0.94, 1.00, and 1.10, respectively.]

Suitability requirements

Resolution: NLT 0.9 between dextrose and xylose; NLT 1.0 between xylose and galactose; NLT 1.5 between galactose and mannose, *System suitability solution*

Tailing factor: 0.8–1.8 for the galactose and mannose peaks, *Standard solution*

Relative standard deviation: NMT 2.0% for the galactose and mannose peaks, *Standard solution*

Analysis

Samples: *Standard solution*, *Sample solution A*, and *Sample solution B*

In the chromatogram of *Sample solution B*, no galactose and mannose peaks are observed.

Calculate the percentage of galactose (C_G) or mannose (C_M) in the portion of Guar Gum taken:

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$$\text{Result } (C_G \text{ or } C_M) = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of galactose or mannose in *Sample solution A*
r_S = peak response of galactose or mannose in the *Standard solution*
C_S = concentration of USP Galactose RS or USP Mannose RS in the *Standard solution* (mg/mL)
C_U = concentration of Guar Gum in *Sample solution A* (mg/mL)

Calculate the content of galactomannans in the portion of Guar Gum taken:

$$\text{Result} = C_M + C_G$$

Calculate the ratio of constituting mannose and galactose in the portion of Guar Gum taken:

$$\text{Result} = C_M/C_G$$

Acceptance criteria
Content of galactomannan: NLT 66.0%
Ratio of constituting mannose and galactose: 1.4–2.2

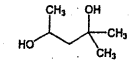
IMPURITIES
- **ARSENIC,** *Method II* ⟨211⟩**:** NMT 3 µg/g
- **LEAD** ⟨251⟩
Analysis: Prepare a *Test Preparation* as directed in the chapter, and use 10 mL of *Diluted Standard Lead Solution* (10 µg of Pb) for the test.
Acceptance criteria: NMT 10 µg/g

SPECIFIC TESTS
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 1.5%
- **ACID-INSOLUBLE MATTER**
Sample: 1.5 g
Analysis: Transfer the *Sample* to a 250-mL beaker containing 150 mL of water and 1.5 mL of sulfuric acid. Cover the beaker with a watch glass, and heat the mixture on a steam bath for 6 h, rubbing down the wall of the beaker frequently with a rubber-tipped stirring rod and replacing any water lost by evaporation. At the end of the 6 h heating period, add 500 mg, accurately weighed, of a filter aid, and pass through a tared, ashless filter. Wash the residue several times with hot water, dry the filter and its contents at 105° for 3 h, cool in a desiccator, and weigh. Determine the amount of acid-insoluble matter by subtracting the weight of the filter aid from that of the residue.
Acceptance criteria: NMT 7.0%
- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic microbial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g. It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*. It is recommended that the enrichment broth contain a 1% cellulase solution additive to optimize the recovery of *Salmonella* from this material.
- **PROTEIN**
Sample: 1.0 g
Analysis: Transfer the *Sample* to a 500-mL Kjeldahl flask, and proceed as directed in *Nitrogen Determination* ⟨461⟩, *Method I*. Determine the percentage of nitrogen. Calculate the amount of protein by multiplying the percentage of nitrogen by 6.25.
Acceptance criteria: NMT 10.0%
- **STARCH**
Analysis: To a dispersion (1 in 10) of Guar Gum add a few drops of iodine TS.
Acceptance criteria: No blue color is produced.
- **LOSS ON DRYING** ⟨731⟩
Analysis: Dry at 105° for 5 h.
Acceptance criteria: NMT 15.0%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Dextrose RS
USP Galactose RS
USP Mannose RS
USP Xylose RS

Hexylene Glycol

Change to read:



$C_6H_{14}O_2$ ▲118.18▲ (NF 1-May-2020)
2,4-Pentanediol, 2-methyl-;
2-Methyl-2,4-pentanediol;
▲2-Methylpentane-2,4-diol▲ (NF 1-May-2020) [107-41-5].

DEFINITION

Change to read:

Hexylene Glycol ▲contains NLT 98.0% and NMT 102.0% of 2-methylpentane-2,4-diol, calculated on the anhydrous basis.▲ (NF 1-May-2020)

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F**▲ (CN 1-May-2020)
Sample: Undried specimen
Acceptance criteria: Meets the requirements

Add the following:

- **▲B. CHROMATOGRAPHIC IDENTITY**
Analysis: Examine the chromatograms from the *Assay*.
Acceptance criteria: The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*.▲ (NF 1-May-2020)

ASSAY

Add the following:

- **▲PROCEDURE**
Diluent: Methanol
Internal standard solution: 1.0 mg/mL of USP Propylene Glycol RS (internal standard) in *Diluent*
System suitability solution: 0.1 mg/mL of diacetone alcohol and 1.0 mg/mL of USP Hexylene Glycol RS in *Internal standard solution*
Standard solution: 1.0 mg/mL of USP Hexylene Glycol RS in *Internal standard solution*

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Sample solution: 1.0 mg/mL of Hexylene Glycol in *Internal standard solution*

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.32-mm × 60-m capillary; bonded with a 1.0-µm layer of phase G1
Temperatures
Injection port: 200°
Detector: 300°
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
40	0	40	3
40	15	200	15

Carrier gas: Helium
Pressure: 15 psi (constant pressure mode)
Injection volume: 1.0 µL
Injection type: Split ratio, 100:1
Liner: Ultra inert liner with glass wool, low pressure drop[1]

System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The approximate relative retention times are listed in *Table 2*.]
Suitability requirements
Resolution: NLT 5 between hexylene glycol and diacetone alcohol, *System suitability solution*
Tailing factor: NMT 2.0, *Standard solution*
Relative standard deviation: NMT 2.0% for the peak response ratio of hexylene glycol to the internal standard, *Standard solution*

Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of hexylene glycol in the portion of the sample taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of hexylene glycol to the internal standard (peak response of hexylene glycol/peak response of the internal standard) from the *Sample solution*
R_S = peak response ratio of hexylene glycol to the internal standard (peak response of hexylene glycol/peak response of the internal standard) from the *Standard solution*
C_S = concentration of USP Hexylene Glycol RS in the *Standard solution* (mg/mL)
C_U = concentration of Hexylene Glycol in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–102.0% on the anhydrous basis ▲ (NF 1-May-2020)

[1] Agilent PN 5190-3165 liner has been suitable. Other equivalent liners are also suitable.

IMPURITIES

Add the following:

▲• **ORGANIC IMPURITIES**
Diluent and **Chromatographic system:** Proceed as directed in the *Assay*.
Sensitivity solution: 0.01 mg/mL of USP Hexylene Glycol RS in *Diluent*
System suitability solution: 0.2 mg/mL of USP Acetone RS and 0.2 mg/mL of USP 2-Propanol RS in *Diluent*
Standard solution: 0.02 mg/mL of USP Hexylene Glycol RS in *Diluent*
Sample solution: 20.0 mg/mL of Hexylene Glycol in *Diluent*
System suitability
Samples: *Sensitivity solution*, *System suitability solution*, and *Standard solution*
[NOTE—The approximate relative retention times are listed in *Table 2*.]
Suitability requirements
Resolution: NLT 1.5 between the acetone peak and the 2-propanol peak, *System suitability solution*
Relative standard deviation: NMT 5.0%, determined from the hexylene glycol peak, *Standard solution*
Signal-to-noise ratio: NLT 10, determined from the hexylene glycol peak, *Sensitivity solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of each individual impurity in the portion of the sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (1/F) \times 100$$

r_U = peak area of each individual impurity from the *Sample solution*
r_S = peak area of hexylene glycol from the *Standard solution*
C_S = concentration of USP Hexylene Glycol RS in the *Standard solution* (mg/mL)
C_U = concentration of Hexylene Glycol in the *Sample solution* (mg/mL)
F = relative response factor (see *Table 2*)

Acceptance criteria: See *Table 2*.

Table 2

Name	Relative Retention Time	Relative Response Factor	Acceptance Criteria, NMT (%)
Acetone	0.42	0.9	0.1
2-Propanol	0.43	0.9	0.1
4-Methylpentan-2-one	0.77	1.3	0.1
4-Methylpentan-2-ol	0.81	1.4	0.1
Diacetone alcohol[a]	0.91	1.0	0.1
Hexylene glycol	1.0	—	—
Any other individual impurity	—	—	0.1
Total impurities	—	—	1.0

[a] 4-Hydroxy-4-methylpentan-2-one. ▲ (NF 1-May-2020)

NF Monographs

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SPECIFIC TESTS
- **SPECIFIC GRAVITY** ⟨841⟩: 0.917–0.923
- **REFRACTIVE INDEX** ⟨831⟩: 1.424–1.430
- **ACIDITY**
  Sample: 10 mL
  Analysis: Mix 1 mL of phenolphthalein TS with 50 mL of water, and add 0.1 N sodium hydroxide until the solution remains pink for 30 s. Add the *Sample*, and titrate with 0.10 N sodium hydroxide until the original pink color returns and remains for 30 s.
  Acceptance criteria: NMT 0.20 mL of 0.10 N sodium hydroxide is required.
- **WATER DETERMINATION** ⟨921⟩, *Method I*: NMT 0.5%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers.

Change to read:

- **USP REFERENCE STANDARDS** ⟨11⟩
  ▲USP Acetone RS▲ (NF 1-May-2020)
  USP Hexylene Glycol RS
  ▲USP 2-Propanol RS
  USP Propylene Glycol RS▲ (NF 1-May-2020)

Purified Honey

DEFINITION
Purified Honey is obtained by purification of honey from the comb of the bee, *Apis mellifera* L. and all subspecies of *Apis mellifera*. The honey is extracted by centrifugation, pressure, or other suitable procedures.

IDENTIFICATION
- **A.**
  Sample solution: 1 in 20 solution
  Standard solution: A 7.5 µg/mL solution of USP L-Proline RS
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Place 0.5 mL of the *Sample solution* and the *Standard solution* in separate reaction vials, and add 0.25 mL of formic acid and 1.0 mL of freshly prepared 3% solution of ninhydrin in peroxide-free ethylene glycol monomethyl ether to each vial. Cap the reaction vials tightly, shake well, and place in boiling water for 15 min. Cool for 5 min in a 22° water bath, remove the caps, and add 5.0 mL of 5% aqueous isopropanol solution.
  Acceptance criteria: The *Sample solution* shows a purple color similar to, or more intense than that of the *Standard solution*. If necessary, scan the solutions being compared using a suitable spectrophotometer against a water blank. The two solutions each exhibit a maximum at the same wavelength, at about 520 nm. The absorbance of the *Sample solution* should be at least as high as that of the *Standard solution*.

IMPURITIES
- **CHLORIDE AND SULFATE,** *Chloride* ⟨221⟩: A 1-g portion shows no more chloride than corresponds to 0.5 mL of 0.020 N hydrochloric acid (0.035%).
- **CHLORIDE AND SULFATE,** *Sulfate* ⟨221⟩: A 2-g portion shows no more sulfate than corresponds to 0.5 mL of 0.020 N sulfuric acid (0.024%).
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩: NMT 0.3%

SPECIFIC TESTS
- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩: The total bacterial count does not exceed 10^3 cfu/g. The total combined molds and yeasts count does not exceed 100 cfu/g.
- **SPECIFIC GRAVITY** ⟨841⟩: 1.400–1.435 at 20°
- **REFRACTIVE INDEX** ⟨831⟩: 1.4900–1.4992 at 20°
- **WATER DETERMINATION,** *Method I* ⟨921⟩: 15.0%–18.6%
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩: Where it is intended for use in preparations for infants under one year of age, it meets the requirements of the *Test for Absence of Clostridium Species*.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.
- **LABELING:** Label it to indicate that it is not intended for use in preparations for infants under one year of age, unless it meets the requirements of the *Test for Absence of Clostridium Species*.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP L-Proline RS

Hydrochloric Acid

HCl 36.46
Hydrochloric acid [7647-01-0].

DEFINITION
Hydrochloric Acid contains NLT 36.5% and NMT 38.0%, by weight, of HCl.

IDENTIFICATION
- **A. IDENTIFICATION TESTS—GENERAL,** *Chloride* ⟨191⟩

ASSAY
- **PROCEDURE**
  Sample: 3 mL
  Titrimetric system
  (See *Titrimetry* ⟨541⟩.)
  Mode: Direct titration
  Titrant: 1 N sodium hydroxide VS
  Blank: 45 mL of water
  Endpoint detection: Visual
  Analysis: Place the *Sample* in a glass-stoppered flask, previously tared while containing about 20 mL of water, and weigh again to obtain the weight of the substance under assay. Dilute with 25 mL of water, and add methyl red TS. Titrate with 1 N sodium hydroxide VS.
  Calculate the percentage of hydrochloric acid (HCl) in the *Sample* taken:

$$\text{Result} = [(V_S - V_B) \times N \times F]/W \times 100$$

V_S = volume of *Titrant* consumed by the *Sample* (mL)
V_B = volume of *Titrant* consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 36.46 mg/mEq
W = weight of the *Sample* (mg)

Acceptance criteria: 36.5%–38.0%

IMPURITIES
- **RESIDUE ON IGNITION** ⟨281⟩
  Sample: 20 mL
  Analysis: To the *Sample*, add 2 drops of sulfuric acid, evaporate to dryness, and ignite.
  Acceptance criteria: NMT 2 mg of residue remains (about 0.008%).

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SPECIFIC TESTS
- **BROMIDE OR IODIDE**
  Sample solution: 1 part Hydrochloric Acid to 2 parts water
  Analysis: Add 1 mL of chloroform to 10 mL of the *Sample solution*. Cautiously add, dropwise, with constant agitation, chlorine TS that has been diluted with an equal volume of water.
  Acceptance criteria: The chloroform remains free from even a transient yellow, orange, or violet color.
- **FREE BROMINE OR CHLORINE**
  Sample solution: 1 part Hydrochloric Acid to 2 parts water
  Analysis: Add 1 mL of potassium iodide TS and 1 mL of chloroform to 10 mL of the *Sample solution*, and agitate the mixture.
  Acceptance criteria: The chloroform remains free from any violet color for at least 1 min.
- **SULFATE**
  Sample solution: 1 part Hydrochloric Acid to 2 parts water
  Analysis: To a mixture of 3 mL of the *Sample solution* and 5 mL of water, add 5 drops of barium chloride TS.
  Acceptance criteria: Neither turbidity nor precipitate appears within 1 h.
- **SULFITE**
  Sample solution: 1 part Hydrochloric Acid to 2 parts water
  Analysis: Complete the test for *Sulfate*, then add to the liquid 2 drops of 0.1 N iodine.
  Acceptance criteria: Neither turbidity nor decolorization of the iodine occurs.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers.

Diluted Hydrochloric Acid

DEFINITION
Diluted Hydrochloric Acid contains, in each 100 mL, NLT 9.5 g and NMT 10.5 g of hydrochloric acid (HCl).
Diluted Hydrochloric Acid may be prepared as follows.

Hydrochloric Acid	226 mL
Purified Water, a sufficient quantity to make	1000 mL

Mix the ingredients.

IDENTIFICATION
- **A. IDENTIFICATION TESTS—GENERAL,** *Chloride* ⟨191⟩

ASSAY
- **PROCEDURE**
  Sample: 10.0 mL
  Blank: 20 mL of water
  Titrimetric system
  (See *Titrimetry* ⟨541⟩.)
  Mode: Direct titration
  Titrant: 1 N sodium hydroxide VS
  Endpoint detection: Visual
  Analysis: Transfer the *Sample* to a conical flask, and add about 20 mL of water. Add 3 drops of methyl red TS. Titrate with *Titrant*. Perform a blank determination. Calculate the amount of hydrochloric acid (HCl) in the portion of sample taken:

$$\text{Result} = (V_S - V_B) \times N \times F$$

V_S = *Titrant* volume consumed by the *Sample* (mL)
V_B = *Titrant* volume consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 3.646×10^{-2} g/mEq

Acceptance criteria: 9.5–10.5 g of HCl in each 100 mL

IMPURITIES
- **RESIDUE ON IGNITION** ⟨281⟩
  Sample: 20 mL
  Analysis: To the *Sample* add 2 drops of sulfuric acid, evaporate to dryness, and ignite.
  Acceptance criteria: NMT 2 mg
- **FREE BROMINE OR CHLORINE**
  Sample: 10 mL
  Analysis: To the *Sample* add 1 mL of potassium iodide TS and 1 mL of chloroform, and agitate the mixture.
  Acceptance criteria: The chloroform remains free from any violet coloration for at least 1 min.
- **SULFATE**
  Sample solution: Mix 3 mL of Diluted Hydrochloric Acid and 5 mL of water.
  Analysis: To the *Sample solution* add 5 drops of barium chloride TS.
  Acceptance criteria: Neither turbidity nor precipitate appears within 1 h.
- **SULFITE**
  Analysis: Complete the test for *Sulfate*, then add to the liquid 2 drops of 0.1 N iodine.
  Acceptance criteria: Neither turbidity nor decolorization of the iodine occurs.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers.

Hydroxyethyl Cellulose

Cellulose, 2-hydroxyethyl ether [9004-62-0].

DEFINITION
Hydroxyethyl Cellulose is a partially substituted poly(hydroxyethyl) ether of cellulose. It is available in several grades, varying in viscosity and degree of substitution, and some grades are modified to improve their dispersion in water. It may contain suitable anticaking agents.

IDENTIFICATION
- **A.**
  Sample solution: 1 g in 100 mL of water
  Analysis: Stir the *Sample solution*.
  Acceptance criteria: It dissolves completely to produce a colloidal solution that remains clear when heated to 60°.
- **B.**
  Sample: 1 mL of the solution from *Identification* test *A*
  Analysis: Place the *Sample* on a glass plate, and allow to evaporate.
  Acceptance criteria: A thin, self-sustaining film is formed.
- **C.**
  Sample solution: 1 in 2000
  Analysis: To 1 mL of the *Sample solution* add 1 mL of phenol solution (50 mg/mL), then add 5 mL of sulfuric acid, shake, and allow to cool.
  Acceptance criteria: The color of the solution becomes orange.

IMPURITIES
- **RESIDUE ON IGNITION** ⟨281⟩: NMT 5.0%
- **LEAD** ⟨251⟩: NMT 10 µg/g

SPECIFIC TESTS
- **PH** ⟨791⟩
  Sample solution: 10 mg/mL

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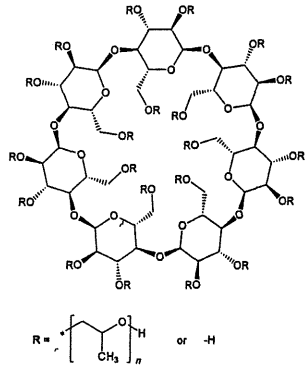
Acceptance criteria: 6.0–8.5

- **LOSS ON DRYING** ⟨731⟩
  Analysis: Dry at 105° for 3 h.
  Acceptance criteria: NMT 10.0%
- **VISCOSITY—CAPILLARY METHODS** ⟨911⟩ or **VISCOSITY—ROTATIONAL METHODS** ⟨912⟩**:** When determined at the concentration and under the conditions specified in the labeling, its viscosity is NLT 50% and NMT 150% of the labeled viscosity, where stated as a single value, or it is between the maximum and minimum values, where stated as a range of viscosities.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **LABELING:** The labeling indicates its viscosity, under specified conditions, in aqueous solution. The indicated viscosity may be in the form of a range encompassing 50%–150% of the average value.

Hydroxypropyl Betadex



$C_{42}H_{70}O_{35}(C_3H_6O)_x$ where $x = 7 \times MS$, MS being molar substitution

Beta cyclodextrin, 2-hydroxypropyl ether [94035-02-6].

DEFINITION

Hydroxypropyl Betadex is a partially substituted poly(hydroxypropyl) ether of Betadex. The number of hydroxypropyl groups per anhydroglucose unit expressed as molar substitution (MS) is NLT 0.40 and NMT 1.50 and is within 10% of the value stated on the label.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K.▲** (CN 1-May-2020) USP Hydroxypropyl Betadex RS
- **B.** It meets the requirements of the test for *Clarity of Solution*.

ASSAY

- **MOLAR SUBSTITUTION**
  (See *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩ .)
  The molar substitution (MS) is calculated from the ratio between the signal from the three protons of the methyl group, contained in the hydroxypropyl functional group, and the signal from the proton attached to the carbon C_1 (glycosidic proton) of the anhydroglucose units.
  Use a Fourier-transform nuclear magnetic resonance (NMR) spectrometer having a magnetic field strength of at least 6 Tesla and that is capable of performing quantitative analysis using proton NMR spectroscopy at a temperature of at least 25°.
  Sample solution: Mix NLT the equivalent of 10.0 mg of dried Hydroxypropyl Betadex with 0.75 mL of deuterium oxide thoroughly in an NMR tube. Place the tube into an NMR probe.
  Analysis: Adjust the spectrometer settings so that a high-resolution proton NMR spectrum can be acquired that will provide quantitative data. Acquire a free induction decay (FID) with at least 8 transients using a spectral window from at least 0–6.2 ppm, with the solvent peak located at 4.8 ppm at 25°. Zero fill the spectrum at least three times, and Fourier transform the FID with no Gaussian line broadening and no more than 0.2 Hz of Lorenzian line broadening.
  Determine the peak areas of the doublet from the methyl protons of the hydroxypropyl functional group at 1.2 ppm (A_1) and the peak areas from the glycosidic protons, which are located between 5 and 5.4 ppm (A_2).
  Calculate the MS:

$$\text{Result} = A_1/(3A_2)$$

A_1 = area of the methyl group of hydroxypropyl
A_2 = area of the glycosidic proton

The degree of substitution is the number of hydroxypropyl groups per molecule of betadex and is obtained by multiplying the MS by 7.

Acceptance criteria: 0.40–1.50 and within 10% of the value stated on the label

IMPURITIES

- **LIMIT OF BETADEX, PROPYLENE GLYCOL, AND OTHER RELATED SUBSTANCES**
  Mobile phase: Water
  Standard solution A: 15 mg/mL of USP Beta Cyclodextrin RS and 25 mg/mL of USP Propylene Glycol RS
  Standard solution B: 1.0 mL of *Standard solution A* diluted with water to 10.0 mL
  Sample solution: Dissolve 2.50 g of Hydroxypropyl Betadex, accurately weighed and calculated on the dried basis, in water with the aid of heat. Cool, and dilute with water to 25.0 mL. The resulting solution is 100 mg/mL of Hydroxypropyl Betadex, calculated on the dried basis, in water.
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: LC
  Detector: Differential refractometer
  Columns
  Guard: Packing L11
  Analytical: 3.9-mm × 30-cm; packing L11
  Column temperature: 40°
  Flow rate: 1.5 mL/min
  Injection volume: 20 µL
  System suitability
  Samples: *Standard solution A* and *Standard solution B*
  [NOTE—The retention time of propylene glycol is about 2.5 min, and the relative retention times with reference to that of propylene glycol for betadex and hydroxypropyl betadex are about 4.2 and about 6, respectively; Hydroxypropyl Betadex elutes as a very wide peak or several peaks.]
  Suitability requirements
  Resolution: NLT 4 between betadex and propylene glycol, *Standard solution A*
  Relative standard deviation: NMT 2.0%, *Standard solution B*

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Analysis
Samples: *Standard solution B* and *Sample solution*
Acceptance criteria: Disregard any peaks eluting before propylene glycol and after the hydroxypropyl betadex peak.
Betadex: NMT 1.5%; the area of the betadex peak in the *Sample solution* is NMT the area of the corresponding peak from *Standard solution B*.
Propylene glycol: NMT 2.5%; the area of the propylene glycol peak in the *Sample solution* is NMT the area of the corresponding peak from *Standard solution B*.
Any other single impurity: NMT 0.25%; the area from any other single impurity peak is NMT 0.1 times the area of propylene glycol in the chromatogram of *Standard solution B*.
Total impurities excluding betadex and propylene glycol: NMT 1%; the total area from all impurity peaks, excluding betadex and propylene glycol, is NMT 0.4 times the area of propylene glycol from *Standard solution B*.
Disregard limit: 0.1%; disregard any peaks that are less than 0.04 times the area of propylene glycol from *Standard solution B*.

• **LIMIT OF PROPYLENE OXIDE**
Ether stock solution: Add 75 µL of ether to 30 mL of dimethylacetamide in a 50-mL volumetric flask, dilute with dimethylacetamide to volume, and mix. This solution contains 1.0 mg/mL of ether.
Internal standard solution: Add 30 µL of *Ether stock solution* to 70 mL of dimethylacetamide in a 100-mL volumetric flask, dilute with dimethylacetamide to volume, and mix.
Propylene oxide stock solution
[**CAUTION**—Propylene oxide is toxic and flammable. Prepare this solution in a well-ventilated fume hood.]
Add 30 mL of dimethylacetamide into a 50-mL volumetric flask. Weigh the flask and contents accurately, add 60 µL of propylene oxide (cooled in a refrigerator) into the flask with a 100-µL cooled microsyringe, weigh again, and calculate the weight of propylene oxide added, by difference. Dilute with dimethylacetamide to volume, and mix. This solution contains 1.0 mg/mL of propylene oxide.
[NOTE—Propylene oxide is a gas at room temperature. It is usually stored in a lecture-type gas cylinder or small metal pressure bomb. Chill the cylinder in a refrigerator before use. Transfer 5 mL of the liquid propylene oxide to a 100-mL beaker chilled in wet ice. Use a gas-tight syringe that has been chilled in a refrigerator.]
System suitability solution: Add 30 µL of *Ether stock solution* and 20 µL of *Propylene oxide stock solution* to 70 mL of dimethylacetamide in a 100-mL volumetric flask, dilute with dimethylacetamide to volume, and mix.
Standard stock solutions: Add 7 mL of dimethylacetamide into each of four 10-mL volumetric flasks. Transfer the following amounts of *Propylene oxide stock solution* into each of the four flasks using a microsyringe, with one amount per flask: 40, 100, 200, and 400 µL. Dilute with dimethylacetamide to volume, and mix. The *Standard stock solutions* contain about 4, 10, 20, and 40 µg/mL of propylene oxide, respectively.
Standard solutions: Into each of four 10-mL headspace vials, transfer 200 ± 5 mg of Hydroxypropyl Betadex, calculated on the dried basis. Pipet 1.0 mL of the *Internal standard solution* into each vial, and close the vial with septum and cap. Into each of the vials add 10 µL of each of the *Standard stock solutions* using a 10-µL syringe, respectively. Allow each vial to stand, and gently shake until the sample is dissolved. The *Standard solutions* contain, respectively, about 0.04, 0.1, 0.2, and 0.4 µg/mL of propylene oxide.
Sample solution: Transfer 200 ± 5 mg of Hydroxypropyl Betadex, calculated on the dried basis, into a 10-mL headspace autosampler vial. Pipet 1.0 mL of the *Internal standard solution* into the vial, and close the vial with a septum and cap. Add 10 µL of dimethylacetamide using a 10-µL syringe. Allow the vial to stand, and gently shake until the sample is dissolved.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC with a balanced pressure automatic headspace sampler
Detector: Flame ionization
Column: 0.32-mm × 10-m fused-silica capillary; coated with a 10-µm layer of stationary phase S3
Temperatures
Injection port: 120°
Detector: 250°
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
50	—	50	10
50	10	100	10
100	20	220	4

Transfer line: 120°
Carrier gas: Helium
Flow rate: 2 mL/min, corresponding to the linear velocity of 44 cm/s
Injection volume: 1.0 mL
Injection type: Split injection; the split ratio is 1:1.
System suitability
Sample: *System suitability solution*
[NOTE—The relative retention times for propylene oxide and ether are about 1.0 and 1.3, respectively.]
Suitability requirements
Resolution: NLT 2.0 between ether and propylene oxide
Analysis
Samples: *Standard solutions* and *Sample solution*
Separately place the vials containing the *Standard solutions* and the *Sample solution* in the automated sampler, and start the sequence so that the vial is heated at a temperature of 100° for 30 min before a suitable portion of its headspace is injected into the chromatograph.
Using a 2-mL gas syringe preheated in an oven at 110°, separately inject the headspace from each vial into the chromatograph. Chromatograph the *Standard solutions* and the *Sample solution*, record the chromatograms, and measure the area ratios of the peak responses of propylene oxide and ether.
Determine, based on a retention time comparison, whether propylene oxide is detected in the *Sample solution*. Plot the area ratios of the peak responses of propylene oxide and ether of the *Sample solution* and the *Standard solutions* versus the content, in µg, of propylene oxide in each vial, as furnished by the *Standard stock solutions*, draw the straight line best fitting the five points, and calculate the correlation coefficient for the line. [NOTE—The *Sample solution* should be plotted as if it had a content of added propylene oxide equivalent to 0 µg.]
A suitable system is one that yields a line having a correlation coefficient of NLT 0.99. Extrapolate the line until it meets the content axis on the negative side. The

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distance between this point and the intersection of the axes represents the total amount, T_U, in µg, of propylene oxide in the *Sample solution*.

Calculate the percentage of propylene oxide in the portion of sample taken:

$$\text{Result} = (T_U/W) \times 100$$

T_U = total amount of propylene oxide from the graph in the *Sample solution* (µg)
W = weight of Hydroxypropyl Betadex taken to prepare the *Sample solution* (µg)

Acceptance criteria: NMT 0.0001%

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩: The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.
- **LOSS ON DRYING** ⟨731⟩
  Sample: 1 g
  Analysis: Dry the *Sample* at 120° for 2 h.
  Acceptance criteria: NMT 10.0%
- **CLARITY OF SOLUTION**
  Sample: 1.0 g
  Analysis: Dissolve the *Sample* in 2.0 mL of water, and heat.
  Acceptance criteria: The resulting solution is clear and remains transparent after cooling to room temperature.
- **BACTERIAL ENDOTOXINS TEST** ⟨85⟩: The level of bacterial endotoxins is such that the requirement under the relevant dosage form monograph(s) in which Hydroxypropyl Betadex is used can be met. Where the label states that Hydroxypropyl Betadex must be subjected to further processing during the preparation of injectable dosage forms, the level of bacterial endotoxins is such that the requirement under the relevant dosage form monograph(s) in which Hydroxypropyl Betadex is used can be met.
- **STERILITY TESTS** ⟨71⟩: Where the label states that Hydroxypropyl Betadex is sterile, it meets the requirements for *Sterility Tests* ⟨71⟩ in the relevant dosage form monograph(s) in which Hydroxypropyl Betadex is used.
- **CONDUCTIVITY**
  Sample solution: 100 mg/mL of Hydroxypropyl Betadex, calculated on the dried basis in previously boiled and cooled to room temperature water
  Apparatus: Use a conductivity meter or resistivity meter that measures the resistance of the column of liquid between the electrodes of the immersed measuring device. The apparatus is supplied with alternating current to avoid the effects of electrode polarization. It is equipped with a temperature compensation device or a precision thermometer.
  Standard solutions: Prepare three standard solutions of potassium chloride containing 0.7455, 0.0746, and 0.0149 g, respectively, of potassium chloride per 1000.0 g of solution. These solutions should be prepared using water, which has been previously boiled and cooled to room temperature and has conductivity that does not exceed 2 µS · cm^{-1}. The conductivity and resistivity of these three *Standard solutions* at 20° are given in *Table 2*.

Table 2

Concentration of Solution (g/1000.0 g)	Conductivity (µS · cm^{-1})	Resistivity (Ω · cm)
0.7455	1330	752
0.0746	133.0	7519
0.0149	26.6	37,594

Calibration
Samples: *Standard solutions*
Choose a conductivity cell that is appropriate for the conductivity of the solution to be examined. The higher the expected conductivity, the higher the cell constant that must be chosen. Commonly used conductivity cells have cell constants on the order of 0.1, 1, and 10 cm^{-1}.
Use a *Standard solution* of potassium chloride that is appropriate for the measurement. The conductivity value of the *Standard solution* of potassium chloride should be near the expected conductivity value of the *Sample solution*.
Rinse the cell several times with water, which has been previously boiled and cooled to room temperature, and at least twice with the *Standard solution* (potassium chloride solution) used for the determination of the cell constant of the conductivity cell.
Measure the resistance of the conductivity cell using the *Standard solution* (potassium chloride solution) at 20 ± 0.1°.
Calculate the constant of the conductivity cell, C (in cm^{-1}):

$$C = R_{KCl} \times K_{KCl}$$

R_{KCl} = measured resistance, expressed in mega-ohms
K_{KCl} = conductivity of the *Standard solution* of potassium chloride used, expressed in µS · cm^{-1}

Calibration acceptance criteria: The measured constant, C, of the conductivity cell must be within 5% of the given value.

Analysis
Sample: *Sample solution*
Rinse the conductivity cell several times with water, which has been previously boiled and cooled to room temperature, and at least twice with the *Sample solution*. Measure the conductivity of the *Sample solution*, while gently stirring with a magnetic stirrer.
Acceptance criteria: NMT 200 µS · cm^{-1}

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. Store at room temperature.
- **LABELING:** Label it to indicate the molar substitution (MS). Where Hydroxypropyl Betadex is intended for use in the manufacture of injectable dosage forms, it is so labeled. Where Hydroxypropyl Betadex must be subjected to further processing during the preparation of injectable dosage forms to ensure acceptable levels of bacterial endotoxins, it is so labeled. Where Hydroxypropyl Betadex is sterile, it is so labeled.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Beta Cyclodextrin RS
  USP Hydroxypropyl Betadex RS
  USP Propylene Glycol RS

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Hydroxypropyl Cellulose

Portions of this monograph that are national *USP* text, and are not part of the harmonized text, are marked with symbols (‣ ‣) to specify this fact.

Cellulose, 2-hydroxypropyl ether [9004-64-2].

DEFINITION

Hydroxypropyl Cellulose is partly *O*-(2-hydroxypropylated) cellulose. It contains NLT 53.4% and NMT 80.5% of hydroxypropoxy groups, calculated on the dried basis. It may contain suitable anti-caking agents, such as silica.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K.▲** (CN 1-May-2020) [NOTE—Disregard any peak at about 1719 cm^{-1}.]

• **B.**

Sample: 1 g of Hydroxypropyl Cellulose

Analysis: Dissolve the *Sample* in 100 mL of water. Transfer 1 mL of this solution to a glass plate, and allow the water to evaporate.

Acceptance criteria: A thin film is formed.

ASSAY

• **HYDROXYPROPOXY GROUPS**

Internal standard solution: Methylcyclohexane in *o*-xylene (1 in 50)

Standard solution: Weigh accurately 60 mg of adipic acid in a reaction vial, add 2.00 mL of *Internal standard solution*, and 1.0 mL of hydriodic acid. Stopper the vial tightly, and weigh accurately. Inject 25 µL of isopropyl iodide through the septum, and again weigh accurately. Mix well. After phase separation, pierce through the septum of the vial with a cooled syringe, and withdraw a sufficient volume of the upper phase as the *Standard solution*.

Sample solution: Weigh accurately 30 mg of hydroxypropylcellulose (dried substance), and transfer to a reaction vial. Add 60 mg of adipic acid, 2.00 mL of *Internal standard solution*, and 1.0 mL of hydriodic acid. Stopper the vial tightly with the valve, and weigh accurately the reaction vial (total mass before heating). Place the vial in an oven or heat in a suitable heater with continuous stirring, maintaining an internal temperature of 115 ± 2° for 70 min. Allow the vial to cool, and weigh accurately the reaction vial (total mass after heating). If the difference of the total mass before heating to the total mass after heating is more than 10 mg, prepare a new *Sample solution*. After phase separation, pierce through the septum of the vial with a cooled syringe, and withdraw a sufficient volume of the upper phase as the *Sample solution*.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.53-mm × 30-m fused silica capillary, coated with a 3-µm layer of phase G1

Temperatures

Detector: 280°

Injection port: 180°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
40	0	40	3
40	10	100	—
100	50	250	3

Carrier gas: Helium

Linear velocity: 52 cm/s

Injection volume: 2 µL

Injection type: Split; split ratio, 50:1

Run time: 15 min

System suitability

Sample: *Standard solution*

[NOTE—The relative retention time for isopropyliodide is about 0.8 with reference to methylcyclohexane (retention time about 8 min).]

Suitability requirements

Resolution: NLT 2 between isopropyl iodide and methylcyclohexane

Relative standard deviation: NMT 2.0%, using the response factor calculation (*F*) for six injections

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the response factor (*F*):

$$F = (A_1 \times W_1 \times C)/(A_2 \times 100)$$

A_1 = peak area of the internal standard from the *Standard solution*

W_1 = weight of isopropyl iodide in the *Standard solution* (mg)

C = content of isopropyl iodide (%)

A_2 = peak area of isopropyl iodide from the *Standard solution*

Calculate the percentage content (m/m) of the hydroxypropoxy group:

$$\text{Result} = (A_4 \times F \times M_1 \times 1.15 \times 100)/(A_3 \times W_2 \times M_2)$$

A_4 = peak area of isopropyl iodide from the *Sample solution*

F = response factor calculated from above

M_1 = molar mass of hydroxypropoxy group, 75.1

1.15 = correction factor to correlate results to previous assay method replaced by this method

A_3 = peak area of the internal standard from the *Sample solution*

W_2 = weight of the sample (dried substance) in the *Sample solution* (mg)

M_2 = molar mass of isopropyl iodide, 170.0

Acceptance criteria: 53.4%–80.5% of hydroxypropoxy groups

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩

Sample: 1.0 g

Analysis: Proceed as directed in the chapter, using a platinum crucible.

Acceptance criteria: NMT 0.8%

• **SILICA**

[**CAUTION**—Perform the mixing and heating of the mixtures containing hydrofluoric acid in a well-ventilated hood.]

Analysis: If the addition of silica is stated on the label, and if more than 0.2% residue is found from the *Residue on*

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Ignition test, moisten the residue with water, and add 5 mL of hydrofluoric acid in small portions. Evaporate on a steam bath to dryness, and cool. Add 5 mL of hydrofluoric acid and 0.5 mL of sulfuric acid, and evaporate to dryness. Slowly increase the temperature until all of the acids have been volatilized, and ignite at 1000 ± 25°. Cool in a desiccator, and weigh. The difference between the final weight and the weight of the initially ignited portion represents the weight of silica.

Acceptance criteria: The weight of silica is NMT 0.6%.

• ‣ **LEAD** ⟨251⟩: NMT 10 ppm.‣

SPECIFIC TESTS

• **PH** ⟨791⟩: 5.0–8.0, in a solution (1 in 100), prepared by evenly distributing the powder into boiling carbon dioxide-free water and stirring the mixture with a magnetic stirrer

• **LOSS ON DRYING** ⟨731⟩

Analysis: Dry at 105° for 4 h.

Acceptance criteria: NMT 5.0%

• ‣ **VISCOSITY—ROTATIONAL METHODS** ⟨912⟩: Determine the apparent viscosity at the concentration and temperature specified on the label with a suitable rotational viscometer (see *Labeling*).‣

ADDITIONAL REQUIREMENTS

• ‣**PACKAGING AND STORAGE:** Store in well-closed containers.‣

• ‣**LABELING:** Label it to indicate the viscosity in an aqueous solution of stated concentration and temperature. The indicated viscosity may be in the form of a range encompassing 50%–150% of the average value. Suitable anti-caking agents, such as silica, should be stated on the label.‣

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Hydroxypropyl Cellulose RS

Low-Substituted Hydroxypropyl Cellulose

Add the following:

▲Portions of this monograph that are national *USP* text, and are not part of the harmonized text, are marked with symbols (‣‣) to specify this fact.▲ (NF 1-May-2019)

Add the following:

▲Cellulose, 2-hydroxypropyl ether [9004-64-21].▲ (NF 1-May-2019)

Change to read:

DEFINITION

Low-Substituted Hydroxypropyl Cellulose is a low-substituted ▲*O*-(2-hydroxypropylated)▲ (NF 1-May-2019) cellulose. ▲It▲ (NF 1-May-2019) contains NLT 5.0% and NMT 16.0% of hydroxypropoxy groups (–$OCH_2CHOHCH_3$), ▲calculated on the dried basis.▲ (NF 1-May-2019)

IDENTIFICATION

Delete the following:

▲• **A.**

Sample: 20 mg

Analysis: Shake the *Sample* with 2 mL of water, and cautiously add 1 mL of a solution of anthrone in sulfuric acid (350 µg/mL).

Acceptance criteria: A blue to greenish-blue color develops at the zone of contact.▲ (NF 1-May-2019)

Change to read:

▲• **A.** ▲**SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K.**▲ (CN 1-May-2020) Meets the requirements▲ (NF 1-May-2019)

Change to read:

• **B.**

Sample: 0.1 g

Analysis: Shake the *Sample* thoroughly with 10 mL of water.▲▲ (NF 1-May-2019)

Acceptance criteria: ▲It does not dissolve.▲ (NF 1-May-2019)

Change to read:

• **C.**

Sample solution: ▲To the suspension obtained in *Identification B* add 1 g of sodium hydroxide and shake until it becomes homogeneous.▲ (NF 1-May-2019)

Analysis: ▲Transfer 5 mL of *Sample solution* to a suitable container, add▲ (NF 1-May-2019) 10 mL of a mixture of acetone and methanol (4:1), ▲and shake.▲ (NF 1-May-2019)

Acceptance criteria: A white, flocculent precipitate is formed.

ASSAY

Change to read:

• ▲**HYDROXYPROPOXY GROUPS**▲ (NF 1-May-2019)

[**CAUTION**—Hydriodic acid and its reaction byproducts are highly toxic. Perform all steps of the *Standard solution* and the *Sample solution* in a properly functioning hood. Specific safety practices to be followed are to be identified to the analyst performing this test.]

Apparatus: For the reaction vial, use a 5-mL pressure-tight serum vial, 50 mm in height, 20 mm in outside diameter, and 13 mm in inside diameter at the mouth. The vial is equipped with a pressure-tight septum with a polytetrafluoroethylene-faced butyl rubber and an air-tight seal using an aluminum crimp or any sealing system that provides sufficient air-tightness. Use a heater with a heating module that has a square-shape aluminum block with holes 20 mm in diameter and 32 mm in depth, into which the reaction vial fits. The heating module is also equipped with a magnetic stirrer capable of mixing the contents of the vial, or use a reciprocal shaker that performs a reciprocating motion of approximately 100 times/min.

Hydriodic acid: Use a reagent with a typical concentration of hydrogen iodide (HI) of about 57%.

Internal standard solution: 30 mg/mL of *n*-octane in *o*-xylene

Standard solution: Into a suitable serum vial, weigh between 60 and 100 mg of adipic acid, and add 2.0 mL of *Hydriodic acid* and 2.0 mL of *Internal standard solution* into the vial. Close the vial securely with a suitable septum stopper. Weigh the vial and contents, add 15–22 µL of isopropyl iodide through the septum with a syringe, weigh again, and calculate the weight of isopropyl iodide added, by difference. ▲Shake the reaction vial well, and use▲ (NF 1-May-2019) the upper layer as the *Standard solution*.

NF Monographs

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Sample solution: Transfer 0.065 g of ▲▲ (NF 1-May-2019) Low-Substituted Hydroxypropyl Cellulose to a 5-mL, thick-walled reaction vial equipped with a pressure-tight septum-type closure, add between 60 and 100 mg of adipic acid, and pipet 2.0 mL of *Internal standard solution* into the vial. Cautiously pipet 2.0 mL of *Hydriodic acid* into the mixture, immediately cap the vial tightly, and weigh. Using the magnetic stirrer equipped in the heating module, or using a reciprocal shaker, mix the contents of the vial continuously, heating and maintaining the temperature of the contents at 130 ± 2° for 60 min. If a reciprocal shaker or magnetic stirrer cannot be used, shake the vial well by hand at 5-min intervals during the initial 30 min of the heating time. Allow the vial to cool, and weigh. ▲If the weight loss is less than 26 mg and there is no evidence of a leak, use the upper layer of the mixture as the *Sample solution*.▲ (NF 1-May-2019)

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Thermal conductivity or hydrogen flame ionization

Column: ▲0.53-mm × 30-m fused silica capillary, coated with a 3-µm layer of phase G1. Use a guard column if necessary.

Temperatures

Detector: 280°

Injection port: 250°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
50	0	50	3
50	10	100	—
100	34.9	250	8▲ (NF 1-May-2019)

Carrier gas: Helium▲▲ (NF 1-May-2019)

Flow rate: With the *Standard solution*, adjust the flow rate so that the retention time of the internal standard is about 10 min ▲(about 4.3 mL/min). [NOTE—The relative retention time for isopropyl iodide (with reference to the *n*-octane) is about 0.8.]▲ (NF 1-May-2019)

Injection volume: 1–2 µL

▲Injection type: Split; split ratio, 40:1

Run time: 20.3 min

System suitability

Sample: *Standard solution*

Suitability requirements

Resolution: NLT 5 between isopropyl iodide and *n*-octane

Relative standard deviation: NMT 2.0%, using the peak area ratio between isopropyl iodide and the internal standard for 6 injections▲ (NF 1-May-2019)

Analysis

Samples: Upper layer of the *Standard solution* and the *Sample solution*

Calculate the percentage of hydroxypropoxy ▲▲ (NF 1-May-2019) in the sample taken:

$$\text{Result} = (Q_{Tb}/Q_{Sb}) \times (W_{Sb}/W_U) \times 44.17$$

Q_{Tb} = ratio of the peak area of isopropyl iodide to *n*-octane in the *Sample solution*

Q_{Sb} = ratio of the peak area of isopropyl iodide to *n*-octane in the *Standard solution*

W_{Sb} = weight of isopropyl iodide in the *Standard solution* (mg)

W_U = weight of Low-Substituted Hydroxypropyl Cellulose calculated on the dried basis, taken for the *Sample solution* (mg)

▲44.17 = molar mass of hydroxypropoxy group/molar mass of isopropyl iodide × 100▲ (NF 1-May-2019)

Acceptance criteria: 5.0%–16.0% on the dried basis

IMPURITIES

Change to read:

• **RESIDUE ON IGNITION** ⟨281⟩

▲Sample: 1.0 g

Acceptance criteria: NMT 0.8%▲ (NF 1-May-2019)

Change to read:

• ▲•▲ (NF 1-May-2019) **CHLORIDE AND SULFATE** ⟨221⟩, *Chloride*

Sample solution: Shake 0.50 g of Low-Substituted Hydroxypropyl Cellulose thoroughly with 30 mL of boiling water, heat on a water bath for 10 min, and filter the supernatant by decantation while hot. Wash the residue thoroughly with 50 mL of boiling water, combine the washings with the filtrate, and add water to make 100 mL after cooling.

Control solution: 0.25 mL of 0.02 N hydrochloric acid

Analysis: Using 10 mL of the *Sample solution* and the *Control solution*, proceed as directed in the chapter, starting with the addition of the nitric acid.

Acceptance criteria: NMT 0.36%; the *Sample solution* shows no more chloride than the *Control solution*.▲•▲ (NF 1-May-2019)

SPECIFIC TESTS

Add the following:

▲• **pH** ⟨791⟩

Sample solution: 10 mg/mL suspension, prepared by evenly distributing 1.0 g of the powder with 100 mL of carbon dioxide-free water and stirring the mixture with a magnetic stirrer

Acceptance criteria: 5.0–7.5▲ (NF 1-May-2019)

Change to read:

• **LOSS ON DRYING** ⟨731⟩

▲Sample: 1 g▲ (NF 1-May-2019)

Analysis: Dry the *Sample* at 105° for 1 h.

Acceptance criteria: NMT 5.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers.

Add the following:

▲• **USP REFERENCE STANDARDS**

USP Low-Substituted Hydroxypropyl Cellulose RS▲ (NF 1-May-2019)

Hydroxypropyl Methylcellulose—*see Hypromellose General Monographs*

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Hymetellose

Methylhydroxyethylcellulose;
Cellulose 2-hydroxyethyl methyl ether [9032-42-2].

DEFINITION
Hymetellose is a partly *O*-(methylated) and *O*-(2-hydroxyethylated) cellulose.

IDENTIFICATION
• **A.**
Sample solution: Use the *Sample solution* prepared in the test for *Color of Solution*.
Analysis: Heat the *Sample solution* in a water bath while stirring.
Acceptance criteria: At a temperature above 50°, the solution becomes cloudy or a flocculent precipitate is formed. The solution becomes clear again on cooling.
• **B.**
Sample solution: 1 mL of the solution from *Identification* test *A*
Analysis: Transfer the *Sample solution* to a glass plate, and allow the water to evaporate.
Acceptance criteria: A thin film is formed.
• **C.**
Sample solution: 10 mL of the solution from *Identification* test *A*
Analysis: To the *Sample solution* add 0.3 mL of 2 N acetic acid and 2.5 mL of tannic acid TS.
Acceptance criteria: A yellowish-white, flocculent precipitate is formed that dissolves in ammonia TS.
• **D.**
Sample: 1 g
Diethanolamine–sodium nitroprusside solution: 50 mg/mL of sodium nitroprusside solution adjusted with 1 N hydrochloric acid to a pH of 9.8. Mix 11 mL of this solution with 1 mL of a diethanolamine solution (200 mg/mL) in water.
Analysis: In a test tube about 160 mm long, thoroughly mix the *Sample* with 2 g of finely powdered manganese sulfate. Introduce, to a depth of 2 cm into the upper part of the tube, a strip of filter paper impregnated with a freshly prepared *Diethanolamine–sodium nitroprusside solution*. Insert the tube 8 cm into a silicone-oil bath at 190°–200°. Perform a blank test without the addition of Hymetellose.
Acceptance criteria: The filter paper becomes blue within 10 min.
• **E.**
Sample: 0.2 g
Analysis: Dissolve the *Sample* completely, without heating, in 15 mL of 70% sulfuric acid. Pour the solution while stirring into 100 mL of ice water, and dilute with ice water to 250 mL. Transfer 1 mL of this solution to a test tube, and while cooling in ice water, add dropwise 8 mL of sulfuric acid, and mix thoroughly. Heat in a water bath for exactly 3 min, and immediately cool in ice water. While the mixture is cold, carefully add 0.6 mL of ninhydrin TS, and mix well. Allow to stand at 25°.
Acceptance criteria: A pink color is produced immediately and does not become violet within 100 min.

IMPURITIES
• **Residue on Ignition** ⟨281⟩
Sample: 1.0 g
Acceptance criteria: NMT 1.0%

Change to read:

• **Chloride and Sulfate,** *Chloride* ⟨221⟩
Sample solution: Dilute 1.0 mL of the *Sample solution* from the test for *Color of Solution* with water to 20 mL, and add 1 mL of nitric acid and 1 mL of silver nitrate TS. Mix, and allow to stand for 5 min, protected from direct sunlight.
Control solution: Dilute 0.71 mL of 0.020 N hydrochloric acid to 100 mL. Mix 10 mL of this solution with water to 20 mL, and add 1 mL of nitric acid and 1 mL of silver nitrate TS. Mix, and allow to stand for 5 min, protected from direct sunlight.
Analysis
(See ▲*Visual Comparison* ⟨630⟩▲ (CN 1-May-2019).)
Compare the turbidity of the *Sample solution* and *Control solution*, if any.
Acceptance criteria: 0.5%. Any turbidity produced by the *Sample solution* does not exceed that of the *Control solution*.

SPECIFIC TESTS
• **pH** ⟨791⟩
Sample solution: Use the *Sample solution* from the test for *Color of Solution*.
Acceptance criteria: 5.5–8.0
• **Loss on Drying** ⟨731⟩
Sample: 1.0 g
Analysis: Dry the *Sample* at 105° to constant weight.
Acceptance criteria: NMT 10.0%
• **Viscosity—Rotational Methods** ⟨912⟩
Sample: An amount equivalent to 6.0 g of dried Hymetellose
Analysis: While stirring, add the *Sample* to 150 g of carbon dioxide-free water heated to 90°. Stir with a propeller-type stirrer for 10 min, place the flask in a bath of ice water, continue the stirring, and allow to remain in the bath of ice water for 40 min to ensure that dissolution is complete. Adjust the mass of the solution to 300 g, and centrifuge the solution to expel any entrapped air. Adjust the temperature of the solution to 20 ± 0.1°, and determine the viscosity using a rotational viscometer with a shear rate of 10/s.
Acceptance criteria: The apparent viscosity is 75%–140% of the value stated on the label.
• **Color of Solution**
Diluent: 27.5 mL of hydrochloric acid in 1000 mL of water
Standard solution: Prepare immediately before use. Mix 2.4 mL of ferric chloride CS and 0.6 mL of cobaltous chloride CS with *Diluent* to make 10 mL, and dilute 5 mL of this solution with *Diluent* to make 100 mL.
Sample solution: While stirring, add a portion equivalent to 1.0 g of dried Hymetellose to 50 g of carbon dioxide-free water heated to 90°. Allow to cool, adjust the weight of the solution to 100 g with carbon dioxide-free water, and stir until dissolution is complete.
Analysis: Make the comparison by viewing the substance and the solution downward in matched color-comparison tubes against a white surface (see *Color and Achromicity* ⟨631⟩).
Acceptance criteria: The *Sample solution* is not more intensely colored than the *Standard solution*.

Change to read:

• **Clarity of Solution**
Hydrazine sulfate solution: 10 mg/mL of hydrazine sulfate. Allow to stand for 4–6 h before use.
[**Caution**—Hydrazine sulfate is highly toxic. Avoid skin contact.]
Methenamine solution: Transfer 2.5 g of methenamine to a 100-mL glass-stoppered flask, add 25.0 mL of water, insert the glass stopper, and mix to dissolve.

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Primary opalescent mixture: To the flask containing *Methenamine solution* add 25.0 mL of *Hydrazine sulfate solution*, mix, and allow to stand for 24 h. This suspension is stable for 2 months. Mix before use, and do not use if it adheres to the container.
Opalescence standard: Dilute 15.0 mL of *Primary opalescent mixture* with water to 1000.0 mL. Use this suspension within 24 h after preparation.
Reference suspension: Transfer 30.0 mL of *Opalescence standard* to a 100-mL volumetric flask, and dilute with water to volume.
Sample solution: Use the solution from the test for *Color of Solution*.
Analysis: Transfer a sufficient portion of the *Sample solution* to a test tube of colorless, transparent, neutral glass with a flat base and an internal diameter of 15–25 mm to obtain a depth of 40 mm. Similarly transfer a portion of the *Reference suspension* to a separate matching test tube. Compare the *Sample solution* and the *Reference suspension* in diffused daylight, viewing vertically against a black background (see ▲*Visual Comparison* ⟨630⟩▲ (CN 1-May-2019)) 5 min after preparation of the *Reference suspension*.
Acceptance criteria: The *Sample solution* is not more opalescent than the *Reference suspension*.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements are specified.
- **LABELING:** Label it to indicate the viscosity of the solution (1 in 50) at 20°.

Hypophosphorous Acid

H_3PO_2 66.00
Phosphinic acid;
Hypophosphorous acid [6303-21-5].

DEFINITION
Hypophosphorous Acid contains NLT 30.0% and NMT 32.0% of H_3PO_2.

IDENTIFICATION
- **A.** Hypophosphorous Acid yields a white precipitate with mercuric chloride TS. This precipitate becomes gray when an excess of hypophosphite is present.
- **B.** Hypophosphorous Acid acidified with sulfuric acid and warmed with cupric sulfate TS yields a red precipitate.

ASSAY
- **PROCEDURE**

Sample solution: Pour 7 mL of Hypophosphorous Acid into a tared, glass-stoppered flask, and weigh. Dilute with about 25 mL of water, and add phenolphthalein TS.
Analysis: Titrate with 1 N sodium hydroxide VS. Each mL of 1 N sodium hydroxide is equivalent to 66.00 mg of H_3PO_2.
Acceptance criteria: 30.0%–32.0%

SPECIFIC TESTS
- **LIMIT OF BARIUM AND OXALATE**

Sample solution: Hypophosphorous Acid and water (1:3)
Analysis 1: Neutralize 30 mL of the *Sample solution* with 6 N ammonium hydroxide: the mixture exhibits little or no precipitation. Filter, acidify 10 mL of the filtrate with hydrochloric acid, and add 2 mL of potassium sulfate TS.
Acceptance criteria 1: No turbidity is produced (barium).
Analysis 2: To a 10-mL portion of the filtrate obtained in *Analysis 1*, add 1 mL of calcium chloride TS.
Acceptance criteria 2: The filtrate shows no turbidity upon the addition of the calcium chloride TS (oxalate).

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers.

Hypromellose Acetate Succinate

Hydroxypropyl methylcellulose acetate succinate;
Cellulose, 2-hydroxypropyl methyl ether, acetate hydrogen butanedioate;
Cellulose, 2-hydroxypropyl methyl ether, acetate succinate [71138-97-1].

DEFINITION
Hypromellose Acetate Succinate is a mixture of acetic acid and monosuccinic acid esters of hydroxypropyl methylcellulose. It contains NLT 12.0% and NMT 28.0% of methoxy groups ($-OCH_3$), NLT 4.0% and NMT 23.0% of hydroxypropoxy groups ($-OCH_2CHOHCH_3$), NLT 2.0% and NMT 16.0% of acetyl groups ($-COCH_3$), and NLT 4.0% and NMT 28.0% of succinoyl groups ($-COC_2H_4COOH$), calculated on the dried basis.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197A▲** (CN 1-May-2020)

Sample: Neat. Do not dry specimen.
Analysis: Use a Fourier transform IR spectrophotometer fitted with a suitable accessory for single bounce attenuated total reflectance (see *Mid-Infrared Spectroscopy* ⟨854⟩) with a diamond or germanium crystal. Acquire a background single-beam spectrum with a clean diamond or germanium crystal sampling plate in place. Place the sample on the diamond or germanium crystal sampling surface with a microspatula or equivalent. For best results, the sample should cover the crystal surface under the pressure point tip. Using the pressure device, apply pressure to the sample, making sure the sample remains centered under the pressure tip. Acquire a single-beam spectrum of the sample, and make the necessary corrections for the background. Release the pressure device, and clear it from the sample area. Wipe the sample off the crystal and pressure device tip, and rinse both with acetone.
Acceptance criteria: The IR spectrum of the *Sample* exhibits maxima only at the same wavelengths as a similarly obtained spectrum of USP Hypromellose Acetate Succinate RS.

ASSAY
- **ACETYL AND SUCCINOYL GROUPS**

Phosphoric acid solution: 1.25 M phosphoric acid and water (2:98)
Buffer: 2.72 g/L of monobasic potassium phosphate
Diluent: Adjust the *Buffer* with 1 N sodium hydroxide to a pH of 7.5.
Acetic acid stock solution: Add approximately 20 mL of water to a stoppered, 100-mL volumetric flask, place the flask on a balance, and tare. Transfer 2.0 mL of glacial acetic acid to the flask, and record the weight of the acid added. Fill the flask with water to volume. Transfer 6 mL of the resulting solution into a 100-mL volumetric flask, and dilute with water to volume.
Succinic acid stock solution: 1.3 mg/mL of succinic acid

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Mobile phase: Adjust the *Buffer* to a pH of 2.8 by the dropwise addition of 6 M phosphoric acid. Pass through a 0.22-µm nylon filter.
Standard solution 1: Transfer 4.0 mL of the *Acetic acid stock solution* and 4.0 mL of the *Succinic acid stock solution* to a 25-mL volumetric flask. Dilute with *Mobile phase* to volume, and mix.
Standard solution 2: Prepare as directed for *Standard solution 1*. This solution is prepared as a duplicate.
Sample solution: Weigh 12.4 mg of Hypromellose Acetate Succinate into a glass vial. Transfer 4.0 mL of 1.0 N sodium hydroxide to the vial, and stir the solution for 4 h. Then, add 4.0 mL of 1.25 M phosphoric acid to the same vial to bring the pH of the solution to 3 or less. Invert the test *Sample solution* vial several times to ensure complete mixing, and pass through a filter of 0.22-µm pore size. Use the clear filtrate.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 215 nm
Column: 4.6-mm × 15-cm; 5-µm packing L1
Column temperature: 20°–30°
Flow rate: 1 mL/min
Run time: 15 min
Injection volume: 10 µL
System suitability
Samples: *Standard solution 1* and *Standard solution 2*
Suitability requirements
Column efficiency: NLT 8000 theoretical plates, determined from the succinic acid peak, *Standard solution 1*
Tailing factor: 0.9–1.5 for the succinic acid peak, *Standard solution 1*
Relative standard deviation: NMT 2.0% for each peak from six replicate injections, *Standard solution 1*
Peak difference: The difference in peak areas between *Standard solution 1* and *Standard solution 2* for both acetic and succinic acids peaks does not exceed 2%.
[NOTE—After each run sequence, the column should be flushed first by 50% water and 50% acetonitrile for 60 min and then by 100% methanol for 60 min. The column should be stored in 100% methanol.]
Analysis
Samples: *Standard solution 1* and *Sample solution*
Calculate the percentage of acetic acid, *A*, in the portion of Hypromellose Acetate Succinate taken:

$$A = (r_{UA}/r_{SA}) \times (C_A/C_U) \times 100$$

r_{UA} = peak response for acetic acid from the *Sample solution*
r_{SA} = peak response for acetic acid from *Standard solution 1*
C_A = concentration of acetic acid in *Standard solution 1* (mg/mL)
C_U = concentration of Hypromellose Acetate Succinate in the *Sample solution* (mg/mL)

Calculate the percentage of acetyl groups ($-COCH_3$) in the portion of Hypromellose Acetate Succinate taken:

$$\text{Result} = (A - A_{free}) \times (M_{r1}/M_{r2})$$

A = defined above
A_{free} = percentage of free acetic acid, as determined in the test for *Limit of Free Acetic and Succinic Acids*
M_{r1} = molecular weight of the acetyl group, 43.04
M_{r2} = molecular weight of acetic acid, 60.05

Calculate the percentage of succinic acid, *S*, in the portion of Hypromellose Acetate Succinate taken:

$$S = (r_{US}/r_{SS}) \times (C_S/C_U) \times 100$$

r_{US} = peak response for succinic acid from the *Sample solution*
r_{SS} = peak response for succinic acid from *Standard solution 1*
C_S = concentration of succinic acid in *Standard solution 1* (mg/mL)
C_U = concentration of Hypromellose Acetate Succinate in the *Sample solution* (mg/mL)

Calculate the percentage of succinoyl groups ($-COC_2H_4COOH$) in the portion of Hypromellose Acetate Succinate taken:

$$\text{Result} = (S - S_{free}) \times (M_{r1}/M_{r2})$$

S = defined above
S_{free} = percentage of free succinic acid, as determined in the test for *Limit of Free Acetic and Succinic Acids*
M_{r1} = molecular weight of the succinoyl group, 101.08
M_{r2} = molecular weight of succinic acid, 118.09

Acceptance criteria
Acetyl groups ($-COCH_3$): 2.0%–16.0% on the dried basis
Succinoyl groups ($-COC_2H_4COOH$): 4.0%–28.0% on the dried basis

• **CONTENT OF METHOXY AND 2-HYDROXYPROPOXY GROUPS**
[**CAUTION**—Hydriodic acid and its reaction byproducts are highly toxic. Perform all steps in the preparation of the *Sample solution* and the *Standard solution* in a properly functioning hood. Specific safety practices to be followed are to be identified to the analyst performing this test.]
Hydriodic acid: Use a reagent having a specific gravity of at least 1.69, equivalent to 55% hydrogen iodide.
Solution A: Methanol and water (10:90)
Solution B: Methanol and water (85:15)
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	70	30
8	40	60
10	15	85
17	15	85

[NOTE—These gradient elution times are established on an HPLC system with a dwell volume of approximately 2.0 mL. The injection time can be adjusted relative to the start of a run to accommodate the change in dwell volume from one HPLC system to another to achieve the separation described.]
Standard stock solution: Transfer 2 mL of *o*-xylene into a stoppered, 10-mL volumetric flask, place the flask on a balance, and tare. Add 200 µL of methyl iodide, insert the stopper into the flask, and accurately weigh: the weight of methyl iodide is about 350 mg. Tare the flask again, add 34 µL of isopropyl iodide, and weigh the flask: the recorded weight of isopropyl iodide is 50 mg. Dilute with *o*-xylene to volume, and mix.
Standard solution: Transfer 85 mg of adipic acid into an 8-mL vial (or other suitable container), add 2 mL of *Hydriodic acid*, and add 2.0 mL of the *Standard stock*

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solution. Shake and allow the phases to separate. Carefully transfer approximately 1.5 mL of the *o*-xylene (top) layer to a small vial, making sure that the bottom aqueous layer is not disturbed. Transfer 1.0 mL of the resulting solution to a 10-mL volumetric flask, and dilute with methanol to volume. [NOTE—This solution is stable for 8 h at 5°.]

Sample solution: [**CAUTION**—Use a cap that has a top safety relief valve, such as a Minniert valve, to prevent accidental explosion of the vial under high pressure when heated.] Weigh 65 mg of Hypromellose Acetate Succinate into a 5-mL reaction vial, and add 2.0 mL of *o*-xylene and about 100 mg of adipic acid. Add 2.0 mL of *Hydriodic acid*, and close the vial tightly with a cap.

Weigh the vial before heating, and place the vial into a heating block at 150°. Shake the vial after 5 min and after 30 min of heating. Remove the vial from the heating block after 1 h of heating, and cool. Weigh the vial. If the weight loss is greater than 10 mg, discard the mixture, and prepare another reaction solution. Carefully transfer approximately 1.5 mL of the top *o*-xylene layer into a small glass vial, making sure that the bottom aqueous layer is not disturbed. Transfer 1.0 mL of this solution into a 10-mL volumetric flask, and dilute with methanol to volume. [NOTE—This solution is stable for 8 h at 5°.]

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Column temperature: 30°

Flow rate: 1 mL/min

Injection volume: 10 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Column efficiency: NLT 10,000 theoretical plates, determined from the methyl iodide peak

Tailing factor: 0.9–1.5 for the methyl iodide peak

Relative standard deviation: NMT 2.0% for each peak

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of methoxy groups ($-OCH_3$) in the portion of Hypromellose Acetate Succinate taken:

$$\text{Result} = (r_{UM}/r_{SM}) \times (C_S/C_U) \times (M_{r1}/M_{r2})$$

r_{UM} = peak response for methyl iodide from the *Sample solution*

r_{SM} = peak response for methyl iodide from the *Standard solution*

C_S = concentration of methyl iodide in the *Standard solution* (mg/mL)

C_U = concentration of Hypromellose Acetate Succinate in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of the methoxy group, 31.03

M_{r2} = molecular weight of methyl iodide, 141.94

Calculate the percentage of 2-hydroxypropoxy groups ($-OCH_2CHOHCH_3$) in the portion of Hypromellose Acetate Succinate taken:

$$\text{Result} = (r_{UI}/r_{SI}) \times (C_S/C_U) \times (M_{r1}/M_{r2})$$

r_{UI} = peak response for isopropyl iodide from the *Sample solution*

r_{SI} = peak response for isopropyl iodide from the *Standard solution*

C_S = concentration of isopropyl iodide in the *Standard solution* (mg/mL)

C_U = concentration of Hypromellose Acetate Succinate in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of the 2-hydroxypropoxy group, 75.09

M_{r2} = molecular weight of isopropyl iodide, 169.99

Acceptance criteria

Methoxy groups ($-OCH_3$): 12.0%–28.0% on the dried basis

Hydroxypropoxy groups ($-OCH_2CHOHCH_3$): 4.0%–23.0% on the dried basis

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩

Analysis: Determine at 600 ± 50°.

Acceptance criteria: NMT 0.20%

• **LIMIT OF FREE ACETIC AND SUCCINIC ACIDS**

Phosphoric acid solution, Buffer, Diluent, Acetic acid stock solution, Succinic acid stock solution, Mobile phase, Standard solution, and **Chromatographic system:** Proceed as directed in the *Assay* for *Acetyl and Succinoyl Groups*.

Sample solution: Weigh 102 mg of Hypromellose Acetate Succinate into a glass vial. Transfer 4.0 mL of *Diluent* to the vial, and stir the content for 2 h. Then, transfer 4.0 mL of the *Phosphoric acid solution* to the same vial to bring the pH of the *Sample solution* to 3 or less. Invert the vial several times to ensure complete mixing, centrifuge, and use the clear supernatant.

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of free acetic acid, A_{free}, in the portion of Hypromellose Acetate Succinate taken:

$$A_{free} = (r_{UA}/r_{SA}) \times (C_A/C_U) \times 100$$

r_{UA} = peak response for acetic acid from the *Sample solution*

r_{SA} = peak response for acetic acid from the *Standard solution*

C_A = concentration of acetic acid in the *Standard solution* (mg/mL)

C_U = concentration of Hypromellose Acetate Succinate in the *Sample solution* (mg/mL)

Calculate the percentage of free succinic acid, S_{free}, in the portion of Hypromellose Acetate Succinate taken:

$$S_{free} = (r_{US}/r_{SS}) \times (C_S/C_U) \times 100$$

r_{US} = peak response for succinic acid from the *Sample solution*

r_{SS} = peak response for succinic acid from the *Standard solution*

C_S = concentration of succinic acid in the *Standard solution* (mg/mL)

C_U = concentration of Hypromellose Acetate Succinate in the *Sample solution* (mg/mL)

Acceptance criteria: The sum of free acetic acid and free succinic acid is NMT 1.0%.

SPECIFIC TESTS

• **LOSS ON DRYING** ⟨731⟩

Analysis: Dry at 105° for 1 h.

Acceptance criteria: NMT 5.0%

• **VISCOSITY—CAPILLARY METHODS** ⟨911⟩

Sodium hydroxide solution: Immediately before use, prepare 4.3 mg/mL of sodium hydroxide in carbon dioxide-free water.

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Analysis: To 2.00 g of Hypromellose Acetate Succinate, previously dried, add *Sodium hydroxide solution* to make 100.0 g, insert a stopper into the vessel, and dissolve by constant shaking for 30 min. Adjust the temperature of the solution to 20 ± 0.1°, and determine the viscosity in a suitable viscometer.

Acceptance criteria: 80%–120% of that stated on the label

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.
- **LABELING:** Label it to indicate its nominal viscosity type.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Hypromellose Acetate Succinate RS

Hypromellose Phthalate

Add the following:

▲Portions of this monograph that are national *USP* text, and are not part of the harmonized text, are marked with symbols (•▲) to specify this fact.▲ (NF 1-May-2019)

DEFINITION

Hypromellose Phthalate is a monophthalic acid ester of hydroxypropyl methylcellulose. It contains methoxy ($-OCH_3$), hydroxypropoxy ($-OCH_2CHOHCH_3$), and phthalyl (*o*-carboxybenzoyl; $C_8H_5O_3$) groups. It contains NLT 21.0% and NMT 35.0% of phthalyl groups, calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

- •▲▲ (NF 1-May-2019) **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K.**▲ (CN 1-May-2020) Do not dry specimens.▲•▲ (NF 1-May-2019)

ASSAY

Change to read:

- **PHTHALYL CONTENT**

  Sample: 1 g

  Analysis: Transfer the *Sample* to a conical flask, dissolve in 50 mL of a mixture of alcohol, acetone, and water (2:2:1), add phenolphthalein TS, and titrate with 0.1 N sodium hydroxide VS. Perform a blank determination (see *Titrimetry* ⟨541⟩).

  Calculate the percentage of phthalyl ▲groups in the portion of Hypromellose Phthalate▲ (NF 1-May-2019) taken:

$$\text{Result} = [0.01 \times M_{r1} \times (V/W)] - [2 \times (M_{r1}/M_{r2}) \times P]$$

M_{r1} = molecular weight of the phthalyl group, 149.1
V = volume of 0.1 N sodium hydroxide consumed after correction for the blank (mL)
W = weight of Hypromellose Phthalate taken, calculated on the anhydrous basis (g)
M_{r2} = molecular weight of phthalic acid, 166.1
P = percentage of free phthalic acid found as directed in the test for *Limit of Free Phthalic Acid*

Acceptance criteria: 21.0%–35.0% ▲▲ (NF 1-May-2019) on the anhydrous basis

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.20%

Change to read:

- **CHLORIDE AND SULFATE** ⟨221⟩, *Chloride*

  Sample solution: Dissolve 1.0 g in 40 mL of 0.2 N sodium hydroxide, add 1 drop of phenolphthalein TS, and add 2 N nitric acid dropwise, with stirring, until the red color is discharged. Add an additional 20 mL of 2 N nitric acid with stirring. Heat on a water bath, with stirring, until the gel-like precipitate formed becomes granular. Cool the mixture, and centrifuge. Separate the liquid phase, and wash the residue with three successive 20-mL portions of water, separating the washings by centrifuging. Dilute the combined liquids with water to 200 mL, mix, and filter.

  Standard solution: Treat 0.50 mL of 0.01 N hydrochloric acid with 10 mL of 0.2 N sodium hydroxide, add 7 mL of 2 N nitric acid, and dilute with water to 50 mL.

  ▲Analysis: Add 1 mL of silver nitrate TS to the *Standard solution*. ▲Add 1 mL of silver nitrate TS to a 50-mL portion of the *Sample solution*. After mixing, allow each solution to stand for 5 min protected from direct sunlight.▲ (ERR 1-May-2019) Compare the turbidity of the solutions.▲ (NF 1-May-2019)

  Acceptance criteria: A 50-mL portion of the *Sample solution* shows no more chloride than the *Standard solution* (0.07%).

Change to read:

- **LIMIT OF FREE PHTHALIC ACID**

  Mobile phase: ▲0.1% trifluoroacetic acid and acetonitrile (90:10)▲ (NF 1-May-2019)

  Standard solution: ▲Weigh accurately and transfer▲ (NF 1-May-2019) 12.5 mg of phthalic acid to a 250-mL volumetric flask, and add 125 mL of acetonitrile. Add 25 mL of water, and dilute with acetonitrile to volume.

  Sample solution: ▲Weigh accurately and transfer▲ (NF 1-May-2019) 200 mg of Hypromellose Phthalate to a 100-mL volumetric flask. Add 50 mL of acetonitrile, and sonicate to dissolve partially. Add 10 mL of water, and sonicate to dissolve. Cool to room temperature, and dilute with acetonitrile to volume.

  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: LC
  Detector: UV 235 nm
  Column: 4.6-mm × 25-cm; packing L1 with a high carbon load
  Flow rate: 2 mL/min
  Injection volume: 10 µL

  System suitability
  Sample: *Standard solution*
  Suitability requirements
  Relative standard deviation: NMT 1.0% for replicate injections

  Analysis
  Samples: *Standard solution* and *Sample solution*
  Calculate the percentage of phthalic acid in the portion of Hypromellose Phthalate taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of phthalic acid from the *Sample solution*
r_S = peak response of phthalic acid from the *Standard solution*

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C_S = concentration of phthalic acid in the *Standard solution* (mg/mL)
C_U = concentration of Hypromellose Phthalate in the *Sample solution* (mg/mL)

Acceptance criteria: NMT 1.0%

SPECIFIC TESTS
- **WATER DETERMINATION** ⟨921⟩, *Method I*: NMT 5.0%
- **VISCOSITY—CAPILLARY METHODS** ⟨911⟩
  Sample solution: Dissolve 10 g, previously dried at 105° for 1 h, in 90 g of a mixture of methanol and methylene chloride (1:1 w/w) by mixing and shaking.
  Analysis: Determine the viscosity at 20 ± 0.1°.
  Acceptance criteria: 80%–120% of that indicated by the label

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers.

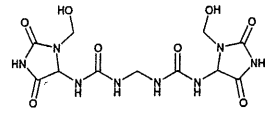
Change to read:

- ▲◆▲ (NF 1-May-2019) **LABELING:** Label it to indicate its viscosity and nominal phthalyl content.▲◆▲ (NF 1-May-2019)

Change to read:

- ▲◆▲ (NF 1-May-2019) **USP REFERENCE STANDARDS** ⟨11⟩
  USP Hypromellose Phthalate RS
  ▲◆▲ (NF 1-May-2019)

Imidurea



$C_{11}H_{16}N_8O_8$ 388.29
N,N″-Methylenebis[*N′*-[3-(hydroxymethyl)-2,5-dioxo-4-imidazolidinyl]urea];
1,1′-Methylenebis[3-[3-(hydroxymethyl)-2,5-dioxo-4-imidazolidinyl]urea] [39236-46-9].

DEFINITION
Imidurea contains NLT 26.0% and NMT 28.0% of nitrogen (N), calculated on the dried basis.

IDENTIFICATION

Change to read:

- **A.** ▲**SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

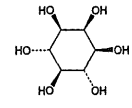
OTHER COMPONENTS
- **NITROGEN CONTENT**
  Sample: 150 mg
  Titrimetric system
  (See *Titrimetry* ⟨541⟩.)
  Mode: Direct titration
  Titrant: 0.1 N hydrochloric acid VS
  Endpoint detection: Visual
  Analysis: Place the *Sample* in a 500-mL Kjeldahl flask, and add 8.0 g of anhydrous sodium sulfate, 0.5 g of anhydrous cupric sulfate, 0.1 g of yellow mercuric oxide, and 11 mL of sulfuric acid. Incline the flask at an angle of 45°, and gently heat the mixture, keeping the temperature below the boiling point until frothing has ceased. Increase the heat until the acid boils briskly, and continue the heating until the solution has become clear green in color or practically colorless for 30 min. Allow to cool, cautiously add 225 mL of water, mix the contents of the flask, and again cool. Add cautiously 1.0 g of zinc metal dust, 2.0 g of sodium thiosulfate, and 18.0 g of sodium hydroxide pellets, and without delay connect the flask to a Kjeldahl connecting bulb (trap), previously attached to a condenser, the delivery tube of which dips beneath the surface of 50 mL of boric acid solution (40 mg/mL) in a 300-mL conical flask. Mix the contents of the Kjeldahl flask by gentle rotation, and distill 125 mL into the receiver. Add NLT 3 drops of methyl red–methylene blue TS to the contents of the receiving vessel, and determine the ammonia by titration with *Titrant*. Perform a blank determination, and make any necessary correction.
  Calculate the percentage of nitrogen in the portion of Imidurea taken. Each mL of 0.1 N hydrochloric acid is equivalent to 1.401 mg of nitrogen.
  Acceptance criteria: 26.0%–28.0% of nitrogen (N) on the dried basis

IMPURITIES
- **RESIDUE ON IGNITION** ⟨281⟩: NMT 3.0%

SPECIFIC TESTS
- **pH** ⟨791⟩
  Sample solution: 10 mg/mL
  Acceptance criteria: 6.0–7.5
- **LOSS ON DRYING** ⟨731⟩
  Analysis: Dry under vacuum over phosphorus pentoxide for 48 h.
  Acceptance criteria: NMT 3.0%
- **COLOR AND CLARITY OF SOLUTION**
  Sample: 3.0 g
  Analysis: Dissolve the *Sample* in 7.0 mL of water in a test tube.
  Acceptance criteria: The solution is clear and colorless.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Imidurea RS

Inositol



$C_6H_{12}O_6$ 180.16
cis-1,2,3,5-*trans*-4,6-Cyclohexanehexol;
myo-Inositol [87-89-8].

DEFINITION
Inositol contains NLT 97.0% and NMT 102.0% of Inositol ($C_6H_{12}O_6$), calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

- **A.** ▲**SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

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ASSAY

• **PROCEDURE**

Mobile phase: Water

System suitability solution: 0.05 mg/mL of USP Inositol RS and 0.05 mg/mL of USP Mannitol RS

Standard solution: 50 mg/mL of USP Inositol RS

Sample solution: 50 mg/mL of Inositol

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 7.8-mm × 30-cm or equivalent; packing L19

Temperature

Column: 85°

Detector: Constant temperature of 30°–35°

Flow rate: 0.5 mL/min

Injection volume: 10 µL

System suitability

Samples: *System suitability solution* and *Standard solution*

[NOTE—The relative retention times for inositol and mannitol are 1.0 and 1.3, respectively.]

Suitability requirements

Resolution: NLT 4.0 between inositol and mannitol, *System suitability solution*

Relative standard deviation: NMT 2.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

[NOTE—Record the chromatograms over a period of two times the retention time of inositol, and measure the peak responses.]

Calculate the percentage of Inositol ($C_6H_{12}O_6$) in the portion of sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of inositol from the *Sample solution*

r_S = peak response of inositol from the *Standard solution*

C_S = concentration of USP Inositol RS in the *Standard solution* (mg/mL)

C_U = concentration of Inositol in the *Sample solution* (mg/mL)

Acceptance criteria: 97.0%–102.0% on the anhydrous basis

IMPURITIES

• **BARIUM**

Sample solution: Use the *Sample solution* prepared in the test for *Clarity of Solution*. To 10 mL of the *Sample solution* add 1 mL of diluted sulfuric acid.

Acceptance criteria: When examined immediately and after 1 h, any opalescence in the solution is not more intense than that in a mixture of 1 mL of water and 10 mL of the *Sample solution* from the test for *Clarity of Solution*.

Change to read:

• **LIMIT OF LEAD**

Lead nitrate stock solution: Dissolve 159.8 mg of lead nitrate in 100 mL of water to which has been added 1 mL of nitric acid, then dilute with water to 1000 mL. Prepare and store this solution in glass containers free from soluble lead salts.

Standard lead solution: On the day of use, dilute 10.0 mL of the *Lead nitrate stock solution* with water to 100.0 mL. Each mL of the *Standard lead solution* contains the equivalent of 10 µg of lead. ▲▲ (ERR 1-May-2019)

Sample solution: Dissolve 20.0 g of Inositol in diluted acetic acid, and dilute with diluted acetic acid to 100 mL. Add 2.0 mL of a saturated ammonium pyrrolidinedithiocarbamate solution (containing about 10 g of ammonium pyrrolidinedithiocarbamate per L) and 10.0 mL of methyl isobutyl ketone, and shake for 30 s. Protect from bright light. Allow the two layers to separate, and use the methyl isobutyl ketone layer.

Blank solution: Prepare as directed for the *Sample solution*, except omit the use of Inositol.

Standard solutions: Prepare as directed for the *Sample solution*, except prepare three *Standard solutions* by adding 0.5, 1.0, and 1.5 mL, respectively, of the *Standard lead solution* in addition to the 20.0 g of Inositol to be examined.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption

Analytical wavelength: 283.3 nm

Lamp: Lead hollow-cathode

Flame: Air–acetylene

Analysis: Set the atomic absorption spectrometer to zero, using the *Blank solution*. Introduce the *Sample solution* and each of the three *Standard solutions* into the instrument, and record the steady absorbance reading. Plot the absorbance readings against the known concentrations of added lead (in µg), and draw a straight line. Extrapolate the line until it meets the concentration axis to obtain the concentration, in mg/kg, of lead in the sample.

Acceptance criteria: NMT 0.5 mg/kg

• **ORGANIC IMPURITIES**

Mobile phase, System suitability solution, and **Sample solution:** Proceed as directed in the *Assay*.

Standard solution: Transfer 2.0 mL of the *Standard solution*, prepared as directed in the *Assay*, to a 100-mL volumetric flask, and dilute with water to volume.

[NOTE—This solution contains 1 mg/mL of inositol.]

Chromatographic system: Proceed as directed in the *Assay*, except use an injection volume of 20 µL.

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of each impurity in the portion of Inositol taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of any impurity from the *Sample solution*

r_S = peak response of inositol from the *Standard solution*

C_S = concentration of USP Inositol RS in the *Standard solution* (mg/mL)

C_U = concentration of Inositol in the *Sample solution* (mg/mL)

Acceptance criteria

Individual impurities: NMT 0.3%

Total impurities: NMT 1.0%. [NOTE—Disregard any impurity peak that is less than 0.05%.]

SPECIFIC TESTS

Change to read:

• **CLARITY OF SOLUTION**

[NOTE—The *Sample solution* is to be compared to *Reference suspension A* in diffused daylight 5 min after preparation of *Reference suspension A*.]

Solution A: 10 mg/mL of hydrazine sulfate. Allow to stand for 4–6 h before use.

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Solution B: 100 mg/mL of methenamine prepared in a glass-stoppered flask.
Primary opalescent suspension: [NOTE—This suspension is stable for 2 months, provided it is stored in a glass container free from surface defects. The suspension must not adhere to the glass and must be well mixed before use.] *Solution A* and *Solution B* (1:1). Allow to stand for 24 h.
Opalescence standard: Transfer 15.0 mL of the *Primary opalescent suspension* to a 1000-mL volumetric flask, dilute with water to volume. [NOTE—This suspension should not be used beyond 24 h after preparation.]
Reference suspensions
Reference suspension A: Transfer 5.0 mL of the *Opalescence standard* to a 100-mL volumetric flask, and dilute with water to volume.
Reference suspension B: Transfer 10.0 mL of the *Opalescence standard* to a 100-mL volumetric flask, and dilute with water to volume.
Sample solution: 100 mg/mL of Inositol
Analysis: Transfer a sufficient portion of the *Sample solution* to a sample tube of colorless, transparent, neutral glass, with a flat base and an internal diameter of 15–25 mm, to obtain a depth of 40 mm. Similarly transfer portions of *Reference suspension A*, *Reference suspension B*, and water to separate matching sample tubes. Compare the *Sample solution*, *Reference suspension A*, *Reference suspension B*, and water in diffused daylight, viewing vertically against a black background (see ▲*Visual Comparison* ⟨630⟩▲ (CN 1-May-2019)). [NOTE—The diffusion of light must be such that *Reference suspension A* can readily be distinguished from water, and that *Reference suspension B* can readily be distinguished from *Reference suspension A*.]
Acceptance criteria: The *Sample solution* shows the same clarity as that of water.

Change to read:

• **COLOR OF SOLUTION**
Standard stock solutions: Prepare three solutions, *A*, *B*, and *C*, containing, respectively, the following parts of ferric chloride CS, cobaltous chloride CS, cupric sulfate CS, and diluted hydrochloric acid.
Standard stock solution A: 2.4:0.6:0:7.0
Standard stock solution B: 2.4:1.0:0.4:6.2
Standard stock solution C: 9.6:0.2:0.2:0
Standard solutions: [NOTE—Prepare the *Standard solutions* immediately before use.]
Standard solution A: Transfer 2.5 mL of *Standard stock solution A* to a 100-mL volumetric flask, dilute with diluted hydrochloric acid to volume, and mix.
Standard solution B: Transfer 2.5 mL of *Standard stock solution B* to a 100-mL volumetric flask, dilute with diluted hydrochloric acid to volume, and mix.
Standard solution C: Transfer 0.75 mL of *Standard stock solution C* to a 100-mL volumetric flask, dilute with diluted hydrochloric acid to volume, and mix.
Sample solution: Use the *Sample solution* prepared in *Clarity of Solution*.
Analysis: Transfer a sufficient portion of the *Sample solution* to a sample tube of colorless, transparent, neutral glass, with a flat base and an internal diameter of 15–25 mm, to obtain a depth of 40 mm. Similarly transfer portions of *Standard solution A*, *Standard solution B*, *Standard solution C*, and water to separate matching sample tubes. Compare the *Sample solution*, *Standard solution A*, *Standard solution B*, *Standard solution C*, and water in diffused daylight, viewing vertically against a white background (see ▲*Visual Comparison* ⟨630⟩▲ (CN 1-May-2019)).
Acceptance criteria: The *Sample solution* is not more intensely colored than *Standard solution A*, *Standard solution B*, *Standard solution C*, or water.
• **CONDUCTIVITY**
Sample solution: 0.2 g/mL of Inositol in water (previously boiled and cooled to room temperature).
Apparatus: Use a conductivity meter or a resistivity meter that measures the resistance of the column of liquid between the electrodes of the immersed measuring device. The apparatus is supplied with alternating current to avoid the effects of electrode polarization. It is equipped with a temperature compensation device or a precision thermometer.
Reagents: Prepare three *Standard solutions* of potassium chloride containing 0.7455, 0.0746, and 0.0149 g, respectively, of potassium chloride per 1000.0 g of solution. These solutions should be prepared with water that has been previously boiled and cooled to room temperature and whose conductivity does not exceed 2 µS/cm. The conductivity and resistivity of these three solutions at 20° are provided in *Table 1*.

Table 1

Concentration of Solution (g/1000.0 g)	Conductivity (µS/cm)	Resistivity (Ω-cm)
0.7455	1330	752
0.0746	133.0	7519
0.0149	26.6	37,594

Calibration: Choose a conductivity cell that is appropriate for the conductivity of the solution to be examined. The higher the expected conductivity, the higher the cell constant that must be chosen. Commonly used conductivity cells have cell constants of the order 0.1, 1, and 10 cm^{-1}. Use a standard solution of potassium chloride that is appropriate for the measurement. The conductivity value of the standard solution of potassium chloride should be near the expected conductivity value of the *Sample solution*. Rinse the cell several times with water that has been previously boiled and cooled to room temperature, and rinse at least twice with the potassium chloride solution used for the determination of the cell constant of the conductivity cell. Measure the resistance of the conductivity cell, using the potassium chloride solution at 20 ± 0.1°. Calculate the constant, in cm^{-1}, of the conductivity cell:

$$\text{Result} = R_{KCl} \times K_{KCl}$$

R_{KCl} = measured resistance, expressed in mega-ohms
K_{KCl} = conductivity of the standard solution of potassium chloride used, expressed in µS/cm. The measured constant of the conductivity cell must be within 5% of the given value.

Analysis: Rinse the conductivity cell several times with water that has been previously boiled and cooled to room temperature, and rinse at least twice with the *Sample solution*. Measure the conductivity of the *Sample solution*, while gently stirring with a magnetic stirrer.
Acceptance criteria: NMT 20 µS/cm
• **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 0.5% determined on a 1.0-g sample

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at room temperature.

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• **USP REFERENCE STANDARDS** ⟨11⟩
USP Inositol RS
USP Mannitol RS

Invert Sugar

Invert Sugar Syrup [8013-17-0].

DEFINITION
Invert Sugar is an aqueous solution of inverted or partly inverted, refined or partly refined sucrose containing dextrose (glucose), fructose, and sucrose. It is produced by the hydrolysis or partial hydrolysis of sucrose with suitable acids or enzymes. Total Invert Sugar contains NLT 90.0% of dextrose and fructose, and medium Invert Sugar contains NLT 45.0% and NMT 55.0% of dextrose and fructose, both calculated on the total solids basis.

IDENTIFICATION
• **A.**
Mobile phase: Acetonitrile and water (80:20, v/v)
Standard solution: 10 mg/mL each of USP Fructose RS, USP Dextrose RS, and USP Sucrose RS
Sample solution: 50 mg/mL of Invert Sugar
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Refractive index
Column: 4.6-mm × 15-cm; 5-µm packing L8
Temperatures
Column: 45°
Detector: 40°
Flow rate: 2.0 mL/min
Injection volume: 15 µL
Analysis
Samples: *Standard solution* and *Sample solution*
[NOTE—The relative retention times for fructose, dextrose, and sucrose are about 0.5, 0.6, and 1.0, respectively.]
Compare the retention times for frucose, dextrose, and sucrose given in the two chromatograms.
Acceptance criteria: The retention times of the fructose, dextrose, and sucrose peaks of the *Sample solution* correspond to those of the *Standard solution*.

ASSAY
• **PROCEDURE**
Apparatus: Mount a ring support on a ring stand 1–2 in above a gas burner and mount a second ring 6–7 in above the first. Place a 6-in open-wire gauze on the lower ring to support a 400-mL conical flask, and place a 4-in watch glass with a center hole on the upper ring to deflect heat. Attach a 50-mL buret to the ring stand so that the tip just passes through the watch glass centered above the flask. Place an indirectly lighted white surface behind the assembly for observing the endpoint.
Sample solution: 2.5–4.0 mg/mL of Invert Sugar
Analysis: Conduct a preliminary test to ascertain the volume of water to be added to the 20.0 mL of standardized cupric tartrate, alkaline, solution VS to obtain a final total volume of 75.0 mL when the endpoint of the titration is reached. The invert sugar content of the *Sample solution* should be between 250 and 400 mg per 100.0 mL so that a titer between 25 and 40 mL is needed to achieve the endpoint. Calculate the amount of water to be added to the cupric tartrate, alkaline, solution VS as the difference:

Result = 75.0 – (20.0 mL of cupric tartrate, alkaline, solution VS + number of mL of preliminary titer)

Transfer 20.0 mL of cupric tartrate, alkaline, solution VS to a 400-mL flask containing a few glass beads or boiling chips. Rapidly add the *Sample solution* to within 0.5 mL of the endpoint, and mix by swirling at ambient temperature. Immediately place the flask on the wire gauze of the *Apparatus*, and adjust the burner flame so that the boiling point of the solution is reached in about 2 min. Boil gently but steadily for 2 min. As boiling continues, add 3–4 drops of methylene blue solution (1 in 100). Complete the titration within 1 min by adding the *Sample solution* dropwise until the blue color disappears. Allow a 5-s reaction time between drops at the end of titration.
Calculate the percentage of invert sugar in the sample taken:

$$P_1 = [W/(C_S \times V_S)] \times 100$$

W = amount of invert sugar equivalent to 20.0 mL of cupric tartrate, alkaline, solution VS, 100 mg
C_S = concentration of the *Sample solution* (mg/mL)
V_S = volume of the *Sample solution* used in the titration (mL)

Calculate the percentage of invert sugar on the total solids basis:

$$P_2 = (P_1/D) \times 100$$

D = percentage of total solids corrected for the percentage of invert sugar and temperature as obtained in *Total Solids*

Acceptance criteria
Total Invert Sugar: NLT 90.0% of dextrose and fructose on the total solids basis
Medium Invert Sugar: 45.0%–55.0% of dextrose and fructose on the total solids basis

IMPURITIES
• **RESIDUE ON IGNITION** ⟨281⟩
Analysis: Weigh a portion of Invert Sugar in a platinum dish. Heat gently until the sample ignites, then allow the sample to burn until it self-extinguishes. Cool, then wet the residue with 2 mL of concentrated sulfuric acid, and heat the sample over a low flame until dry. Ignite to constant weight in a muffle furnace at 800 ± 25° for 30 min, or longer if necessary for complete ignition; cool in a desiccator; and weigh.
Acceptance criteria: NMT 0.2%

SPECIFIC TESTS
• **pH** ⟨791⟩: 3.0–5.5
• **TOTAL SOLIDS**
Instrumental conditions
(See *Refractive Index* ⟨831⟩.)
Mode: Refractometer, operating at 589 nm, equipped with a jacket for water circulation or some other mechanism for maintaining the sample at 20 ± 0.1° or some other fixed temperature. Before proceeding with measurements, ensure that the sample and the prism have reached the equilibrium temperature and that the instrument has been properly checked and calibrated against a standard provided by the manufacturer.
Analysis: Measure the refractive index of Invert Sugar, and convert the value to the approximate percentage of solids (uncorrected for invert sugar) by using *Table 1*. Correct for invert sugar and temperature:

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$$D = (S + T) + (P_1 \times F)$$

S = approximate percent solids determined from the refractive index table (see *Table 1*)
T = temperature correction derived from the temperature correction table (see *Table 2*) if the refractometer was operated at other than 20°
P_1 = percentage of invert sugar determined as directed in the *Assay*
F = deWhalley factor, 0.022

Acceptance criteria
Total Invert Sugar: 71.7%–77.3%
Medium Invert Sugar: 76.2%–77.2%

ADDITIONAL REQUIREMENTS
- **LABELING:** Label to indicate the percentage of total solids and Invert Sugar.
- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Dextrose RS
  USP Fructose RS
  USP Sucrose RS

Isobutane



C_4H_{10} 58.12

DEFINITION
Isobutane contains NLT 95.0% of isobutane (C_4H_{10}).
[**CAUTION**—Isobutane is highly flammable and explosive.]

IDENTIFICATION
- **A. IR ABSORPTION:** Exhibits maxima, among others, at about the following wavelengths (µm): 3.4 (vs), 6.8 (s), 7.2 (m), 8.5 (m), and 10.9 (m).
- **B.** The vapor pressure of a test specimen obtained as directed for *Propellants* ⟨602⟩, and determined at 21° by means of a suitable pressure gauge, is between 303 and 331 kPa absolute (44 and 48 psia).

ASSAY
- **PROCEDURE**
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: GC
  Detector: Thermal conductivity
  Column: 3-mm × 6-m aluminum; packed with 10 weight percent of liquid phase G30 on support S1D
  Column temperature: 33°
  Carrier gas: Helium
  Flow rate: 50 mL/min
  Injection volume: 2 µL
  System suitability
  Sample: Isobutane
  Suitability requirements
  Sample response comparison: Peak responses for isobutane from duplicate injections agree within 1%.
  Analysis
  Sample: Isobutane
  Connect one Isobutane cylinder to the chromatograph through a suitable sampling valve and a flow control valve downstream from the sampling valve. Flush the liquid specimen through the sampling valve, taking care to avoid entrapment of gas or air in the sampling valve.
  Calculate the percentage purity of Isobutane:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of isobutane
r_T = sum of all the peak responses

Acceptance criteria: NLT 95.0%

SPECIFIC TESTS
- **WATER:** NMT 0.001%, determined as directed in *Propellants* ⟨602⟩
- **HIGH-BOILING RESIDUES:** NMT 5 µg/mL, determined as directed in *Propellants* ⟨602⟩
- **ACIDITY OF RESIDUE**
  Sample: Residue from the test for *High-Boiling Residues*
  Analysis: Add 10 mL of water to the *Sample*, mix by swirling for about 30 s, add 2 drops of methyl orange TS, insert the stopper in the tube, and shake vigorously.
  Acceptance criteria: No pink or red color appears in the aqueous layer.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight cylinders, and prevent exposure to excessive heat.

Isobutyl Alcohol



$CH_3CH(CH_3)CH_2OH$ 74.12
2-Methyl-1-propanol;
2-Methylpropyl alcohol;
1-Isobutanol [78-83-1].

DEFINITION
Isobutyl Alcohol contains NLT 98.0% of 2-methyl-1-propanol ($C_4H_{10}O$).

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F** ▲ (CN 1-May-2020)
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the 2-methyl-1-propanol peak of the *System suitability solution*, as obtained in the *Assay*.

ASSAY
- **PROCEDURE**
  System suitability solution: USP 1-Butanol RS and USP 2-Methyl-1-Propanol RS (1:1)
  Reference solution: 0.1% of Isobutyl Alcohol in water
  Sample solution: Isobutyl Alcohol (neat)
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: GC
  Detector: Flame ionization
  Column: 0.53-mm × 30-m; coated with a 3.0-µm layer of thickness phase G43
  Temperatures
  Detector: 250°
  Injection port: 140°
  Column: See *Table 1*.

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Table 1[a] (Invert Sugar)

Sucrose (g/100 g)[b]	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
56	1.4329	4332	4334	4336	4338	4340	4343	4345	4347	4349
57	1.4352	4354	4356	4358	4360	4363	4365	4367	4369	4372
58	1.4374	4376	4378	4380	4383	4385	4387	4389	4392	4394
59	1.4396	4398	4401	4403	4405	4407	4410	4412	4414	4417
60	1.4419	4421	4423	4426	4428	4430	4432	4435	4437	4439
61	1.4442	4444	4446	4448	4451	4453	4455	4458	4460	4462
62	1.4464	4467	4469	4471	4474	4476	4478	4481	4483	4485
63	1.4488	4490	4492	4495	4497	4499	4502	4504	4506	4509
64	1.4511	4513	4516	4518	4520	4523	4525	4527	4530	4532
65	1.4534	4537	4539	4541	4544	4546	4548	4551	4553	4556
66	1.4558	4560	4563	4565	4567	4570	4572	4575	4577	4579
67	1.4582	4584	4586	4589	4591	4594	4596	4598	4601	4603
68	1.4606	4608	4610	4613	4615	4618	4620	4623	4625	4627
69	1.4630	4632	4635	4637	4639	4642	4644	4647	4649	4652
70	1.4654	4657	4659	4661	4664	4666	4669	4671	4674	4676
71	1.4679	4681	4683	4686	4688	4691	4693	4696	4698	4701
72	1.4703	4706	4708	4711	4713	4716	4718	4721	4723	4726
73	1.4728	4730	4733	4735	4738	4740	4743	4745	4748	4750
74	1.4753	4756	4758	4761	4763	4766	4768	4771	4773	4776
75	1.4778	4781	4783	4786	4788	4791	4793	4796	4798	4801
76	1.4804	4806	4809	4811	4814	4816	4819	4821	4824	4826
77	1.4829	4832	4834	4837	4839	4842	4844	4847	4850	4852
78	1.4855	4857	4860	4862	4865	4868	4870	4873	4875	4878
79	1.4881	4883	4886	4888	4891	4894	4896	4899	4901	4904
80	1.4907	4909	4912	4914	4917	4920	4922	4925	4928	4930
81	1.4933	4935	4938	4941	4943	4946	4949	4951	4954	4957
82	1.4959	4962	4964	4967	4970	4972	4975	4978	4980	4983
83	1.4986	4988	4991	4994	4996	4999	5002	5004	5007	5010
84	1.5012	5015	5018	5020	5023	5026	5029	5031	5034	5037
85	1.5039	—	—	—	—	—	—	—	—	—

[a] SOURCE: "International Refractive Index Scale of ICUMSA (1974) for Pure Sucrose Solutions at 20°C and 589 nm." Adapted from "Refractometry and Tables—Official" (ICUMSA SPS-3 1994), International Commission for Uniform Methods of Sugar Analysis (ICUMSA), c/o British Sugar Technical Centre, Colney, Norwich NR4 7UB, England. No rounding has been carried out; therefore, values given may be too low by a maximum of 1×10^{-4}.

[b] The refractive index of sugar solutions is used as a rapid method for the approximate determination of dry substance content. For the determination of dry substance content in aqueous solutions of mixtures of sucrose and invert sugar the *Refractive Index Scale for Pure Sucrose Solutions* is typically used. The sucrose content is considered to be an equivalent to the approximate dry substance content.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
40	—	40	20
40	10	240	20

Carrier gas: Helium
Flow rate: 4.8–4.9 mL/min
Injection volume: 1 µL

Injection type: Split injection. The split ratio is 30:1. [NOTE—A needle wash with the *Sample solution* is recommended to minimize the carry over.]

System suitability

Sample: *System suitability solution*

[NOTE—The 2-methyl-1-propanol peak typically elutes at about 11 min, and 1-butanol at about 15 min. The relative retention times for 2-methyl-1-propanol and 1-butanol are 0.7 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 2.0 between 2-methyl-1-propanol and 1-butanol

Relative standard deviation: NMT 2.0%

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Table 2[a] (Invert Sugar)

Temperature (°)	Measured Sucrose (% solids)																	
	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85
Subtract from the measured value																		
15	0.29	0.30	0.32	0.33	0.34	0.35	0.36	0.37	0.37	0.38	0.38	0.38	0.38	0.38	0.38	0.38	0.37	0.37
16	0.24	0.25	0.26	0.27	0.28	0.28	0.29	0.30	0.30	0.30	0.31	0.31	0.31	0.31	0.31	0.30	0.30	0.30
17	0.18	0.19	0.20	0.20	0.21	0.21	0.22	0.22	0.23	0.23	0.23	0.23	0.23	0.23	0.23	0.23	0.23	0.22
18	0.12	0.13	0.13	0.14	0.14	0.14	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
19	0.06	0.06	0.07	0.07	0.07	0.07	0.07	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.07
Add to the measured value																		
21	0.06	0.07	0.07	0.07	0.07	0.07	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.07
22	0.13	0.14	0.14	0.14	0.15	0.15	0.15	0.15	0.16	0.16	0.16	0.16	0.16	0.16	0.15	0.15	0.15	0.15
23	0.20	0.21	0.21	0.22	0.22	0.23	0.23	0.23	0.23	0.24	0.24	0.24	0.24	0.23	0.23	0.23	0.23	0.22
24	0.27	0.28	0.29	0.29	0.30	0.30	0.31	0.31	0.31	0.32	0.32	0.32	0.32	0.31	0.31	0.31	0.30	0.30
25	0.34	0.35	0.36	0.37	0.38	0.38	0.39	0.39	0.40	0.40	0.40	0.40	0.40	0.39	0.39	0.38	0.38	0.37
26	0.42	0.43	0.44	0.45	0.46	0.46	0.47	0.47	0.48	0.48	0.48	0.48	0.48	0.47	0.47	0.46	0.46	0.45
27	0.50	0.51	0.52	0.53	0.54	0.55	0.55	0.56	0.56	0.56	0.56	0.56	0.56	0.55	0.55	0.54	0.53	0.52
28	0.58	0.59	0.60	0.61	0.62	0.63	0.64	0.64	0.64	0.65	0.65	0.64	0.64	0.63	0.63	0.62	0.61	0.60
29	0.66	0.67	0.68	0.70	0.71	0.71	0.72	0.73	0.73	0.73	0.73	0.73	0.72	0.72	0.71	0.70	0.69	0.67
30	0.74	0.76	0.77	0.78	0.79	0.80	0.81	0.81	0.82	0.82	0.81	0.81	0.80	0.80	0.79	0.78	0.76	0.75
31	0.83	0.84	0.85	0.87	0.88	0.89	0.89	0.90	0.90	0.90	0.90	0.89	0.89	0.88	0.87	0.86	0.84	0.82
32	0.92	0.93	0.94	0.96	0.97	0.98	0.98	0.99	0.99	0.99	0.99	0.98	0.97	0.96	0.95	0.93	0.92	0.90
33	1.01	1.02	1.03	1.05	1.06	1.07	1.07	1.08	1.08	1.08	1.07	1.07	1.06	1.04	1.03	1.01	1.00	0.98
34	1.10	1.11	1.13	1.14	1.15	1.16	1.16	1.17	1.17	1.16	1.16	1.15	1.14	1.13	1.11	1.09	1.07	1.05
35	1.19	1.21	1.22	1.23	1.24	1.25	1.25	1.26	1.26	1.25	1.25	1.24	1.23	1.21	1.19	1.17	1.15	1.13
36	1.29	1.30	1.31	1.33	1.34	1.34	1.35	1.35	1.35	1.34	1.34	1.33	1.31	1.29	1.28	1.25	1.23	1.20
37	1.39	1.40	1.41	1.42	1.43	1.44	1.44	1.44	1.44	1.43	1.43	1.41	1.40	1.38	1.36	1.33	1.31	1.28
38	1.49	1.50	1.51	1.52	1.53	1.53	1.54	1.54	1.53	1.53	1.52	1.50	1.48	1.46	1.44	1.42	1.39	1.36
39	1.59	1.60	1.61	1.62	1.63	1.63	1.63	1.63	1.63	1.62	1.61	1.59	1.57	1.55	1.52	1.50	1.47	1.43
40	1.69	1.70	1.71	1.72	1.73	1.73	1.73	1.73	1.72	1.71	1.70	1.68	1.66	1.63	1.61	1.58	1.54	1.51

[a] SOURCE: Adapted from "Refractometry and Tables—Official" (ICUMSA SPS-3 1994), International Commission for Uniform Methods of Sugar Analysis (ICUMSA), c/o British Sugar Technical Centre, Colney, Norwich NR4 7UB, England.

Analysis

Samples: *Reference solution* and *Sample solution*

Calculate the percentage of 2-methyl-1-propanol ($C_4H_{10}O$) in the portion of Isobutyl Alcohol taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of isobutyl alcohol

r_S = sum of all the peaks except those each of which with an area less than 0.1 times the area of the major peak from the *Reference solution*

Acceptance criteria: NLT 98.0%

IMPURITIES

• **LIMIT OF ISOBUTYRALDEHYDE, BUTYRALDEHYDE, 2-BUTANOL, 1-BUTANOL, AND OTHER VOLATILE IMPURITIES**

Sample solution and **Chromatographic system:** Proceed as directed in the *Assay*.

Reference solution: 0.1% of Isobutyl Alcohol in water

Standard solution: 0.2% of USP Isobutyraldehyde RS, 0.2% of USP Butyraldehyde RS, 0.1% of USP 1-Butanol RS, and 0.1% of USP 2-Butanol RS in the *Sample solution*

System suitability

Sample: *Standard solution*

[NOTE—See *Table 2* for relative retention times.]

Table 2

Component	Relative Retention Time
Isobutyraldehyde	0.4
Butyraldehyde	0.5
2-Butanol	0.6
2-Methyl-1-propanol	0.8
1-Butanol	1.0

Suitability requirements

Resolution: NLT 1.5 between all adjacent peaks

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Analysis
Samples: *Sample solution*, *Reference solution*, and *Standard solution*
If any peaks of the *Sample solution* have the same retention times as the peaks due to isobutyraldehyde, butyraldehyde, 2-butanol, and 1-butanol, subtract the areas of any such peaks from the peak areas of the *Standard solution* at these retention times. The difference is calculated below:

$$\text{Result } (\Delta r) = r_S - r_U$$

r_S = peak response of each individual impurity (isobutyraldehyde, butyraldehyde, 2-butanol, or 1-butanol) from the *Standard solution*
r_U = peak response of each individual impurity (isobutyraldehyde, butyraldehyde, 2-butanol, or 1-butanol), if present, from the *Sample solution*

Calculate the percentage of each impurity other than isobutyraldehyde, butyraldehyde, 2-butanol, and 1-butanol in the portion of Isobutyl Alcohol taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of each impurity other than isobutyraldehyde, butyraldehyde, 2-butanol, and 1-butanol from the *Sample solution*
r_T = sum of all the peaks from the *Sample solution*, except those each of which with an area less than 0.1 times the area of the major peak from the *Reference solution*)

Acceptance criteria: See *Table 3*. Disregard any peak with an area less than 0.1 times the area of the major peak from the *Reference solution*, corresponding to 0.01%.

Table 3

Impurity	Percentage (%)
Isobutyraldehyde	The area of any peak of the *Sample solution* corresponding to isobutyraldehyde, r_U, is NMT half of the difference (Δr) between the area of the peak due to isobutyraldehyde in the *Standard solution* and the area of the peak due to isobutyraldehyde in the *Sample solution*, corresponding to NMT 0.1%.
Butyraldehyde	The area of any peak of the *Sample solution* corresponding to butyraldehyde, r_U, is NMT half of the difference (Δr) between the area of the peak due to butyraldehyde in the *Standard solution* and the area of the peak due to butyraldehyde in the *Sample solution*, corresponding to NMT 0.1%.
2-Butanol	The area of any peak of the *Sample solution* corresponding to 2-butanol, r_U, is NMT the difference (Δr) between the area of the peak due to 2-butanol in the *Standard solution* and the area of the peak due to 2-butanol in the *Sample solution*, corresponding to NMT 0.1%.
1-Butanol	The area of any peak of the *Sample solution* corresponding to 1-butanol, r_U, is NMT the difference (Δr) between the area of the peak due to 1-butanol in the *Standard solution* and the area of the peak due to 1-butanol in the *Sample solution*, corresponding to NMT 0.1%.
Total Impurities	NMT 2.0%

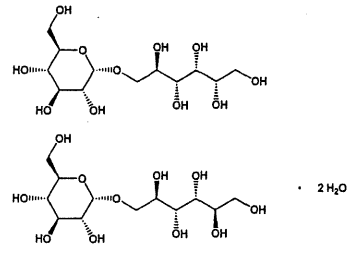
• **LIMIT OF NONVOLATILE RESIDUE**
Sample: 100 mL
Analysis: Evaporate the *Sample* in a tared porcelain dish on a steam bath, and dry at 105° for 30 min.
Acceptance criteria: The weight of the residue does not exceed 4 mg, corresponding to NMT 0.004%.

SPECIFIC TESTS
• **ACIDITY**
Sample: 74 mL (60 g)
Analysis: Titrate the *Sample* with 0.020 N alcoholic potassium hydroxide, using phenolphthalein TS as the indicator, until a pink color persists for NLT 15 s.
Acceptance criteria: NMT 2.5 mL is consumed.
• **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 0.5%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight containers, and prevent exposure to excessive heat.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP 1-Butanol RS
USP 2-Butanol RS
USP Butyraldehyde RS
USP Isobutyraldehyde RS
USP 2-Methyl-1-Propanol RS

Isomalt

Portions of this monograph that are national *USP* text, and are not part of the harmonized text, are marked with symbols (‣ ◂) to specify this fact.



$C_{12}H_{24}O_{11}$ 344.31
$C_{12}H_{24}O_{11} \cdot 2H_2O$ 380.32
6-*O*-α-D-Glucopyranosyl-D-sorbitol and 1-*O*-α-D-glucopyranosyl-D-mannitol dihydrate;
6-*O*-α-D-Glucopyranosyl-D-glucitol and 1-*O*-α-D-glucopyranosyl-D-mannitol dihydrate [64519-82-0].

DEFINITION
Isomalt contains NLT 98.0% and NMT 102.0% of a mixture of 6-*O*-α-D-glucopyranosyl-D-sorbitol (1,6-GPS) and 1-*O*-α-D-glucopyranosyl-D-mannitol (1,1-GPM), and neither of the two components is less than 3.0% of the mixture, calculated on the anhydrous basis.

IDENTIFICATION
• ‣**A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩
Standard solution: 5 mg/mL of USP Isomalt RS
Sample solution: 5 mg/mL
Chromatographic system
Adsorbent: 0.25-mm layer of chromatographic silica gel mixture containing a fluorescent indicator having optimal intensity at 254 nm
Application volume: 1 µL
Developing solvent system: Ethyl acetate, pyridine, water, acetic acid, and propionic acid (10:10:2:1:1)
Analysis
Samples: *Standard solution* and *Sample solution*
Proceed as directed in the chapter. Thoroughly dry the starting points in warm air. Develop over 10 cm using the *Developing solvent system*, dry the plate in a current of hot air, and dip for 3 s in a 1-mg/mL solution of sodium periodate. Dip the plate for 3 s in a mixture of dehydrated

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alcohol, sulfuric acid, acetic acid, and anisaldehyde (90:5:1:1). Dry the plate in a current of hot air until colored spots become visible. The background color may be brightened by exposure to warm steam. Examine in daylight.

Acceptance criteria: The principal spots of the *Sample solution* are similar in position and color to those of the *Standard solution*.

• **B.** The retention times of the two principal peaks of the *Sample solution* correspond to those of the *Standard solution*, as obtained in the *Assay*.

ASSAY

• **PROCEDURE**

Mobile phase: Water

Standard solution: 20 mg/mL of USP Isomalt RS

Sample solution: 20 mg/mL of Isomalt

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Refractive index, maintained at a constant temperature (40° for example)

Columns

Guard: 4.6-mm × 3-cm; packing L19

Analytical: 7.8-mm × 30-cm; packing L19

Column temperature: 80 ± 3°

Flow rate: 0.5 mL/min

Injection volume: 20 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for 1,1-GPM and 1,6-GPS are about 1.0 and 1.2, respectively.]

Suitability requirements

Resolution: NLT 2.0 between 1,1-GPM and 1,6-GPS

Relative standard deviation: NMT 2.0% for the 1,6-GPS and 1,1-GPM peaks

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of 1,6-GPS in the portion of Isomalt taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of 1,6-GPS from the *Sample solution*

r_S = peak response of 1,6-GPS from the *Standard solution*

C_S = concentration of 1,6-GPS in the *Standard solution*, with calculation based on the declared 1,6-GPS content of USP Isomalt RS (mg/mL)

C_U = concentration of Isomalt in the *Sample solution* (mg/mL)

Calculate the percentage of 1,1-GPM in the portion of Isomalt taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of 1,1-GPM from the *Sample solution*

r_S = peak response of 1,1-GPM from the *Standard solution*

C_S = concentration of 1,1-GPM in the *Standard solution*, with calculation based on the declared 1,1-GPM content of USP Isomalt RS (mg/mL)

C_U = concentration of Isomalt in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–102.0% of a mixture of 6-*O*-α-D-glucopyranosyl-D-sorbitol (1,6-GPS) and 1-*O*-α-D-glucopyranosyl-D-mannitol (1,1-GPM), and neither of the two components is less than 3.0% of the mixture, calculated on the anhydrous basis

IMPURITIES

• **LIMIT OF NICKEL**

[NOTE—The purity of the reagents and the water used must be suitable for trace analysis, and the reagents and water must be free of nickel.]

Sample solution: Dissolve 10.0 g of Isomalt in 30 mL of dilute acetic acid (115–125 g/L), add water, and shake to dissolve. Dilute with water to 100.0 mL. Add 2.0 mL of saturated ammonium pyrrolidinedithiocarbamate TS and 10.0 mL of water-saturated methyl isobutyl ketone ($C_6H_{12}O$, 4-methyl-2-pentanone), and then shake for 30 s, protected from bright light. Allow the layers to separate and use the methyl isobutyl ketone layer.

Standard solutions: Prepare three reference solutions in the same manner as the *Sample solution* except add 0.5 mL, 1.0 mL, and 1.5 mL, respectively, of nickel standard solution TS (10 ppm Ni) in addition to the 10.0 g of the substance to be examined.

Blank solution: Treat water-saturated methyl isobutyl ketone as described for preparation of the *Sample solution* omitting the Isomalt.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: 232.0 nm

Lamp: Nickel hollow-cathode

Flame: Air–acetylene

Analysis

Samples: *Sample solution*, *Standard solutions*, and *Blank solution*

Set the zero of the instrument using the *Blank solution*. Record the average of the steady readings for each of the *Standard solutions* and the *Sample solution*. Between each measurement, rinse with water and ascertain that the reading returns to zero with the *Blank solution*. Plot the absorbances of the *Standard solutions* and the *Sample solution* versus the added quantity of nickel. Extrapolate the line joining the points on the graph until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of nickel in the *Sample solution*.

Acceptance criteria: NMT 1 µg/g, calculated on the anhydrous basis

• **ORGANIC IMPURITIES**

Mobile phase, Sample solution, and **Chromatographic system:** Proceed as directed in the *Assay*.

System suitability solution: 20 mg/mL of USP Isomalt RS and 0.1 mg/mL each of USP Mannitol RS and USP Sorbitol RS in water

Standard solution: 0.1 mg/mL each of USP Sorbitol RS and USP Mannitol RS

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for 1,1-GPM, 1,6-GPS, mannitol, and sorbitol are about 1.0, 1.2, 1.6, and 2.0, respectively. The typical retention time for 1,1-GPM is about 12.3 min.]

Suitability requirements

Resolution: NLT 2.0 between 1,1-GPM and 1,6-GPS

Analysis

Samples: *Sample solution* and *Standard solution*

Calculate the percentage of mannitol or sorbitol in the portion of Isomalt taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

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r_U = peak response of mannitol or sorbitol from the *Sample solution*
r_S = peak response of mannitol or sorbitol from the *Standard solution*
C_S = concentration of USP Mannitol RS or USP Sorbitol RS in the *Standard solution* (mg/mL)
C_U = concentration of Isomalt in the *Sample solution* (mg/mL)

Calculate the percentage of any unknown impurity in the portion of Isomalt taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of each unknown impurity from the *Sample solution*
r_S = peak response of sorbitol from the *Standard solution*
C_S = concentration of USP Sorbitol RS in the *Standard solution* (mg/mL)
C_U = concentration of Isomalt in the *Sample solution* (mg/mL)

Acceptance criteria: See *Table 1*. [NOTE—Disregard any impurity peak that is less than 0.1%.]

Table 1

Name	Acceptance Criteria, NMT (%)
Mannitol	0.5
Sorbitol	0.5
Any unknown impurity	0.5
Total impurities	2.0

• **REDUCING SUGARS**
Sample solution: Dissolve 3.3 g in 10 mL of Purified Water with the aid of gentle heat. Cool and add 20 mL of cupric citrate TS and a few glass beads. Heat so that boiling begins after 4 min, and maintain boiling for 3 min. Cool rapidly, and add 100 mL of a 2.4% (v/v) solution of glacial acetic acid and 20 mL of 0.025 M iodine VS. With continuous shaking, add 25 mL of a mixture of hydrochloric acid and water (6:94).
Analysis: After the precipitate has dissolved, titrate the excess iodine with 0.05 N sodium thiosulfate VS, using 1 mL of starch TS, added toward the end of the titration as an indicator.
Acceptance criteria: NLT 12.8 mL of 0.05 N sodium thiosulfate VS is required, corresponding to NMT 0.3% of reducing sugars, determined on the anhydrous basis as glucose.

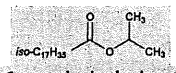
SPECIFIC TESTS
• **WATER DETERMINATION** ⟨921⟩, *Method I*
Sample: 0.3 g
Analysis: Add the *Sample* to a mixture of anhydrous methanol and formamide (1:1) at 50 ± 5°.
Acceptance criteria: NMT 7.0%
• **CONDUCTIVITY**
Sample solution: Dissolve 20 g in carbon dioxide-free water with gentle heating (40°–50°), cool, and dilute with the same solvent to 100 mL.
Analysis: Using an appropriate conductivity meter that has been standardized with a potassium chloride conductivity calibration standard, measure the conductivity of the *Sample solution* while gently stirring with a magnetic stirrer.
Acceptance criteria: NMT 20 µS/cm

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements are specified.
• **LABELING:** Label it to indicate the percentage content of 1,6-GPS and 1,1-GPM.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Isomalt RS
USP Mannitol RS
USP Sorbitol RS

Isopropyl Alcohol—*see Isopropyl Alcohol General Monographs*

Add the following:

Isopropyl Isostearate



Isooctadecanoic acid, 1-methylethyl ester contains mainly isomers of isopropyl branched stearates.

$C_{21}H_{42}O_2$ 326.56
Isooctadecanoic acid, 1-methylethyl ester [68171-33-5].

DEFINITION
Isopropyl Isostearate is obtained by esterification of isostearic acid, which is a mixture mainly of saturated branched 18 carbon-chain (C18) fatty acids and linear hexadecanoic (C16:0) and octadecanoic acids (C18:0), with isopropyl alcohol.

IDENTIFICATION

Change to read:

• **A.** **SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197A** or **197F.** (CN 1-May-2020) Disregard a weak absorption band at about 1780 cm^{-1}.
• **B.** It meets the requirements of the test for *Content of Isopropyl Fatty Esters*.

ASSAY
• **CONTENT OF ISOPROPYL FATTY ESTERS**
System suitability solution: 5.0 mg/mL of USP Isopropyl Palmitate RS and 0.5 mg/mL of USP Isopropyl Myristate RS in *n*-hexane
Reference solution: 2.0 mg/mL of isopropyl stearate in *n*-hexane
Sample solution: 5.0 mg/mL of Isopropyl Isostearate in *n*-hexane
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.32-mm × 15-m fused silica; coated with a 1.0-µm layer of stationary phase G27
Temperatures
Injection port: 240°
Detector: 280°
Column: See *Table 1*.

NF Monographs

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Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
150	—	150	1
150	6	230	21

Carrier gas: Helium
Flow rate: 1.5 mL/min
Injection volume: 2.0 µL
Injection type: Split injection, split ratio, 10:1
Run time: About 35 min

System suitability
Samples: *System suitability solution* and *Reference solution*
[NOTE—The relative retention times for isopropyl myristate, isopropyl palmitate, and isopropyl stearate are 0.8, 1.0, and 1.3, respectively.]
Suitability requirements
Resolution: NLT 6.0 between the isopropyl myristate and isopropyl palmitate peaks, *System suitability solution*
Tailing factor: NMT 2 for the isopropyl palmitate peak, *System suitability solution*
Relative standard deviation: NMT 2.0%, *System suitability solution*

Analysis
Samples: *System suitability solution*, *Reference solution*, and *Sample solution*
Identify the isopropyl fatty ester peaks in the chromatogram of the *Sample solution* by comparing the retention times of these peaks with those obtained in the chromatograms of the *System suitability solution* and *Reference solution*, and measure the peak areas for all of the fatty acid ester peaks in the chromatogram obtained from the *Sample solution*.
Calculate the percentage of each fatty acid ester component in the portion of Isopropyl Isostearate:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak area for each individual fatty acid ester component
r_T = sum of the peak areas, excluding the solvent peak, from the *Sample solution*

Acceptance criteria
Sum of the contents of the isopropyl fatty esters eluting between isopropyl palmitate and isopropyl stearate (excluding isopropyl palmitate and isopropyl stearate): NLT 65.0%
Sum of the contents of isopropyl myristate, isopropyl palmitate, and isopropyl stearate: NMT 11.0%

IMPURITIES
• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%

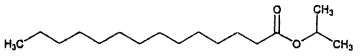
SPECIFIC TESTS
• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Acid Value*: NMT 1.0, determined on 20.0 g
• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Hydroxyl Value*: NMT 5.0
• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Iodine Value*: NMT 3.0, determined on 3.0 g
• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Peroxide Value*: NMT 5.0
• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Saponification Value*: 165–180, determined on 2.0 g
• **WATER DETERMINATION** ⟨921⟩, *Method I*: NMT 0.1%, determined on 5.0 g[1]

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight containers. Store at room temperature.

Change to read:

• **USP REFERENCE STANDARDS** ⟨11⟩
▲USP Isopropyl Isostearate RS▲ (ERR 1-Oct-2018)
USP Isopropyl Myristate RS
USP Isopropyl Palmitate RS▲ 1S (NF36)

Isopropyl Myristate



$C_{17}H_{34}O_2$ 270.45
Tetradecanoic acid, 1-methylethyl ester;
Isopropyl myristate [110-27-0].

DEFINITION
Isopropyl Myristate consists of esters of isopropyl alcohol and saturated high molecular weight fatty acids, principally myristic acid. It contains NLT 90.0% of isopropyl myristate ($C_{17}H_{34}O_2$).

IDENTIFICATION
• **A.** The retention times of the major peaks of the *Sample solution* correspond to those of the *System suitability solution*, as obtained in the *Assay*.

ASSAY
• **PROCEDURE**
System suitability solution: 5.0 mg/mL of USP Isopropyl Myristate RS and 0.5 mg/mL of USP Isopropyl Palmitate RS in *n*-hexane
Sample solution: 5.0 mg/mL of Isopropyl Myristate in *n*-hexane
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.32-mm × 15-m fused silica coated with a 1.0-µm layer of stationary phase G27
Temperatures
Injection port: 240°
Detector: 280°
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
150	—	150	1
150	6	230	8

Carrier gas: Helium
Flow rate: 1.5 mL/min
Injection volume: 2.0 µL
Injection type: Split injection, split ratio 10:1
Run time: 22 min

[1] Hydranal composite 5 is suitable.

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System suitability
Sample: *System suitability solution*
[NOTE—The relative retention times for isopropyl myristate and isopropyl palmitate are 1.0 and 1.3, respectively.]
Suitability requirements
Resolution: NLT 6.0 between the isopropyl myristate and isopropyl palmitate peaks
Tailing factor: NMT 2 for the isopropyl myristate peak
Relative standard deviation: NMT 2.0%
Analysis
Sample: *Sample solution*
Calculate the percentage of isopropyl myristate ($C_{17}H_{34}O_2$) in the portion of Isopropyl Myristate taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak area of isopropyl myristate
r_T = sum of the peak areas of all the peaks, except the solvent peak

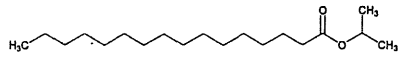
Acceptance criteria: NLT 90.0%

IMPURITIES
• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%

SPECIFIC TESTS
• **SPECIFIC GRAVITY** ⟨841⟩: 0.846–0.854
• **REFRACTIVE INDEX** ⟨831⟩: 1.432–1.436 at 20°
• **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩: NMT 1
• **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩: NMT 1
• **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩: 202–212

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Isopropyl Myristate RS
USP Isopropyl Palmitate RS

Isopropyl Palmitate



$C_{19}H_{38}O_2$ 298.50
Hexadecanoic acid, 1-methylethyl ester;
Isopropyl palmitate [142-91-6].

DEFINITION
Isopropyl Palmitate consists of esters of isopropyl alcohol and saturated high molecular weight fatty acids. It contains NLT 90.0% of isopropyl palmitate ($C_{19}H_{38}O_2$).

IDENTIFICATION
• **A.** The retention times of the major peaks of the *Sample solution* correspond to those of the *System suitability solution*, as obtained in the *Assay*.

ASSAY
• **PROCEDURE**
System suitability solution: 5.0 mg/mL of USP Isopropyl Palmitate RS and 0.5 mg/mL of USP Isopropyl Myristate RS in *n*-hexane
Sample solution: 5.0 mg/mL of Isopropyl Palmitate in *n*-hexane
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.32-mm × 15-m fused silica; coated with a 1.0-µm layer of stationary phase G27
Temperatures
Injection port: 240°
Detector: 280°
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
150	—	150	1
150	6	230	8

Carrier gas: Helium
Flow rate: 1.5 mL/min
Injection volume: 2.0 µL
Injection type: Split injection, split ratio 10:1
Run time: 22 min
System suitability
Sample: *System suitability solution*
[NOTE—The relative retention times for isopropyl myristate and isopropyl palmitate are 0.8 and 1.0, respectively.]
Suitability requirements
Resolution: NLT 6.0 between the isopropyl myristate and isopropyl palmitate peaks
Tailing factor: NMT 2 for the isopropyl palmitate peak
Relative standard deviation: NMT 2.0%
Analysis
Sample: *Sample solution*
Calculate the percentage of isopropyl palmitate ($C_{19}H_{38}O_2$) in the portion of Isopropyl Palmitate taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak area of isopropyl palmitate
r_T = sum of the peak areas of all the peaks, except the solvent peak

Acceptance criteria: NLT 90.0%

IMPURITIES
• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%

SPECIFIC TESTS
• **SPECIFIC GRAVITY** ⟨841⟩: 0.850–0.855
• **REFRACTIVE INDEX** ⟨831⟩: 1.435–1.438
• **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩: NMT 1
• **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩: NMT 1
• **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩: 183–193

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Isopropyl Myristate RS
USP Isopropyl Palmitate RS

Isostearyl Isostearate

DEFINITION
Isostearyl Isostearate is obtained by esterification of isostearic acid which is a mixture mainly of saturated branched 18 carbon-chain (C18) fatty acids, linear hexadecanoic (C16:0)

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and octadecanoic acids (C18:0), with isostearyl alcohol. It contains NLT 85.0% of isostearyl isostearate.

IDENTIFICATION

• **A. ESTER**

Analysis 1: Proceed as directed in *Fats and Fixed Oils* ⟨401⟩, *Procedures, Saponification Value.*

Sample: 3 g

Analysis: Proceed as directed in the chapter. Heat under reflux for 1 h.

Acceptance criteria: 90–110

Analysis 2: Proceed as directed in *Fats and Fixed Oils* ⟨401⟩, *Procedures, Acid Value.*

Sample: Sample amount (see *Fats and Fixed Oils* ⟨401⟩, *Table 1*)

Acceptance criteria: NMT 6.0

• **B. CHROMATOGRAPHIC IDENTITY**

Analysis: Proceed as directed in the *Assay.*

Acceptance criteria: The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution,* as obtained in the *Assay.*

ASSAY

• **PROCEDURE**

Mobile phase: Tetrahydrofuran

Reference solution: 2 mg/mL of isostearic acid in *Mobile phase*

Standard solution: 40 mg/mL of USP Isostearyl Isostearate RS in *Mobile phase*

Sample solution: 40 mg/mL of Isostearyl Isostearate in *Mobile phase*

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability.*)

Mode: LC

Detector: Differential refractive index

Column: Two 7.5-mm × 30-cm columns in tandem; 3-µm packing L21

Temperatures

Column: 35°

Detector: 35°

Flow rate: 1.0 mL/min

Injection volume: 20 µL

Run time: 30 min

System suitability

Samples: *Reference solution* and *Standard solution*

[NOTE—The relative retention times for isostearyl isostearate, isostearic acid, and isostearyl alcohol are 1.00, 1.07, and 1.09, respectively.]

Suitability requirements

Resolution: NLT 1.3 between the isostearyl isostearate peak and the adjacent peak at the retention time 1.09 relative to isostearyl isostearate, *Standard solution*

Relative standard deviation: NMT 2.0%, determined from the isostearyl isostearate peak, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of isostearyl isostearate in the portion of Isostearyl Isostearate taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of isostearyl isostearate from the *Sample solution*

r_S = peak response of isostearyl isostearate from the *Standard solution*

C_S = concentration of USP Isostearyl Isostearate RS in the *Standard solution*

C_U = concentration of Isostearyl Isostearate in the *Sample solution*

Acceptance criteria: NLT 85.0%

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.2%

SPECIFIC TESTS

• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Hydroxyl Value*

Sample: 4 g

Analysis: Proceed as directed in the chapter. A sand bath can be used.

Acceptance criteria: NMT 25.0

• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Iodine Value*

Sample: 3 g

Acceptance criteria: NMT 5.0

• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Peroxide Value*

Sample: 3 g

Acceptance criteria: NMT 5.0

• **WATER DETERMINATION** ⟨921⟩, *Method I*

Sample: 3 g

Acceptance criteria: NMT 0.5%

• **REFRACTIVE INDEX** ⟨831⟩: 1.454–1.464 at 25°

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light and moisture. Store at room temperature, and avoid exposure to excessive heat.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Isostearyl Isostearate RS

Juniper Tar—*see Juniper Tar General Monographs*

Kaolin—*see Kaolin General Monographs*

Alpha-Lactalbumin

$C_{626}H_{958}N_{162}O_{196}S_9$ 14178

[9051-29-0].

DEFINITION

Alpha-Lactalbumin is a lyophilized or spray-dried powder of compact globular metalloprotein that may contain a single bound calcium ion and is capable of binding zinc and other metals. Alpha-Lactalbumin is isolated either from bovine milk or from whey, both of which should be from edible sources suitable for human use. All materials derived from bovine sources must originate from countries free of bovine spongiform encephalopathy. It contains alpha-lactalbumin at NLT 90.0% of the labeled total protein content. The remainder consists mostly of beta-lactoglobulin. It may contain suitable stabilizers.

IDENTIFICATION

• **A. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS**

Gel fixing solution: In a 1-L Pyrex bottle, thoroughly mix 500 mL of water, 400 mL of alcoholic TS, and 100 mL of glacial acetic acid.

Gel staining solution: Prepare a solution of Coomassie blue G-250 having a concentration of 0.25 g/L in a 10.0% (v/v) acetic acid solution.[1] Store at room temperature.

[1] A suitable gel staining solution is available from, e.g., Bio-Rad. Coomassie brilliant blue G-250 is available from Bio-Rad, Cat. # 161-0406.

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Destaining solution: 10.0% (v/v) acetic acid in water [NOTE—This solution may be stored at room temperature for up to 6 months from the date prepared.]

Sample buffer: Prepare a solution containing 200 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), 2% (w/v) sodium dodecyl sulfate (SDS), 40% (v/v) glycerol, and 0.04% (w/v) Coomassie blue G-250. If necessary, adjust with hydrochloric acid or sodium hydroxide to a pH of 6.8.[2]

Running buffer: Prepare a solution containing 100 mM tris(hydroxymethyl)aminomethane, 100 mM *N*-tris(hydroxymethyl)methylglycine (tricine), and 0.1% (w/v) SDS in water. If necessary, adjust with hydrochloric acid or sodium hydroxide to a pH of 8.3.[3] In a 400-mL beaker, thoroughly mix 35 mL of the solution so obtained (or 10× Tris/Tricine/SDS buffer)[4] with 315 mL of water.

Molecular weight marker: Use a suitable molecular weight marker containing protein bands between 3.5 and 27 kDa.

Molecular weight standard solution: Transfer 16 µL of the *Sample buffer* into a 0.5-mL microcentrifuge tube. Pipet 4 µL of the *Molecular weight marker* into the microcentrifuge tube, and mix. Incubate the mixture in the closed microcentrifuge tube for 5 min at 95°. After incubation, allow the tube to stand for 5 min at room temperature. Centrifuge at 5000 rpm for 1 min.

Alpha-Lactalbumin standard stock solution: 1.0% (w/v) of USP Alpha-Lactalbumin RS in water in a 2-mL centrifuge tube.

Alpha-Lactalbumin standard working solution: Pipet 21 µL of the *Sample buffer* and 3 µL of the *Alpha-Lactalbumin standard stock solution* into a 0.5-mL microcentrifuge tube, and mix. Proceed as directed for *Molecular weight standard solution* beginning with "Incubate the mixture".

Sample stock solution: 1.0% (w/v) of Alpha-Lactalbumin in water in a 2-mL centrifuge tube.

Sample solution: Pipet 21 µL of the *Sample buffer* and 3 µL of the *Sample stock solution* into a 0.5-mL microcentrifuge tube, and mix. Proceed as directed for *Molecular weight standard solution* beginning with "Incubate the mixture".

SDS-PAGE gel and apparatus set-up: Following the manufacturer's instructions, assemble and fill a 16.5% Tris-Tricine Ready Gel[5] in the Mini-Protean III Electrophoresis Module,[6] or in an equivalent module. Add *Running buffer* appropriately to this apparatus.

Analysis

Gel loading: Load 10 µL of the *Molecular weight standard solution*, 2.5 µL of the *Alpha-Lactalbumin standard working solution*, and 2.5 µL of the *Sample solution*, respectively, into the 16.5% Tris-Tricine SDS-PAGE gel. [NOTE—The loaded samples contain approximately 3 µg of protein based on the sample weight.]

Running the gel: Set the voltage to 100 V, and run at a constant voltage. Run the gel until the tracking dye front is approximately 10 mm from the bottom of the gel (approximately 80–90 min).

Gel fixing: Remove the gel, transfer to a plastic container, and soak in the *Gel fixing solution* for 30 min on a shaking rack. Decant the *Gel fixing solution*. Rinse with water, and decant.

Gel staining: Pour approximately 100 mL of the *Gel staining solution* into the staining container. Place the gel into the staining container, and allow the stain to completely cover the gel. Place the staining container on an appropriate shaker, and stain the gel for 60–90 min with gentle shaking.

Destaining: Drain the *Gel staining solution* into an appropriate waste container, and add 100 mL of *Destaining solution* to the container to cover the gel. Place the container on an appropriate shaker, and shake with gentle agitation for 30 min. Discard the used *Destaining solution*, and repeat destaining as necessary. Repeat rinsing with *Destaining solution* three to four times at 30-min intervals or until the gel is destained to the desired clarity.

Acceptance criteria: The Alpha-Lactalbumin has one major band at 14 kDa, a minor band at 16 kDa, and a molecular weight that is similar to that of USP Alpha-Lactalbumin RS.

• **B.** The retention time of the major peak for alpha-lactalbumin from the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Alpha-Lactalbumin* in the *Assay*.

ASSAY

• **CONTENT OF ALPHA-LACTALBUMIN**

Mobile phase: Prepare a solution of 0.02 M Tris-HCl, 0.5% SDS, and 0.1 N sodium chloride. Adjust the pH of the solution to 5.95 ± 0.05. Pass this solution through a filter having a 0.5-µm or finer porosity, and degas.

Standard solution: 1.0 mg/mL of USP Alpha-Lactalbumin RS in *Mobile phase*. [NOTE—Prepare it immediately before use.]

System suitability solutuion: 0.5 mg/mL of USP Alpha-Lactalbumin RS and 0.5 mg/mL of beta-lactoglobulin in *Mobile phase*

Sample solution: 1.0 mg/mL of Alpha-Lactalbumin in *Mobile phase*

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 7.8-mm × 30-cm analytical column, packing L33 [NOTE—Equilibrate the column for approximately 90 min at 0.6 mL/min of *Mobile phase* or until a stable baseline is achieved.]

Flow rate: 0.6 mL/min

Injection size: 20 µL

System suitability

Sample: *System suitability solution* [NOTE—The relative retention times for beta-lactoglobulin and alpha-lactalbumin are 0.91 and 1.00, respectively.]

Suitability requirements

Resolution: NLT 1.65 between beta-lactoglobulin and alpha-lactalbumin

Tailing factor: Not greater than 1.1 for the alpha-lactalbumin peak

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the purity of Alpha-Lactalbumin as a percentage of total protein:

$$\text{Result} = (r_U/r_S) \times [C_S/(C_U \times P)] \times 100$$

r_U = peak response for alpha-lactalbumin from the *Sample solution*

r_S = peak response for alpha-lactalbumin from the *Standard solution*

C_S = concentration of USP Alpha-Lactalbumin RS in the *Standard solution* (mg/mL)

C_U = concentration of Alpha-Lactalbumin in the *Sample solution* (mg/mL)

P = percentage of total protein content

[2] A suitable sample buffer is available as Tricine sample buffer from Bio-Rad, Cat. # 161-0739.

[3] An undiluted suitable running buffer is available as 10× Tris/Tricine/SDS buffer from Bio-Rad, Cat. # 161-0744.

[4] Available as 10× Tris/Tricine/SDS buffer from Bio-Rad, Cat. # 161-0744.

[5] Available from Bio-Rad, Cat. # 161-1107 or 161-1179.

[6] Available from Bio-Rad, Cat. # 165-3302.

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Acceptance criteria: Content of alpha-lactalbumin is NLT 90.0% of the labeled total protein content.

• **TOTAL PROTEIN CONTENT**

Sample: 250 mg of Alpha-Lactalbumin

Analysis: Combust the *Sample* in the presence of pure oxygen (99.9%) in an airtight oven at 950° with a suitable nitrogen analyzer. The components such as carbon dioxide, sulfur dioxide, and moisture are absorbed by various in-line chemical filters. All nitrogenous matter is converted into nitrogen in the presence of catalytic converters. The weight percent of nitrogen is measured by a thermal conductivity detector. Blank the system by analyzing a suitable nitrogen blank material, such as powdered cellulose, and obtaining a zero reading. Calibrate and qualify the system by using EDTA. The relative standard deviation for replicate runs is NMT 0.5%. Calculate the weight percent of total protein content in Alpha-Lactalbumin by multiplying the percentage of nitrogen found by 6.23.

Acceptance criteria: Total protein content is NLT 90.0%.

OTHER COMPONENTS

• **LIMIT OF BETA-LACTOGLOBULIN**

Mobile phase, System suitability solution, Sample solution, Chromatographic system, and **System suitability:** Prepare as directed in the test for *Content of Alpha-Lactalbumin*.

Standard solution: 1.0 mg/mL of beta lactoglobulin in *Mobile phase*. [NOTE—Prepare it immediately before use.]

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of beta-lactoglobulin as a percentage of total protein:

$$\text{Result} = (r_U/r_S) \times [C_S/(C_U \times P)] \times 100$$

r_U = peak response for beta-lactoglobulin from the *Sample solution*

r_S = peak response for beta-lactoglobulin from the *Standard solution*

C_S = concentration of beta-lactoglobulin in the *Standard solution* (mg/mL)

C_U = concentration of Alpha-Lactalbumin in the *Sample solution* (mg/mL)

P = percentage of total protein content

Acceptance criteria: NMT 6.5%, calculated on the total protein basis

• **CONTENT OF CALCIUM**

Standard stock solution: Dissolve 1.249 g of calcium carbonate in 270 mL of 3 N hydrochloric acid (dilute 250 mL of hydrochloric acid with water to 1000 mL) in a 1000-mL volumetric flask. Dilute with water to volume, and mix. Dilute 50 mL of the solution so obtained to 1000 mL. The *Standard stock solution* contains 25 µg/mL of calcium.[7]

Lanthanum chloride solution: Weigh 11.7 g (± 100 mg) of lanthanum oxide, and transfer to a 1000-mL volumetric flask. Add enough water to wet the powder, and then slowly add 50 mL of hydrochloric acid. [CAUTION—Exothermic reaction.] Let the test specimen dissolve, dilute with water to volume, and mix. This solution contains 1% (w/v) of lanthanum and is stable for up to 6 months when stored at room temperature.

Blank solution: 10-fold dilution of *Lanthanum chloride solution*

Working standard solutions: To five identical 25-mL volumetric flasks add 0, 5, 10, 15, and 20 mL, respectively, of *Standard stock solution*. Add 2.5 mL of *Lanthanum chloride solution*, and dilute with water to volume. The *Working standard solutions* contain 0, 5, 10, 15, and 20 µg/mL of calcium, each containing 0.1% (w/v) of lanthanum.

Sample solution: Transfer 1.0 g of Alpha-Lactalbumin to a 100-mL volumetric flask, add 10 mL of *Lanthanum chloride solution*, and dilute with water to volume.

Spectrometric conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: 422.7 nm

Lamp: Calcium hollow-cathode lamp

Flame: Reduced air–acetylene

Analysis

Samples: *Working standard solutions* and *Sample solution*

Concomitantly determine the absorbances of the *Samples* using the *Blank solution*. [NOTE—Optimize flame parameters in accordance with the instrument manufacturer's instructions.]

Plot the absorbances of the *Working standard solutions* versus the concentration, in µg/mL, of calcium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C, in µg/mL, of calcium in the *Sample solution*.

Calculate the quantity of calcium (Ca), in mg, in each g of Alpha-Lactalbumin taken:

$$\text{Result} = (V \times C)/W \times F \times 100$$

V = volume of the *Sample solution*, 100 mL

C = as determined above

W = weight of Alpha-Lactalbumin taken to prepare the *Sample solution* (g)

F = conversion factor for µg to mg (10^{-3} mg/µg)

Acceptance criteria: NMT 1 mg/g of calcium

IMPURITIES

INORGANIC IMPURITIES

• **Ash:** Ignite 1 g of Alpha-Lactalbumin at NMT 550° until free from carbon. Cool in a desiccator, and weigh: NMT 3.5% is found.

• **LIMIT OF PHOSPHORUS**

Hydrochloric acid solution: Pipet 250 mL of hydrochloric acid into a 1000-mL volumetric flask, dilute with water to volume, and mix.

Molybdovanadate reagent: Dissolve 20 g of ammonium molybdate in 200 mL of water with the aid of heat, and then allow the molybdate solution to cool. Dissolve 1.0 g of ammonium vanadate in 125 mL of water with the aid of heat, cool, and add 160 mL of hydrochloric acid. Gradually add, with stirring, the molybdate solution to the vanadate solution, and dilute with water to 1000 mL.

Phosphorus standard stock solution I: Transfer 8.8 g of monobasic potassium phosphate (KH_2PO_4), previously dried for 2 h at 105°, to a 1000-mL volumetric flask, and add 750 mL of water to dissolve. Dilute with water to volume. This solution contains 2 mg/mL of phosphorus. [NOTE—Store the solution in a refrigerator.]

Phosphorus standard stock solution II: Immediately before use, dilute 50 mL of *Phosphorus standard stock solution I* with water to 1000 mL. [NOTE—Store in a refrigerator.]

Standard solutions: Transfer 0.0 mL, 5.0 mL, 8.0 mL, 10.0 mL, and 15.0 mL of *Phosphorus standard stock solution II*, respectively, to five identical 100-mL volumetric flasks. Proceed as directed in the *Analysis*: after treatment

[7] A commercially prepared, certificated AA standard is available as Calcium AA, ICP standards, 1000 ppm Ca in dilute hydrochloric acid, Cat. # ACA1KH, from RICCA. Make an appropriate dilution to obtain a final concentration of 25 µg/mL of calcium.

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with the *Molybdovanadate reagent*, the resulting final phosphorus concentrations for the *Standard solutions* are 0.0, 5.0, 8.0, 10.0, and 15.0 µg/mL, respectively.

Sample solution: Transfer 4.0 g of Alpha-Lactalbumin to an ashing dish. Dry the test specimen on a hot plate or steam bath. Ignite in a muffle furnace at a maximum temperature of 600° until free of carbon. Cool, add 40 mL of *Hydrochloric acid solution* and several drops of nitric acid, and bring to boil on a hot plate. Cool, transfer to a 100-mL volumetric flask by rinsing the ashing dish with water, dilute with water to volume, and mix. Pipet 20.0 mL of the *Sample solution* into a 100-mL volumetric flask.

Spectrometric conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Mode: UV-Vis

Analytical Wavelength: 400 nm

Analysis

Samples: *Standard solutions* and the *Sample solution*

To each of the flasks containing the *Samples*, add 20.0 mL of *Molybdovanadate reagent*, dilute with water to volume, mix, and allow to stand for exactly 10 min for maximum color development. The *Standard solutions* and the *Sample solution* are treated identically. Concomitantly determine the absorbances of each of the *Samples* in 1-cm cells using the *Spectrometric conditions* described above. Use one of the *Standard solutions* with phosphorus concentration at 0.0 µg/mL to zero the spectrophotometer. Plot the absorbances of the *Standard solutions* versus concentration, in µg/mL, of phosphorus, and draw the straight line best fitting the four plotted points. From the graph so obtained, determine the concentration, C, in µg/mL, of phosphorus in the *Sample solution*.

Calculate the quantity, in µg, of phosphorus in each g of Alpha-Lactalbumin taken:

$$\text{Result} = (V \times C)/W \times D$$

V = volume of the *Sample solution*, 100 mL
C = as determined above
W = weight of Alpha-Lactalbumin taken to prepare the *Sample solution* (g)
D = dilution factor, 5

Acceptance criteria: NMT 700 µg/g of phosphorus

ORGANIC IMPURITIES

• Procedure 1: Limit of Lipid (Fat)

Weighing dish preparation: Pre-dry the clean dishes under the same conditions that will be used for final drying after fat extraction. Ensure that all surfaces where weighing dishes will be placed (i.e., hot plate, desiccator, etc.) are clean and free of particulates. At the end of oven drying, place the weighing dishes in a desiccator, and cool to room temperature. Immediately before use, weigh the dishes to the nearest 0.1 mg, and record the weights. Check the balance zero after weighing each dish. Protect the weighed dishes from contamination with extraneous matter.

Analysis

Transfer 0.5 g of Alpha-Lactalbumin to a Mojonnier-style ether extraction flask that has the capacity to hold a volume of 21–23 mL in the lower bulb plus neck at the bottom of the flask. The flask has a smooth, round opening at the top that can be sealed when closed with cork. Add 10 mL of water at a temperature of 40°, and mix. Add 1.5 mL of ammonium hydroxide to the Alpha-Lactalbumin, and mix thoroughly. Add 3 drops of phenolphthalein TS to help sharpen the visual appearance of the interface between the ether and the aqueous layers during extraction. Add 10 mL of alcohol, close with the cork stopper that has been water soaked, and shake the flask for 15 s.

For the first extraction, add 25 mL of ether, replace the cork stopper, and shake the flask very vigorously for approximately 1 min, releasing built-up pressure by loosening the stopper as necessary. Add 25 mL of petroleum ether, replace the cork stopper, and repeat vigorous shaking for about 1 min. Centrifuge the flask at about 600 rpm for NLT 30 s to obtain a clean separation of the aqueous (bright pink) and the ether phases. Decant the ether solution into a suitable weighing dish prepared as directed for *Weighing dish preparation*. When the ether solution is decanted into the dish, be careful not to pour any suspended solids or aqueous phase into the weighing dish. Ether can be evaporated at NMT 100° from the dish while conducting the second extraction.

For the second extraction, add 5 mL of alcohol to the original flask, close with the cork stopper, and shake vigorously for 15 s. Add 15 mL of ether, replace the cork, and shake the flask vigorously for about 1 min. Add 15 mL of petroleum ether, replace the cork stopper, and repeat vigorous shaking for about 1 min. Centrifuge the flask at about 600 rpm for NLT 30 s to obtain a clean separation of the aqueous (bright pink) and the ether phases. If the interface is below the neck of the flask, add water to bring the level about halfway up to the neck. Add water slowly down the inside surface of the flask so that there is minimum disturbance of the interface. Decant the ether solution for the second extraction into the same weighing dish used for the first extraction.

For the third extraction, omit addition of the alcohol and repeat the procedure used for the second extraction. Completely evaporate the solvents in a hood on a hot plate at NMT 100°, and avoid spattering. Dry the extracted fat and the weighing dish to constant weight in a forced air oven at 100° ± 1° for NLT 30 min or in a vacuum oven at 70° to 75° at more than 50.8 cm (20 inches) of vacuum for NLT 7 min. Remove the weighing dish from the oven, and place in a desiccator to cool to room temperature. Record the weight of the weighing dish containing the fat.

Run a blank determination using water, and record the weight of any dry residue collected. The reagent blank should be less than 2.0 mg of residue. [NOTE—A negative number is not acceptable.]

Calculate the weight percent of lipid (fat) in the portion of Alpha-Lactalbumin taken:

$$\text{Result} = [(W_2 - W_1) - W_3]/W \times 100$$

W_1 = weight of empty weighing dish (g)
W_2 = weight of the weighing dish containing fat (g)
W_3 = weight of the reagent blank residue (g)
W = weight of the Alpha-Lactalbumin taken for the fat extraction (g)

Acceptance criteria: NMT 1.0% is found. The difference between duplicate runs is NMT 0.03% fat.

• Procedure 2: Limit of Lactose

Carrez I solution: Transfer 3.60 g of potassium ferrocyanide [$K_4Fe(CN)_6 \cdot 3H_2O$] to a 100-mL volumetric flask, dissolve in and dilute with water to volume, and mix.

Carrez II solution: Transfer 7.20 g of zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$) to a 100-mL volumetric flask, dissolve in and dilute with water to volume, and mix.

[NOTE—The following four test reagents are included in a test kit.[8]]

[8] Available from Boehringer-Mannheim (R-Biopharm, Inc., 7950 Old US 27S, Marshall, MI 49068 USA; Tel: +1-877-789-3033 or +1-269-789-3033; Fax: +1-269-789-3070; www.r-biopharm.com).

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Test reagent 1: About 600 mg of lyophilisate consisting of a mixture of citrate buffer (pH 6.6), nicotinamide adenine dinucleotide (NAD) (35 mg), anhydrous magnesium sulfate, and stabilizers (added if necessary). Dissolve lyophilisate in 7.0 mL of water before use.
Test reagent 2: About 1.7 mL of an enzyme suspension of β-galactosidase (approximately 100 Units).
Test reagent 3: 34 mL of a solution consisting of 0.51 M potassium diphosphate buffer (pH 8.6), and stabilizers (added if necessary).
Test reagent 4: About 1.7 mL of an enzyme suspension of galactose dehydrogenase (about 40 Units).
Sample solution: Transfer 1.0 g of Alpha-Lactalbumin to a 100-mL volumetric flask, add about 60 mL of water, and mix. Add 5 mL of *Carrez I solution*, and mix. Add 5 mL of *Carrez II solution*, and mix. Add 10 mL of 0.1 N sodium hydroxide solution, and mix vigorously. Dilute with water to volume, and mix. Pass through a filter paper, and use the clear filtrate. [NOTE—This procedure breaks emulsions, absorbs some colors, and precipitates proteins.]
Analysis
Label one glass or disposable plastic cuvet as "blank" and the second glass or disposable plastic cuvet as "test". [NOTE—These two cuvets should be equivalent.] To each cuvet, pipet 0.20 mL of *Test reagent 1* and 0.05 mL of *Test reagent 2*. Pipet 0.10 mL of the *Sample solution* into the cuvet that is labeled "test". Mix both cuvets with their stirrers, and incubate at 20°–25° for 20 min. Pipet 1.00 mL of *Test reagent 3* into each cuvet. Pipet 2.00 mL of water into the cuvet that is labeled "blank" and 1.90 mL of water into the cuvet containing the *Sample solution*. Mix, and incubate at 20°–25° for about 2 min.
Determine the absorbances, A_{S1} and A_{B1}, at 340 nm, for the *Sample solution* and the blank, respectively. Add 0.05 mL of *Test reagent 4* to each cuvet. Mix and incubate at 20°–25° until the reaction has stopped (about 10–15 min). Determine the absorbances A_{S2} and A_{B2}, at 340 nm, again for the *Sample solution* and the blank, respectively. If the reaction has not stopped after 15 min, continue to read the absorbances at 2-min intervals until the absorbance for the *Sample solution* remains constant for two successive measurements.
Calculate the percentage of lactose in the portion of Alpha-Lactalbumin taken:

$$\text{Result} = V_1 \times V_2 \times M_r \times [(A_{S2} - A_{S1}) - (A_{B2} - A_{B1})]/(\varepsilon \times L \times V_3 \times W) \times 100$$

V_1 = volume of the *Sample solution*, 0.1 L
V_2 = volume of the final sample solution in the cuvet, 3.30 mL
M_r = molecular weight of lactose monohydrate, 360.32 g/mol
ε = absorption coefficient of nicotinamide adenine dinucleotide reduced form (NADH) at 340 nm, 6300 $L \cdot mol^{-1} \cdot cm^{-1}$
L = light path of the cuvet, 1.0 cm
V_3 = volume of the *Sample solution* taken into the cuvet, 0.1 mL
W = weight of Alpha-Lactalbumin taken to prepare the *Sample solution* (g)

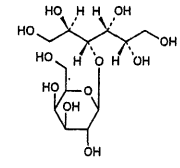
Acceptance criteria: NMT 1.0% of lactose

SPECIFIC TESTS
- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic bacterial count does not exceed 1000 cfu/g. The total combined molds and yeasts count does not exceed 100 cfu/g. It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.
- **DENATURATION TEMPERATURE**
Sample solution: Prepare a protein dough by mixing 3 g of Alpha-Lactalbumin powder with 2 g of water. Place the dough into a well-sealed sample container.
Analysis: Perform two measurements on the dough sample using a differential scanning calorimeter. Heat to 140°, and scan. Cool rapidly to below room temperature, and rescan. Apply a scan rate of 10°/min. Weigh pans before and after scanning to verify that no moisture loss occurs during the scanning process. Measure and record the denaturation temperatures as peak temperatures. The formation of two peaks indicates the presence of both the apo form and the holo form of Alpha-Lactalbumin.
Acceptance criteria: The denaturation temperature for Alpha-Lactalbumin in the apo form is between 50° and 52°; the denaturation temperature for Alpha-Lactalbumin in the holo form is between 58° and 61°.
- **pH** ⟨791⟩**:** NMT 7.5, in a solution (1 in 10)
- **LOSS ON DRYING** ⟨731⟩**:** Dry 1.0–1.5 g in a vacuum oven at 100°, at a pressure of 660 mm of mercury, and with continuous dry air feed for 5 h: it loses NMT 6.5% of its weight.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers, and store at the temperature indicated on the label.
- **LABELING:** Label it to state the protein content, expressed as a total protein percentage. Indicate the type of source material, expressed as bovine milk, whey, or both, used to manufacture the final product. Label it to indicate the storage conditions, the expiration date, and the name and concentration of any added stabilizers.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Alpha-Lactalbumin RS

Lactic Acid—*see Lactic Acid General Monographs*

Lactitol



$C_{12}H_{24}O_{11}$ 344.31
$C_{12}H_{24}O_{11} \cdot H_2O$ 362.34
$C_{12}H_{24}O_{11} \cdot 2H_2O$ 380.35
4-*O*-β-D-Galactopyranosyl-D-glucitol [585-86-4].
Monohydrate [81025-04-9].
Dihydrate [81025-03-8].

DEFINITION
Lactitol contains NLT 98.0% and NMT 101.0% of $C_{12}H_{24}O_{11}$, calculated on the anhydrous basis.

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IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K.▲** (CN 1-May-2020)

ASSAY

• **PROCEDURE**

Mobile phase: Water

Standard solution: 10.0 mg/mL of USP Lactitol RS

Sample solution: 10.0 mg/mL of Lactitol

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 7.8-mm × 30-cm; packing L34

Column temperature: 85°

Flow rate: 0.7 mL/min

Injection size: 25 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 1.0% for lactitol

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of lactitol ($C_{12}H_{24}O_{11}$) in the portion of Lactitol taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Lactitol RS in the *Standard solution* (mg/mL)

C_U = concentration of Lactitol in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–101.0% on the anhydrous basis

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.5%

• **RELATED COMPOUNDS**

Standard solution: 0.3 mg/mL of USP Lactitol RS

Sample solution: Prepare as directed in the *Assay*.

Chromatographic system: Proceed as directed in the *Assay*.

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for lactose, glucose, galactose, lactulitol, lactitol, galactitol, and sorbitol are about 0.53, 0.58, 0.67, 0.72, 1.0, 1.55, and 1.68, respectively.]

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentages of galactitol, sorbitol, lactulitol, lactose, glucose, and galactose in the portion of Lactitol taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of the relevant related compound, if observed, from the *Sample solution*

r_S = peak response of lactitol from the *Standard solution*

C_S = concentration of USP Lactitol RS in the *Standard solution* (mg/mL)

C_U = concentration of Lactitol in the *Sample solution* (mg/mL)

Acceptance criteria: The total of the percentages of all related compounds is NMT 1.5%.

• **REDUCING SUGARS**

Standard solution: Pipet 2 mL of a dextrose solution containing 0.5 mg/mL into a 10-mL conical flask.

Sample solution: Disolve 500 mg in 2.0 mL of water in a 10-mL conical flask.

Analysis: Concomitantly add 1 mL of alkaline cupric tartrate TS to each solution, heat to boiling, and cool.

Acceptance criteria: NMT 0.2%, calculated as dextrose. The *Sample solution* shows no more turbidity than that produced in the *Standard solution*, in which a reddish brown precipitate forms.

SPECIFIC TESTS

• **WATER DETERMINATION,** *Method I* ⟨921⟩: For the monohydrate form, 4.5%–5.5%; for the dihydrate form, 9.5%–10.5%; and for the anhydrous form, NMT 0.5%.

ADDITIONAL REQUIREMENTS

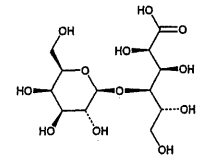
• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

• **LABELING:** Label it to indicate whether it is the monohydrate, the dihydrate, or the anhydrous form.

• **USP REFERENCE STANDARDS** ⟨11⟩

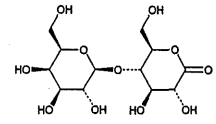
USP Lactitol RS

Lactobionic Acid



$C_{12}H_{22}O_{12}$ (acid form) 358.30

[96-82-2].



$C_{12}H_{20}O_{11}$ (δ-lactone) 340.28

[5965-65-1].

4-*O*-β-Galactopyranosyl-D-gluconic acid.

DEFINITION

Lactobionic Acid is a mixture in variable proportions of 4-*O*-β-D-galactopyranosyl-D-gluconic acid and 4-*O*-β-D-galactopyranosyl-D-glucono-1,5-lactone. It contains NLT 98.0% and NMT 102.0%, on the anhydrous basis.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K.▲** (CN 1-May-2020) [NOTE—If the spectra obtained show differences, dissolve the test substance and USP Lactobionic Acid RS separately in water, dry at 105°, and record new spectra using the residues.]

• **B. THIN-LAYER CHROMATOGRAPHY** ⟨621⟩

Standard solution: 10 mg/mL of USP Lactobionic Acid RS

Sample solution: 10 mg/mL of Lactobionic Acid

NF Monographs

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Adsorbent: Chromatographic silica gel mixture with an average particle size of 10–15 µm (TLC plates)
Developing solvent: Methanol, ethyl acetate, ammonium hydroxide, and water (2:1:1:1)
Application volume: 5 µL
Spray reagent: Slowly add 10 mL of sulfuric acid to about 40 mL of water. Mix, and allow to cool. Dilute with water to 100 mL, and mix. Add 2.5 g of ammonium molybdate and 1 g of ceric sulfate, and shake for 15 min to dissolve.
Analysis: Develop the chromatograms until the solvent front has moved about three-fourths the length of the plate, and allow to dry. Spray the plate with *Spray reagent*, and allow to dry. Repeat two more times, heat at 110° for 15 min, and examine.
Acceptance criteria: The principal spot from the *Sample solution* is similar in position and color to the principal spot from the *Standard solution*.

ASSAY

- **PROCEDURE**

Sample: 0.350 g of Lactobionic Acid
Analysis: Dissolve the *Sample* in 50 mL of carbon dioxide-free water, previously heated to 30°. Immediately titrate with 0.1 N sodium hydroxide, and determine the two equivalence points potentiometrically. (*See Titrimetry* ⟨541⟩.)
Each mL of 0.1 N sodium hydroxide consumed to the first equivalency point is equivalent to 35.83 mg of $C_{12}H_{22}O_{12}$ (corresponds to the acid form), and each mL of 0.1 N sodium hydroxide consumed between the first and second equivalency points is equivalent to 34.03 mg of $C_{12}H_{20}O_{11}$ (corresponds to the δ-lactone form).
Calculate the content, expressed as a percentage, of the lactobionic acid as the sum of both results.
Acceptance criteria: 98.0%–102.0% on the anhydrous basis

SPECIFIC TESTS

- **WATER DETERMINATION,** *Method Ia* ⟨921⟩

Sample solution: 0.50 g in a mixture of methanol and formamide (2:1)
Acceptance criteria: NMT 5.0%

- **APPEARANCE OF SOLUTION**

Sample solution: 120 mg/mL of Lactobionic Acid
Standard stock solution: Pipet 24.0 mL of ferric chloride CS and 6.0 mL of cobaltous chloride CS into a 100-mL volumetric flask. Dilute with 1% (w/v) hydrochloric acid to volume.
Reference solution: Pipet 12.5 mL of the *Standard stock solution* into a 100-mL volumetric flask. Dilute with 1% (w/v) hydrochloric acid to volume.
Acceptance criteria: The *Sample solution* is clear and not more intensely colored than the *Reference solution*.

- **OPTICAL ROTATION,** *Specific Rotation* ⟨781S⟩

Sample solution: 10 mg/mL of Lactobionic Acid. Allow to stand for 24 h.
Acceptance criteria: +23.0° to +29.0° (anhydrous substance)

- **REDUCING SUGARS**

Sample solution: Dissolve 5.0 g of Lactobionic Acid in 25 mL of water with the aid of gentle heat, and cool.
Analysis: To the *Sample solution* add 20 mL of cupric citrate TS and a few glass beads. Heat so that boiling begins after 4 min, and maintain boiling for 3 min. Cool rapidly, and add 100 mL of a 2.4% solution of glacial acetic acid and 20.0 mL of 0.025 M iodine VS. With continuous shaking, add 25 mL of a mixture of 6 mL of hydrochloric acid and 94 mL of water. When the precipitate has dissolved, titrate the excess iodine with 0.05 M sodium thiosulfate VS using 1 mL of starch TS, added toward the end of the titration as an indicator.
Acceptance criteria: NLT 12.8 mL of 0.05 M sodium thiosulfate VS is required, corresponding to NMT 0.2% of reducing sugars, as glucose.

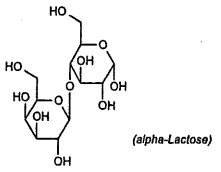
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩: NMT 0.2%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Lactobionic Acid RS

Anhydrous Lactose

Portions of the monograph text that are national *USP* text, and are not part of the harmonized text, are marked with symbols (‣ ‣) to specify this fact.



DEFINITION

Anhydrous Lactose is
O-β-D-galactopyranosyl-(1→4)-β-D-glucopyranose (β-lactose), or a mixture of
O-β-D-galactopyranosyl-(1→4)-β-D-glucopyranose and
O-β-D-galactopyranosyl-(1→4)-α-D-glucopyranose (α-lactose).

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
- **‣B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩

Adsorbent: 0.25-mm layer of chromatographic silica gel
Diluent: Methanol and water (3:2)
Standard solution A: 0.5 mg/mL of USP Anhydrous Lactose RS in *Diluent*
Standard solution B: Contains 0.5 mg/mL of USP Dextrose RS, 0.5 mg/mL of USP Anhydrous Lactose RS, 0.5 mg/mL of USP Fructose RS, and 0.5 mg/mL of USP Sucrose RS in *Diluent*
Sample solution: 0.5 mg/mL of Anhydrous Lactose in *Diluent*
Application volume: 2 µL
Developing solvent system: Ethylene dichloride, glacial acetic acid, methanol, and water (10:5:3:2)
Spray reagent: 5 mg/mL of thymol in a mixture of alcohol and sulfuric acid (19:1)
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Allow the spots to dry, and develop the plate in a paper-lined chromatographic chamber equilibrated with the *Developing solvent system* for about 1 h prior to use. Allow the chromatogram to develop until the solvent front has moved about three-quarters of the length of the plate. Remove the plate from the chamber, dry in a current of

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warm air, and redevelop the plate in fresh *Developing solvent system*. Remove the plate from the chamber, mark the solvent front, and dry the plate in a current of warm air. Spray the plate evenly with *Spray reagent*. Heat the plate at 130° for 10 min.

System suitability: The test is not valid unless *Standard solution B* shows four clearly discernible spots, disregarding any spots at the origin.

Acceptance criteria: The principal spot from the *Sample solution* corresponds in appearance and R_F value to that from *Standard solution A*.

OTHER COMPONENTS

• **Content of Alpha and Beta Anomers**

Silylation reagent: Dimethyl sulfoxide, pyridine, and trimethylsilylimidazole (19.5: 58.5: 22)

Standard solution: Prepare a mixture of alpha-lactose monohydrate and beta-lactose having an anomeric ratio of about 1:1 based on the labeled anomeric contents of the alpha-lactose monohydrate and the beta-lactose. Introduce 10 mg of this mixture into a vial with a screw cap. Add 4 mL of *Silylation reagent*. Sonicate for 20 min at room temperature. Transfer 400 µL to an injection vial. Add 1 mL of pyridine. Close the vial, and mix well.

Sample solution: Introduce 10 mg of Anhydrous Lactose into a vial with a screw cap. Add 4 mL of *Silylation reagent*. Sonicate for 20 min at room temperature. Transfer 400 µL to an injection vial. Add 1 mL of pyridine. Close the vial, and mix well.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Columns

Precolumn:[1] 0.53-mm × 2-m intermediate polarity deactivated fused silica

Analytical:[2] 0.25-mm × 15-m G27 on fused silica; film thickness 0.25 µm

Temperatures

Detector: 325°

Injection port: 275° or use cold on-column injection

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
80	—	80	1
80	35	150	—
150	12	300	2

Carrier gas: Helium

Flow rate: 2.8 mL/min

Injection volume: 0.5 µL

Injection type: Splitless or by cold on-column injection

System suitability

Sample: *Standard solution*

Suitability requirements

Resolution: NLT 3.0 between the peaks due to alpha-lactose and beta-lactose

Analysis

Sample: *Sample solution*

[NOTE—The relative retention time with reference to beta-lactose is about 0.9 for alpha-lactose (retention time = about 12 min).]

Calculate the percentage content of alpha-lactose:

$$\text{Result} = S_a/(S_a + S_b) \times 100$$

S_a = area of the peak due to alpha-lactose
S_b = area of the peak due to beta-lactose

Calculate the percentage content of beta-lactose:

$$\text{Result} = S_b/(S_a + S_b) \times 100$$

S_a = area of the peak due to alpha-lactose
S_b = area of the peak due to beta-lactose

IMPURITIES

• **Residue on Ignition** ⟨281⟩: NMT 0.1%

SPECIFIC TESTS

• **Clarity and Color of Solution**

Hydrazine sulfate solution: Dissolve 1.0 g of hydrazine sulfate in water, and dilute to 100.0 mL. Allow to stand for 4–6 h.

Hexamethylenetetramine solution: In a 100-mL ground-glass stoppered flask dissolve 2.5 g of hexamethylenetetramine in 25.0 mL of water.

Primary opalescent suspension: To the *Hexamethylenetetramine solution* in the flask add 25.0 mL of the *Hydrazine sulfate solution*. Mix and allow to stand for 24 h. This suspension is stable for 2 months, provided it is stored in a glass container free from surface defects. The suspension must not adhere to the glass and must be well mixed before use.

Standard opalescence: Dilute 15.0 mL of the *Primary opalescent suspension* to 1000.0 mL with water. This suspension is freshly prepared and may be stored for up to 24 h.

Reference suspension: To 5.0 mL of the *Standard opalescence* add 95.0 mL of water. Mix and shake before use.

Reference solution: To 6.0 mL of ferric chloride CS, 2.5 mL of cobaltous chloride CS, and 1.0 mL of cupric sulfate CS add hydrochloric acid (10 g/L HCl) to make 1000 mL.

Sample solution: 1 g in 10 mL of boiling water. Allow to cool.

Instrumental conditions

Mode: Vis

Analytical wavelength: 400 nm

Acceptance criteria: NMT 0.04 for the absorbance divided by the path length in centimeters; and the clarity of the *Sample solution* is the same as that of water or its opalescence is not more pronounced than that of the *Reference suspension*, and it is not more colored than the *Reference solution*.

• **Loss on Drying** ⟨731⟩

Analysis: Dry a sample at 80° for 2 h.

Acceptance criteria: NMT 0.5%

• **Water Determination,** *Method I* ⟨921⟩

Sample solution: Anhydrous Lactose in a mixture of methanol and formamide (2:1)

Acceptance criteria: NMT 1.0%

• **Microbial Enumeration Tests** ⟨61⟩ and **Tests for Specified Microorganisms** ⟨62⟩: The total aerobic microbial count is NMT 10^2 cfu/g and the total combined molds and yeasts count is NMT 50 cfu/g. It meets the requirements of the test for absence of *Escherichia coli*.

[1] Restek Guard column is suitable.
[2] Varian CP-Sil 8 CB is suitable.

Supplement 4

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• **PROTEIN AND LIGHT-ABSORBING IMPURITIES**
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Sample solution: 1% solution (w/v)
Instrumental conditions
Mode: UV
Wavelength range: 210–300 nm
Acceptance criteria: NMT 0.25 for the absorbance divided by the path length in centimeters at 210–220 nm; NMT 0.07 for the absorbance divided by the path length in centimeters at 270–300 nm

• **ACIDITY OR ALKALINITY**
Sample solution: Dissolve 6 g by heating in 25 mL of carbon dioxide-free water, cool, and add 0.3 mL of phenolphthalein TS.
Acceptance criteria: The solution is colorless, and NMT 0.4 mL of 0.1 N sodium hydroxide is required to produce a pink or red color.

• **OPTICAL ROTATION,** *Specific Rotation* ⟨781S⟩
Sample solution: Dissolve 10 g by heating in 80 mL of water to 50°. Allow to cool, and add 0.2 mL of 6 N ammonium hydroxide. Allow to stand for 30 min, and dilute with water to 100 mL.
Acceptance criteria: +54.4° to +55.9°, calculated on the anhydrous basis, at 20°

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers.

• **LABELING:** Where the labeling indicates the relative quantities of alpha- and beta-lactose, determine compliance using *Content of Alpha and Beta Anomers*. Where the labeling states the particle size distribution, it also indicates the d_{10}, d_{50}, and d_{90} values and the range for each.

• **USP REFERENCE STANDARDS** ⟨11⟩
USP Dextrose RS
USP Fructose RS
USP Anhydrous Lactose RS
USP Sucrose RS

Lactose Monohydrate

Portions of the monograph text that are national *USP* text, and are not part of the harmonized text, are marked with symbols (• •) to specify this fact.

DEFINITION

Lactose Monohydrate is the monohydrate of O-β-D-galactopyranosyl-(1→4)-α-D-glucopyranose. [NOTE—Lactose Monohydrate may be modified as to its physical characteristics. It may contain varying proportions of amorphous lactose.]

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

• **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩
Diluent: Methanol and water (3:2)
Standard solution A: 0.5 mg/mL of USP Lactose Monohydrate RS in *Diluent*
Standard solution B: 0.5 mg/mL each of USP Dextrose RS, USP Lactose Monohydrate RS, USP Fructose RS, and USP Sucrose RS in *Diluent*
Sample solution: 0.5 mg/mL of Lactose Monohydrate in *Diluent*
Adsorbent: 0.25-mm layer of chromatographic silica gel
Application volume: 2 µL
Developing solvent system: Ethylene dichloride, glacial acetic acid, methanol, and water (10:5:3:2)
Spray reagent: 5 mg/mL of thymol in a mixture of alcohol and sulfuric acid (19:1)
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Allow the spots to dry, and develop the plate in a paper-lined chromatographic chamber equilibrated with *Developing solvent system* for about 1 h prior to use. Allow the chromatogram to develop until the solvent front has moved about three-quarters of the length of the plate. Remove the plate from the chamber, dry in a current of warm air, and redevelop the plate in fresh *Developing solvent system*. Remove the plate from the chamber, mark the solvent front, and dry the plate in a current of warm air. Spray the plate evenly with *Spray reagent*. Heat the plate at 130° for 10 min.
System suitability: The test is not valid unless the chromatogram of *Standard solution B* shows four clearly discernible spots, disregarding any spots at the origin.
Acceptance criteria: The principal spot from the *Sample solution* corresponds in appearance and R_F value to that from *Standard solution A*.•

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩
Analysis: A sample ignited at a temperature of 600 ± 50°
Acceptance criteria: NMT 0.1%

SPECIFIC TESTS

• **CLARITY AND COLOR OF SOLUTION**
Sample solution: 1 g in 10 mL of boiling water
Analysis: The *Sample solution* is clear and nearly colorless. Determine the absorbance of this solution at a wavelength of 400 nm.
Acceptance criteria: The absorbance divided by the path length, in cm, is NMT 0.04.

• • **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic microbial count does not exceed 1×10^2 cfu/g, the total combined molds and yeasts count does not exceed 5×10^1 cfu/g, and it meets the requirements of the test for absence of *Escherichia coli*.•

• **OPTICAL ROTATION,** *Specific Rotation* ⟨781S⟩
Sample solution: Dissolve 10 g by heating in 80 mL of water to 50°. Allow to cool, and add 0.2 mL of 6 N ammonium hydroxide. Allow to stand for 30 min, and dilute with water to 100 mL.
Acceptance criteria: +54.4° to +55.9°, calculated on the anhydrous basis, determined at 20°

• **ACIDITY OR ALKALINITY**
Sample solution: Dissolve 6 g by heating in 25 mL of carbon dioxide-free water, cool, and add 0.3 mL of phenolphthalein TS.
Acceptance criteria: The solution is colorless, and NMT 0.4 mL of 0.1 N sodium hydroxide VS is required to produce a pink or red color.

• • **LOSS ON DRYING** ⟨731⟩
Analysis: Dry a sample at 80° for 2 h.
Acceptance criteria
Monohydrate: NMT 0.5%
Monohydrate, modified: NMT 1.0%•

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• **WATER DETERMINATION,** *Method I* ⟨921⟩
Sample solution: Prepare a preparation containing Lactose Monohydrate in a mixture of methanol and formamide (2:1).
Acceptance criteria: 4.5%–5.5%
• **PROTEIN AND LIGHT-ABSORBING IMPURITIES**
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Sample solution: 1% (w/v)
Analysis: Measure the light absorption of the *Sample solution* in the range of 210–300 nm.
Acceptance criteria: The absorbance divided by the path length, in cm, is NMT 0.25 in the range of 210–220 nm and is NMT 0.07 in the range of 270–300 nm.

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight containers.
• **LABELING:** Where the labeling states the particle size distribution, it also indicates the d_{10}, d_{50}, and d_{90} values and the range for each. For modified Lactose Monohydrate, also label it to indicate the method of modification.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Dextrose RS
USP Fructose RS
USP Lactose Monohydrate RS
USP Sucrose RS

Lanolin, Anhydrous
—see Lanolin General Monographs

Lanolin, Modified—*see Modified Lanolin General Monographs*

Lanolin Alcohols

[8027-33-6].

DEFINITION
Lanolin Alcohols is a mixture of aliphatic alcohols, triterpenoid alcohols, and sterols, obtained by the hydrolysis of Lanolin. It may contain NMT 0.1% of a suitable antioxidant.

IDENTIFICATION
• **A.**
Sample: 0.5 g
Analysis: Dissolve the *Sample* in 5 mL of chloroform, and add 1 mL of acetic anhydride and 2 drops of sulfuric acid.
Acceptance criteria: A green color is produced.

ASSAY
• **CONTENT OF STEROLS** (as cholesterol)
Sample: 20 g
Analysis: Melt the *Sample* on a water bath, mix, and allow to cool. Dissolve 100 mg in 12 mL of warm (60°) 90% alcohol. Allow to stand for 18 h, pass through a medium-porosity, sintered-glass filter, and wash the residue with two 15-mL portions of 90% alcohol. Combine the filtrate and washings, add 20 mL of a freshly prepared 1-in-100 solution of digitonin in 90% alcohol, and warm to 60°. Allow to cool, pass through a medium-porosity, sintered-glass filter with the aid of gentle vacuum, wash the residue with 10 mL of 90% alcohol, and dry at 105° to constant weight. Each g of residue is equivalent to 0.239 g of cholesterol.
Acceptance criteria: NLT 30.0% of sterols, calculated as cholesterol

IMPURITIES
• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.15%
• **COPPER**
Solution A: 1 mg/mL of sodium diethyldithiocarbamate
Sample: 5.0 g
Control: Add 1 mL of *Solution A* and a few drops of 6 N ammonium hydroxide to 2.5 mL of a 39.3-ppm solution of cupric sulfate. Dilute with water to 50 mL.
Analysis: Heat the *Sample* over a small flame until charred, ignite the residue at 550°, and dissolve the ash in 5 mL of hydrochloric acid, with the aid of heat. Cool, dilute with water, render alkaline with ammonium hydroxide, boil to remove the excess ammonia, add a few drops of bromine TS, boil again, and filter. To the filtrate add 1 mL of *Solution A*, a few drops of 6 N ammonium hydroxide, and sufficient water to bring the volume to 50 mL.
Acceptance criteria: The *Sample* is not darker than the *Control* (5 ppm).

SPECIFIC TESTS
• **MELTING RANGE OR TEMPERATURE,** *Class II* ⟨741⟩: NLT 56°
• **ACIDITY AND ALKALINITY**
Analysis: Boil 10 g with 100 mL of water for 5 min, with frequent stirring. Remove the source of heat, add 0.5 mL of phenolphthalein TS, and stir.
Acceptance criteria: No pink color is produced. Add 0.5 mL of methyl orange TS, and stir: no red color is produced.
• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry at 105° for 1 h.
Acceptance criteria: NMT 0.5%
• **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩: NMT 2.0.
• **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩: 120–180
• **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩
Sample: Take wedge-shaped pieces with bases that contain part of the surface.
Analysis: Melt the pieces before carrying out the determination. Before adding the 0.5 mL of saturated potassium iodide solution, cool the solution obtained to room temperature.
Acceptance criteria: NMT 15
• **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩
Sample: 5 g of molten Lanolin Alcohols
Analysis: Reflux the *Sample* with the alcoholic potassium hydroxide for 4 h.
Acceptance criteria: NMT 12

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed, light-resistant containers, and store at controlled room temperature.
• **LABELING:** Label it to indicate the name and quantity of any antioxidant added.

Hydrogenated Lanolin

[8031-44-5].

DEFINITION
A mixture of higher aliphatic alcohols, hydrocarbons, and sterols is obtained from the direct high-pressure, high-temperature hydrogenation of lanolin. During the hydrogenation process, the esters and acids present are reduced to the corresponding alcohols. Alcoholic derivatives

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may be further reduced to hydrocarbons. It may contain antioxidants.

IDENTIFICATION

• **A.**

Sample: 50 mg

Analysis: Dissolve the *Sample* in 5 mL of methylene chloride, and add 1 mL of acetic anhydride and 0.1 mL of sulfuric acid.

Acceptance criteria: A green color is produced.

• **B. CHROMATOGRAPHIC IDENTITY**

Analysis: Proceed as directed in the test for *Chromatographic Profile of Fatty Alcohols, Hydrocarbons, and Sterols* in the *Assay*.

Acceptance criteria: The retention times of the cetyl alcohol, stearyl alcohol, cholestane, cholestanol, and 24,25-dihydrolanosterol peaks of the *Sample solution* correspond to those of *Standard solution A*, as obtained in the *Assay*.

ASSAY

• **CHROMATOGRAPHIC PROFILE OF FATTY ALCOHOLS, HYDROCARBONS, AND STEROLS**

Standard solution A: 2.5 mg/mL of USP Hydrogenated Lanolin RS in dehydrated alcohol

Standard solution B: 0.5 mg/mL of cholestane, 0.5 mg/mL of cholestanol, and 0.25 mg/mL of 24,25-dihydrolanosterol in dehydrated alcohol

Standard solution C: 0.5 mg/mL of USP Cetyl Alcohol RS in dehydrated alcohol. [NOTE—Vortex or sonification helps standard preparation.]

Standard solution D: 0.5 mg/mL of USP Stearyl Alcohol RS in dehydrated alcohol. [NOTE—Vortex or sonification helps standard preparation.]

Sample solution: 2.5 mg/mL of Hydrogenated Lanolin in dehydrated alcohol

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.25-mm × 30-m fused-silica capillary; 0.25-µm layer of phase G2

Temperatures

Detector: 350°

Injection port: 325°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
100	—	100	5
100	5	300	15

Carrier gas: Helium

Flow rate: 1 mL/min

Injection volume: 1 µL

System suitability

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Standard solution D*

[NOTE—See *Table 2* for relative retention times.]

Table 2

Component	Relative Retention Time
Cetyl alcohol	1.00
Stearyl alcohol	1.15
Cholestane	1.63
Cholestanol	1.77
24,25-Dihydrolanosterol	1.85

System suitability requirements

Resolution: NLT 30 between cetyl alcohol and stearyl alcohol, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, *Standard solution D*, and *Sample solution*

Identify the peaks from *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Standard solution D*.

Calculate the percentage of cetyl alcohol (stearyl alcohol, cholestane, cholestanol, or 24,25-dihydrolanosterol) in the portion of Hydrogenated Lanolin taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of cetyl alcohol (stearyl alcohol, cholestane, cholestanol, or 24,25-dihydrolanosterol) from the *Sample solution*

r_S = peak response of cetyl alcohol (stearyl alcohol, cholestane, cholestanol, or 24,25-dihydrolanosterol) from *Standard solution C*, *Standard solution D*, or *Standard solution B*

C_S = concentration of USP Cetyl Alcohol RS (USP Stearyl Alcohol RS, cholestane, cholestanol, or 24,25-dihydrolanosterol) in *Standard solution C*, *Standard solution D*, or *Standard solution B* (mg/mL)

C_U = concentration of Hydrogenated Lanolin in the *Sample solution* (mg/mL)

Acceptance criteria

Sample solution: Chromatogram exhibits a profile similar to that in the chromatogram of *Standard solution A*.

Cetyl alcohol, stearyl alcohol, cholestane, cholestanol, and 24,25-dihydrolanosterol: See *Table 3*.

Table 3

Name	Acceptance Criteria (%)
Cetyl alcohol	2–15
Stearyl alcohol	0.5–10
Cholestane	2–13
Cholestanol	3–11
24,25-Dihydrolanosterol	3–15

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%, determined on 5.0 g

SPECIFIC TESTS

• **MELTING RANGE OR TEMPERATURE** ⟨741⟩: 45°–55°. Allow to stand at 20° for 16 h.

• **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩: NMT 1.0, determined on 5.0 g

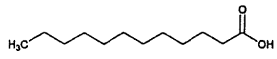
• **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩: 140–180

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- **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩**:** NMT 8.0.
  Heat under reflux for 4 h.
- **LOSS ON DRYING** ⟨731⟩
  Sample: 2.0 g
  Analysis: Dry the *Sample* in an oven at 105° for 1 h.
  Acceptance criteria: NMT 3.0%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light. Do not store above 45°.
- **LABELING:** Label it to indicate the name and amount of the antioxidant added.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Cetyl Alcohol RS
  USP Hydrogenated Lanolin RS
  USP Stearyl Alcohol RS

Lauric Acid



$C_{12}H_{24}O_2$ 200.32
Dodecanoic acid;
1-Dodecanoic acid;
1-Undecanecarboxylic acid;
n-Dodecanoic acid [143-07-7].

DEFINITION
Lauric Acid contains NLT 98.0% and NMT 102.0% of dodecanoic acid ($C_{12}H_{24}O_2$), calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

- **A.** ▲**SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

ASSAY
- **PROCEDURE**
  Solvent A: 0.1% (v/v) trifluoroacetic acid in water
  Mobile phase: Acetonitrile and *Solvent A* (85:15, v/v)
  Standard solution: 15 mg/mL of USP Lauric Acid RS in *Mobile phase*
  Sample solution: 15 mg/mL of Lauric Acid in *Mobile phase*
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: LC
  Detector: UV 212 nm
  Column: 4.6-mm × 15-cm analytical; 5-µm packing L1
  Column temperature: Ambient
  Flow rate: 1.0 mL/min
  Injection volume: 10 µL
  System suitability
  Sample: *Standard solution*
  Suitability requirements
  Relative standard deviation: NMT 3.0%, *Standard solution*
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Calculate the percentage of lauric acid ($C_{12}H_{24}O_2$) in the portion of Lauric Acid taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of lauric acid from the *Sample solution*
r_S = peak response of lauric acid from the *Standard solution*
C_S = concentration of USP Lauric Acid RS in the *Standard solution* (mg/mL)
C_U = concentration of Lauric Acid in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–102.0% on the anhydrous basis

IMPURITIES
- **RESIDUE ON IGNITION** ⟨281⟩
  Sample: 10 g
  Acceptance criteria: NMT 0.1%

SPECIFIC TESTS
- **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩**:** NMT 3.0
- **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩**:** NMT 10.0
- **WATER DETERMINATION,** *Method Ia* ⟨921⟩**:** NMT 0.2%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers. Store at room temperature.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Lauric Acid RS

Lauroyl Polyoxylglycerides

DEFINITION
Lauroyl Polyoxylglycerides is a mixture of monoesters, diesters, and triesters of glycerol and monoesters and diesters of polyethylene glycols. The polyethylene glycols used have a mean molecular weight between 300 and 1500. The article is produced by partial alcoholysis of saturated oils, mainly containing triglycerides of lauric acid with polyethylene glycols, by esterification of glycerol and polyethylene glycols with fatty acids, or as a mixture of glycerol esters and ethylene oxide condensate with the fatty acids of the hydrogenated oils. It may contain free polyethylene glycols.

IDENTIFICATION

Change to read:

- **A.** ▲**SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
- **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩
  Standard solution: 50 mg/mL of USP Lauroyl Polyoxylglycerides RS in methylene chloride
  Sample solution: 50 mg/mL of Lauroyl Polyoxylglycerides in methylene chloride
  Application volume: 10 µL
  Developing solvent system: Ether and hexanes (70:30)
  Spray reagent: 0.1 mg/mL of rhodamine B in alcohol
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Proceed as directed in the chapter. Then spray the plate with *Spray reagent*, and locate the spots on the plate by examination under UV light at a wavelength of 365 nm.
  Acceptance criteria: The R_F values of the principal spots of the *Sample solution* correspond to those of the *Standard solution*.

Supplement 1

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• **C.** It meets the requirements in *Specific Tests* (see *Table 1*) for *Fats and Fixed Oils, Fatty Acid Composition* ⟨401⟩.

IMPURITIES

• **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 0.1%

• **ALKALINE IMPURITIES**

Sample: 5.0 g

Analysis: Heat the *Sample* slightly until the test substance melts, add 10 mL of alcohol and 0.05 mL of bromophenol blue TS, and mix well. While the solution is still warm, titrate with 0.01 N hydrochloric acid VS to change the color to yellow.

Acceptance criteria: NMT 1.0 mL of 0.01 N hydrochloric acid is required.

• **LIMIT OF FREE ETHYLENE OXIDE AND DIOXANE**

Analysis: Proceed as directed in *Ethylene Oxide and Dioxane* ⟨228⟩, *Method I.*

Acceptance criteria

Ethylene oxide: NMT 1 µg/g

Dioxane: NMT 10 µg/g

• **LIMIT OF FREE GLYCEROL**

Sample: 1.2 g

Periodic acetic acid solution: Dissolve 0.446 g of sodium periodate in 2.5 mL of a 25% (v/v) solution of sulfuric acid, diluting to 100.0 mL with glacial acetic acid.

Potassium iodide solution: 75 mg/mL of potassium iodide

Blank: 25 mL of methylene chloride

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Residual titration

Titrant: 0.1 M sodium thiosulfate VS

Endpoint detection: Visual

Analysis: Dissolve the *Sample* in 25 mL of methylene chloride, heating if necessary. Cool, and add 100 mL of water and 25.0 mL of *Periodic acetic acid solution.* Shake, and allow to stand for 30 min. Add 40 mL of *Potassium iodide solution,* and allow to stand for 1 min. Add 1 mL of starch TS, and titrate the liberated iodine with 0.1 M sodium thiosulfate VS. Perform a blank determination, and make any necessary correction.

Calculate the percentage of glycerol in the sample taken:

$$\text{Result} = \{[(V_B - V_S) \times N \times F]/W\} \times 100$$

V_B = *Titrant* volume consumed by the *Blank* (mL)
V_S = *Titrant* volume consumed by the *Sample* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 23.0 mg/mEq
W = *Sample* weight (mg)

Acceptance criteria: NMT 5.0%

SPECIFIC TESTS

• **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩

Sample: 2.0 g

Acceptance criteria: NMT 2.0

• **FATS AND FIXED OILS,** *Fatty Acid Composition* ⟨401⟩**:** Lauroyl Polyoxylglycerides exhibits the composition profile of fatty acids shown in *Table 1.*

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
8	0	≤15.0
10	0	≤12.0
12	0	30.0–50.0
14	0	5.0–25.0
16	0	4.0–25.0
18	0	5.0–35.0

• **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩

Sample: 1.0 g

Acceptance criteria: Within the range specified in *Table 2* for the labeled type

Table 2

Type of Polyethylene Glycols	Hydroxyl Value
300	65–85
400	60–80
600	50–70
1500	36–56

• **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩**:** NMT 2.0

• **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩

Sample: 2.0 g

Acceptance criteria: NMT 6.0

• **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩

Sample: 2.0 g

Acceptance criteria: Within the range specified in *Table 3* for the labeled type

Table 3

Type of Polyethylene Glycols	Saponification Value
300	190–204
400	170–190
600	150–170
1500	79–93

• **WATER DETERMINATION,** *Method I* ⟨921⟩

Sample: 1.0 g

Analysis: Instead of using methanol as the solvent, one of two solvent systems can be used: a mixture of methylene chloride and anhydrous methanol (70:30 v/v), or anhydrous pyridine.

Acceptance criteria: NMT 1.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light and moisture. Store at controlled room temperature.

• **LABELING:** Label it to indicate the type and the average nominal molecular weight of polyethylene glycols used as part of the official title.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Lauroyl Polyoxylglycerides RS

Lecithin

[8002-43-5].

DEFINITION

Lecithin is a complex mixture of acetone-insoluble phosphatides, which consist chiefly of phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and

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phosphatidic acid, present in conjunction with various amounts of other substances such as triglycerides, fatty acids, and carbohydrates, as separated from the crude vegetable oil source. The content of each of the phospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidic acid) is indicated on the certificate of analysis.

IDENTIFICATION

• **A. Identification of Phospholipids by Thin-Layer Chromatography**

Mobile phase: Chloroform, methanol, water (65:25:4, v/v/v)

Standard solution A: 10 mg/mL of USP Phosphatidic Acid (Soy) Monosodium RS and 10 mg/mL of USP Phosphatidylcholine (Soy) RS in *Mobile phase*

Standard solution B: 7 mg/mL of USP Phosphatidylethanolamine (Soy) RS and 7 mg/mL of USP Lysophosphatidylcholine (Soy) RS in *Mobile phase*

Sample solution: 20 mg/mL of Lecithin in *Mobile phase*

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Mode: TLC

Plate: 20-cm × 20-cm, silica gel 60 on aluminum foil, 0.2-mm layer

Application volume: 20 µL

Spray reagent: Dilute 80 mL of phosphoric acid (85%) with 600 mL of water in a 1-L volumetric flask. [Note—Add water to the flask first.] While stirring, add 100 g of anhydrous cupric sulfate. After stirring for 10 min, most of the cupric sulfate is dissolved. Add water to volume and continue stirring until the solid completely dissolves.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Fill the chromatography chamber with the *Mobile phase* to a height of about 0.5 cm. Place a fat-free, U-shaped filter paper in the glass trough and press it against the wall. Sufficient saturation is reached once the *Mobile phase* has permeated to the upper rim of the filter paper. Apply the *Samples* in different bands to the previously marked starting point on a TLC plate. Place the TLC plate in the saturated chromatography chamber. When the *Mobile phase* front has reached the mark (12 cm above the starting point), remove the TLC plate, and dry it using a dryer. Spray or immerse the TLC plate in the *Spray reagent*, and dry it again with a dryer (a current of hot air). Heat the plate to 170° for 10 min. Develop all lipids by charring as dark brown spots.

Acceptance criteria: The R_f values of the spots for phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid, and lysophosphatidylcholine from the *Sample solution* correspond to those from *Standard solution A* and *Standard solution B*. [Note—Depending on the sample tested, if a phospholipid component presents in a low amount in the sample, the corresponding spot in the *Sample solution* on the TLC may not be visualized.]

ASSAY

• **Content of Phospholipids**

[Note—Perform the test for lysophosphatidylcholine for Lecithin intended for use in the manufacture of injectable dosage forms.]

Solution A: Mix 1342 g (2.0 L) of *n*-hexane, 334.1 g (425 mL) of isopropanol (2-propanol), 39.4 g (38 mL) of glacial acetic acid, and 2.0 mL of triethylamine.

Solution B: Mix 663.5 g (850 mL) of isopropanol, 15.8 g (15 mL) of glacial acetic acid, 140 g (140 mL) of water, and 0.8 mL of triethylamine.

Solvent: *n*-Hexane, isopropanol, and water (46:46:8, v/v/v). [Note—To avoid the formation of two phases, mix the isopropanol and water first, and then add the *n*-hexane.]

Mobile phase: See *Table 1*.

Table 1

Program Step	Time (min)	Flow Rate (mL/min)	Solution A (%)	Solution B (%)
1	0	1.0	95	5
2	5.0	1.0	80	20
3	8.5	1.0	60	40
4	14.0	1.0	55	45
5	15.0	1.0	0	100
6	17.5	1.0	0	100
7	17.6	1.0	95	5
8	21.0	1.0	95	5
9	22.0	2.0	95	5
10	27.0	2.0	95	5
11	29.0	1.0	95	5

Phospholipids standard stock solution (2X): 0.8 mg/mL of USP Phosphatidylcholine (Soy) RS, 0.4 mg/mL of USP Phosphatidylethanolamine (Soy) RS, 0.4 mg/mL of phosphatidylinositol prepared from USP Phosphatidylinositol (Soy) Sodium RS, and 0.2 mg/mL of phosphatidic acid prepared from USP Phosphatidic Acid (Soy) Monosodium RS in *Solvent*. [Note—Due to the highly hydroscopic nature of phospholipids, take special precaution in the Standard preparation.]

Phospholipids standard solutions: Prepare as directed in *Table 2*.

Table 2

Concentration	Phospholipids Standard Stock Solution (2X): Solvent (v/v)
0.6X	3:7
0.8X	4:6
1.0X	5:5
1.2X	6:4
1.4X	7:3

System suitability solution: *Phospholipids standard solution 1.0X*

Lysophosphatidylcholine standard stock solution (2X): 60 µg/mL of USP Lysophosphatidylcholine (Soy) RS in *Solvent*

Resolution solution: *Phospholipids standard stock solution (2X)* and *Lysophosphatidylcholine standard stock solution (2X)* (1:1)

Lysophosphatidylcholine standard solution: 30 µg/mL of USP Lysophosphatidylcholine (Soy) RS in *Solvent*

Sample solution: 1 mg/mL of Lecithin in *Solvent*. [Note—If necessary, adjust the concentration of the *Sample solution* to obtain the concentration of each of the phospholipids within the calibration range.]

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Evaporative light-scattering

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Column: 4-mm × 12.5-cm; 5-µm packing L20
Temperatures
Detector: 50°
Column: 55°
Flow rate: 1.0 mL/min with step gradient at 2.0 mL/min (see *Table 1*)
Injection volume: 20 µL
[NOTE—Depending on the different settings of the *Detector*, the *Detector* temperature and *Flow rate* can be adjusted as long as system suitability requirements are met.]
System suitability
Samples: *System suitability solution* and *Resolution solution*
[NOTE—The relative retention times for phosphatidic acid, phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol, and lysophosphatidylcholine are 0.4, 0.9, 1.0, 1.2, and 1.3, respectively, for the *Resolution solution*.]
Suitability requirements
Resolution: NLT 2.0, *System suitability solution*
Relative standard deviation: NMT 5.0%, *System suitability solution*
Analysis
Samples: *Phospholipids standard solutions*, *Lysophosphatidylcholine standard solution*, and *Sample solution*
Identify the peaks of the relevant phospholipids from the *Sample solution* by comparison with the *Phospholipids standard solutions*. Measure the areas of the phospholipid peaks. Plot the logarithms of the relevant responses versus the logarithms of the concentrations, in mg/mL, of each of the phospholipids from the *Standard solutions*, and determine the linear regression line using a least-squares analysis. The correlation coefficient for the linear regression line is NLT 0.995.
From the graphs, determine the concentration (C), in mg/mL, of the relevant phospholipid in the *Sample solution*.
Calculate the percentage of each of the phospholipids (phosphatidic acid, phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositol) in the portion of Lecithin taken:

$$\text{Result} = (C_U/C_S) \times 100$$

C_U = concentration of each of the phospholipids in the *Sample solution* (mg/mL)
C_S = concentration of Lecithin (mg/mL)

Based on the *Lysophosphatidylcholine standard solution*, identify the peak of lysophosphatidylcholine. Compare the peak area of lysophosphatidylcholine from the *Lysophosphatidylcholine standard solution* and the *Sample solution*, respectively.
Acceptance criteria
Content of each of the phospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidic acid): Within the respective ranges stated on the label
For Lecithin intended for use in the manufacture of injectable dosage forms
Content of lysophosphatidylcholine: The peak area of lysophosphatidylcholine in the *Sample solution* is NMT the peak area of lysophosphatidylcholine in the *Lysophosphatidylcholine standard solution*, corresponding to NMT 3.0% of lysophosphatidylcholine in Lecithin.
Content of phosphatidylcholine: NLT 70.0%

IMPURITIES

• **LEAD** ⟨251⟩: NMT 10 ppm

• **HEXANE-INSOLUBLE MATTER**
Sample: If the substance under test is plastic or semisolid, soften the Lecithin by warming it at a temperature not exceeding 60°, and then mix. Weigh 10.0 g into a 250-mL conical flask.
Analysis: To the *Sample* add 100 mL of hexane. Shake until solution is apparently complete or until no more residue seems to be dissolving. Pass through a coarse-porosity filtering funnel that previously has been heated at 105° for 1 h, cooled, and weighed. Wash the flask with two 25-mL portions of hexane, and pour both washings through the funnel. Dry the funnel at 105° for 1 h. [**CAUTION**—Hexane is flammable.] Cool to room temperature, and determine the gain in weight.
Acceptance criteria: NMT 0.3%
For Sunflower Lecithin: NMT 1.0%

SPECIFIC TESTS

• **CONTENT OF ACETONE-INSOLUBLE MATTER**
Sample: If the substance under test is plastic or semisolid, soften the Lecithin by warming it briefly at a temperature not exceeding 60°, and then mix. Transfer 2 g to a 40-mL centrifuge tube that previously has been tared along with a stirring rod, cool, and weigh.
Analysis: To the *Sample* add 15.0 mL of acetone, and warm carefully in a water bath to melt the test specimen without evaporating the acetone. Stir to help dissolve completely, and place in an ice-water bath for 5 min. De-oiled lecithin and fractions are suspended in acetone by stirring. Add acetone that has been previously chilled to 0°–5° to the 40-mL mark on the tube, stirring during the addition. Cool in an ice-water bath for 15 min, stir, remove the rod, clarify by centrifuging at about 2000 rpm for 5 min, and decant. Break up the residue with the stirring rod, and refill the centrifuge tube to the 40-mL mark with chilled acetone, while stirring. Cool in an ice-water bath for 15 min, stir, remove the rod, centrifuge, and decant. Break up the residue with the stirring rod. Place the tube in a horizontal position until most of the acetone has evaporated. Mix again, and heat the tube containing the acetone-insoluble residue and the stirring rod at 105° to constant weight. [**CAUTION**—Acetone is flammable.]
Determine the weight of the residue, and calculate the percentage of acetone-insoluble matter.
Acceptance criteria: NLT 50.0%
For Lecithin intended for use in the manufacture of injectable dosage forms: NLT 80.0%

• **FATS AND FIXED OILS** ⟨401⟩, *Acid Value*
Sample: If the substance under test is plastic or semisolid, soften the Lecithin by warming it briefly at a temperature not exceeding 60°, and then mix. Transfer 2 g to a 250-mL conical flask.
Analysis: Dissolve the *Sample* in 50 mL of petroleum ether with 100°–120° boiling range. To this solution add 50 mL of alcohol, previously neutralized to phenolphthalein with 0.1 N sodium hydroxide, and mix. Add phenolphthalein TS. Titrate with 0.1 N sodium hydroxide VS to a pink endpoint that persists for 5 s.
Calculate the amount, in mg, of potassium hydroxide required to neutralize the free acids in 1.0 g of Lecithin:

$$\text{Result} = (M_r \times N \times V)/W$$

M_r = molecular weight of potassium hydroxide, 56.11
N = normality of the sodium hydroxide VS
V = volume of the sodium hydroxide VS consumed in the titration (mL)
W = weight of Lecithin taken (g)

Acceptance criteria: NMT 36

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• **PEROXIDE VALUE**
Sample: 5 g of Lecithin
Analysis: Transfer the *Sample* into a 250-mL Erlenmeyer flask with a ground-glass stopper, add 35 mL of a mixture of chloroform and glacial acetic acid (2:1), and mix. Completely dissolve the test specimen while shaking gently. The solution becomes transparent. Completely replace the air in the flask with nitrogen. While purging with nitrogen, add 1 mL of potassium iodide solution (165 mg/mL of potassium iodide), then stop the flow of the nitrogen, and immediately place a stopper in the flask. Shake for 1 min, and allow to stand in a dark place for 5 min. Add 75 mL of water, replace the stopper again, and shake vigorously. Titrate with 0.01 N sodium thiosulfate VS, adding starch TS as the endpoint is approached, and continue the titration until the blue starch color has just disappeared. Perform a blank determination (see *Titrimetry* ⟨541⟩), and make any necessary correction.
Calculate the peroxide value, as mEq of peroxide per 1000 g of Lecithin:

$$\text{Result} = (S \times N/W) \times 1000$$

S = net volume of sodium thiosulfate solution required for titration (mL)
N = normality of the sodium thiosulfate solution
W = weight of Lecithin taken (g)

Acceptance criteria: NMT 10
For Lecithin intended for use in the manufacture of injectable dosage forms: NMT 3
• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g. It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.
• **WATER DETERMINATION** ⟨921⟩, *Method I*: NMT 2.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed, light-resistant containers. Store at the temperature indicated on the label. Protect from excess heat and moisture.
• **LABELING:** Label to indicate the content of each of the phospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidic acid). The labeling also indicates the natural source of lecithin. Where Lecithin is intended for use in the manufacture of injectable dosage forms, it is so labeled. Label it to indicate the storage conditions.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Lysophosphatidylcholine (Soy) RS
USP Phosphatidic Acid (Soy) Monosodium RS
USP Phosphatidylcholine (Soy) RS
USP Phosphatidylethanolamine (Soy) RS
USP Phosphatidylinositol (Soy) Sodium RS

Lemon Oil

DEFINITION

Lemon Oil is the volatile oil obtained by expression, without the aid of heat, from the fresh peel of the fruit of *Citrus x limon* (L.) Osbeck (Fam. Rutaceae), with or without the previous separation of the pulp and the peel. The total aldehyde content, calculated as citral ($C_{10}H_{16}O$), is NLT 2.2% and NMT 3.8% for California-type Lemon Oil, and NLT 3.0% and NMT 5.5% for Italian-type Lemon Oil.
[NOTE—Do not use Lemon Oil that has a terebinthine odor.]

ASSAY

• **TOTAL ALDEHYDE CONTENT**
Reagent solution: Dissolve 4.5 g of hydroxylamine hydrochloride in 13 mL of water. Add 85 mL of tertiary butyl alcohol, mix, and adjust with 0.5 N potassium hydroxide to a pH of 3.4.
Sample: 5 mL
Analysis: Pipet 50 mL of the *Reagent solution* into a conical flask containing the *Sample*. Insert the stopper in the flask, and allow to stand at room temperature for 30 min, with occasional shaking. Titrate the liberated hydrochloric acid with 0.5 N alcoholic potassium hydroxide VS to a pH of 3.4. Each mL of 0.5 N alcoholic potassium hydroxide consumed in the titration is equivalent to 76.12 mg of total aldehydes, calculated as citral ($C_{10}H_{16}O$).
Acceptance criteria: The total aldehyde content, calculated as citral ($C_{10}H_{16}O$), is 2.2%–3.8% for California-type Lemon Oil or 3.0%–5.5% for Italian-type Lemon Oil.

SPECIFIC TESTS

• **SPECIFIC GRAVITY** ⟨841⟩**:** 0.849–0.855
• **OPTICAL ROTATION,** *Angular Rotation* ⟨781A⟩**:** +57° to +65.6°
• **REFRACTIVE INDEX** ⟨831⟩**:** 1.473–1.476 at 20°
• **ULTRAVIOLET ABSORBANCE**
Sample solution: Dilute 250 mg of Oil to 100 mL with alcohol
Blank: Alcohol
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: UV-Vis
Spectral range: 260–400 nm
Analysis
Samples: *Sample solution* and *Blank*
Record the spectrum in a 1-cm cell, and determine the absorbance at the wavelength of maximum absorbance at about 315 nm using the line drawn tangent to the curves appearing as minima in the spectrum in wavelength regions above and below the maximum wavelength as the baseline.
Acceptance criteria: The absorbance, calculated on the basis of a 250-mg specimen, is NLT 0.20 for California-type Lemon Oil or NLT 0.49 for Italian-type Lemon Oil.
• **FOREIGN OILS:** Place 50 mL of Oil in a four-bulb Ladenburg flask having the following dimensions: the lower or main bulb is about 6 cm in diameter, and the smaller condensing bulbs are about 3.5, 3.0, and 2.5 cm in diameter; the distance from the bottom of the flask to the side-arm is about 20 cm. Distill Oil at a rate of 1 drop/s until the distillate measures 5 mL: the angular rotation of the first 5 mL is NMT 6° less than that of the original Oil. The refractive index at 20° of this same portion is 0.001–0.003 lower than that of the original Oil.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-filled, tight containers, and avoid exposure to excessive heat.
• **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant source from which the article was derived. Label it to also indicate whether it is California-type or Italian-type Lemon Oil. The label indicates that Oil is not to be used if it has a terebinthine odor.

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Lemon Tincture

DEFINITION

Lemon Tincture is prepared from lemon peel, which is the outer yellow rind of the fresh, ripe fruit of *Citrus* × *Limon* Osbeck (Fam. Rutaceae).

Prepare Lemon Tincture as follows.

Lemon Peel	500 g
Alcohol	900 mL
Alcohol, a sufficient quantity to make	1000 mL

Macerate the *Lemon Peel* in 900 mL of *Alcohol* in a closed container, and store in a warm place. Agitate the container frequently for 3 days or until the soluble matter is dissolved. Transfer the mixture to a filter, using talc as the filtering medium, and when most of the liquid has drained away, wash the residue on the filter with a sufficient amount of *Alcohol*, and combine the filtrates so that the preparation is brought to a final volume of 1000 mL.

OTHER COMPONENTS

- **Alcohol Determination,** *Method I* ⟨611⟩**:** 62%–72% of the labeled amount

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Package in tight, light-resistant containers, and avoid exposure to direct sunlight and to excessive heat. Store at controlled room temperature.
- **Labeling:** The label states the Latin binomial and, following the official name, the part of the plant source from which the article was derived.

Licorice Fluidextract

DEFINITION

Prepare Licorice Fluidextract as follows (see *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩). To 1000 g of coarsely ground Licorice, add about 3000 mL of boiling Purified Water, mix, and allow to macerate in a suitable, covered percolator for 2 h. Allow the percolation to proceed at a rate of 1–3 mL/min, gradually adding boiling Purified Water until the Licorice is exhausted. Add enough diluted ammonia solution to the percolate to impart a distinctly ammoniacal odor, and boil the liquid actively under normal atmospheric pressure until it is reduced in volume to about 1500 mL. Filter the liquid, evaporate the filtrate on a steam bath until the residue measures 750 mL, cool, gradually add 250 mL of Alcohol and enough Purified Water to make the product measure 1000 mL, and mix.

OTHER COMPONENTS

- **Alcohol Determination,** *Method I* ⟨611⟩ **:** 20.0%–24.0%

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in tight, light-resistant containers, and avoid exposure to direct sunlight and to excessive heat.
- **Labeling:** The label states the Latin binomial and, following the official name, the part of the plant source from which the article was derived.

Linoleoyl Polyoxylglycerides

DEFINITION

Linoleoyl Polyoxylglycerides is a mixture of monoesters, diesters, and triesters of glycerol and monoesters and diesters of polyethylene glycols. The polyethylene glycols used have a mean molecular weight between 300 and 400. The article is produced by partial alcoholysis of unsaturated oils, mainly containing triglycerides of linoleic acid with polyethylene glycol, by esterification of glycerol and polyethylene glycol with fatty acids, or as a mixture of glycerol esters and ethylene oxide condensate with the fatty acids of the unsaturated oils. It may contain free polyethylene glycols.

IDENTIFICATION

Change to read:

- **A. ▲Spectroscopic Identification Tests** ⟨197⟩, *Infrared Spectroscopy*: **197F** ▲ (CN 1-May-2020)
- **B. Thin-Layer Chromatographic Identification Test** ⟨201⟩

Standard solution: 50 mg/mL of USP Linoleoyl Polyoxylglycerides RS in methylene chloride

Sample solution: 50 mg/mL of Linoleoyl Polyoxylglycerides in methylene chloride

Application volume: 10 µL

Developing solvent system: Ether and hexanes (70:30)

Spray reagent: 0.1 mg/mL of rhodamine B in alcohol

Analysis

Samples: *Standard solution* and *Sample solution*

Proceed as directed in the chapter. Then spray the plate with *Spray reagent*, and locate the spots on the plate by examination under UV light at a wavelength of 365 nm.

Acceptance criteria: The R_F values of the principal spots of the *Sample solution* correspond to those of the *Standard solution*.

- **C.** It meets the requirements in *Specific Tests* (see *Table 1*) for *Fats and Fixed Oils, Fatty Acid Composition* ⟨401⟩.

IMPURITIES

- **Articles of Botanical Origin,** *Total Ash* ⟨561⟩**:** NMT 0.1%
- **Alkaline Impurities**

Sample: 5.0 g

Analysis: To the *Sample* add 10 mL of alcohol and 0.05 mL of bromophenol blue TS, and mix well. Titrate with 0.01 N hydrochloric acid VS to change the color to yellow.

Acceptance criteria: NMT 1.0 mL of 0.01 N hydrochloric acid is required.

- **Limit of Free Ethylene Oxide and Dioxane**

Analysis: Proceed as directed in *Ethylene Oxide and Dioxane* ⟨228⟩, *Method I*.

Acceptance criteria

Ethylene oxide: NMT 1 µg/g

Dioxane: NMT 10 µg/g

- **Limit of Free Glycerol**

Sample: 1.2 g

Periodic acetic acid solution: Dissolve 0.446 g of sodium periodate in 2.5 mL of a 25% (v/v) solution of sulfuric acid. Dilute with glacial acetic acid to 100.0 mL.

Potassium iodide solution: 75 mg/mL of potassium iodide

Blank: 25 mL of methylene chloride

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Residual titration

Titrant: 0.1 M sodium thiosulfate VS

Endpoint detection: Visual

Analysis: Dissolve the *Sample* in 25 mL of methylene chloride, heating if necessary. Cool, and add 100 mL of

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water and 25.0 mL of *Periodic acetic acid solution*. Shake, and allow to stand for 30 min. Add 40 mL of *Potassium iodide solution*, and allow to stand for 1 min. Add 1 mL of starch TS, and titrate the liberated iodine with 0.1 M sodium thiosulfate VS. Perform a blank determination, and make any necessary correction.
Calculate the percentage of glycerol in the sample taken:

$$\text{Result} = \{[(V_B - V_S) \times N \times F]/W\} \times 100$$

V_B = *Titrant* volume consumed by the *Blank* (mL)
V_S = *Titrant* volume consumed by the *Sample* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 23.0 mg/mEq
W = *Sample* weight (mg)

Acceptance criteria: NMT 5.0%

SPECIFIC TESTS

- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩
  Sample: 2.0 g
  Acceptance criteria: NMT 2.0
- **FATS AND FIXED OILS,** *Fatty Acid Composition* ⟨401⟩**:**
  Linoleoyl Polyoxylglycerides exhibits the composition profile of fatty acids shown in *Table 1*.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
16	0	4.0–20.0
18	0	≤6.0
18	1	20.0–35.0
18	2	50.0–65.0
18	3	≤2.0
20	0	≤1.0
20	1	≤1.0

- **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩
  Sample: 1.0 g
  Acceptance criteria: 45–65
- **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩**:** 90–110
- **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩
  Sample: 2.0 g
  Acceptance criteria: NMT 12.0
- **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩
  Sample: 2.0 g
  Acceptance criteria: 150–170
- **WATER DETERMINATION,** *Method I* ⟨921⟩
  Sample: 1.0 g
  Analysis: Instead of using methanol as the solvent, one of two solvent systems can be used: a mixture of methylene chloride and anhydrous methanol (70:30 v/v), or anhydrous pyridine.
  Acceptance criteria: NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light and moisture. Store at controlled room temperature.
- **LABELING:** Label it to indicate the type and the average nominal molecular weight of polyethylene glycol used as part of the official title.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Linoleoyl Polyoxylglycerides RS

Magnesium Aluminometasilicate

DEFINITION

Magnesium Aluminometasilicate is a synthetic material that exists in two forms, Type I-A and Type I-B, having different pH requirements. The required contents for both forms are the same: NLT 29.1% and NMT 35.5% of aluminum oxide (Al_2O_3), NLT 11.4% and NMT 14.0% of magnesium oxide (MgO), and NLT 29.2% and NMT 35.6% of silicon dioxide (SiO_2), calculated on the dried basis.

IDENTIFICATION

- **A. IDENTIFICATION TESTS—GENERAL,** *Aluminum* ⟨191⟩
  Sample solution: Transfer 0.5 g of Magnesium Aluminometasilicate to a suitable container, add 5 mL of a sulfuric acid solution (1 in 3), and heat until white fumes are observed. Cool, add 20 mL of water, and filter. Neutralize the filtrate with ammonia TS, and retain for use in *Identification* test *B*. Collect the precipitate, and dissolve in 3 N hydrochloric acid.
  Acceptance criteria: The *Sample solution* meets the requirements.
- **B. IDENTIFICATION TESTS—GENERAL,** *Magnesium* ⟨191⟩
  Sample solution: Use the filtrate retained from *Identification* test *A*.
  Acceptance criteria: The *Sample solution* meets the requirements.
- **C.**
  Analysis: Prepare a bead by fusing a few crystals of sodium ammonium phosphate on a platinum loop in the flame of a Bunsen burner. Place the hot, transparent bead in contact with Magnesium Aluminometasilicate, and again fuse.
  Acceptance criteria: Silica floats about in the bead producing, upon cooling, an opaque bead with a web-like structure.

ASSAY

- **ALUMINUM OXIDE**
  Edetate disodium titrant: Prepare a solution with a concentration of 18.6 g/L of edetate disodium in water and standardize as follows. Weigh 2 g of aluminum wire, transfer to a 1000-mL volumetric flask, and add 50 mL of a mixture of hydrochloric acid and water (1:1). Swirl the flask to ensure contact of the aluminum and the acid, and allow the reaction to proceed until all of the aluminum has dissolved. Dilute with water to volume. Pipet 10 mL of this solution into a 250-mL beaker and add, in the order named and with continuous stirring, 25.0 mL of *Edetate disodium titrant* and 20 mL of acetic acid–ammonium acetate buffer TS, and boil gently for 5 min. Cool, and add 50 mL of alcohol and 2 mL of dithizone TS. Titrate with 0.05 M zinc sulfate VS to a bright rose-pink color. Perform a blank determination, substituting 10 mL of water for the aluminum solution, and make any necessary correction.
  Calculate the molarity of the solution taken:

$$\text{Result} = W/(A_r \times V)$$

W = weight of aluminum in the portion of solution taken (mg)
A_r = atomic weight of aluminum, 26.98
V = volume of *Edetate disodium titrant* consumed (mL)

Sample solution: Transfer 1.25 g of Magnesium Aluminometasilicate to a conical flask, add 10 mL of 3 N hydrochloric acid and 50 mL of water, and heat on a water bath for 15 min. To this solution add 8 mL of hydrochloric acid, and heat on a water bath for 10 min. After cooling, transfer the solution to a 250-mL volumetric flask, rinse the

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conical flask with water, and add the washings to the volumetric flask. Dilute with water to volume. Centrifuge, and use the supernatant as the *Sample solution.* Retain a portion for use in the *Assay* for *Magnesium Oxide.*

Analysis: Transfer 20.0 mL of the *Sample solution* to a beaker and add 20.0 mL of *Edetate disodium titrant.* To this solution add 15 mL of acetic acid–ammonium acetate buffer TS and 20 mL of water, and boil for 5 min. After cooling, add 50 mL of alcohol and 2 mL of dithizone TS, and titrate with 0.05 M zinc sulfate VS until the color of the solution changes from green-violet to rose-pink. Perform a blank determination. Each mL of 0.05 M *Edetate disodium titrant* is equivalent to 2.5490 mg of Al_2O_3.

Acceptance criteria: 29.1%–35.5% of aluminum oxide (Al_2O_3) on the dried basis

- **MAGNESIUM OXIDE**

Sample solution: Use the *Sample solution* prepared for use in the *Assay* for *Aluminum Oxide.*

Analysis: Transfer 50.0 mL of the *Sample solution* to a suitable container, add 50 mL of water and 25 mL of a trolamine solution (1 in 2), and shake well. Add 25 mL of ammonia–ammonium chloride buffer TS and 0.04 g of eriochrome black T trituration as the indicator. Titrate with 0.05 M edetate disodium VS until the red-purple color changes to blue and persists for 30 s. Each mL of 0.05 M edetate disodium VS is equivalent to 2.0152 mg of MgO.

Acceptance criteria: 11.4%–14.0% of magnesium oxide (MgO) on the dried basis

- **SILICON DIOXIDE**

Sample: 1 g

Analysis: To the *Sample* add 30 mL of 3 N hydrochloric acid, and evaporate on a water bath to dryness. Moisten the residue with hydrochloric acid, and evaporate again on a water bath to dryness. To the residue add 8 mL of hydrochloric acid and 25 mL of hot water, and stir. Allow to stand, and then decant the supernatant through an ashless filter paper. To the residue in the container add 10 mL of hot water, stir, and decant the supernatant through the filter paper. Wash the residue in the container with three additional 10-mL portions of hot water, stir, and decant as described above. Treat the residue in the container with 50 mL of water, and heat on a water bath for 15 min. Filter, and rinse the residue on the filter paper with hot water until no precipitate is obtained when 1 mL of silver nitrate TS is added to 5 mL of the washing. Transfer the filter paper and its contents to a tared platinum crucible, heat to dryness, incinerate, and continue to heat at 800 ± 25° for 1 h. Cool, and weigh. Moisten the residue with 6 mL of hydrofluoric acid, evaporate to dryness, and ignite for 5 min. Cool, and weigh. The loss in weight represents the weight of silicon dioxide (SiO_2).

Acceptance criteria: 29.2%–35.6% of silicon dioxide (SiO_2) on the dried basis

IMPURITIES

- **CHLORIDE AND SULFATE,** *Chloride* ⟨221⟩

Sample: A 20-mL portion of the diluted filtrate retained from the test for *Soluble Salts*

Control: 0.75 mL of 0.020 N hydrochloric acid

Acceptance criteria: NMT 0.053%; the *Sample* shows no more chloride than corresponds to the *Control.*

- **CHLORIDE AND SULFATE,** *Sulfate* ⟨221⟩

Sample: A 2-mL portion of the diluted filtrate retained from the test for *Soluble Salts*

Control: 0.5 mL of 0.020 N sulfuric acid

Acceptance criteria: NMT 0.480%; the *Sample* shows no more sulfate than corresponds to the *Control.*

- **ARSENIC,** *Method I* ⟨211⟩**:** NMT 3 µg/g
- **IRON** ⟨241⟩

Sample solution: To 0.11 g of Magnesium Aluminometasilicate add 8 mL of 2 N nitric acid, boil for 1 min, and cool. Dilute with water to 100 mL, and centrifuge. Dilute 30 mL of the supernatant with water to 45 mL.

Acceptance criteria: NMT 0.03%

SPECIFIC TESTS

- **ACID-CONSUMING CAPACITY**

Sample solution: Transfer 0.2 g of Magnesium Aluminometasilicate to a glass-stoppered flask, and add 100.0 mL of 0.1 N hydrochloric acid VS. Stopper the flask tightly, shake at 37 ± 2° for 1 h, and filter. Use the filtrate.

Analysis: Transfer 50.0 mL of the *Sample solution* to a beaker, and while stirring, titrate the excess hydrochloric acid with 0.1 N sodium hydroxide VS to attain a pH of 3.5. Perform a blank determination.

Acceptance criteria: NLT 210 mL of 0.1 N hydrochloric acid is consumed per g of Magnesium Aluminometasilicate, calculated on the dried basis.

- **pH** ⟨791⟩

Sample: 2 g

Analysis: Add 50 mL of water to the *Sample.* While stirring, immerse the pH electrodes in the suspension, and after 2 min, record the pH.

Acceptance criteria

Type I-A: 6.5–8.5

Type I-B: 8.5–10.5

- **LOSS ON DRYING** ⟨731⟩

Analysis: Dry at 110° for 7 h.

Acceptance criteria: NMT 20.0%

- **SOLUBLE SALTS**

Sample: 10.0 g

Analysis: Transfer the *Sample* to a suitable container, add 150 mL of water, and boil gently for 15 min, with shaking. After cooling, dilute with water to 150 mL, and centrifuge. Dilute 75 mL of the clear filtrate with water to 100 mL, and retain the diluted filtrate for use in the tests for *Alkalinity, Chloride,* and *Sulfate.* Evaporate 25 mL of the diluted filtrate on a water bath, and heat at 700° for 2 h.

Acceptance criteria: NMT 0.020 g (NMT 1.6%)

- **ALKALINITY**

Sample: A 20-mL portion of the diluted filtrate retained from the test for *Soluble Salts*

Analysis: Add 2 drops of phenolphthalein TS to the *Sample.*

Acceptance criteria: If a pink color is produced, NMT 0.50 mL of 0.1 N hydrochloric acid is required to discharge it.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and prevent exposure to excessive heat.
- **LABELING:** Label it to indicate whether it is Type I-A or Type I-B.

Magnesium Aluminosilicate

DEFINITION

Magnesium Aluminosilicate is a synthesized material that contains NLT 20.5% and NMT 27.7% of magnesium oxide (MgO), NLT 27.0% and NMT 34.3% of aluminum oxide (Al_2O_3), and NLT 14.4% and NMT 21.7% of silicon dioxide (SiO_2), calculated on the dried basis.

IDENTIFICATION

- **A. IDENTIFICATION TESTS—GENERAL,** *Aluminum* ⟨191⟩

Sample: 0.5 g

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Analysis: Transfer the *Sample* to a suitable container, add 5 mL of a sulfuric acid solution (1 in 3), and heat until white fumes are observed. Cool, add 20 mL of water, and filter. Neutralize the filtrate with ammonia TS, and retain for use in *Identification* test *B*. Collect the precipitate, and dissolve in 3 N hydrochloric acid.

Acceptance criteria: Meets the requirements

• **B. IDENTIFICATION TESTS—GENERAL,** *Magnesium* ⟨191⟩

Sample solution: The filtrate retained from *Identification* test *A*

Acceptance criteria: Meets the requirements

• **C.**

Analysis: Prepare a bead by fusing a few crystals of sodium ammonium phosphate on a platinum loop in the flame of a Bunsen burner. Place the hot, transparent bead in contact with Magnesium Aluminosilicate, and again fuse.

Acceptance criteria: The silica floats about in the bead, producing, upon cooling, an opaque bead with a weblike structure.

ASSAY

• **ALUMINUM OXIDE**

Edetate disodium titrant: Prepare a solution with a concentration of 18.6 g/L of edetate disodium in water, and standardize as follows. Weigh 2 g of aluminum wire, transfer to a 1000-mL volumetric flask, and add 50 mL of a mixture of hydrochloric acid and water (1:1). Swirl the flask to ensure contact of the aluminum and the acid, and allow the reaction to proceed until all the aluminum has dissolved. Dilute with water to volume. Pipet 10 mL of this solution into a 250-mL beaker, and add, in the order named and with continuous stirring, 25.0 mL of *Edetate disodium titrant* and 20 mL of acetic acid–ammonium acetate buffer TS. Boil gently for 5 min. Cool, and add 50 mL of alcohol and 2 mL of dithizone TS. Titrate with 0.05 M zinc sulfate VS to a bright rose-pink color. Perform a blank determination, substituting 10 mL of water for the aluminum solution, and make any necessary correction. Calculate the molarity of the solution taken:

$$\text{Result} = W/(A_r \times V)$$

W = weight of aluminum in the portion of solution taken (g)
A_r = atomic weight of aluminum, 26.98 g/mol
V = volume of *Edetate disodium titrant* consumed (mL)

Sample solution: Transfer 1.25 g of Magnesium Aluminosilicate to a conical flask, add 10 mL of 3 N hydrochloric acid and 50 mL of water, and heat on a water bath for 15 min. To this solution add 8 mL of hydrochloric acid, and heat on a water bath for 10 min. After cooling, transfer the solution to a 250-mL volumetric flask, rinse the conical flask with water, and add the washings to the volumetric flask. Dilute with water to volume. Centrifuge, and use the supernatant as the *Sample solution*.

[NOTE—Retain a portion of the *Sample solution* for use in the *Assay* for *Magnesium Oxide*.]

Blank: 10 mL of 3 N hydrochloric acid and 50 mL of water

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Residual titration

Titrant: *Edetate disodium titrant*

Back-titrant: 0.05 M zinc sulfate VS

Endpoint detection: Visual

Analysis: Transfer 20.0 mL of the *Sample solution* to a beaker, and add 20.0 mL of *Titrant*. To this solution add 15 mL of acetic acid–ammonium acetate buffer TS and 20 mL of water, and boil for 5 min. After cooling, add 50 mL of alcohol and 2 mL of dithizone TS, and titrate with the *Back-titrant* until the color of the solution changes from green-violet to rose-pink. Perform a blank determination, and make the necessary correction. Each mL of 0.05 M *Edetate disodium titrant* is equivalent to 2.5490 mg of aluminum oxide (Al_2O_3).

Acceptance criteria: 27.0%–34.3% on the dried basis

• **MAGNESIUM OXIDE**

Sample solution: Use the portion retained from the *Sample solution* prepared in the *Assay* for *Aluminum Oxide*.

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Direct titration

Titrant: 0.05 M edetate disodium VS

Endpoint detection: Visual

Analysis: Transfer 50.0 mL of the *Sample solution* to a suitable container, add 50 mL of water and 25 mL of a trolamine solution (500 mg/mL), and shake well. Add 25 mL of ammonia–ammonium chloride buffer TS and 0.04 g of eriochrome black TS trituration as the indicator. Titrate with *Titrant* until the red-purple color changes to blue and persists for 30 s. Each mL of 0.05 M edetate disodium VS is equivalent to 2.0152 mg of magnesium oxide (MgO).

Acceptance criteria: 20.5%–27.7% on the dried basis

• **SILICON DIOXIDE**

Sample: 1 g

Analysis: To the *Sample* add 30 mL of 3 N hydrochloric acid, and evaporate on a water bath to dryness. Moisten the residue with hydrochloric acid, and again evaporate on a water bath to dryness. To the residue add 8 mL of hydrochloric acid and 25 mL of hot water, and stir. Allow to stand, then decant the supernatant through an ashless filter paper. To the residue in the container add 10 mL of hot water, stir, and decant the supernatant through the filter paper. Wash the residue in the container with three additional 10-mL portions of hot water, stir, and decant as described above. Treat the residue in the container with 50 mL of water, and heat on a water bath for 15 min. Filter, and rinse the residue on the filter paper with hot water until no precipitate is obtained when 1 mL of silver nitrate TS is added to 5 mL of the washing. Transfer the filter paper and its contents to a tared platinum crucible, heat to dryness, incinerate, and continue to heat at 800 ± 25° for 1 h. Cool, and weigh. Moisten the residue with 6 mL of hydrofluoric acid, evaporate to dryness, and ignite for 5 min. Cool, and weigh. The loss in weight represents the weight of SiO_2.

Acceptance criteria: 14.4%–21.7% on the dried basis

IMPURITIES

• **CHLORIDE AND SULFATE,** *Chloride* ⟨221⟩

Analysis: A 20-mL portion of the diluted filtrate retained from the test for *Soluble Salts* shows no more chloride than corresponds to 0.75 mL of 0.020 N hydrochloric acid.

Acceptance criteria: NMT 0.053%

• **CHLORIDE AND SULFATE,** *Sulfate* ⟨221⟩

Analysis: A 2-mL portion of the diluted filtrate retained from the test for *Soluble Salts* shows no more sulfate than corresponds to 0.5 mL of 0.020 N sulfuric acid.

Acceptance criteria: NMT 0.480%

• **ARSENIC,** *Method I* ⟨211⟩**:** NMT 3 µg/g

• **IRON** ⟨241⟩

Sample: 0.11 g

Analysis: To the *Sample* add 8 mL of 2 N nitric acid, boil for 1 min, and cool. Dilute with water to 100 mL, and centrifuge. Dilute 30 mL of the supernatant with water to 45 mL.

Acceptance criteria: NMT 0.03%

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SPECIFIC TESTS

• **ACID-CONSUMING CAPACITY**

Sample solution: Transfer 0.2 g of Magnesium Aluminosilicate to a glass-stoppered flask, and add 100.0 mL of 0.1 N hydrochloric acid VS. Stopper the flask tightly, shake at 37 ± 2° for 1 h, and filter. Use the filtrate.

Analysis: Transfer 50.0 mL of the filtrate from the *Sample solution* to a beaker, and while stirring, titrate the excess hydrochloric acid with 0.1 N sodium hydroxide VS to a pH of 3.5. Perform a blank determination, and make any necessary corrections.

Acceptance criteria: NLT 250 mL of 0.1 N hydrochloric acid is consumed per g of Magnesium Aluminosilicate, calculated on the dried basis.

• **PH** ⟨791⟩

Sample: 2 g

Analysis: Add 50 mL of water to the *Sample*. While stirring, immerse the pH electrodes in the suspension, and after 2 min, record the pH.

Acceptance criteria: 8.5–10.5

• **LOSS ON DRYING** ⟨731⟩

Analysis: Dry at 110° for 7 h.

Acceptance criteria: NMT 20.0%

• **SOLUBLE SALTS**

Sample: 10.0 g

Analysis: Transfer the *Sample* to a suitable container, add 150 mL of water, and boil gently for 15 min, with shaking. After cooling, dilute with water to 150 mL, and centrifuge. Dilute 75 mL of the clear filtrate with water to 100 mL, and retain the diluted filtrate for use in the tests for *Chloride*, *Sulfate*, and *Alkalinity*. Evaporate 25 mL of the diluted filtrate on a water bath, and heat at 700° for 2 h.

Acceptance criteria: NMT 1.6%; the residue weighs NMT 0.020 g.

• **ALKALINITY**

Sample: 20 mL of diluted filtrate retained from the test for *Soluble Salts*

Analysis: Add 2 drops of phenolphthalein TS to the *Sample*, containing 1 g of Magnesium Aluminosilicate.

Acceptance criteria: If a pink color is produced, NMT 0.50 mL of 0.1 N hydrochloric acid is required to discharge it.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers, and prevent exposure to excessive heat.

Magnesium Aluminum Silicate

DEFINITION

Magnesium Aluminum Silicate is a blend of colloidal montmorillonite and saponite that has been processed to remove grit and nonswellable ore components.

The requirements for viscosity and ratio of aluminum content to magnesium content differ for the several types of Magnesium Aluminum Silicate, as set forth in the table below.

Type	Viscosity (mPa · s)		Al Content/ Mg Content		Appearance
	Min.	Max.	Min.	Max.	
IA	225	600	0.5	1.2	Fine granules or flakes
IB	150	450	0.5	1.2	Microfine powder
IC	800	2200	0.5	1.2	Fine granules or flakes
IIA	100	300	1.4	2.8	Fine granules or flakes

IDENTIFICATION

• **A.**

Sample: 2 g

Analysis 1: Add the *Sample* in small portions to 100 mL of water with intense agitation. Allow to stand for 12 h to ensure complete hydration. Place 2 mL of the resulting mixture on a suitable glass slide, and allow to air-dry at room temperature to produce an oriented film. Place the slide in a vacuum desiccator over a free surface of ethylene glycol. Evacuate the desiccator, and close the stopcock so that the ethylene glycol saturates the desiccator chamber. Allow to stand for 12 h. Record the X-ray diffraction pattern (see *X-Ray Diffraction* ⟨941⟩), and calculate the *d* values.

Acceptance criteria 1: The largest peak corresponds to a *d* value between 15.0 and 17.2 Å.

Analysis 2: Prepare a random powder specimen of Magnesium Aluminum Silicate, record the X-ray diffraction pattern, and determine the *d* values in the region between 1.48 and 1.54 Å.

Acceptance criteria 2: Peaks are found at 1.492–1.504 Å and at 1.510–1.540 Å.

• **B.** It meets the requirements of the test for *Viscosity* in *Specific Tests*.

• **C.** It meets the requirements for *Ratio of aluminum content to magnesium content* in the test for *Aluminum Content and Magnesium Content*.

• **D.** Its appearance corresponds to the description in the *table* in the *Definition*.

ASSAY

• **ALUMINUM CONTENT AND MAGNESIUM CONTENT**

Aluminum content

Aluminum standard stock solution: Dissolve 1.000 g of aluminum in a mixture of 10 mL of hydrochloric acid and 10 mL of water by gentle heating. Transfer the solution to a 1000-mL volumetric flask, dilute with water to volume, and mix. This solution contains the equivalent of 1 mg/mL of aluminum.

Aluminum standard solutions: Transfer 2-, 5-, and 10-mL aliquots of the *Aluminum standard stock solution* to separate 100-mL volumetric flasks containing 200 mg of sodium chloride, dilute each with water to volume, and mix.

Sample stock solution: Transfer 0.200 g of Magnesium Aluminum Silicate to a 25-mL platinum crucible containing 1.0 g of lithium metaborate, and mix. Using a muffle furnace or a suitable burner, heat slowly at first, and ignite at 1000°–1200° for 15 min. Cool, place the crucible in a 100-mL beaker containing 25 mL of dilute nitric acid (50 mg/mL), and add an additional 50 mL of the dilute acid, filling and submerging the upright crucible. Place a polyfluorocarbon-coated magnetic stirring bar into the crucible, and stir gently with a magnetic stirrer to dissolve. Pour the contents into a 250-mL beaker, and remove the crucible. Warm the solution, transfer through a rapid-flow filter paper with the aid of water into a 200-mL volumetric flask, dilute with water to volume, and mix.

Sample solution: Pipet 20 mL of the *Sample stock solution* into a 100-mL volumetric flask. Add 20 mL of a solution

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of sodium chloride (10 mg/mL), dilute with water to volume, and mix.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometer equipped with a single-slot burner

Analytical wavelength: 309 nm

Lamp: Aluminum hollow-cathode

Flame: Oxidizing acetylene–air–nitrous oxide

Analysis

Samples: *Aluminum standard solutions* and *Sample solution*

Determine the absorbances of the *Aluminum standard solutions* and the *Sample solution*. From a linear regression equation calculated from the absorbances and concentrations of the *Aluminum standard solutions*, determine the aluminum content in the magnesium aluminum silicate.

Magnesium content

Lanthanum solution: Stir 88.30 g of lanthanum chloride ($LaCl_3$) with 500 mL of 6 N hydrochloric acid to dissolve, transfer with the aid of water to a 1000-mL volumetric flask, dilute with water to volume, and mix.

Magnesium standard stock solution: Place 1.000 g of magnesium in a 250-mL beaker containing 20 mL of water, and carefully add 20 mL of hydrochloric acid, warming, if necessary, to complete the reaction. Transfer the solution to a 1000-mL volumetric flask, dilute with water to volume, and mix. This solution contains the equivalent of 1 mg/mL of magnesium. Transfer 10.0 mL of this solution to a 1000-mL volumetric flask, dilute with water to volume, and mix.

Magnesium standard solutions: Transfer 5-, 10-, 15-, and 20-mL aliquots of the *Magnesium standard stock solution* to separate 100-mL volumetric flasks. To each flask add 20.0 mL of *Lanthanum solution*, dilute with water to volume, and mix.

Sample stock solution: Use the *Sample stock solution* prepared as directed for *Aluminum content*.

Sample solution: Transfer 25 mL of the *Sample stock solution* to a 50-mL volumetric flask, dilute with water to volume, and mix. Transfer 5.0 mL of this solution to a 100-mL volumetric flask, add 20.0 mL of *Lanthanum solution*, dilute with water to volume, and mix.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption

Analytical wavelength: 285 nm

Lamp: Magnesium hollow-cathode

Flame: Reducing flame of acetylene–air

Analysis

Samples: *Magnesium standard solutions* and *Sample solution*

Determine the absorbances of the *Sample solution* and the *Magnesium standard solutions*. From a linear regression equation calculated from the absorbances and concentrations of the *Magnesium standard solutions*, determine the magnesium content in the magnesium aluminum silicate.

Ratio of aluminum content to magnesium content

Analysis: Using the results from the *Aluminum content* and the *Magnesium content*, determine the ratio of aluminum content to magnesium content.

Acceptance criteria

Type IA: 0.5–1.2

Type IB: 0.5–1.2

Type IC: 0.5–1.2

Type IIA: 1.4–2.8

IMPURITIES

• **ARSENIC** ⟨211⟩, *Method I*

Standard preparation: Transfer 5.0 mL (5 µg of arsenic) of the *Standard Arsenic Solution* to a 25-mL volumetric flask, and add dilute hydrochloric acid (1:25) to volume.

Test preparation: Transfer 13.3 g of Magnesium Aluminum Silicate to a 250-mL beaker containing 100 mL of dilute hydrochloric acid (1:25), mix, cover with a watch glass, and boil gently with occasional stirring for 15 min without allowing excessive foaming. Allow the insoluble material to settle, and decant the hot supernatant through a rapid-flow filter paper into a 200-mL volumetric flask, retaining as much sediment as possible in the beaker. Add 25 mL of hot dilute hydrochloric acid (1:25) to the residue in the beaker, stir, and heat to boiling. Allow the insoluble material to settle, and decant the supernatant through the filter into the 200-mL volumetric flask. Repeat the extraction with four additional 25-mL portions of hot dilute hydrochloric acid (1:25), decanting each hot supernatant through the filter into the volumetric flask. At the last extraction, transfer as much of the insoluble material as possible onto the filter. Cool the combined filtrates to room temperature, add dilute hydrochloric acid (1:25) to volume, and mix. Use 25 mL for the test.

Acceptance criteria: NMT 3 µg/g; the absorbance due to any red color from the *Test preparation* does not exceed that produced by the *Standard preparation*.

• **LEAD**

Standard preparation: On the day of use, dilute 3.0 mL of lead nitrate stock solution TS with water to 100 mL. Each mL contains the equivalent of 3 µg of lead.

Sample: 10.0 g

Test preparation: Transfer the *Sample* to a 250-mL beaker containing 100 mL of dilute hydrochloric acid (1:25), stir, cover with a watch glass, and boil for 15 min. Cool to room temperature, and allow the insoluble matter to settle. Decant the supernatant through a rapid-flow filter paper into a 400-mL beaker. Add 25 mL of hot water to the insoluble matter in the 250-mL beaker, and stir. Allow the insoluble matter to settle, and decant the supernatant through the filter into the 400-mL beaker. Repeat the extraction with two additional 25-mL portions of water, decanting each supernatant portion through the filter into the 400-mL beaker. Wash the filter with 25 mL of hot water, collecting this filtrate in the 400-mL beaker. Concentrate the combined extracts by gentle boiling to approximately 20 mL. If a precipitate appears, add 2–3 drops of nitric acid, heat to boiling, and cool to room temperature. Filter the concentrated extracts through a rapid-flow filter paper into a 50-mL volumetric flask. Transfer the remaining contents of the 400-mL beaker through the filter paper and into the flask with water. Dilute with water to volume.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometer equipped with a deuterium arc background correction and a single-slot burner

Analytical wavelength: 284 nm

Lamp: Lead hollow-cathode

Flame: Oxidizing flame of air and acetylene

Acceptance criteria: The absorbance of the *Test preparation* is NMT that of the *Standard preparation* (15 µg/g).

SPECIFIC TESTS

• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩: Its total aerobic microbial count does not exceed 10^3 cfu/g, and it meets the requirements of the test for absence of *Escherichia coli*.

• **pH** ⟨791⟩

Sample suspension: 50 mg/mL

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Acceptance criteria: 9.0–10.0

• **Loss on Drying** ⟨731⟩

Analysis: Dry at 110° to constant weight.

Acceptance criteria: NMT 8.0%

• **Viscosity**

Sample: After determining the *Loss on Drying*, weigh a quantity of Magnesium Aluminum Silicate, equivalent to 25.0 g on the dried basis. Over a period of a few seconds, transfer the undried test specimen to a suitable 1-L blender jar containing an amount of water, maintained at a temperature of 25 ± 2°, that is sufficient to produce a mixture weighing 500 g. Blend for 3 min, accurately timed, at 14,000–15,000 rpm (high speed).[1]

[Note—Heat generated during blending causes a temperature rise to above 30°.]

Analysis: Transfer the contents of the blender to a 600-mL beaker, and allow to stand for 5 min. The sample temperature should be 33 ± 3°. Using a suitable rotational viscometer[2] equipped with a spindle as specified below, operate the viscometer at 60 rpm for 6 min, accurately timed, and record the scale reading.

For Type IA, use a spindle with a cylinder 1.87 cm in diameter and 0.69 cm high attached to a shaft 0.32 cm in diameter, the distance from the top of the cylinder to the lower tip of the shaft being 2.54 cm, and the immersion depth being 5.00 cm (No. 2 spindle). If the scale reading is greater than 90% of full scale, repeat the measurement, using a spindle similar to the No. 2 spindle but with the cylinder 1.27 cm in diameter and 0.16 cm high instead (No. 3 spindle).

For Type IC, use a No. 3 spindle. If the scale reading is greater than 90% of full scale, repeat the measurement using a spindle consisting of a cylindrical shaft 0.32 cm in diameter and with an immersion depth of 4.05 cm (No. 4 spindle).

For Types IB and IIA, use a No. 2 spindle.

Acceptance criteria

Type IA: 225–600 mPa · s

Type IB: 150–450 mPa · s

Type IC: 800–2200 mPa · s

Type IIA: 100–300 mPa · s

• **Acid Demand**

Sample: After determining the *Loss on Drying*, weigh a quantity of Magnesium Aluminum Silicate equivalent to 5.00 g.

Analysis: Disperse the *Sample* in 500 mL of water with the aid of a suitable blender fitted with a 1-L jar. Using a stopwatch, designate zero time. With constant mixing, add 3.0-mL portions of 0.100 N hydrochloric acid at 5, 65, 125, 185, 245, 305, 365, 425, 485, 545, 605, 665, and 725 s, and add a 1.0-mL portion at 785 s. Determine the pH potentiometrically at 840 s.

Acceptance criteria: NMT 4.0

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Preserve in tight containers.

• **Labeling:** Label it to indicate its type.

[1] A suitable blender is available from Waring as Waring Commercial Blender Model 7009G or equivalent with 1-L glass jar and tachometer adapter, Model CAC24 or equivalent.

[2] A suitable viscometer is available from Brookfield as Viscometer Model LVF or LVT, or equivalent.

Magnesium Silicate

DEFINITION

Magnesium Silicate is a compound of magnesium oxide and silicon dioxide. It contains NLT 15.0% of magnesium oxide (MgO) and NLT 67.0% of silicon dioxide (SiO_2), calculated on the ignited basis.

IDENTIFICATION

• **A. Identification Tests—General,** *Magnesium* ⟨191⟩

Sample: 500 mg

Analysis: Mix the *Sample* with 10 mL of 3 N hydrochloric acid. Filter, and neutralize the filtrate to litmus paper with 6 N ammonium hydroxide.

Acceptance criteria: The neutralized filtrate meets the requirements.

• **B.**

Analysis: Prepare a bead by fusing a few crystals of sodium ammonium phosphate on a platinum loop in the flame of a Bunsen burner. Place the hot, transparent bead in contact with Magnesium Silicate, and again fuse.

Acceptance criteria: Silica floats about in the bead, producing, upon cooling, an opaque bead with a web-like structure.

ASSAY

• **Magnesium Oxide**

Sample: 1.5 g

Titrimetric system

Mode: Residual titration

Titrant: 1 N sodium hydroxide VS

Endpoint detection: Visual

Analysis: Dissolve the *Sample* in 50.0 mL of 1 N sulfuric acid VS. Digest on a steam bath for 1 h, cool to room temperature, and add methyl orange TS. Titrate the excess acid in the sample with *Titrant*. Each mL of 1 N sulfuric acid is equivalent to 20.15 mg of MgO.

Acceptance criteria: NLT 15.0% on the ignited basis

• **Silicon Dioxide**

Sample: 700 mg

Analysis: Transfer the *Sample* to a small platinum dish. Add 10 mL of 1 N sulfuric acid, and heat on a steam bath to dryness, leaving the dish uncovered. Treat the residue with 25 mL of water, and digest on a steam bath for 15 min. Decant the supernatant through an ashless filter paper, with the aid of suction, and wash the residue, by decantation, three times with hot water, passing the washings through the filter paper. Finally, transfer the residue to the filter, and wash thoroughly with hot water. Transfer the filter paper and its contents to the platinum dish previously used. Heat to dryness, incinerate, ignite strongly for 30 min, cool, and weigh. Moisten the residue with water, and add 6 mL of hydrofluoric acid and 3 drops of sulfuric acid. Evaporate to dryness, ignite for 5 min, cool, and weigh. The loss in weight represents the weight of SiO_2.

Acceptance criteria: NLT 67.0% on the ignited basis

IMPURITIES

• **Fluoride**

Indicator solution: 100 mg/mL of lanthanum alizarin complexan mixture in 60% isopropyl alcohol. Filter the solution if it is not clear.

Standard solution: 2.21 µg/mL of sodium fluoride in 0.1 N hydrochloric acid

Sample solution: Prepare a slurry consisting of 5.0 g of Magnesium Silicate and 45 mL of 0.1 N hydrochloric acid. Stir at room temperature for 15 min, and pass through a filter of 0.45-µm pore size into a 50-mL volumetric flask. Wash the filter with five 1-mL portions of 0.1 N hydrochloric

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acid, collecting the washings in the flask. Dilute with 0.1 N hydrochloric acid to volume.

Instrumental conditions

Mode: Vis

Analytical wavelength: About 620 nm

Cell: 1 cm

Blank: 0.1 N hydrochloric acid, *Indicator solution*, and water (5:5:15)

Analysis: Transfer 5.0 mL of the *Standard solution* and *Sample solution* to separate 25-mL volumetric flasks, add 5.0 mL of *Indicator solution*, dilute with water to volume, and allow to stand for 1 h in diffuse light at ambient temperature. Determine the absorbance of the solutions against the *Blank*.

Acceptance criteria: 10 µg/g; the absorbance of the *Sample solution* is NMT than that of the *Standard solution*.

• **SOLUBLE SALTS**

Sample: 10.0 g

Analysis: Boil the *Sample* with 150 mL of water for 15 min. Cool to room temperature, and allow the mixture to stand for 15 min. Filter with the aid of suction, transfer the filtrate to a 200-mL volumetric flask, and dilute with water to volume. Evaporate 50.0 mL of this solution, representing 2.5 g of the Silicate, in a tared platinum dish to dryness. Ignite gently to constant weight. Retain the remaining diluted solution for the test for *Free Alkali*.

Acceptance criteria: 3.0%; NMT 75.0 mg

• **FREE ALKALI**

Sample: 20 mL of the retained diluted filtrate prepared in the test for *Soluble Salts*

Analysis: Add 2 drops of phenolphthalein TS to the *Sample*, representing 1 g of Magnesium Silicate.

Acceptance criteria: If a pink color is produced, NMT 2.5 mL of 0.1 N hydrochloric acid is required to discharge it.

• **LEAD** ⟨251⟩

Test preparation: Dissolve 1.0 g of Magnesium Silicate in 20 mL of 3 N hydrochloric acid, evaporate on a steam bath to 10 mL, dilute with water to 20 mL, and cool.

Acceptance criteria: NMT 10 µg/g

SPECIFIC TESTS

• **RATIO OF SiO_2 TO MgO**

Analysis: Divide the percentage of SiO_2 obtained in the *Assay* for *Silicon Dioxide* by the percentage of MgO obtained in the *Assay* for *Magnesium Oxide*.

Acceptance criteria: 2.50–4.50

• **LOSS ON DRYING** ⟨731⟩

[NOTE—Retain the dried specimen for the test for *Loss on Ignition*.]

Analysis: Dry at 105° for 2 h.

Acceptance criteria: NMT 15.0%

• **LOSS ON IGNITION** ⟨733⟩

Sample: The specimen retained from the test for *Loss on Drying*

Analysis: Ignite the *Sample* at 900°–1000° for 20 min.

Acceptance criteria: NMT 15%, previously dried

• **pH** ⟨791⟩

Sample solution: A well-mixed aqueous suspension (1 in 10)

Acceptance criteria: 7.0–10.8

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Magnesium Stearate

Portions of the monograph text that are national *USP* text, and are not part of the harmonized text, are marked with symbols (•,) to specify this fact.

Octadecanoic acid, magnesium salt;
Magnesium stearate [557-04-0].

DEFINITION

Magnesium Stearate is a compound of magnesium with a mixture of solid organic acids, and consists chiefly of variable proportions of magnesium stearate and magnesium palmitate. The fatty acids are derived from edible sources. It contains NLT 4.0% and NMT 5.0% of magnesium (Mg), calculated on the dried basis.

IDENTIFICATION

• **A. IDENTIFICATION TESTS—GENERAL** ⟨191⟩, *Magnesium*

Sample solution: Mix 5.0 g with 50 mL of peroxide-free ether, 20 mL of diluted nitric acid, and 20 mL of water in a round-bottom flask. Connect the flask to a reflux condenser, and reflux until dissolution is complete. Allow to cool, and transfer the contents of the flask to a separator. Shake, allow the layers to separate, and transfer the aqueous layer to a flask. Extract the ether layer with two 4-mL portions of water, and add these aqueous extracts to the main aqueous extract. Wash the aqueous extract with 15 mL of peroxide-free ether, transfer the aqueous extract to a 50-mL volumetric flask, and dilute with water to volume. Retain the unused portion of this solution for the chloride and sulfate impurity tests.

Acceptance criteria: The *Sample solution* meets the requirements.

• **B.** The retention times of the peaks corresponding to stearic acid and palmitic acid of the *Sample solution* correspond to those of the *System suitability solution*, as obtained in the test for *Relative Content of Stearic Acid and Palmitic Acid*.

ASSAY

• **PROCEDURE**

Buffer: Dissolve 5.4 g of ammonium chloride in water, add 20 mL of ammonium hydroxide, and dilute with water to 100 mL.

Sample: 500 mg

Analysis: To the *Sample* add 50 mL of a mixture of butyl alcohol and dehydrated alcohol (1:1), 5 mL of ammonium hydroxide, 3 mL of *Buffer*, 30.0 mL of 0.1 M edetate disodium VS, and 1 or 2 drops of eriochrome black TS. Heat at 45°–50° until the solution is clear. Cool, and titrate the excess edetate disodium with 0.1 M zinc sulfate VS until the solution color changes from blue to violet (see *Titrimetry* ⟨541⟩). Perform a blank determination, and make any necessary correction. Each mL of 0.1 M edetate disodium is equivalent to 2.431 mg of magnesium (Mg).

Acceptance criteria: 4.0%–5.0% on the dried basis

IMPURITIES

• **CHLORIDE AND SULFATE** ⟨221⟩, *Chloride*: A 10.0-mL portion of the *Sample solution* prepared in *Identification* test *A* shows no more chloride than corresponds to 1.4 mL of 0.020 N hydrochloric acid (0.1%).

• **CHLORIDE AND SULFATE** ⟨221⟩, *Sulfate*: A 6.0-mL portion of the *Sample solution* prepared in *Identification* test *A* shows no more sulfate than corresponds to 3.0 mL of 0.020 M sulfuric acid (1.0%).

• **LIMIT OF CADMIUM**

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glassware before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin. Select all reagents to

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have as low a content of cadmium, lead, and nickel as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glassware before use by soaking in warm 8 N nitric acid for 30 min, and rinse with deionized water.]

Matrix modifier solution: Prepare a solution containing 200 mg/mL of monobasic ammonium phosphate and 10 mg/mL of magnesium nitrate. Alternatively, use an appropriate matrix modifier as recommended by the manufacturer of the graphite furnace atomic absorption (GFAA) spectrophotometer.

Blank: Nitric acid in water (1 in 4)

Standard solution: 0.00825 µg/mL of cadmium nitrate tetrahydrate in *Blank*, corresponding to a known concentration of 0.0030 µg/mL of cadmium

Sample stock solution: Transfer 0.100 g of Magnesium Stearate to a suitable polytetrafluoroethylene (PTFE)-lined acid-digestion bomb, and add 2.5 mL of nitric acid. Close and seal the bomb according to the manufacturer's operating instructions. [**CAUTION**—When using an acid-digestion bomb, be thoroughly familiar with the safety and operating instructions. Carefully follow the bomb manufacturer's instructions regarding care and maintenance of these acid-digestion bombs. Do not use metal-jacketed bombs or liners that have been used with hydrochloric acid because of contamination from corrosion of the metal jacket by hydrochloric acid.] Heat the bomb in an oven at 170° for 3 h. Air cool the bomb slowly to room temperature as per the bomb manufacturer's instructions. Place the bomb in a hood, and open carefully because corrosive gases may be expelled. Dilute the residue with water to 10.0 mL in a volumetric flask.

Sample solutions: Dilute the *Sample stock solution* with *Blank* (1 in 10). Prepare mixtures of this solution, the *Standard solution*, and the *Blank* with the following proportional compositions, by volume (mL): 1.0/0/1.0, 1.0/0.5/0.5, and 1.0/1.0/0. Add 50 µL of *Matrix modifier solution* to each mixture. These *Sample solutions* contain, respectively, 0, 0.00075, and 0.0015 µg/mL of cadmium from the *Standard solution*. [NOTE—Retain the remaining *Sample stock solution* for use in the tests for *Limit of Lead* and *Limit of Nickel*.]

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry (using a suitable GFAA spectrophotometer equipped with a pyrolytic tube with platform)

Analytical wavelength: Cadmium emission line at 228.8 nm

Lamp: Cadmium hollow-cathode

Temperature: Use the temperature programming recommended for cadmium by the GFAA manufacturer (for examples of temperature parameters for GFAA analysis of cadmium, see *Table 1*).

Table 1

	Drying Stage	Ashing Stage	Atomization Stage
Temperature	110°	600°	1800°
Ramp time	10 s	10 s	0 s
Hold time	20 s	30 s	5 s

Analysis: Use the *Blank* to set the instrument to zero. Plot the absorbances of the *Sample solutions* versus their contents of cadmium, in µg/mL, as furnished by the *Standard solution*, draw the straight line best fitting the three points, using a linear least-squares fit, and extrapolate the line until it intercepts the concentration axis on the negative side. From the intercept determine the concentration, *C*, in µg/mL, of cadmium in the *Sample solution* containing 0 mL of the *Standard solution*. Calculate the content, in ppm, of cadmium in the specimen taken:

$$\text{Result} = (C/W) \times F$$

W = weight of Magnesium Stearate taken to prepare the *Sample stock solution* (g)

F = dilution factor for the sample, 200

Alternatively, the GFAA software can be used to calculate the cadmium content of the sample. For either calculation, the correlation coefficient (r) of the standard additions plot must be at least 0.99.

Acceptance criteria: NMT 3 ppm

• **LIMIT OF LEAD**

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glassware before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin. Select all reagents to have as low a content of cadmium, lead, and nickel as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glassware before use by soaking in warm 8 N nitric acid for 30 min, and rinse with deionized water.]

Matrix modifier solution: Prepare as directed for *Matrix modifier solution* in *Limit of Cadmium*.

Blank: Prepare as directed for *Blank* in *Limit of Cadmium*.

Standard solution: 0.1598 µg/mL of lead nitrate in *Blank*, corresponding to a known concentration of 0.100 µg/mL of lead. Prepare and store any solutions of lead nitrate in glass containers free from soluble lead salts.

Sample stock solution: Use a portion of the *Sample stock solution* retained from the test for *Limit of Cadmium*.

Sample solutions: Prepare mixtures of the *Sample stock solution*, the *Standard solution*, and the *Blank* with the following proportional compositions, by volume (mL): 1.0/0/1.0, 1.0/0.5/0.5, and 1.0/1.0/0. Add 50 µL of the *Matrix modifier solution* to each mixture. These *Sample solutions* contain, respectively, 0, 0.025, and 0.05 µg/mL of lead from the *Standard solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry (using a suitable GFAA spectrophotometer equipped with a pyrolytic tube with platform)

Analytical wavelength: Lead emission line at 283.3 nm

Lamp: Lead hollow-cathode

Temperature: Use the temperature programming recommended for lead by the GFAA manufacturer (for examples of temperature parameters for GFAA analysis of lead, see *Table 1*).

Analysis: Use the *Blank* to set the instrument to zero. Plot the absorbances of the *Sample solutions* versus their contents of lead, in µg/mL, as furnished by the *Standard solution*, draw the straight line best fitting the three points, using a linear least-squares fit, and extrapolate the line until it intercepts the concentration axis on the negative side. From the intercept determine the concentration, *C*, in µg/mL, of lead in the *Sample solution* containing 0 mL of the *Standard solution*. Calculate the content, in ppm, of lead in the specimen taken:

$$\text{Result} = (C/W) \times F$$

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W = weight of Magnesium Stearate taken to prepare the *Sample stock solution* (g)
F = dilution factor for the sample, 20

Alternatively, the GFAA software can be used to calculate the lead content of the sample. For either calculation, the correlation coefficient (r) of the standard additions plot must be at least 0.99.

Acceptance criteria: NMT 10 ppm

• **LIMIT OF NICKEL**

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glassware before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin. Select all reagents to have as low a content of cadmium, lead, and nickel as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glassware before use by soaking in warm 8 N nitric acid for 30 min, and rinse with deionized water.]

Matrix modifier solution: Prepare as directed for *Matrix modifier solution* in *Limit of Cadmium*.

Blank: Prepare as directed for *Blank* in *Limit of Cadmium*.

Standard solution: 0.2477 µg/mL of nickel nitrate hexahydrate in *Blank*, corresponding to a known concentration of 0.050 µg/mL of nickel

Sample stock solution: Use a portion of the *Sample stock solution* retained from the test for *Limit of Cadmium*.

Sample solutions: Prepare mixtures of the *Sample stock solution*, the *Standard solution*, and the *Blank* with the following proportional compositions, by volume (mL): 1.0/0/1.0, 1.0/0.5/0.5, and 1.0/1.0/0. Add 50 µL of the *Matrix modifier solution* to each mixture. These *Sample solutions* contain, respectively, 0, 0.0125, and 0.025 µg/mL of nickel from the *Standard solution*.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry (using a suitable GFAA spectrophotometer equipped with a pyrolytic tube with platform)

Analytical wavelength: Nickel emission line at 232.0 nm

Lamp: Nickel hollow-cathode

Temperature: Use the temperature programming recommended for nickel by the GFAA manufacturer (for examples of temperature parameters for GFAA analysis of nickel, see *Table 1*).

Analysis: Use the *Blank* to set the instrument to zero. Plot the absorbances of the *Sample solutions* versus their contents of nickel, in µg/mL, as furnished by the *Standard solution*, draw the straight line best fitting the three points, using a linear least-squares fit, and extrapolate the line until it intercepts the concentration axis on the negative side. From the intercept determine the concentration, C, in µg/mL, of nickel in the *Sample solution* containing 0 mL of the *Standard solution*.

Calculate the content, in ppm, of nickel in the specimen taken:

$$\text{Result} = (C/W) \times F$$

W = weight of Magnesium Stearate taken to prepare the *Sample stock solution* (g)
F = dilution factor for the sample, 20

Alternatively, the GFAA software can be used to calculate the nickel content of the sample. For either calculation, the correlation coefficient (r) of the standard additions plot must be at least 0.99.

Acceptance criteria: NMT 5 ppm

SPECIFIC TESTS

• ‣ **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic microbial count does not exceed 10^3 cfu/g, the total combined molds and yeasts count does not exceed 5×10^2 cfu/g. It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.‣

• **ACIDITY OR ALKALINITY**

Sample solution: To 1.0 g add 20 mL of carbon dioxide-free water, boil on a steam bath for 1 min with continuous shaking, cool, and filter. Add 0.05 mL of bromothymol blue TS to 10 mL of the filtrate.

Acceptance criteria: NMT 0.05 mL of 0.1 N hydrochloric acid or 0.1 N sodium hydroxide is required to change the color of the indicator.

• ‣ **SPECIFIC SURFACE AREA** ⟨846⟩

[NOTE—In cases where there are no functionality-related concerns regarding the specific surface area of this article, this test may be omitted.]

Where the labeling states the specific surface area, determine the specific surface area value as directed in the chapter in the P/P_0 range of 0.05–0.15, and using outgassing conditions of 2 h at 40°. If the plot deviates from linearity for P/P_0 values of 0.05–0.15, then a suitable range of P/P_0 values should be validated for linearity. In this case, it is necessary to state the range of validated P/P_0 values, the increment of the P/P_0 values, and the outgassing conditions used.‣

• **LOSS ON DRYING** ⟨731⟩

Analysis: Dry at 105° to constant weight.

Acceptance criteria: NMT 6.0%

• **RELATIVE CONTENT OF STEARIC ACID AND PALMITIC ACID**

System suitability solution: Transfer 50 mg each of USP Stearic Acid RS and USP Palmitic Acid RS to a small conical flask fitted with a suitable reflux condenser. Add 5.0 mL of a solution prepared by dissolving 14 g of boron trifluoride in methanol to make 100 mL, swirl to mix, and reflux for 10 min until the solids have dissolved. Add 4 mL of chromatographic *n*-heptane through the condenser, and reflux for 10 min. Cool, add 20 mL of saturated sodium chloride solution, shake, and allow the layers to separate. Pass the *n*-heptane layer through 0.1 g of anhydrous sodium sulfate (previously washed with chromatographic *n*-heptane) into a suitable flask. Transfer 1.0 mL of this solution to a 10-mL volumetric flask, and dilute with chromatographic *n*-heptane to volume.

Sample solution: Transfer 100 mg of Magnesium Stearate to a small conical flask fitted with a suitable reflux condenser, and proceed as directed for *System suitability solution*, beginning with "Add 5.0 mL of a solution prepared by dissolving".

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.32-mm × 30-m fused silica capillary, bonded with a 0.5-µm layer of phase G16

Temperatures

Injector: 220°

Detector: 260°

Column: See *Table 2*.

Table 2

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	—	70	2

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Table 2 *(continued)*

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	5	240	5

Carrier gas: Helium
Flow rate: 2.4 mL/min
Injection volume: 1 µL
Injection type: Splitless injection system

System suitability
Sample: *System suitability solution*
[NOTE—The relative retention times for methyl palmitate and methyl stearate are about 0.9 and 1.0, respectively.]
Suitability requirements
Resolution: NLT 5.0 between methyl palmitate and methyl stearate
Relative standard deviation: NMT 3.0% for the palmitate and stearate peak areas from six replicate injections; NMT 1.0% for the peak area ratio of palmitate to stearate from six replicate injections

Analysis
Sample: *Sample solution*
Measure the peak areas for all the fatty acid esters in the chromatogram.
Calculate the percentage of stearic acid in the fatty acid fraction of the portion of Magnesium Stearate taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak area of methyl stearate from the *Sample solution*
r_T = sum of the peak areas of all the fatty acid esters from the *Sample solution*

Similarly, calculate the percentage of palmitic acid in the portion of Magnesium Stearate taken.

$$\text{Result} = (r_P/r_T) \times 100$$

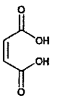
r_P = peak area of methyl palmitate from the *Sample solution*
r_T = sum of the peak areas of all the fatty acid esters from the *Sample solution*

Acceptance criteria: NLT 40% for the stearate peak. The sum of the stearate and palmitate peaks is NLT 90% of the total peak areas of all the fatty acids.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **▲LABELING:** Where the labeling states the specific surface area, it also indicates which method specified in *Specific Surface Area* ⟨846⟩ is used. Label to indicate that the fatty acids are derived from edible sources.▲
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Palmitic Acid RS
  USP Stearic Acid RS

Maleic Acid



$C_4H_4O_4$ 116.07
(*Z*)-Butenedioic acid;
cis-Butenedioic acid [110-16-7].

DEFINITION

Change to read:

Maleic Acid contains NLT ▲98.0%▲ (NF 1-Aug-2019) and NMT ▲102.0%▲ (NF 1-Aug-2019) of maleic acid ($C_4H_4O_4$), calculated on the anhydrous basis.

IDENTIFICATION

Delete the following:

- ▲**A. PROCEDURE**
  Analysis: Dissolve 500 mg of Maleic Acid in 10 mL of water.
  Acceptance criteria: The pH of the solution is less than 2.▲ (NF 1-Aug-2019)

Change to read:

- ▲**A.**▲ (NF 1-Aug-2019) ▲**SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

Change to read:

- **B.** ▲The retention time of the maleic acid peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.▲ (NF 1-Aug-2019)

ASSAY

Change to read:

- **PROCEDURE**
  ▲**Solution A:** 10 mM potassium phosphate buffer, adjusted with phosphoric acid to a pH of 2.3
  Solution B: Acetonitrile and *Solution A* (55:45)
  Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0.0	99	1
8.0	95	5
22.0	55	45
27.0	55	45

Return to original conditions and re-equilibrate the system.
Diluent: Acetonitrile and *Solution A* (2:98)
System suitability solution: 50 µg/mL of USP Fumaric Acid RS and 200 µg/mL each of USP Lactic Acid RS, USP Glacial Acetic Acid RS, and USP Succinic Acid RS in *Diluent*
Standard solution: 50 µg/mL of USP Maleic Acid RS in *Diluent*
Sample solution: 50 µg/mL of Maleic Acid in *Diluent*
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)

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Mode: LC
Detector: UV 210 nm
Column: 4.6-mm × 15-cm; 3-µm packing L1
Flow rate: 0.8 mL/min
Injection volume: 10 µL
System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The relative retention times for lactic acid, acetic acid, succinic acid, and fumaric acid are about 0.5, 0.6, 0.9, and 1.0, respectively.]
Suitability requirements
Resolution: NLT 2.0 between the lactic acid and acetic acid peaks; NLT 3.0 between the succinic acid and fumaric acid peaks, *System suitability solution*
Tailing factor: NMT 1.5, *Standard solution*
Relative standard deviation: NMT 1.0%, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of maleic acid in the portion of Maleic Acid taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of maleic acid from the *Sample solution*
r_S = peak area of maleic acid from the *Standard solution*
C_S = concentration of USP Maleic Acid RS in the *Standard solution* (µg/mL)
C_U = concentration of Maleic Acid in the *Sample solution* (µg/mL)▲ (NF 1-Aug-2019)

Acceptance criteria: ▲98.0%–102.0%▲ (NF 1-Aug-2019) on the anhydrous basis

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%, determined on a 1.0-g portion
• **LIMIT OF IRON**
Solution A: Dissolve 9.7 g of potassium thiocyanate in 100 mL of water.
Diluted standard iron solution: Immediately before use, dilute 1 volume of *Standard Iron Solution*, prepared as directed in *Iron* ⟨241⟩, with 9 volumes of water. [NOTE—This solution contains the equivalent of 1 µg/mL of iron.]
Standard solution: Add 6 mL of water to 5 mL of *Diluted standard iron solution*. Add 1 mL of diluted hydrochloric acid and 0.05 mL of bromine TS. After 5 min, remove the excess of bromine with the aid of a current of air, add 3 mL of *Solution A*, and shake well.
Sample solution: Dissolve 1 g of Maleic Acid in 10 mL of water. Add 2 mL of diluted hydrochloric acid and 0.05 mL of bromine TS. After 5 min, remove the excess of bromine with the aid of a current of air, add 3 mL of *Solution A*, and shake well.
Analysis: Allow the *Standard solution* and *Sample solution* to stand for 5 min.
Acceptance criteria: Any red color in the *Sample solution* is not more intense than that in the *Standard solution* (NMT 5 ppm).

Change to read:

• **LIMIT OF FUMARIC ACID ▲AND MALIC ACID**
Solution A, Solution B, Mobile phase, Diluent, System suitability solution, and **Chromatographic system:** Proceed as directed in the *Assay*.
Standard solution: 50 µg/mL of USP Fumaric Acid RS and 25 µg/mL of USP Malic Acid RS
Sample solution: 5 mg/mL of Maleic Acid in *Diluent*
System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The relative retention times for lactic acid, acetic acid, succinic acid, and fumaric acid are about 0.5, 0.6, 0.9, and 1.0, respectively.]
Suitability requirements
Resolution: NLT 2.0 between the lactic acid and acetic acid peaks; NLT 3.0 between the succinic acid and fumaric acid peaks, *System suitability solution*
Tailing factor: NMT 1.5 for malic acid and fumaric acid, *Standard solution*
Relative standard deviation: NMT 3.0% for the malic acid and fumaric acid peaks, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of fumaric acid or malic acid in the portion of Maleic Acid taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of fumaric acid or malic acid from the *Sample solution*
r_S = peak area of fumaric acid or malic acid from the *Standard solution*
C_S = concentration of USP Fumaric Acid RS or USP Malic Acid RS in the *Standard solution* (µg/mL)
C_U = concentration of Maleic Acid in the *Sample solution* (mg/mL)
F = conversion factor, 0.001 mg/µg

Acceptance criteria
Fumaric acid: NMT 1.0%
Malic acid: NMT 0.5%▲ (NF 1-Aug-2019)

SPECIFIC TESTS

Delete the following:

▲• **COLOR AND CLARITY OF SOLUTION**
Dilute hydrochloric acid solution: Dilute 27.5 mL of hydrochloric acid with water to 1000 mL.
Reference solution: Mix 2.4 mL of ferric chloride CS and 0.6 mL of cobaltous chloride CS with *Dilute hydrochloric acid solution* to make 10 mL. Dilute 5 mL of this solution with *Dilute hydrochloric acid solution* to make 100 mL.
Sample solution: 100 mg/mL of Maleic Acid
Analysis: Place the *Reference solution* and the *Sample solution* in matched color-comparison tubes, and compare the solutions by viewing them downward against a white surface (see *Color and Achromicity* ⟨631⟩).
Acceptance criteria: The *Sample solution* is clear and not more intensely colored than the *Reference solution*.▲ (NF 1-Aug-2019)

Change to read:

• **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT ▲0.5%▲ (NF 1-Aug-2019)

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight glass containers, protected from light. Store at room temperature.

Add the following:

▲• **LABELING:** Where Maleic Acid is intended for use in the manufacture of injectable dosage forms, it is so labeled.

NF Monographs

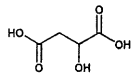
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Where Maleic Acid must be subjected to further processing during the preparation of injectable dosage forms to ensure acceptable levels of bacterial endotoxins, it is so labeled. ▲ (NF 1-Aug-2019)

Change to read:

• **USP REFERENCE STANDARDS** ⟨11⟩
USP Fumaric Acid RS
▲USP Glacial Acetic Acid RS
USP Lactic Acid RS ▲ (NF 1-Aug-2019)
USP Maleic Acid RS
▲USP Malic Acid RS
USP Succinic Acid RS ▲ (NF 1-Aug-2019)

Malic Acid



$C_4H_6O_5$ 134.09
Hydroxybutanedioic acid, (±)-;
(±)-Malic acid;
(±)-Hydroxysuccinic acid [617-48-1].

DEFINITION
Malic Acid contains NLT 99.0% and NMT 100.5% of $C_4H_6O_5$.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K** ▲ (CN 1-May-2020): On undried sample

ASSAY
• **PROCEDURE**
Sample: 2 g
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 1 N sodium hydroxide VS
Endpoint detection: Visual
Analysis: Dissolve the *Sample* in 40 mL of recently boiled and cooled water. Add phenolphthalein TS, and titrate to the first appearance of a faint pink color that persists for NLT 30 s. Perform a blank determination.
Calculate the percentage of malic acid ($C_4H_6O_5$) in the *Sample* taken:

$$\text{Result} = [(V - B) \times N \times F \times 100]/W$$

V = volume of *Titrant* consumed by the *Sample* (mL)
B = volume of *Titrant* consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 67.04 mg/mEq
W = weight of the *Sample* (mg)

Acceptance criteria: 99.0%–100.5%

IMPURITIES
• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%
• **FUMARIC AND MALEIC ACIDS**
Mobile phase: 0.01 N sulfuric acid
System suitability solution: 1 mg/mL of Malic Acid, 10 µg/mL of USP Fumaric Acid RS, and 4 µg/mL of USP Maleic Acid RS in *Mobile phase*
Standard solution: 5.0 µg/mL of USP Fumaric Acid RS and 2.0 µg/mL of USP Maleic Acid RS in *Mobile phase*
Sample solution: 1.0 mg/mL of Malic Acid in *Mobile phase*
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 210 nm
Column: 6.5-mm × 30-cm; packing L17
Column temperature: 37 ± 1°
Flow rate: 0.6 mL/min
Injection size: 20 µL
System suitability
Sample: *System suitability solution*
[NOTE—The relative retention times for maleic acid, malic acid, and fumaric acid are 0.6, 1.0, and 1.5, respectively.]
Suitability requirements
Resolution: NLT 2.5 for the maleic acid and malic acid peaks; NLT 7.0 for the malic acid and fumaric acid peaks
Relative standard deviation: NMT 2.0% for the maleic acid peak
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of maleic acid and of fumaric acid in the portion of Malic Acid taken:

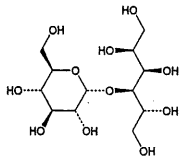
$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of maleic acid or fumaric acid from the *Sample solution*
r_S = peak response of maleic acid or fumaric acid from the *Standard solution*
C_S = concentration of USP Maleic Acid RS or USP Fumaric Acid RS in the *Standard solution* (mg/mL)
C_U = concentration of Malic Acid in the *Sample solution* (mg/mL)

Acceptance criteria
Fumaric acid: NMT 1.0%
Maleic acid: NMT 0.05%
• **WATER-INSOLUBLE SUBSTANCES**
Sample: 25 g
Analysis: Dissolve the *Sample* in 100 mL of water, filter the solution through a tared filtering crucible, wash the filter with hot water, and dry at 100° to constant weight.
Acceptance criteria: The increase in weight is NMT 25 mg (0.1%).

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Fumaric Acid RS
USP Maleic Acid RS
USP Malic Acid RS

Maltitol



$C_{12}H_{24}O_{11}$ 344.31

NF Monographs

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D-Glucopyranosyl-D-glucitol [585-88-6].

DEFINITION

Maltitol contains NLT 92.0% and NMT 100.5% of D-maltitol ($C_{12}H_{24}O_{11}$), calculated on the anhydrous basis. The amounts of total sugars, other polyhydric alcohols, and any polyol anhydrides, if detected, are not included in the requirements or in the calculated amount in *General Notices and Requirements, 5.60.10 Other Impurities in USP and NF Articles.*

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

ASSAY

- **PROCEDURE**

Mobile phase: Water. [NOTE—Degas the *Mobile phase* before use.]

System suitability solution: 4.8 mg/g of USP Maltitol RS and 4.8 mg/g of sorbitol

Standard solution: 10 mg/g of USP Maltitol RS and 1.6 mg/g of sorbitol

Sample solution: Dissolve 0.20 g of Maltitol in water, and dilute with water to 20 g. Record the final solution weight, and mix thoroughly. The solution is 10 mg/g of Maltitol.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Refractive index
Column: 7.8-mm × 10-cm; packing L34
Temperatures
Column: 60 ± 2°
Detector: 35°
Flow rate: 0.5 mL/min
Injection volume: 10 µL

System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The relative retention times for maltitol and sorbitol are 0.48 and 1.0, respectively.]
Suitability requirements
Resolution: NLT 2.0 between maltitol and sorbitol, *System suitability solution*
Relative standard deviation: NMT 2.0%, *Standard solution*

Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of D-maltitol ($C_{12}H_{24}O_{11}$) in the portion of Maltitol taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times [100/(100 - W)] \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP Maltitol RS in the *Standard solution* (mg/g)
C_U = concentration of Maltitol in the *Sample solution* (mg/g)
W = percentage obtained in the test for *Water Determination*

Acceptance criteria: 92.0%–100.5% on the anhydrous basis

IMPURITIES

- **LIMIT OF NICKEL**

Sample solution: Dissolve 20.0 g of Maltitol in diluted acetic acid, and dilute with diluted acetic acid to 150 mL.
Blank solution: 150 mL of diluted acetic acid
Standard solutions: Prepare three solutions by adding 0.5, 1.0, and 1.5 mL of nickel standard solution TS to 20.0 g of Maltitol dissolved in diluted acetic acid, and dilute with the same solvent to 150 mL.
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Analytical wavelength: Maxima at about 232.0 nm
Lamp: Nickel hollow-cathode
Flame: Air–acetylene
Analysis
Samples: *Standard solutions* and *Sample solution*
To each sample, add 2.0 mL of a saturated ammonium pyrrolidinedithiocarbamate solution (containing 10 g/L of ammonium pyrrolidinedithiocarbamate) and 10.0 mL of methyl isobutyl ketone, and shake for 30 s. Protect from bright light. Allow the two layers to separate, and use the methyl isobutyl ketone layer. Set the instrument to zero using the organic layer from the *Blank solution*. Concomitantly determine the absorbances of the organic layer from the *Samples* at least three times each. Record the average of the steady readings for each of the *Standard solutions* and the *Sample solution*. Between each measurement, aspirate the organic layer from the *Blank solution*, and ascertain that the reading returns to zero. Plot the absorbances of the *Standard solutions* and the *Sample solution* versus the added quantity of nickel. Extrapolate the line joining the points on the graph until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of nickel in the *Sample solution*.
Acceptance criteria: NMT 1 µg/g

- **REDUCING SUGARS**

Sample: 3.3 g
Titrimetric system
Mode: Residual titration
Titrant: 0.05 N sodium thiosulfate VS
Endpoint detection: Visual
Analysis: Dissolve the *Sample* in 3 mL of water with the aid of gentle heat. Cool, and add 20.0 mL of cupric citrate TS and a few glass beads. Heat so that boiling begins after 4 min, and maintain boiling for 3 min. Cool rapidly, and add 40 mL of diluted acetic acid, 60 mL of water, and 20.0 mL of 0.05 N iodine VS. With continuous shaking, add 25 mL of hydrochloric acid in water solution (6:94). When the precipitate has dissolved, titrate the excess of iodine with *Titrant* using 2 mL of starch TS, added towards the end of the titration, as an indicator.
Acceptance criteria: NLT 12.8 mL of *Titrant* is required, corresponding to NMT 0.3% of reducing sugars, as glucose. [NOTE—The amount determined in this test is not included in the calculated amount in *General Notices and Requirements, 5.60.10 Other Impurities in USP and NF Articles.*]

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩: The total aerobic microbial count using the *Plate Method* does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.
- **CONDUCTIVITY**

Sample solution: 200 mg/mL
Analysis: Using an appropriate conductivity meter, choose a conductivity cell that is appropriate for the properties and

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conductivity of the solution to be examined. Use a certified reference material, for example a solution of potassium chloride, that is appropriate for the measurement.[1] The conductivity value of the certified reference material should be near the expected conductivity value of the solution to be examined. After calibrating the apparatus with a certified reference material solution, rinse the conductivity cell several times with water and at least twice with the aqueous solution to be examined. Measure the conductivity of the *Sample solution* at a temperature of 20°, while gently stirring with a magnetic stirrer.

Acceptance criteria: NMT 20 µS/cm

• **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 1.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements are specified.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Maltitol RS

Maltitol Solution

DEFINITION

Maltitol Solution is a water solution containing, on the anhydrous basis, NLT 50.0% of D-maltitol ($C_{12}H_{24}O_{11}$) (w/w) and NMT 8.0% of D-sorbitol ($C_6H_{14}O_6$) (w/w). The amounts of total sugars, other polyhydric alcohols, and any polyol anhydrides, if detected, are not included in the requirements nor in the calculated amount under *Other Impurities*.

IDENTIFICATION

• **A. PROCEDURE**

Sample: 1.4 g of Maltitol Solution

Analysis: Dissolve the *Sample* in 75 mL of water. Transfer 3 mL of this solution to a 15-cm test tube, add 3 mL of freshly prepared catechol (1 in 10), and mix. Add 6 mL of sulfuric acid, and mix. Gently heat the tube in a flame for about 30 s.

Acceptance criteria: A deep pink or wine-red color appears.

• **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

• **C. LIMIT OF DIETHYLENE GLYCOL AND ETHYLENE GLYCOL**

Diluent: Acetone and water (96:4)

Standard stock solution: 0.5 mg/mL of USP Diethylene Glycol RS and 0.5 mg/mL of USP Ethylene Glycol RS in *Diluent*

Internal standard stock solution: 0.5 mg/mL of 1,3-butanediol (internal standard) in *Diluent*

Standard solution: 0.04 mg/mL of USP Diethylene Glycol RS, 0.04 mg/mL of USP Ethylene Glycol RS, and 0.04 mg/mL of 1,3-butanediol, in *Diluent*, prepared from the *Standard stock solution* and *Internal standard stock solution*

Sample solution: Transfer 1.0 g of Maltitol Solution to a 25-mL volumetric flask. Add 1.0 mL of water to the flask, and mix on a vortex mixer for 3 min. Add 2.0 mL of the *Internal standard stock solution* and 5 mL of *Diluent*, and mix on a vortex mixer for 3 min. Add the remaining *Diluent* to the flask to volume in two equal portions. Mix the contents for about 3 min after each addition of *Diluent*. Pass a portion of the supernatant layer through a nylon filter of 0.45-µm pore size. Discard the first 2 mL of the filtrate, and collect the rest of the filtrate for analysis. [NOTE—Acetone is used to precipitate maltitol.]

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.32-mm × 15-m fused-silica capillary column; 0.25-µm layer of phase G46

Temperature

Detector: 300°

Injection port: 240°

Column: See temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	—	70	2
70	50	300	5

Carrier gas: Helium

Flow rate: 3.0 mL/min

Injection size: 1.0 µL

Injection type: Split injection. The split ratio is about 10:1. [NOTE—A general purpose split/splitless, taper, glass wool, and deactivated liner is used.]

System suitability

Sample: *Standard solution*

[NOTE—See the relative retention time table below. Relative retention times are provided for information only, and the standards should be used to ensure appropriate peak identification.]

Name	Relative Retention Time
Ethylene glycol	1.0
1,3-Butanediol (internal standard)	2.2
Diethylene glycol	2.8

Suitability requirements

Resolution: NLT 15 between ethylene glycol and 1,3-butanediol

Analysis

Samples: *Standard solution* and *Sample solution*

Based on the *Standard solution*, identify the peaks of ethylene glycol, 1,3-butanediol (internal standard), and diethylene glycol. Compare peak area ratios of ethylene glycol to the internal standard and of diethylene glycol to the internal standard in the *Standard solution* and *Sample solution*, respectively.

Acceptance criteria

Diethylene glycol: The peak area ratio of diethylene glycol to the internal standard in the *Sample solution* is NMT the peak area ratio of diethylene glycol to the internal standard in the *Standard solution*, corresponding to NMT 0.10% of diethylene glycol in Maltitol Solution.

Ethylene glycol: The peak area ratio of ethylene glycol to the internal standard in the *Sample solution* is NMT the peak area ratio of ethylene glycol to the internal standard in the *Standard solution*, corresponding to NMT 0.10% of ethylene glycol in Maltitol Solution.

[1] Commercially available conductivity calibration solutions for conductivity meter standardization, standardized by methods traceable to the National Institute of Standards and Technology (NIST), may be used. Solutions prepared according to the instructions given in ASTM Standard D1125 may be used, provided the conductivity of the resultant solution is the same as that of the solution prepared from the NIST-certified material.

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ASSAY

• **PROCEDURE**

Mobile phase: Water

Standard solution: 10 mg/g of USP Maltitol RS and 1.6 mg/g of USP Sorbitol RS

Sample solution: 20 mg/g of Maltitol Solution in water

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 7.8-mm × 10-cm; packing L34

Temperature

Column: 60 ± 2°

Detector: 35°

Flow rate: 0.5 mL/min

Injection size: 10 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for maltotriitol, maltitol, and sorbitol are 0.38, 0.48, and 1.0, respectively.]

Suitability requirements

Tailing factor: NMT 1.2 for maltitol and sorbitol

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage, on the anhydrous basis, of $C_{12}H_{24}O_{11}$ and $C_6H_{14}O_6$ in the portion taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times [100/(100 - W)] \times 100$$

r_U = peak response of D-maltitol or D-sorbitol from the *Sample solution*

r_S = peak response of D-maltitol or D-sorbitol from the *Standard solution*

C_S = concentration of the appropriate USP Reference Standard in the *Standard solution* (mg/g)

C_U = concentration of Maltitol Solution in the *Sample solution* (mg/g)

W = percentage in the test for *Water Determination*

Acceptance criteria: NLT 50.0% of D-maltitol (w/w) and NMT 8.0% of D-sorbitol (w/w), on the anhydrous basis

IMPURITIES

INORGANIC IMPURITIES

• **Residue on Ignition** ⟨281⟩: NMT 0.1%, calculated on the anhydrous basis, determined on a 2-g portion

• **LIMIT OF NICKEL**

Solution A: 10 mg/mL of ammonium pyrrolidine dithiocarbamate

Sample solution: Dissolve and dilute 20.0 g of Maltitol Solution with diluted acetic acid to 100 mL. Add 2.0 mL of *Solution A* and 10.0 mL of methyl isobutyl ketone, and shake for 30 s. Protect from bright light. Allow the two layers to separate, and use the methyl isobutyl ketone layer.

Standard solutions: Prepare as directed for the *Sample solution*, except to prepare three solutions by adding 0.5, 1.0, and 1.5 mL of nickel standard solution TS.

Blank solution: Prepare as directed for the *Sample solution*, except to omit the use of the Maltitol Solution.

Spectrometric conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: 232.0 nm (maximum absorbance)

Lamp: Nickel hollow-cathode

Flame: Air–acetylene

Analysis

Samples: *Sample solution*, *Standard solutions*, and *Blank solution*

Set the instrument to zero using the *Blank solution*. Concomitantly determine the absorbances of the *Standard solutions* and the *Sample solution* at least three times each. Record the average of the steady readings for each of the *Standard solutions* and the *Sample solution*. Between each measurement, aspirate the *Blank solution*, and ascertain that the reading returns to zero. Plot the absorbances of the *Standard solutions* and the *Sample solution* versus the added quantity of nickel. Extrapolate the line joining the points on the graph until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of nickel in the *Sample solution*.

Acceptance criteria: NMT 1 ppm, calculated on the anhydrous basis

ORGANIC IMPURITIES

• **Procedure: Reducing Sugars**

Sample: An amount of Maltitol Solution equivalent to 3.3 g on the anhydrous basis

Analysis: To the *Sample* add 3 mL of water, 20.0 mL of cupric citrate TS, and a few glass beads. Heat so that boiling begins after 4 min, and maintain boiling for 3 min. Cool rapidly, and add 40 mL of diluted acetic acid, 60 mL of water, and 20.0 mL of 0.05 N iodine VS. With continuous shaking, add 25 mL of a mixture of 6 mL of hydrochloric acid and 94 mL of water. When the precipitate has dissolved, titrate the excess of iodine with 0.05 N sodium thiosulfate VS using 2 mL of starch TS, added toward the end of the titration, as an indicator.

[NOTE—The amount determined in this test is not included in the calculated amount under *Other Impurities*.]

Acceptance criteria: NLT 12.8 mL of 0.05 N sodium thiosulfate VS is required, corresponding to NMT 0.3% of reducing sugars, on the anhydrous basis, as glucose.

SPECIFIC TESTS

• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIC MICROORGANISMS** ⟨62⟩: The total aerobic microbial count using the *Plate Method* is NMT 1000 cfu/mL, and the total combined molds and yeasts count is NMT 100 cfu/mL.

• **pH** ⟨791⟩: 5.0–7.5, in a 14% (w/w) solution of Maltitol Solution in carbon dioxide-free water

• **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 31.5%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements are specified.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Diethylene Glycol RS

USP Ethylene Glycol RS

USP Maltitol RS

USP Sorbitol RS

Maltodextrin

DEFINITION

Maltodextrin is a nonsweet, nutritive saccharide mixture of polymers that consists of D-glucose units, with a Dextrose Equivalent less than 20. It is prepared by the partial hydrolysis of a food grade starch with suitable acids and/or enzymes. It may be physically modified to improve its physical and functional characteristics.

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ASSAY

• **DEXTROSE EQUIVALENT**

Standard solution: 10 mg/mL of USP Dextrose RS

Sample solution: Transfer 5 g of Maltodextrin with the aid of hot water to a 100-mL volumetric flask, cool, add water to volume, and mix.

Analysis: Transfer 25.0-mL portions of alkaline cupric tartrate TS to each of two boiling flasks. Bring the contents of one flask to boiling within 2 min while titrating with the *Standard solution* to within 0.5 mL of the anticipated endpoint. Boil gently for 2 min. Continue to boil gently, add 2 drops of methylene blue solution (1 in 100), and complete the titration within 1 min by adding the *Standard solution* dropwise or in small increments until the blue color disappears, determined by viewing against a white background in daylight or under equivalent illumination. If more than 0.5 mL of the titrant was required after the addition of the indicator, repeat the titration, adding the necessary volume of titrant before adding the indicator. Bring the contents of the second flask to boiling, and similarly titrate with the *Sample solution*.

Calculate the Dextrose Equivalent, on the dried basis, in the portion of Maltodextrin taken:

$$\text{Result} = [100/(1 - 0.01 \times A)] \times (C_S/C_U) \times (V_S/V_U)$$

A = percentage *Loss on Drying* of the Maltodextrin taken

C_S = concentration of USP Dextrose RS in the *Standard solution* (mg/mL)

C_U = concentration of Maltodextrin in the *Sample solution* (mg/mL)

V_S = titrated volume of the *Standard solution* (mL)

V_U = titrated volume of the *Sample solution* (mL)

Acceptance criteria: Less than 20

[NOTE—This is a limit test. For Maltodextrins with lower reducing values, other procedures may give other results.]

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 0.5%

• **LIMIT OF PROTEIN**

Sample: 10 g

Analysis: Transfer the *Sample* to an 800-mL Kjeldahl flask, and add 10 g of anhydrous potassium sulfate or sodium sulfate, 300 mg of copper selenite or mercuric oxide, and 60 mL of sulfuric acid. Gently heat the mixture, keeping the flask inclined at about a 45° angle, and after frothing has ceased, boil briskly until the solution has remained clear for about 1 h. Cool, and very cautiously add about 50 mL of water while swirling to dissipate the resulting heat. Add an additional 150–250 mL of water, mix, and cool again. Cautiously pour 75 mL (or enough to make the mixture strongly alkaline) of sodium hydroxide solution (2 in 5) down the inside of the flask so that it forms a layer under the acid solution, and then add a few pieces of granular zinc. Immediately connect the flask to a distillation apparatus consisting of a Kjeldahl connecting bulb and a condenser, the delivery tube of which extends well beneath the surface of an accurately measured excess of 0.1 N sulfuric acid contained in a 500-mL flask. Gently rotate the contents of the Kjeldahl flask to mix, and distill until all ammonia has passed into the absorbing acid solution (about 250 mL of distillate). To the receiving flask add 0.25 mL of methyl red–methylene blue TS, and titrate the excess acid with 0.1 N sodium hydroxide. Perform a blank determination, substituting pure sucrose or dextrose for the test specimen, and make any necessary correction. Each mL of 0.1 N sulfuric acid consumed is equivalent to 1.401 mg of nitrogen (*N*).

Calculate the percentage of *N* in the specimen taken, and then calculate the percentage of protein by multiplying the percentage of *N* by 6.25.

Acceptance criteria: NMT 0.1%

• **LIMIT OF SULFUR DIOXIDE**

Hydrogen peroxide solution: Dilute 30% hydrogen peroxide with water to obtain a 3% solution. Just before use, add 3 drops of methyl red TS, and neutralize to a yellow endpoint with 0.01 N sodium hydroxide. Do not exceed the endpoint.

Nitrogen: Use high-purity nitrogen, with a flow regulator that will maintain a flow of 200 ± 10 mL/min. Guard against the presence of oxygen by passing the nitrogen through a scrubber, such as alkaline pyrogallol, prepared as follows. Add 4.5 g of pyrogallol to a gas-washing bottle, purge the bottle with nitrogen for 3 min, and add a solution containing 85 mL of water and 65 g of potassium hydroxide, while maintaining an atmosphere of nitrogen in the bottle.

Apparatus: The apparatus (see *Figure 1*) is designed to effect the selective transfer of sulfur dioxide from the specimen in boiling aqueous hydrochloric acid to the *Hydrogen peroxide solution*. The backpressure is limited to the unavoidable pressure due to the height of the *Hydrogen peroxide solution* above the tip of the bubbler, *F*. Keeping the backpressure as low as possible reduces the likelihood that sulfur dioxide will be lost through leaks. Preboil vinyl and silicone tubing. Apply a thin film of stopcock grease to the sealing surfaces of all of the joints except the joint between the separatory funnel and the flask, and clamp the joints to ensure tightness. The separatory funnel, *B*, has a capacity of 100 mL or greater. The inlet adapter, *A*, with a hose connector provides a means of applying headpressure over the solution. [NOTE—A pressure-equalizing dropping funnel is not recommended because condensate, which may contain sulfur dioxide, is deposited in the funnel and the side arm.]

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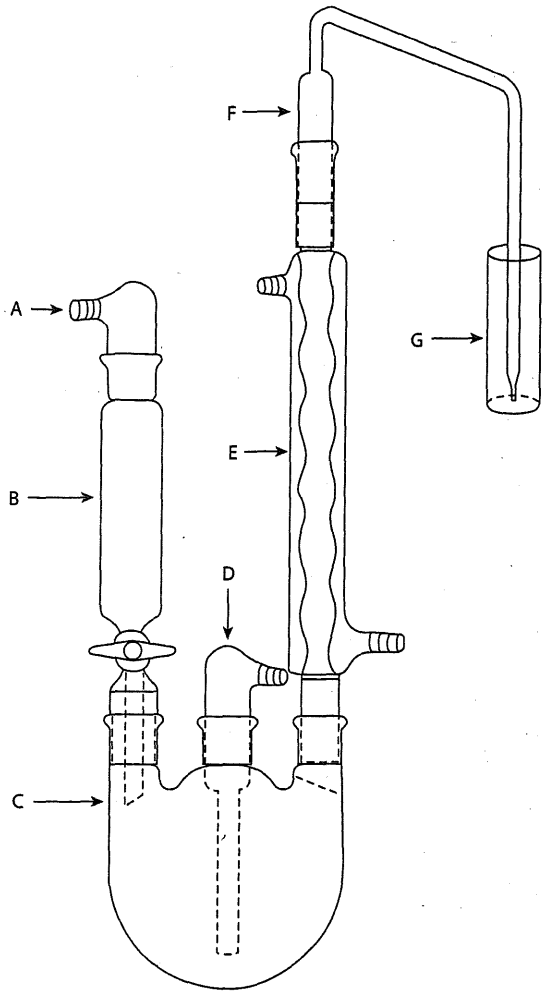


Figure 1. Apparatus for the Sulfur Dioxide Test.

The round-bottomed flask, *C*, is a 1000-mL flask with three 24/40 tapered joints. The gas inlet tube, *D*, is long enough to permit introduction of the nitrogen within 2.5 cm of the bottom of the flask. The Allihn condenser, *E*, has a jacket length of 300 mm. The bubbler, *F*, is fabricated from glass according to the dimensions given in *Figure 2*. The *Hydrogen peroxide solution* is contained in a vessel, *G*, having an inside diameter of about 2.5 cm and a depth of about 18 cm. Circulate coolant, such as a mixture of water and methanol (4:1) maintained at 5°, to chill the condenser.

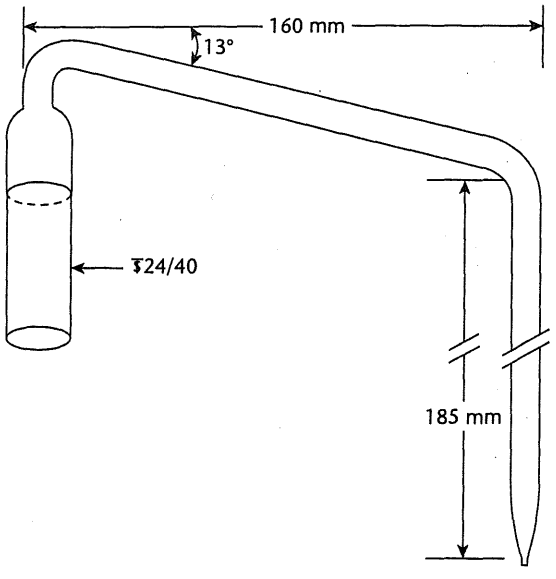


Figure 2. Bubbler (*F*) for the Sulfur Dioxide Apparatus.

Analysis: Position the *Apparatus* in a heating mantle controlled by a power-regulating device. Add 400 mL of water to the flask. Close the stopcock of the separatory funnel, and add 90 mL of 4 N hydrochloric acid to the separatory funnel. Begin the flow of *Nitrogen* at a rate of 200 ± 10 mL/min. Start the condenser coolant flow. Add 30 mL of the *Hydrogen peroxide solution* to vessel G. After 15 min, remove the separatory funnel, and transfer a mixture of 50.0 g of Maltodextrin and 100 mL of alcohol solution (5 in 100). Apply stopcock grease to the outer joint of the separatory funnel, return the separatory funnel to the tapered joint flask, and concomitantly resume the nitrogen flow. Apply headpressure above the hydrochloric acid solution in the separatory funnel with a rubber bulb equipped with a valve. Open the stopcock of the separatory funnel to permit the hydrochloric acid solution to flow into the flask. Continue to maintain sufficient pressure above the hydrochloric acid solution to force it into the flask. [NOTE—The stopcock may be temporarily closed, if necessary, to pump up the pressure.]

To guard against escape of sulfur dioxide (SO_2) into the separatory funnel, close the stopcock before the last few mL of hydrochloric acid drain out. Apply power to the heating mantle sufficient to cause about 85 drops of reflux/min. After refluxing for 1.75 h, remove vessel G, add 3 drops of methyl red TS, and titrate the contents with 0.01 N sodium hydroxide VS, using a 10-mL buret with an overflow tube and a hose connection to a carbon dioxide–absorbing tube, to a yellow endpoint that persists for at least 20 s. Perform a blank determination, and make any necessary correction (see *Titrimetry* ⟨541⟩).

Calculate the quantity, in µg/g, of SO_2 in the portion of Maltodextrin taken:

$$\text{Result} = 1000 \times F \times V \times N/W$$

F = milliequivalent weight of sulfur dioxide, 32.03
V = titrant volume consumed (mL)
N = actual normality of the titrant
W = weight of Maltodextrin taken (g)

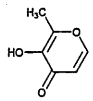
Acceptance criteria: NMT 40 µg/g (ppm)

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SPECIFIC TESTS
- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩: It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.
- **PH** ⟨791⟩: 4.0–7.0, in a 0.2-g/mL solution in carbon dioxide-free water.
- **LOSS ON DRYING** ⟨731⟩: Dry a sample at 105° for 2 h in a forced-air oven: it loses NMT 6.0% of its weight.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers, or in well-closed containers, at a temperature not exceeding 30° and a relative humidity not exceeding 50%.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Dextrose RS

Maltol



$C_6H_6O_3$ 126.11
3-Hydroxy-2-methyl-4-pyrone [118-71-8].

DEFINITION
Maltol contains NLT 99.0% of maltol ($C_6H_6O_3$), calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

Change to read:

- **B. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Ultraviolet-Visible Spectroscopy*: **197U**▲ (CN 1-May-2020)
  Sample solution: 0.01 mg/mL in 0.1 N hydrochloric acid
  Blank: 0.1 N hydrochloric acid

ASSAY
- **PROCEDURE**
  Standard solution: 0.01 mg/mL of USP Maltol RS in 0.1 N hydrochloric acid
  Sample solution: 0.01 mg/mL of Maltol in 0.1 N hydrochloric acid
  Instrumental conditions
  Mode: UV
  Analytical wavelength: Maximum at about 274 nm
  Blank: 0.1 N hydrochloric acid
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Calculate the percentage of maltol ($C_6H_6O_3$) in the portion of Maltol taken:

$$\text{Result} = (A_U/A_S) \times (C_S/C_U) \times 100$$

A_U = absorbance of the *Sample solution*
A_S = absorbance of the *Standard solution*
C_S = concentration of USP Maltol RS in the *Standard solution* (mg/mL)
C_U = concentration of the *Sample solution* (mg/mL)

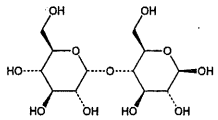
Acceptance criteria: NLT 99.0% on the anhydrous basis

IMPURITIES
- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.2%, determined on 1.0 g
- **LEAD** ⟨251⟩: NMT 10 ppm

SPECIFIC TESTS
- **MELTING RANGE OR TEMPERATURE,** *Class Ia* ⟨741⟩: 160°–164°
- **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 0.5%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light. No storage requirements are specified.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Maltol RS

Maltose



$C_{12}H_{22}O_{11} \cdot H_2O$ 360.31
$C_{12}H_{22}O_{11}$ 342.30
4-*O*-α-D-Glucopyranosyl-β-D-glucopyranose [69-79-4].
4-*O*-α-D-Glucopyranosyl-β-D-glucopyranose monohydrate [6363-53-7].

DEFINITION
Maltose is a sugar. It contains one molecule of water of hydration or is anhydrous. It contains NLT 92.0% of maltose, calculated on the anhydrous basis. The amounts of other sugars, if detected, are not included in the requirements or the calculated amount in *General Notices, 5.60.10 Other Impurities in USP and NF Articles*.

IDENTIFICATION
- **A.** Perform this test for Anhydrous Maltose only.
  Sample solution: 50 mg/mL
  Analysis: Add 2–3 drops of the *Sample solution* to 5 mL of hot alkaline cupric tartrate TS.
  Acceptance criteria: A red precipitate is formed.
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

Change to read:

- **C. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020): USP Maltose Monohydrate RS

ASSAY
- **PROCEDURE**
  Mobile phase: Water
  System suitability solution: 10 mg/g each of maltotriose, maltose, and glucose
  Standard solution: Dissolve USP Maltose Monohydrate RS in water to obtain a solution having a concentration of about 10 mg/g. Calculate the exact concentration on the anhydrous basis.
  Sample solution: Dissolve 0.10 g of Maltose in water, and dilute with water to 10 g. Record the final solution weight, and mix thoroughly.

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Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Refractive index
Column: 7.8-mm × 30-cm; packing L58
Temperatures
Column: 80 ± 2°
Detector: 40°
Flow rate: 0.35 mL/min; adjust so that the resolution between maltotriose and maltose is NLT 1.6.
Injection volume: 20 µL
System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The relative retention times for maltotriose, maltose, and glucose are about 0.9, 1.0, and 1.2, respectively.]
Suitability requirements
Resolution: NLT 1.6 between maltotriose and maltose, *System suitability solution*
Relative standard deviation: NMT 2.0%, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate, on the anhydrous basis, the percentage of Maltose taken:

$$\text{Result} = [(r_U/r_S) \times (C_S/C_U)]/[(100 - W)/100] \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP Maltose Monohydrate RS in the *Standard solution*, on the anhydrous basis (mg/g)
C_U = concentration of Maltose in the *Sample solution* (mg/g)
W = percentage of water from the test for *Water Determination*

Acceptance criteria: NLT 92.0% on the anhydrous basis

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩
Sample: 2 g
Acceptance criteria: NMT 0.05%

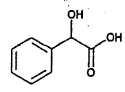
SPECIFIC TESTS

• **DEXTRIN, STARCH, AND SULFITE**
Sample solution: 1.0 g of Maltose in 10 mL of water
Analysis: Add 1 drop of iodine TS to the *Sample solution*.
Acceptance criteria: A yellow color develops. Then add 1 drop of starch TS; a blue color develops.
• **PH** ⟨791⟩
Sample solution: 100 mg/mL in carbon dioxide-free water
Acceptance criteria
Anhydrous form: 3.7–4.7
Monohydrate form: 4.0–5.5
• **WATER DETERMINATION,** *Method I* ⟨921⟩
Anhydrous: NMT 1.5%
Monohydrate: 4.5%–6.5%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Maltose Monohydrate RS

Mandelic Acid



$C_8H_8O_3$ 152.15
Benzeneacetic acid, α-hydroxy-;
(*RS*)-2-Hydroxy-2-phenylacetic acid;
(±)-α-Hydroxyphenylacetic acid;
2-Hydroxy-2-phenylacetic acid [90-64-2].

DEFINITION

Mandelic Acid contains NLT 98.0% and NMT 102.0% of α-hydroxyphenylacetic acid ($C_8H_8O_3$), calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
• **B. CHROMATOGRAPHIC IDENTITY**
Analysis: Proceed as directed in the *Assay*.
Acceptance criteria: The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

ASSAY

• **PROCEDURE**
Solvent A: 0.01 M phosphoric acid
Mobile phase: Acetonitrile, methanol, and *Solvent A* (17:3:80)
Standard stock solution A: Prepare a solution having known concentrations of 0.2 mg/mL of acetophenone, 0.5 mg/mL of benzoylformic acid, and 0.25 mg/mL of USP Benzaldehyde RS, respectively, in *Mobile phase*.
Standard stock solution B: Transfer 25 mg of USP Benzoic Acid RS to a 250-mL volumetric flask, add 5.0 mL of *Standard stock solution A*, dilute with *Mobile phase* to volume, and mix.
Standard stock solution C: Use USP Mandelic Acid RS to prepare a solution having a known concentration of 5 mg/mL in *Mobile phase*.
System suitability solution: Transfer 5.0 mL of *Standard stock solution B* and 20.0 mL of *Standard stock solution C* to a 100-mL volumetric flask, dilute with *Mobile phase* to volume, and mix. The solution contains 1 mg/mL of USP Mandelic Acid RS, 0.2 µg/mL of acetophenone, 0.5 µg/mL of benzoylformic acid, 0.25 µg/mL of USP Benzaldehyde RS, and 5 µg/mL of USP Benzoic Acid RS.
Standard solution: 1 mg/mL of USP Mandelic Acid RS in *Mobile phase* prepared from *Standard stock solution C*
Sample solution: 1 mg/mL of Mandelic Acid in *Mobile phase*
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 240 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: Ambient
Flow rate: 0.8 mL/min
Injection volume: 20 µL
System suitability
Sample: *System suitability solution*
[NOTE—See *Table 1* for relative retention times.]

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Table 1

Component	Relative Retention Time
Benzoylformic acid	0.9
Mandelic acid	1.0
Benzoic acid	3.2
Benzaldehyde	3.6
Acetophenone	4.8

Suitability requirements
Resolution: NLT 3.0, between the benzoylformic acid peak and the mandelic acid peak and between the benzoic acid peak and the benzaldehyde peak
Relative standard deviation: NMT 1% for the mandelic acid peak
Tailing factor: NMT 2.0 for each peak
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of mandelic acid ($C_8H_8O_3$) in the portion of Mandelic Acid taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of mandelic acid from the *Sample solution*
r_S = peak response of mandelic acid from the *Standard solution*
C_S = concentration of USP Mandelic Acid RS in the *Standard solution* (mg/mL)
C_U = concentration of Mandelic Acid in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–102.0% on the anhydrous basis

IMPURITIES
• **Residue on Ignition** ⟨281⟩**:** NMT 0.1%
• **Limit of Benzoylformic Acid, Benzaldehyde, Benzoic Acid, and Acetophenone**
Standard stock solution A, Standard stock solution B, Chromatographic system, and **System suitability:** Proceed as directed in the *Assay*.
Standard solution: Transfer 5.0 mL of *Standard stock solution B* to a 100-mL volumetric flask, dilute with *Mobile phase* to volume, and mix. The *Standard solution* contains 0.2 µg/mL of acetophenone, 0.5 µg/mL of benzoylformic acid, 0.25 µg/mL of USP Benzaldehyde RS, and 5 µg/mL of USP Benzoic Acid RS.
Sample solution: 2.5 mg/mL of Mandelic Acid in *Mobile phase*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of each related compound (benzoylformic acid, benzaldehyde, benzoic acid, or acetophenone) in the portion of Mandelic Acid taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of the relevant related compound (benzoylformic acid, benzaldehyde, benzoic acid, or acetophenone) from the *Sample solution*
r_S = peak response of the relevant related compound (benzoylformic acid, benzaldehyde, benzoic acid, or acetophenone) from the *Standard solution*
C_S = concentration of the relevant related compound (benzoylformic acid, USP Benzaldehyde RS, USP Benzoic Acid RS, or acetophenone) in the *Standard solution* (mg/mL)
C_U = concentration of Mandelic Acid in the *Sample solution* (mg/mL)

Acceptance criteria
Benzoylformic acid: NMT 0.1%
Benzoic acid: NMT 1.0%
Benzaldehyde: NMT 0.05%
Acetophenone: NMT 0.01%
• **Limit of Chloride**
Sample: 1 g
Analysis: Proceed as directed for *Chloride and Sulfate* ⟨221⟩, *Chloride*.
Acceptance criteria: 0.01%; a 1-g portion shows no more than corresponds to 0.15 mL of 0.020 N hydrochloric acid.

SPECIFIC TESTS
• **Melting Range or Temperature** ⟨741⟩**:** 118°–121°
• **Water Determination,** *Method Ia* ⟨921⟩**:** NMT 0.5%

Change to read:

• **Turbidity**
(See ▲*Nephelometry and Turbidimetry* ⟨855⟩▲ (CN 1-May-2019).)
Sample solution: 50 mg/mL in water
Blank: Reserve a portion of the water used to prepare the *Sample solution*.
Fixed reproducible standards: See *Determination of Turbidity* in *Elastomeric Closures for Injections* ⟨381⟩.
Analysis: Measure the turbidity of the *Sample solution* and *Blank* as directed in ▲*Nephelometry and Turbidimetry* ⟨855⟩▲ (CN 1-May-2019) against the *Fixed reproducible standards*.
Acceptance criteria: The turbidity is the difference between the values obtained for the *Blank* and the *Sample solution*, expressed in Nephelos units. The *Sample solution* shows no more turbidity than the 10 Nephelos units standard.

ADDITIONAL REQUIREMENTS
• **Packaging and Storage:** Preserve in well-closed containers. Store in a dry and well-ventilated place. Protect from light.
• **USP Reference Standards** ⟨11⟩
USP Benzaldehyde RS
USP Benzoic Acid RS
USP Mandelic Acid RS

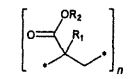
Mannitol—*see Mannitol General Monographs*

Meglumine—*see Meglumine General Monographs*

Menthol—*see Menthol General Monographs*

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Methacrylic Acid and Ethyl Acrylate Copolymer



R_1	R_2
CH_3	H
or H	C_2H_5

Poly(methacrylic acid, ethyl acrylate);
Methacrylic acid–ethyl acrylate copolymer [25212-88-8].

DEFINITION

Methacrylic Acid and Ethyl Acrylate Copolymer consists of methacrylic acid and ethyl acrylate monomers arranged in a random distribution. Methacrylic acid units in Methacrylic Acid and Ethyl Acrylate Copolymer, previously dried, are NLT 46.0% and NMT 50.6%. It may contain suitable surface-active agents.

IDENTIFICATION

Change to read:

- **A. ▲Spectroscopic Identification Tests ⟨197⟩, *Infrared Spectroscopy*: 197K▲** (CN 1-May-2020)**:** Use USP Methacrylic Acid and Ethyl Acrylate Copolymer (1:1) RS for Methacrylic Acid and Ethyl Acrylate Copolymer having a range of 46.0%–50.6% for methacrylic acid units.
- **B.** It meets the requirements of the *Assay*.

ASSAY

- **Procedure**

Sample: 1 g, previously dried

Analysis: Dissolve the *Sample* in 100 mL of neutralized acetone, and titrate with 0.1 N sodium hydroxide VS, determining the endpoint potentiometrically (see *Titrimetry* ⟨541⟩). Each mL of 0.1 N sodium hydroxide is equivalent to 8.609 mg of methacrylic acid ($C_4H_6O_2$) units.

Acceptance criteria: 46.0%–50.6%

IMPURITIES

- **Residue on Ignition ⟨281⟩:** NMT 0.4%
- **Limit of Methacrylic Acid and Ethyl Acrylate**

Sodium perchlorate solution: 35 mg/mL of sodium perchlorate. This solution has a concentration of 0.25 M.

Mobile phase: Add phosphoric acid dropwise to water to obtain a solution with a pH of 2.0. Prepare a mixture of this acidified water and methanol (80:20), and degas.

Standard solution: Dissolve 0.01 g of methacrylic acid and 0.01 g of ethyl acrylate in 5 mL of butanol, and add methanol to exactly 100 mL. Transfer 1.0 mL of this solution to a 100-mL volumetric flask. Dilute with methanol to volume. Mix 5.0 mL of this solution with 5.0 mL of *Sodium perchlorate solution*. This solution contains about 0.5 µg/mL each of methacrylic acid and ethyl acrylate.

Sample solution: Transfer about 3 g of Methacrylic Acid and Ethyl Acrylate Copolymer to a 50-mL volumetric flask, dilute with methanol to volume, and mix. Add 5.0 mL of this solution dropwise, while continuously stirring, to a beaker that contains 5.0 mL of *Sodium perchlorate solution*. Remove the precipitated polymer to obtain a clear supernatant by centrifugation (e.g., NLT 5000 × *g* for NLT 5 min). Use the clear supernatant.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 202 nm

Column: 4.0-mm × 12.5-cm; 7-µm packing L1

Flow rate: 2 mL/min

Injection volume: 20 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for methacrylic acid and ethyl acrylate are 1.0 and 2.6, respectively.]

Suitability requirements

Resolution: NLT 2.0 between methacrylic acid and ethyl acrylate

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of each monomer (methacrylic acid or ethyl acrylate) in the portion of Methacrylic Acid and Ethyl Acrylate Copolymer taken:

$$\text{Result} = (r_U/r_S) \times (C/W) \times V_F \times D \times F \times 100$$

r_U = peak response of the monomer (methacrylic acid or ethyl acrylate) from the *Sample solution*

r_S = peak response of the monomer (methacrylic acid or ethyl acrylate) from the *Standard solution*

C = concentration of the monomer (methacrylic acid or ethyl acrylate) in the *Standard solution* (µg/mL)

W = weight of Methacrylic Acid and Ethyl Acrylate Copolymer taken to prepare the *Sample solution* (g)

V_F = final volume of the *Sample solution*, 10 mL

D = dilution factor for preparation of the *Sample solution*, 10

F = conversion factor, 10^{-6} g/µg

Acceptance criteria: NMT 0.01% for the total amount of monomers

SPECIFIC TESTS

- **Viscosity—Rotational Methods ⟨912⟩**

Analysis: Place 254.6 g of isopropyl alcohol and 7.9 g of water in a test flask. Add a quantity of Methacrylic Acid and Ethyl Acrylate Copolymer, equivalent to 37.5 g of solids on the dried basis, while stirring by means of a magnetic stirrer. Close the flask, and continue stirring until the polymer has dissolved completely. Adjust the temperature to 20 ± 0.1°. Equip a rotational rheometer[1] following *Method II*. The shear rate under the test condition is NLT 1 s^{-1} and NMT 100 s^{-1}. Validations demonstrate that equivalent viscosity value is also obtained using a rotational viscometer with a cylindrical spindle 1.9 cm in diameter and 6.5 cm high, attached to a shaft 0.3 cm in diameter.[2] The spindle rotates at 30 rpm at an immersion depth of 8.15 cm (see *Method I*). Follow the instrument manufacturer's directions to measure the apparent viscosity.

Acceptance criteria: 100–200 mPa · s, for Methacrylic Acid and Ethyl Acrylate Copolymer with a range of 46.0%–50.6% for methacrylic acid units

- **Loss on Drying ⟨731⟩**

Analysis: Dry at 110° for 6 h.

Acceptance criteria: NMT 5.0%

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in tight containers, and store at controlled room temperature.

[1] A suitable rheometer is available from Physica Messtechnik GmbH as the Coaxial-Cylinder 27 or the Double-Gap-Cylinder 26.7, or any other equivalent rheometer.

[2] A suitable spindle is available from Brookfield as an LV1 spindle, or the equivalent.

Supplement 4

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• **LABELING:** Label it to indicate the range of methacrylic acid units. The labeling also indicates the name and quantity of any added surface-active agent.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Methacrylic Acid and Ethyl Acrylate Copolymer (1:1) RS (USP Methacrylic Acid Copolymer, Type C RS)

Methacrylic Acid and Ethyl Acrylate Copolymer Dispersion

DEFINITION

Methacrylic Acid and Ethyl Acrylate Copolymer Dispersion is an aqueous dispersion of Methacrylic Acid and Ethyl Acrylate Copolymer. It contains, on the basis of the calculated amount of dry substance in the Dispersion, NLT 46.0% and NMT 50.6% of methacrylic acid units. It may contain suitable surface-active agents.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020): *Loss on Drying*
• **B.** It meets the requirements in the *Assay*.

ASSAY

• **PROCEDURE**
Sample: 2.5 g of the Dispersion
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.1 N sodium hydroxide VS
Endpoint detection: Potentiometric
Analysis: Dissolve the *Sample* in 100 mL of neutralized acetone. Titrate the solution as directed in *Titrimetric system*. Each mL of 0.1 N sodium hydroxide is equivalent to 8.609 mg of methacrylic acid ($C_4H_6O_2$) units.
Calculate, on the dried basis, the percentage of methacrylic acid units in the portion of Dispersion taken:

$$\text{Result} = (V \times N)/[W \times (100 - L)] \times 860.9$$

V = volume of *Titrant* consumed (mL)
N = normality of the *Titrant*
W = weight of Dispersion taken (g)
L = percentage of the *Loss on Drying* value for the Dispersion

Acceptance criteria: 46.0%–50.6% based on the calculated amount of dry substance in the Dispersion

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩
Analysis: Using mild heating conditions (e.g., steam bath, sand bath) to avoid loss of material, evaporate the Dispersion to dryness prior to ignition.
Acceptance criteria: NMT 0.2% residue is obtained, calculated on the undried Dispersion basis.
• **LIMIT OF MONOMERS**
Mobile phase: Add phosphoric acid dropwise to water to obtain a solution with a pH of 2.0. Prepare a mixture of this acidified water and methanol (80:20), and degas.
Sodium perchlorate solution: Dissolve 3.5 g of sodium perchlorate in 100 mL of water. This solution has a concentration of 0.25 M.
Standard solution: Dissolve 0.01 g of methacrylic acid and 0.01 g of ethyl acrylate in 5 mL of butanol, and add methanol to make exactly 100 mL. Transfer 1.0 mL of this solution to a 100-mL volumetric flask, and dilute with methanol to volume. Mix 10.0 mL of this solution with 5.0 mL of *Sodium perchlorate solution*, accurately measured. This solution contains about 0.67 µg/mL each of methacrylic acid and ethyl acrylate.
Sample solution: Transfer a quantity of Dispersion, equivalent to 3 g of solids on the dried basis, to a 50-mL volumetric flask, dilute with methanol to volume, and mix. Add 10.0 mL of this solution dropwise while continuously stirring into a beaker that contains 5.0 mL of *Sodium perchlorate solution*, accurately measured. Remove the precipitated polymer by centrifugation (e.g., NLT 5000 × *g* for NLT 5 min). Use the clear supernatant.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 202 nm
Column: 4.0-mm × 12.5-cm; 7-µm packing L1
Flow rate: 2 mL/min
Injection volume: 20 µL
System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for methacrylic acid and ethyl acrylate are 1.0 and 2.6, respectively.]
Suitability requirements
Resolution: NLT 2.0 between methacrylic acid and ethyl acrylate
Relative standard deviation: NMT 5.0%, determined for each analyte
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of each monomer in the weight of the Dispersion taken:

$$\text{Result} = (r_U/r_S) \times (C/W) \times V_F \times D \times F \times 100$$

r_U = peak response of the monomer (methacrylic acid or ethyl acrylate) from the *Sample solution*
r_S = peak response of the monomer (methacrylic acid or ethyl acrylate) from the *Standard solution*
C = concentration of the monomer (methacrylic acid or ethyl acrylate) in the *Standard solution* (µg/mL)
W = weight of the Dispersion taken to prepare the *Sample solution* (g)
V_F = final volume of the *Sample solution*, 15 mL
D = dilution factor for preparation of the *Sample solution*, 5
F = conversion factor, 10^{-6} g/µg

Acceptance criteria: NMT 0.01% of total monomers, based on the weight of the Dispersion taken

SPECIFIC TESTS

• **COAGULUM CONTENT**
Analysis: Weigh a stainless steel sieve having 90-µm openings or a suitable single-woven wire cloth with a mesh width of 90 µm, and filter 100 g of the Dispersion through it. [NOTE—Suitable single-woven wire cloth mesh meets the requirements set in ISO 9044.] Wash the sieve or the cloth with distilled water until a clear filtrate is obtained, and dry the sieve or the cloth to constant weight at 110°.
Acceptance criteria: The weight of the residue does not exceed 1000 mg (1%).
• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry at 110° for 6 h.
Acceptance criteria: 68.5%–71.5%
• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩: The total aerobic

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microbial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.

- **pH** ⟨791⟩: 2.0–3.0
- **Viscosity—Rotational Methods,** *Method II* ⟨912⟩

Analysis: Equip a suitable rotational viscometer with an adapter comprising a cylindrical spindle rotating within an accurately machined chamber (or tube).[1] Mix the Dispersion, pipet the volume of test specimen recommended by the instrument manufacturer into the chamber (or tube), and ensure that the temperature of the test specimen is at 20 ± 0.1°. The shear rate under the test condition is NLT 1 s^{-1} and NMT 100 s^{-1}.[2] Measure the apparent viscosity following the instrument manufacturer's directions.

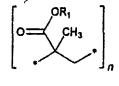
Acceptance criteria: The viscosity is between 2 and 15 mPa · s.

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in tight containers. Store at controlled room temperature. Protect from freezing.
- **Labeling:** The label indicates the name and amount of any substance added as a surface-active agent.
- **USP Reference Standards** ⟨11⟩

USP Methacrylic Acid and Ethyl Acrylate Copolymer (1:1) RS (USP Methacrylic Acid Copolymer, Type C RS)

Methacrylic Acid and Methyl Methacrylate Copolymer



R_1 = H or CH_3

(Ratio of H to CH_3 is either 1:1 or 1:2)
Poly(methacrylic acid, methyl methacrylate);
Methacrylic acid–methyl methacrylate copolymer [25086-15-1].

DEFINITION

Methacrylic Acid and Methyl Methacrylate Copolymer consists of methacrylic acid and methyl methacrylate monomers arranged in a random distribution. Methacrylic acid units in Methacrylic Acid and Methyl Methacrylate Copolymer, previously dried, are NLT 27.6% and NMT 50.6%. It may contain suitable surface-active agents.

IDENTIFICATION

Change to read:

- **A. ▲Spectroscopic Identification Tests** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

Use USP Methacrylic Acid and Methyl Methacrylate Copolymer (1:1) RS for Methacrylic Acid and Methyl Methacrylate Copolymer, with a range of 46.0%–50.6% for methacrylic acid units.

Use USP Methacrylic Acid and Methyl Methacrylate Copolymer (1:2) RS for Methacrylic Acid and Methyl Methacrylate Copolymer, with a range of 27.6%–30.7% for methacrylic acid units.

- **B.** It meets the requirements of the *Assay*.

ASSAY

- **Procedure**

Sample: 1 g, previously dried
Analysis: Dissolve the *Sample* in 100 mL of neutralized acetone, and titrate with 0.1 N sodium hydroxide VS, determining the endpoint potentiometrically (see *Titrimetry* ⟨541⟩). Each mL of 0.1 N sodium hydroxide is equivalent to 8.609 mg of methacrylic acid ($C_4H_6O_2$) units.
Acceptance criteria
Methacrylic acid and methyl methacrylate copolymer (1:2): 27.6%–30.7%
Methacrylic acid and methyl methacrylate copolymer (1:1): 46.0%–50.6%

IMPURITIES

- **Residue on Ignition** ⟨281⟩: NMT 0.1%
- **Limit of Methacrylic Acid and Methyl Methacrylate**

Phosphate buffer: Prepare an aqueous solution containing 17.8 g/L of anhydrous dibasic sodium phosphate and 17.0 g/L of monobasic potassium phosphate. Adjust with phosphoric acid to a pH of 2.0. This buffer has a concentration of 0.125 M.
Mobile phase: Add phosphoric acid dropwise to water to obtain a solution with a pH of 2.0. Prepare a mixture of this acidified water and methanol (80:20), and degas.
Standard solution: Dissolve 0.05 g of methacrylic acid and 0.05 g of methyl methacrylate in 5 mL of butanol, and add methanol to exactly 100 mL. Transfer 1.0 mL of this solution to a 100-mL volumetric flask. Dilute with methanol to volume. Mix 3.0 mL of this solution with 10.0 mL of *Phosphate buffer*. This solution contains 1.15 µg/mL each of methacrylic acid and methyl methacrylate. [Note—Due to volatility of monomers, tightly closed vials should be used.]
Sample solution: Transfer 1 g of Methacrylic Acid and Methyl Methacrylate Copolymer to a 50-mL volumetric flask, dilute with methanol to volume, and mix. Add 3 mL of this solution dropwise, while continuously stirring, to a beaker that contains 10.0 mL of *Phosphate buffer*. Remove the precipitated polymer to obtain a clear supernatant by centrifugation (e.g., NLT 5000 × *g* for NLT 5 min). Use the clear supernatant. [Note—Due to volatility of monomers, tightly closed vials should be used.]
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 202 nm
Column: 4.0-mm × 12.5-cm; 7-µm packing L1
Flow rate: 2 mL/min
Injection volume: 20 µL
System suitability
Sample: *Standard solution*
[Note—The relative retention times for methacrylic acid and methyl methacrylate are 1.0 and 2.8, respectively.]
Suitability requirements
Resolution: NLT 2.0 between methacrylic acid and methyl methacrylate
Relative standard deviation: NMT 5.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of each monomer (methacrylic acid or methyl methacrylate) in the portion of Methacrylic Acid and Methyl Methacrylate Copolymer taken:

[1] A commercial device is available from Brookfield as an ultra-low (UL) viscosity adapter. The adapter comprises a 0.4-cm diameter shaft, an accurately machined chamber (or tube) with an internal diameter of 2.8 cm and a depth of 13.5 cm, and a cylindrical spindle 2.5 cm in diameter and 9.1 cm in height.
[2] The cylindrical spindle rotates at 30 rpm, which corresponds to a shear rate of approximately 37 s^{-1}.

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$$\text{Result} = (r_U/r_S) \times (C/W) \times V_F \times D \times F \times 100$$

r_U = peak response of the monomer (methacrylic acid or methyl methacrylate) from the *Sample solution*
r_S = peak response of the monomer (methacrylic acid or methyl methacrylate) from the *Standard solution*
C = concentration of the monomer (methacrylic acid or methyl methacrylate) in the *Standard solution* (µg/mL)
W = weight of Methacrylic Acid and Methyl Methacrylate Copolymer taken to prepare the *Sample solution* (g)
V_F = final volume of the *Sample solution*, 13 mL
D = dilution factor for preparation of the *Sample solution*, 16.7
F = conversion factor, 10^{-6} g/µg

Acceptance criteria: NMT 0.05% for the total amount of monomers

SPECIFIC TESTS

- **VISCOSITY—ROTATIONAL METHODS** ⟨912⟩

Analysis: Place 254.6 g of isopropyl alcohol and 7.9 g of water in a test flask. Add a quantity of Methacrylic Acid and Methyl Methacrylate Copolymer, equivalent to 37.5 g of solids on the dried basis, while stirring by means of a magnetic stirrer. Close the flask, and continue stirring until the polymer has dissolved completely. Adjust the temperature to 20 ± 0.1°. Equip a rotational rheometer[1] following *Method II*. The shear rate under the test condition is NLT 1 s^{-1} and NMT 100 s^{-1}. Validations demonstrate that an equivalent viscosity value is also obtained using a rotational viscometer with a cylindrical spindle 1.9 cm in diameter and 6.5 cm high, attached to a shaft 0.3 cm in diameter.[2] The spindle rotates at 30 rpm at an immersion depth of 8.15 cm (see *Method I*). Follow the instrument manufacturer's directions to measure the apparent viscosity.

Acceptance criteria

Methacrylic acid and methyl methacrylate copolymer, with a range of 46.0%–50.6% for methacrylic acid units: 60–120 mPa · s

Methacrylic acid and methyl methacrylate copolymer, with a range of 27.6%–30.7% for methacrylic acid units: 50–200 mPa · s

- **LOSS ON DRYING** ⟨731⟩

Analysis: Dry at 110° for 6 h.

Acceptance criteria: NMT 5.0%

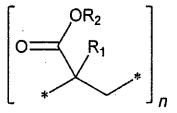
ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and store at controlled room temperature.
- **LABELING:** Label it to indicate the range of methacrylic acid units. The labeling also indicates the name and quantity of any added surface-active agent.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Methacrylic Acid and Methyl Methacrylate Copolymer (1:1) RS (USP Methacrylic Acid Copolymer, Type A RS)
USP Methacrylic Acid and Methyl Methacrylate Copolymer (1:2) RS (USP Methacrylic Acid Copolymer, Type B RS)

[1] A suitable rheometer is available from Physica Messtechnik GmbH as the Coaxial-Cylinder 27 or the Double-Gap-Cylinder 26.7, or any other equivalent rheometer.
[2] A suitable spindle is available from Brookfield as an LV1 spindle, or the equivalent.

Partially-Neutralized Methacrylic Acid and Ethyl Acrylate Copolymer



$R_1 = CH_3$; R_2 = H or
$R_1 = CH_3$; R_2 = Na or
R_1 = H; $R_2 = C_2H_5$

Partially-neutralized poly(methacrylic acid, ethyl acrylate); Partially-neutralized methacrylic acid–ethyl acrylate copolymer [25212-88-8].

DEFINITION

Partially-Neutralized Methacrylic Acid and Ethyl Acrylate Copolymer consists of methacrylic acid and ethyl acrylate monomers arranged in a random distribution, some units of methacrylic acid in the copolymer are neutralized by sodium hydroxide. The non-neutralized methacrylic acid units in the partially-neutralized methacrylic acid and ethyl acrylate copolymer are NLT 43.2% and NMT 47.6%, calculated on the dried basis. It may contain suitable emulsifiers.

IDENTIFICATION

- **A. INFRARED ABSORPTION**

Sample: 100 mg of Partially-Neutralized Methacrylic Acid and Ethyl Acrylate Copolymer

Analysis: Dissolve the *Sample* in 1 mL of dehydrated alcohol, and place 2 drops of the solution on a sodium chloride (or potassium bromide) plate. Dry to evaporate the solvent and allow to form a film and cover with another sodium chloride (or potassium bromide) plate.

Acceptance criteria: The IR absorption spectrum of Partially-Neutralized Methacrylic Acid and Ethyl Acrylate Copolymer exhibits maxima corresponding to the same wavelengths as that of a similar preparation of USP Partially-Neutralized Methacrylic Acid and Ethyl Acrylate Copolymer (1:1) RS, treated in the same manner.

- **B.** It meets the requirements of the *Assay*.

ASSAY

- **PROCEDURE**

Sample: 1 g, calculated on the dried basis

Analysis: Dissolve the *Sample* in 40 mL of water and 60 mL of 2-propanol, and titrate with 0.1 N sodium hydroxide VS, determining the endpoint potentiometrically (see *Titrimetry* ⟨541⟩). Each mL of 0.1 N sodium hydroxide is equivalent to 8.609 mg of methacrylic acid ($C_4H_6O_2$) units.

Acceptance criteria: 43.2%–47.6% for Partially-Neutralized Methacrylic Acid and Ethyl Acrylate Copolymer

IMPURITIES

INORGANIC IMPURITIES

- **Residue on Ignition** ⟨281⟩: 2.0%–3.5%

ORGANIC IMPURITIES

- **Procedure: Limit of Methacrylic Acid and Ethyl Acrylate**

Phosphoric acid solution: 0.1% phosphoric acid prepared from phosphoric acid

Mobile phase: Methanol and *Phosphoric acid solution* (3:7)

Standard solution: 1.0 µg/mL each of methacrylic acid and ethyl acrylate in methanol

Sample solution: Transfer 0.5 g of Partially-Neutralized Methacrylic Acid and Ethyl Acrylate Copolymer to a 25-mL volumetric flask, and dissolve in 20 mL of methanol. Add *Phosphoric acid solution* dropwise to precipitate the polymer

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while continuously stirring until the volume of 25 mL is reached. [NOTE—Stir with a magnetic stirrer for 10 min. Any volume deviation caused by the precipitation is negligible for contents in the ppm range. Use a magnetic stirrer of appropriate size to avoid a large variance from the final volume of the *Sample solution*.] As soon as the solid matter has settled, pass the supernatant through a filter of 0.45-µm pore size. [NOTE—Solution that cannot be filtered is centrifuged at NLT 20,000 × *g* for NLT 30 min.] Use the clear supernatant.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 205 nm
Column: 4.0-mm × 12.5-cm analytical column, 5-µm packing L1 or 4.6-mm × 15.0-cm analytical column, 5-µm packing L1
Flow rate: 1.2 mL/min
Injection size: 20 µL
[NOTE—Where appropriate, the volume must be adapted to the sensitivity of the detector.]
[NOTE—Column switching system may be used for extension of column lifetime.]

System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for methacrylic acid and ethyl acrylate are 1.0 and 2.2, respectively.]
Suitability requirements
Resolution: NLT 5.0 between methacrylic acid and ethyl acrylate
Relative standard deviation: NMT 5.0%

Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of each monomer (methacrylic acid or ethyl acrylate) in the portion of Methacrylic Acid and Ethyl Acrylate Copolymer taken:

$$\text{Result} = (r_U/r_S) \times (C_S/W) \times V_F \times F \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of the *Standard solution* (µg/mL)
W = weight of Partially-Neutralized Methacrylic Acid and Ethyl Acrylate Copolymer taken to prepare the *Sample solution* (g)
V_F = final volume of the *Sample solution*, 25 mL
F = conversion factor, 10^{-6} g/µg

Acceptance criteria: NMT 0.01% for the total amount of monomers

SPECIFIC TESTS
- **LOSS ON DRYING** ⟨731⟩**:** Dry a sample at 110° for 6 h: it loses NMT 5.0% of its weight.
- **VISCOSITY—ROTATIONAL METHODS** ⟨912⟩**:** Weigh 400 g of water into a short form, 600-mL beaker (internal diameter about 80 mm and height 120 mm). After determining the *Loss on Drying*, weigh a quantity of undried Partially-Neutralized Methacrylic Acid and Ethyl Acrylate Copolymer, equivalent to 100 g on the dried basis. Transfer the sample to the beaker very slowly while effectively stirring (avoid lumps). Ensure that the stirring is very effective at the beginning and that the powder is immersed very slowly at the same time. Once the powder is dispersed and no lumps are visible, gentle stirring is then sufficient. Ensure a colloidal dispersion (milky white liquid) by stirring at room temperature for 3 h and taking care to avoid mixing in excess air. Afterwards allow the container to stand for 1 h, control the temperature to 23 ± 0.1°, and let the entrapped air dissipate. [NOTE—Ensure that the concentration of this solution is 20% (w/w).] Determine the viscosity of this solution at 23 ± 0.1° using a suitable rotational viscometer with a cylindrical spindle 1.9 cm in diameter and 6.5 cm high, attached to a shaft 0.3 cm in diameter.[1] The spindle rotates at 50 rpm at an immersion depth of 8.1 cm. Follow the instrument manufacturer's directions to measure the apparent viscosity.
Acceptance criteria: 20–100 mPa · s

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers, and store at controlled room temperature.
- **LABELING:** Label it to indicate the range of non-neutralized methacrylic acid units. The labeling also indicates the name and quantity of any emulsifier if the content is 0.10% or greater.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Partially-Neutralized Methacrylic Acid and Ethyl Acrylate Copolymer (1:1) RS

Methyl Alcohol



CH_4O 32.04
Methanol [67-56-1].

DEFINITION
Methyl Alcohol contains NLT 99.5% of CH_3OH.
[**CAUTION**—Methyl Alcohol is poisonous.]

IDENTIFICATION

Change to read:

- **A.** ▲**SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F**▲ (CN 1-May-2020)
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

ASSAY
- **PROCEDURE**
System suitability solution: Dilute 1.0 mL of USP Methyl Alcohol RS and 1.0 mL of USP Acetone RS with tetrahydrofuran to 50 mL.
Internal standard solution: 2% (v/v) acetonitrile in tetrahydrofuran
Standard solution: 15.8 mg/mL of USP Methyl Alcohol RS in *Internal standard solution*
Sample solution: 15.8 mg/mL of Methyl Alcohol in *Internal standard solution*
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Detector: Flame ionization
Column: 0.32-mm × 30-m fused-silica capillary column, coated with a 1.8-µm layer of phase G43
Temperature
Injector: 200°
Detector: 280°
Column: See *Table 1*.

[1] A suitable spindle is available from Brookfield as an LV1 spindle, or the equivalent.

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Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
40	—	40	5
40	20	240	—

Carrier gas: Helium
Linear velocity: 35 cm/s
Injection type: Split ratio, 20:1
Injection size: 1 µL
System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The relative retention times for methyl alcohol, acetone, and acetonitrile are 1.0, about 1.6, and about 1.8, respectively.]
Suitability requirements
Resolution: NLT 15 between methyl alcohol and acetone, *System suitability solution*
Tailing factor: NMT 1.5 for methyl alcohol, *System suitability solution*
Relative standard deviation: NMT 2.0% for the ratio of the peak area of methyl alcohol to acetonitrile, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of methyl alcohol (CH_3OH) in the portion of Methyl Alcohol taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak area ratio from the *Sample solution*
R_S = peak area ratio from the *Standard solution*
C_S = concentration of USP Methyl Alcohol RS in the *Standard solution* (mg/mL)
C_U = concentration of Methyl Alcohol in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 99.5%

IMPURITIES

• **NONVOLATILE RESIDUE**
Sample: 250 mL of Methyl Alcohol
Analysis: Evaporate the *Sample* in a 600-mL beaker on a steam bath, in a well-ventilated hood, until the volume is reduced to about 100 mL. Cool, transfer a portion of the liquid to a suitable, tared 50-mL platinum dish on a steam bath, and evaporate. Repeat the process until all of the liquid has been transferred, and then evaporate to dryness. Dry at 105° for 30 min, cool, and weigh.
Acceptance criteria: The weight of the residue does not exceed 2 mg, corresponding to NMT 0.001% (w/w).
• **ACETONE AND ALDEHYDES** (as acetone)
Standard solution: Dilute 1.9 mL (1.5 g) of acetone with water to 1000 mL, then dilute 1.0 mL of this solution with water to 100 mL. Dilute 2 mL of the resulting solution with water to 5 mL. The *Standard solution* contains 30 µg of acetone and is freshly prepared.
Sample solution: Dilute 1.25 mL (1 g) of Methyl Alcohol with water to 5 mL.
Analysis: Adjust to and maintain each solution at 20°. Add 5 mL of alkaline mercuric–potassium iodide TS to each of the *Standard solution* and *Sample solution.*
Acceptance criteria: Any turbidity produced in the *Sample solution* is not greater than that produced in the *Standard solution* (NMT 0.003%).
• **READILY CARBONIZABLE SUBSTANCES** ⟨271⟩
Sample: 5 mL
Analysis: Cool 5 mL of sulfuric acid, contained in a small conical flask, to 10°, and add the *Sample* dropwise with constant mixing, maintaining the temperature below 20° throughout the test.
Acceptance criteria: No discoloration develops.
• **READILY OXIDIZABLE SUBSTANCES**
Sample: 20 mL of Methyl Alcohol
Analysis: Cool the *Sample* to 15°, add 0.1 mL of 0.1 N potassium permanganate, and allow to stand at 15°.
Acceptance criteria: The pink color does not completely disappear within 5 min.

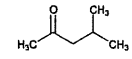
SPECIFIC TESTS

• **ACIDITY**
Sample solution: Mix 25 mL of water with 10 mL of alcohol and 0.5 mL of phenolphthalein TS, and add 0.02 N sodium hydroxide until a slight pink color persists after shaking for 30 s. Taking precautions to avoid absorption of carbon dioxide, add 19 mL (15 g) of Methyl Alcohol.
Analysis: Titrate the *Sample solution* with 0.020 N sodium hydroxide.
Acceptance criteria: NMT 0.45 mL of 0.020 N sodium hydroxide is required to produce a pink color.
• **ALKALINITY** (as ammonia)
Sample: 28.6 mL (22.6 g) of Methyl Alcohol
Analysis: Mix the *Sample* with 25 mL of water, add 1 drop of methyl red TS, and titrate with 0.020 N sulfuric acid.
Acceptance criteria: NMT 0.20 mL of 0.020 N sulfuric acid is required to produce a pink color (3 ppm).
• **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 0.1%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers, remote from heat, sparks, and open flames.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Acetone RS
USP Methyl Alcohol RS

Methyl Isobutyl Ketone



$C_6H_{12}O$ 100.16
2-Pentanone, 4-methyl-;
4-Methyl-2-pentanone [108-10-1].

DEFINITION

Methyl Isobutyl Ketone contains NLT 99.0% of methyl isobutyl ketone ($C_6H_{12}O$).

IDENTIFICATION

• **A.** The IR absorption spectrum of a thin film of it between sodium chloride crystals exhibits maxima, among others, at the following wavelengths, in µm: 5.81 (vs), 6.80 (m), 7.00 (m), 7.09 (m), 7.29 (vs), 7.72 (m), 8.06 (m), 8.31 (sh), 8.53 (s), and 8.91 (m).

IMPURITIES

• **LIMIT OF NONVOLATILE RESIDUE**
Sample: 50 mL
Analysis: Evaporate the *Sample* in a tared porcelain dish on a steam bath, and dry at 105° for 1 h. Weigh the residue.
Acceptance criteria: NMT 4 mg (0.008%)

SPECIFIC TESTS

• **SPECIFIC GRAVITY** ⟨841⟩**:** NMT 0.799, indicating NLT 99.0% of methyl isobutyl ketone ($C_6H_{12}O$)

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• **DISTILLING RANGE,** *Method I* ⟨721⟩**:** Between 114° and 117°, a correction factor of 0.046°/mm Hg being applied as necessary

• **ACIDITY**

Sample: 15.0 mL

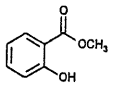
Analysis: Mix the *Sample* with 15 mL of neutralized alcohol, add phenolphthalein TS, and titrate with 0.050 N sodium hydroxide.

Acceptance criteria: NMT 0.40 mL is required for neutralization.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers.

Methyl Salicylate



$C_8H_8O_3$ 152.15

Benzoic acid, 2-hydroxy-, methyl ester;
Methyl salicylate [119-36-8].

DEFINITION

Methyl Salicylate is produced synthetically or is obtained by maceration and subsequent distillation with steam from the leaves of *Gaultheria procumbens* L. (Fam. Ericaceae) or from the bark of *Betula lenta* L. (Fam. Betulaceae). It contains NLT 98.0% and NMT 102.0% of methyl salicylate ($C_8H_8O_3$).

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F**▲ (CN 1-May-2020)

• **B. CHROMATOGRAPHIC IDENTITY**

Analysis: Proceed as directed in the *Assay*.

Acceptance criteria: The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*.

ASSAY

• **PROCEDURE**

Mobile phase: Methanol and 0.1% phosphoric acid (55:45)

Diluent: Methanol

System suitability solution: 150 µg/mL of USP Methyl Salicylate RS and 3 µg/mL of USP Methyl Salicylate Related Compound A RS in *Diluent*

Standard solution: 150 µg/mL of USP Methyl Salicylate RS in *Diluent*

Sample solution: 150 µg/mL of Methyl Salicylate in *Diluent*

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 237 nm

Column: 4.6-mm × 7.5-cm; 3.5-µm packing L7

Column temperature: Ambient

Flow rate: 1.0 mL/min

Injection volume: 10 µL

Run time: 7 min

System suitability

Samples: *System suitability solution* and *Standard solution*

[NOTE—The relative retention times for methyl salicylate and dimethyl 4-hydroxyisophthalate are 1.0 and 1.2, respectively.]

Suitability requirements

Resolution: NLT 1.5 between methyl salicylate and dimethyl 4-hydroxyisophthalate, *System suitability solution*

Tailing factor: NMT 1.5 for the methyl salicylate peak, *Standard solution*

Relative standard deviation: NMT 0.5% for the methyl salicylate peak, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of methyl salicylate in the portion of Methyl Salicylate taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Methyl Salicylate RS in the *Standard solution* (µg/mL)

C_U = concentration of Methyl Salicylate in the *Sample solution* (µg/mL)

Acceptance criteria: 98.0%–102.0%

IMPURITIES

• **LIMIT OF SALICYLIC ACID AND DIMETHYL 4-HYDROXYISOPHTHALATE**

Mobile phase, Diluent, Sample solution, and **Chromatographic system:** Proceed as directed in the *Assay*.

Standard solution: 0.15 µg/mL of USP Salicylic Acid RS, 0.15 µg/mL of USP Methyl Salicylate RS, and 0.75 µg/mL of USP Methyl Salicylate Related Compound A RS in *Diluent*

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for salicylic acid, methyl salicylate, and dimethyl 4-hydroxyisophthalate are 0.6, 1.0, and 1.2, respectively.]

Suitability requirements

Resolution: NLT 4 between salicylic acid and methyl salicylate; NLT 2 between methyl salicylate and dimethyl 4-hydroxyisophthalate

Relative standard deviation: NMT 3% for all three peaks

Analysis

Samples: *Sample solution* and *Standard solution*

Calculate the percentage of each individual impurity in the portion of Methyl Salicylate taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of salicylic acid or dimethyl 4-hydroxyisophthalate from the *Sample solution*

r_S = peak response of salicylic acid or dimethyl 4-hydroxyisophthalate from the *Standard solution*

C_S = concentration of USP Salicylic Acid RS or USP Methyl Salicylate Related Compound A RS in the *Standard solution* (µg/mL)

C_U = concentration of Methyl Salicylate in the *Sample solution* (µg/mL)

Acceptance criteria

Salicylic acid: NMT 0.1%

Dimethyl 4-hydroxyisophthalate: NMT 0.5%

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SPECIFIC TESTS
- **SOLUBILITY IN 70% ALCOHOL:** One volume of synthetic Methyl Salicylate dissolves in seven volumes of 70% alcohol. One volume of natural Methyl Salicylate dissolves in seven volumes of 70% alcohol, the solution shows NMT a slight cloudiness.
- **SPECIFIC GRAVITY** ⟨841⟩: 1.180–1.185 for the synthetic variety; 1.176–1.182 for the natural variety
- **OPTICAL ROTATION** ⟨781A⟩, *Procedures, Angular Rotation*: Synthetic Methyl Salicylate and that from *Betula lenta* are optically inactive. Methyl Salicylate from *Gaultheria procumbens* is slightly levorotatory, the angular rotation not exceeding –1.5° in a 100-mm tube.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **LABELING:** Label it to indicate whether it was made synthetically or distilled from either of the plants of *Gaultheria procumbens* or *Betula lenta*.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Methyl Salicylate RS
  USP Methyl Salicylate Related Compound A RS
  Dimethyl 4-hydroxyisophthalate.
  $C_{10}H_{10}O_5$ 210.18
  USP Salicylic Acid RS

Methylcellulose—*see Methylcellulose General Monographs*

Methylene Chloride

CH_2Cl_2 84.93
Methane, dichloro-;
Dichloromethane [75-09-2].

DEFINITION
Methylene Chloride contains NLT 99.0% of methylene chloride (CH_2Cl_2). [CAUTION—Perform all steps involving evaporation of methylene chloride in a well-ventilated fume hood.]

IDENTIFICATION
- **A.**
  Sample: 5 mL
  Analysis: Place the *Sample* into a glass-stoppered, 10-mL conical flask, and shake for several min. Remove the stopper, quickly withdraw a portion of the vapor into a 50-mL syringe that is not fitted with a needle, and inject the vapor into a suitable evacuated gas cell.
  Acceptance criteria: The IR absorption spectrum of the vapor shows strong doublet peaks at 7.8 and 7.9 µm and at 13.2 and 13.4 µm, and relatively few minor peaks.

ASSAY
- **PROCEDURE**
  System suitability solution: Methylene chloride and chloroform (3:7)
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: GC
  Detector: Thermal conductivity (under typical conditions)
  Column: 4-mm × 1.8-m; packed with 15% liquid phase G18 on 30- to 60-mesh S1C unsilanized support
  Temperatures
  Injection port: 200°
  Detector: 250°
  Column: 60°
  Carrier gas: Helium
  Flow rate: 20 mL/min
  Injection volume: 1 µL
  System suitability
  Sample: *System suitability solution*
  Suitability requirements
  Resolution: NLT 4.0 between methylene chloride and chloroform
  Tailing factor: NMT 1.4
  Relative standard deviation: The peak response ratio does not exceed 2% for five replicate injections.
  Analysis
  Sample: Methylene Chloride
  Inject the *Sample*, and determine the peak responses by any convenient means. [NOTE—The order of elution is amylenes (5 or 6 peaks), if present, and then methylene chloride.]
  Calculate the percentage of methylene chloride (CH_2Cl_2) in the portion of sample taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of methylene chloride
r_T = sum of all the peak responses

Acceptance criteria: NLT 99.0%

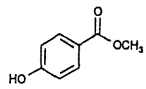
IMPURITIES
- **LIMIT OF NONVOLATILE RESIDUE**
  Sample: 50 g
  Analysis: Evaporate the *Sample* in a platinum or porcelain dish on a steam bath, and dry at 105° for 30 min.
  Acceptance criteria: NMT 0.002%; NMT 1 mg of residue

SPECIFIC TESTS
- **LIMIT OF HYDROGEN CHLORIDE**
  Sample: 20.0 mL
  Analysis: Into each of two glass-stoppered, 50-mL color-comparison cylinders having an internal diameter of 20 mm, place 10 mL of water, 2 drops of phenolphthalein TS, and sufficient 0.010 N sodium hydroxide to produce a pink color that persists after vigorous shaking for 30 s and is of equal intensity in each cylinder.
  [NOTE—In the following step, take special care to avoid contamination with carbon dioxide.]
  Into one of the cylinders, place the *Sample* and 0.70 mL of 0.010 N sodium hydroxide, and shake again.
  Acceptance criteria: NMT 0.001%; the pink color in the sample cylinder is at least as intense as that in the comparison cylinder, and the color persists for NLT 15 min.
- **SPECIFIC GRAVITY** ⟨841⟩: 1.318–1.322
- **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 0.02%
- **FREE CHLORINE**
  Sample: 10 mL
  Analysis: To the *Sample* add 10 mL of water and 0.1 mL of potassium iodide TS, shake for 2 min, and allow the liquids to separate.
  Acceptance criteria: The lower layer does not show a violet tint.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers.

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Methylparaben



$C_8H_8O_3$ 152.15
Benzoic acid, 4-hydroxy-, methyl ester;
Methyl *p*-hydroxybenzoate [99-76-3].

DEFINITION
Methylparaben contains NLT 98.0% and NMT 102.0% of $C_8H_8O_3$.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197M**▲ (CN 1-May-2020)
- **B. MELTING RANGE OR TEMPERATURE** ⟨741⟩: 125°–128°

ASSAY
- **PROCEDURE**

Mobile phase, Sample solution, Standard solution B, and **Chromatographic system:** Proceed as described in the procedure for *Related Substances.*

System suitability
Sample: *Standard solution B*
Suitability requirements
Relative standard deviation: NMT 0.85% for 6 injections

Analysis
Samples: *Sample solution* and *Standard solution B*
Calculate the percentage of Methylparaben in the *Sample solution*:

$$\text{Result} = P \times (r_U \times C_S)/(r_S \times C_U)$$

P	= labeled purity of USP Methylparaben RS expressed as a percentage
r_U	= peak area of methylparaben from the *Sample solution*
C_S	= concentration of methylparaben in *Standard solution B*
r_S	= peak area of methylparaben from *Standard solution B*
C_U	= concentration of Methylparaben in the *Sample solution*

Acceptance criteria: 98.0%–102.0%

IMPURITIES
INORGANIC IMPURITIES
- **Residue on Ignition** ⟨281⟩: NMT 0.1%, determined on 1.0 g

ORGANIC IMPURITIES
- **Procedure: Related Substances**

Mobile phase: Methanol and a 6.8 g/L solution of potassium dihydrogen phosphate (65:35 v/v)
Sample solution: Dissolve 50.0 mg of Methylparaben in 2.5 mL of methanol, and dilute with *Mobile phase* to 50.0 mL. Dilute 10.0 mL of this solution with *Mobile phase* to 100.0 mL.
Standard solution A: 5.0 µg/mL each of *p*-hydroxy benzoic acid and USP Methylparaben RS in *Mobile phase*
Standard solution B: Dissolve 50.0 mg of USP Methylparaben RS in 2.5 mL of methanol, and dilute with *Mobile phase* to 50.0 mL. Dilute 10.0 mL of this solution with *Mobile phase* to 100.0 mL.
Standard solution C: Dilute 1.0 mL of the *Sample solution* with *Mobile phase* to 20.0 mL. Dilute 1.0 mL of this solution with *Mobile phase* to 10.0 mL.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability.*)
Mode: LC
Detector: UV 272 nm
Column: 4.6-mm × 15-cm; 5-µm packing L1
Flow rate: 1.3 mL/min
Injection size: 10 µL
Run time: About 5 times the retention time of methylparaben
System suitability
Sample: *Standard solution A*
[NOTE—The retention time of methylparaben is about 2.3 min; the relative retention time for *p*-hydroxy benzoic acid is about 0.6.]
Suitability requirements
Resolution: NLT 2.0 between the *p*-hydroxybenzoic acid and methylparaben peaks
Analysis
Samples: *Sample solution* and *Standard solution C*
[NOTE—Disregard any limit that is 0.2 times the area of the principal peak in the chromatogram obtained with *Standard solution C* (0.1%).]
Acceptance criteria
***p*-Hydroxybenzoic acid:** The peak area in the *Sample solution*, multiplied by 1.4 to correct for the calculation of content, is NMT the area of the principal peak in *Standard solution C* (0.5%).
Unspecified impurities: The peak area of each impurity in the *Sample solution* is NMT the area of the principal peak in *Standard solution C* (0.5%).
Total impurities: The total peak area for all impurities in the *Sample solution* is NMT twice the area of the principal peak in *Standard solution C* (1.0%).

SPECIFIC TESTS
- **COLOR OF SOLUTION**

Sample solution: 100 mg/mL in alcohol
Comparison solution: Mix 2.4 mL of ferric chloride CS, 1.0 mL of cobaltous chloride CS, and 0.4 mL of cupric sulfate CS with 0.3 N hydrochloric acid to make 10 mL. Dilute 5 mL of this solution with 0.3 N hydrochloric acid to make 100 mL. [NOTE—Prepare and use this solution immediately.]
Analysis
Samples: Alcohol, *Sample solution*, and *Comparison solution*
Make the comparison by viewing the solutions downward in matched color-comparison tubes against a white surface (see *Color and Achromicity* ⟨631⟩).
Acceptance criteria: The *Sample solution* is clear and not more intensely colored than alcohol or the *Comparison solution.*

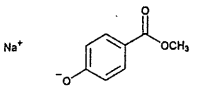
- **ACIDITY**

Sample solution: To 2 mL of the *Sample solution* prepared in the test for *Color of Solution*, add 3 mL of alcohol, 5 mL of carbon dioxide-free water, and 0.1 mL of bromocresol green TS.
Analysis: Titrate with 0.10 N sodium hydroxide.
Acceptance criteria: NMT 0.1 mL is required to produce a blue color.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Methylparaben RS

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Methylparaben Sodium



$C_8H_7NaO_3$ 174.13

Benzoic acid, 4-hydroxy-, methyl ester, sodium salt;
Methyl *p*-hydroxybenzoate, sodium salt;
Sodium 4-methoxycarbonylphenolate [5026-62-0].

DEFINITION

Methylparaben Sodium contains NLT 95.0% and NMT 102.0% of methylparaben sodium ($C_8H_7NaO_3$), calculated on the anhydrous basis.

IDENTIFICATION

• **A.**

Standard: 0.5 g of USP Methylparaben RS

Sample: 0.5 g

Analysis: Dissolve the *Sample* in 5 mL of water. Acidify with hydrochloric acid, and filter the resulting precipitate. Wash the precipitate with water, and dry it over silica gel for 5 h. Repeat with the *Standard*.

Acceptance criteria: The IR absorption spectrum of a mineral oil dispersion of the *Sample* exhibits maxima only at the same wavelengths as those of a similar preparation of the *Standard*.

• **B.**

Sample solution: Ignite 0.3 g of Methylparaben Sodium, cool, and dissolve the residue in about 3 mL of 3 N hydrochloric acid.

Acceptance criteria: A platinum wire dipped in the *Sample solution* imparts an intense, persistent yellow color to a nonluminous flame.

ASSAY

• **PROCEDURE**

Mobile phase: Methanol and a 6.8 g/L solution of potassium dihydrogen phosphate (65:35, v/v)

System suitability solution: 5.0 µg/mL each of *p*-hydroxybenzoic acid and USP Methylparaben RS in *Mobile phase*

Standard solution: Dissolve 50.0 mg of USP Methylparaben RS in 2.5 mL of methanol, and dilute with *Mobile phase* to 50.0 mL. Dilute 10.0 mL of this solution with *Mobile phase* to 100.0 mL.

Sample solution: Dissolve 50.0 mg of Methylparaben Sodium in 2.5 mL of methanol, and dilute with *Mobile phase* to 50.0 mL. Dilute 10.0 mL of this solution with *Mobile phase* to 100.0 mL.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 272 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Flow rate: 1.3 mL/min

Injection volume: 10 µL

Run time: About 5 times the retention time of the methylparaben peak

System suitability

Samples: *System suitability solution* and *Standard solution*

[NOTE—The retention time for methylparaben is about 2.2 min; the relative retention times for *p*-hydroxybenzoic acid and methylparaben are about 0.7 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 2.0 between the *p*-hydroxybenzoic acid and methylparaben peaks, *System suitability solution*

Relative standard deviation: NMT 0.85% for six injections, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of methylparaben sodium ($C_8H_7NaO_3$) in the portion of Methylparaben Sodium taken:

$$\text{Result} = P \times (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2})$$

P = labeled purity of USP Methylparaben RS expressed as a percentage

r_U = peak area of methylparaben from the *Sample solution*

r_S = peak area of methylparaben from the *Standard solution*

C_S = concentration of methylparaben in the *Standard solution*

C_U = concentration of Methylparaben Sodium in the *Sample solution*

M_{r1} = molecular weight of methylparaben sodium, 174.13

M_{r2} = molecular weight of methylparaben, 152.15

Acceptance criteria: 95.0%–102.0% on the anhydrous basis

IMPURITIES

• **RELATED COMPOUNDS**

Mobile phase, System suitability solution, Sample solution, and Chromatographic system: Proceed as directed in the *Assay*.

Standard solution: Dilute 1.0 mL of the *Sample solution* with *Mobile phase* to 20.0 mL. Dilute 1.0 mL of this solution with *Mobile phase* to 10.0 mL.

System suitability

Sample: *System suitability solution*

[NOTE—The retention time for methylparaben is about 2.2 min; the relative retention times for *p*-hydroxybenzoic acid and methylparaben are about 0.7 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 2.0 between the *p*-hydroxybenzoic acid and methylparaben peaks

Analysis

Samples: *Standard solution* and *Sample solution*

Acceptance criteria

***p*-Hydroxybenzoic acid:** NMT 3.0%; the peak area in the *Sample solution*, multiplied by 1.4 to correct for the calculation of content, is NMT 6 times the area of the principal peak in the *Standard solution*.

Unspecified impurities: NMT 0.5%; the peak area of each impurity in the *Sample solution* is NMT the area of the principal peak in the *Standard solution*.

Total impurities: NMT 1.0%; the total peak area for all unspecified impurities in the *Sample solution* is NMT twice the area of the principal peak in the *Standard solution*.

• **CHLORIDE AND SULFATE,** *Chloride* ⟨221⟩

Sample: 0.2 g

Control: 0.10 mL of 0.020 N hydrochloric acid

Acceptance criteria: 0.035%; the *Sample* shows no more chloride than the *Control*.

• **CHLORIDE AND SULFATE,** *Sulfate* ⟨221⟩

Sample: 0.25 g

Control: 0.30 mL of 0.020 N sulfuric acid

Acceptance criteria: 0.12%; the *Sample* shows no more sulfate than the *Control*.

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SPECIFIC TESTS
- **COMPLETENESS OF SOLUTION** ⟨641⟩
  Sample solution: 1 g of Methylparaben Sodium dissolved in water
  Acceptance criteria: Meets the requirements
- **pH** ⟨791⟩
  Sample solution: 1 mg/mL
  Acceptance criteria: 9.5–10.5
- **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 5.0%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Methylparaben RS

Methylpyrrolidone



C_5H_9NO 99.1
1-Methyl-2-pyrrolidinone;
N-Methyl-2-pyrrolidone;
N-Methylpyrrolidone;
1-Methyl-2-pyrrolidone;
Pyrrolidin, 1-methyl-2-one-;
1-Methylpyrrolidin-2-one;
N-Methyl-γ-butyrolactam;
N-Methyl tetrahydropyrrolone;
1-Methyl-2-oxopyrrolidine;
N-Methyl-1-oxotetramethyleneamine;
2-Methyl-2-azacyclopentanone [872-50-4].

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F▲** (CN 1-May-2020)

IMPURITIES
- **ORGANIC IMPURITIES**
  Standard solution: To 1 mL of USP Methylpyrrolidone RS, add 1 mL of pyrrolidone, and dilute with methylene chloride to 20 mL.
  Sample solution: Methylpyrrolidone (neat)
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: GC
  Detector: Flame ionization
  Column: 0.32-mm × 30-m fused-silica capillary; 5-µm layer of phase G2
  Temperatures
  Injector: 280°
  Detector: 280°
  Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
100	—	100	0
100	3	170	30

Carrier gas: Nitrogen
Linear velocity: 20 cm/s
Injection type: Split ratio about 100:1
Injection volume: 1 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Resolution: NLT 2.0 between pyrrolidone and methylpyrrolidone
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of each impurity, excluding any solvent peaks and peaks NMT 0.02%, in the portion of Methylpyrrolidone taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of each individual impurity from the *Sample solution*
r_T = sum of the responses of all the peaks from the *Sample solution*

Acceptance criteria: NMT 0.1% of any individual impurity; and NMT 0.3% of total impurities

SPECIFIC TESTS
- **ALKALINITY**
  Bromothymol blue solution: Dissolve 50 mg of bromothymol blue in a mixture of 4 mL of 0.02 M sodium hydroxide and 20 mL of alcohol, and dilute with water to 100 mL.
  Sample: Methylpyrrolidone (neat)
  Analysis: Add 0.5 mL of *Bromothymol blue solution* as indicator to 50 mL of water, and adjust with 0.02 M potassium hydroxide or 0.02 M hydrochloric acid until a yellow color is obtained. Add 50 mL of the *Sample*. Titrate with 0.02 M hydrochloric acid to the initial coloration.
  Acceptance criteria: NMT 8.0 mL of 0.02 M hydrochloric acid is required.

Change to read:

- **CLARITY OF SOLUTION**
  [NOTE—The *Sample* is to be compared to the *Reference suspension* in diffused daylight 5 min after preparation of the *Reference suspension*.]
  Hydrazine solution: 10 mg/mL of hydrazine sulfate. [NOTE—Allow to stand 4–6 h before use.]
  Methenamine solution: Transfer 2.5 g of methenamine to a 100-mL glass-stoppered flask, add 25.0 mL of water, insert the glass stopper, and mix to dissolve.
  Primary opalescent suspension
  [NOTE—This suspension is stable for 2 months, provided it is stored in a glass container free from surface defects. The suspension must not adhere to the glass and must be well mixed before use.]
  Transfer 25.0 mL of the *Hydrazine solution* to the *Methenamine solution* in the 100-mL glass-stoppered flask. [NOTE—Allow to stand for 24 h.]
  Opalescence standard: Transfer 15.0 mL of the *Primary opalescent suspension* to a 1000-mL volumetric flask, and dilute with water to volume. [NOTE—This suspension should not be used beyond 24 h after preparation.]
  Reference suspension: Transfer 5.0 mL of the *Opalescence standard* to a 100-mL volumetric flask, and dilute with water to volume.
  Sample: Methylpyrrolidone (neat)
  Analysis: Transfer a sufficient portion of the *Sample* to a test tube of colorless, transparent, neutral glass with a flat base and an internal diameter of 15–25 mm to obtain a depth of 40 mm. Similarly transfer portions of the *Reference*

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suspension and water to separate matching test tubes. Compare the *Sample*, *Reference suspension*, and water in diffused daylight, viewing vertically against a black background (see ▲*Visual Comparison* ⟨630⟩▲ (CN 1-May-2019)). [NOTE—The diffusion of light must be such that the *Reference suspension* can readily be distinguished from water.]

Acceptance criteria: The *Sample* shows the same clarity as that of water, or its opalescence is not more pronounced than that of the *Reference suspension*.

Change to read:

- **COLOR OF SOLUTION**

Comparison solution: Mix 3.0 mL of ferric chloride CS, 3.0 mL of cobaltous chloride CS, and 2.4 mL of cupric sulfate CS with 0.3 N hydrochloric acid to make 10 mL. Dilute 1.0 mL of this solution with 0.3 N hydrochloric acid to make 100 mL. [NOTE—Prepare and use this solution immediately.]

Sample: Methylpyrrolidone (neat)

Analysis: Transfer a sufficient portion of the *Sample* to a test tube of colorless, transparent, neutral glass with a flat base and an internal diameter of 15–25 mm to obtain a depth of 40 mm. Similarly transfer a portion of the *Comparison solution* to a separate matching test tube. Compare the color of the *Sample* with that of the *Comparison solution* in diffused daylight, viewing vertically against a white background (see ▲*Visual Comparison* ⟨630⟩▲ (CN 1-May-2019)).

Acceptance criteria: The *Sample* is not more intensely colored than the *Comparison solution*.

- **WATER DETERMINATION,** *Method Ic* ⟨921⟩**:** NMT 0.1%, determined on 1.0 g

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in light-resistant containers.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Methylpyrrolidone RS

Mineral Oil—*see Mineral Oil General Monographs*

Mineral Oil, Rectal—*see Mineral Oil, Rectal General Monographs*

Light Mineral Oil

DEFINITION

Light Mineral Oil is a purified mixture of liquid hydrocarbons obtained from petroleum. It may contain a suitable stabilizer.

IDENTIFICATION

Change to read:

- **A.** ▲**SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F** ▲ (CN 1-May-2020)
- **B.** It meets the requirements in *Specific Tests* for *Viscosity—Capillary Methods* ⟨911⟩.

IMPURITIES

- **LIMIT OF POLYCYCLIC AROMATIC HYDROCARBONS**

Dimethyl sulfoxide: Use spectrophotometric grade dimethyl sulfoxide.

***n*-Hexane:** Use *n*-hexane that has been washed by being shaken previously twice with one-fifth its volume of *Dimethyl sulfoxide*.

Standard solution: 7.0 µg/mL of USP Naphthalene RS in isooctane (2,2,4-trimethylpentane)

Standard blank: 2,2,4-Trimethylpentane

Sample solution: Transfer 25.0 mL of Light Mineral Oil and 25 mL of *n-Hexane* to a 125-mL separator, and mix. [NOTE—Use no lubricants other than water on the stopcock, or use a separator equipped with a suitable polymeric stopcock.]

Add 5.0 mL of *Dimethyl sulfoxide*, and shake the mixture vigorously for 1 min. Allow to stand until the lower layer is clear, transfer the lower layer to another 125-mL separator, add 2 mL of *n-Hexane*, and shake vigorously. Use the lower layer.

Sample blank: *Dimethyl sulfoxide* that has been shaken previously vigorously for 1 min with *n-Hexane* in the ratio of 5 mL of *Dimethyl sulfoxide* to 25 mL of *n-Hexane*

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Mode: UV

Analytical wavelengths

Standard solution: 275 nm

Sample solution: 260–350 nm

Cell: 1 cm

Analysis

Samples: *Standard solution, Standard blank, Sample solution,* and *Sample blank*

Acceptance criteria: The absorbance at any wavelength in the specified range of the *Sample solution* is NMT one-third of the absorbance of the *Standard solution*.

SPECIFIC TESTS

- **SPECIFIC GRAVITY** ⟨841⟩**:** 0.818–0.880
- **VISCOSITY—CAPILLARY METHODS** ⟨911⟩**:** 3.0–34.4 $mm^2 \cdot s^{-1}$ for kinematic viscosity, measured with a capillary viscometer at 40 ± 0.1°
- **ACIDITY**

Sample solution: Combine 10 mL of Light Mineral Oil and 20 mL of boiling water, shake vigorously for 1 min, and allow to cool. Remove, and filter the aqueous layer.

Analysis: To 10 mL of the *Sample solution* add 0.1 mL of phenolphthalein TS.

Acceptance criteria: The solution does not produce a pink color. NMT 1.0 mL of 0.01 N sodium hydroxide is required to produce a pink color.

- **READILY CARBONIZABLE SUBSTANCES TEST** ⟨271⟩

Sample: 5 mL

Standard solution: In a glass-stoppered test tube that previously has been rinsed with hot nitric acid (see *Cleaning Glass Apparatus* ⟨1051⟩), mix 3 mL of ferric chloride CS, 1.5 mL of cobaltous chloride CS, and 0.5 mL of cupric sulfate CS then overlaid with 5 mL of Light Mineral Oil.

Analysis: Place the *Sample* in a glass-stoppered test tube that previously has been rinsed with hot nitric acid (see *Cleaning Glass Apparatus* ⟨1051⟩), then rinsed with water, and dried. Add 5 mL of sulfuric acid containing 94.5%–94.9% of H_2SO_4, and heat in a boiling water bath for 10 min. After the test tube has been in the bath for 30 s, remove it quickly, and, while holding the stopper in place, give three vigorous, vertical shakes over an amplitude of about 5 in. Repeat every 30 s. Do not keep the test tube out of the bath longer than 3 s for each shaking period. At the end of 10 min from the time when first placed in the water bath, remove the test tube.

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Acceptance criteria: The oil portion of the *Sample* may turn hazy, but it remains colorless or shows a slight pink or yellow color, and the acid portion of the *Sample* does not become darker than the *Standard solution.*

• **SOLID PARAFFIN**

Sample: Light Mineral Oil that has been dried previously in a beaker at 105° for 2 h and cooled to room temperature in a desiccator over silica gel

Analysis: Fill a tall, cylindrical, standard oil-sample bottle of colorless glass of 120-mL capacity with the *Sample.* Insert the stopper, and immerse the bottle in a mixture of ice and water for 4 h.

Acceptance criteria: The *Sample* is sufficiently clear that a black line 0.5 mm in width, on a white background, held vertically behind the bottle, is clearly visible.

• **LIMIT OF SULFUR COMPOUNDS**

Solution A: Saturated solution of lead(II) oxide in sodium hydroxide (200 mg/mL)

Sample: 4.0 mL

Analysis: Combine the *Sample,* 2 mL of dehydrated alcohol, and 2 drops of *Solution A,* heat at 70° for 10 min with frequent shaking, and cool.

Acceptance criteria: No dark brown color develops.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers. No storage requirements specified.

• **LABELING:** Label it to indicate the name and quantity of any substance added as a stabilizer, and label packages intended for direct use by the public to indicate that it is not intended for internal use.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Mineral Oil RS

USP Naphthalene RS

Topical Light Mineral Oil—*see Topical Light Mineral Oil General Monographs*

Mono- and Di-glycerides

DEFINITION

Mono- and Di-glycerides is a mixture of glycerol mono- and di-esters, with minor amounts of tri-esters, of fatty acids from edible oils. It contains NLT 40.0% of monoglycerides. The monoglyceride content is NLT 90.0% and NMT 110.0% of the value indicated in the labeling. It may contain suitable stabilizers.

ASSAY

• **MONOGLYCERIDES**

Mobile phase: Tetrahydrofuran

Sample solution: 40 mg/mL of Mono- and Di-glycerides in tetrahydrofuran

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability.*)

Mode: LC

Detector: Refractive index

Column: 7-mm × 60-cm; 5-µm packing L21 (100 Å)

[NOTE—Two or three 7.5-mm × 30-cm L21 columns may be used in place of one 60-cm column provided that *System suitability* requirements are met.]

Temperatures

Column: 40°

Detector: 40°

Flow rate: 1 mL/min

Injection volume: 40 µL

System suitability

Sample: *Sample solution*

[NOTE—The order of elution is triglycerides, diglycerides, monoglycerides, and glycerin.]

Suitability requirements

Relative standard deviation: NMT 1.0%, determined from the monoglycerides peak

Analysis

Sample: *Sample solution*

Calculate the percentage of monoglycerides in the portion of Mono- and Di-glycerides taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response for monoglycerides

r_T = sum of the responses of all the peaks, except the solvent peak

Acceptance criteria: 90.0%–110.0% of the value indicated in the labeling

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 0.1%

• **ARSENIC,** *Method II* ⟨211⟩**:** NMT 3 µg/g

• **LIMIT OF FREE GLYCERIN**

Mobile phase, Sample solution, and **Chromatographic system:** Proceed as directed in the *Assay* for *Monoglycerides.*

Standard solutions: 0.5, 1.0, 2.0, and 4.0 mg/mL of USP Glycerin RS in tetrahydrofuran

Analysis

Samples: *Sample solution* and *Standard solutions*

Measure the responses for the glycerin peaks. Plot the concentration, in mg/mL, of USP Glycerin RS in the *Standard solutions* versus the glycerin peak responses obtained. From the standard curve so obtained, determine the glycerin concentration in the *Sample solution.*

Calculate the percentage of glycerin in the portion of Mono- and Di-glycerides taken:

$$\text{Result} = (C_U/C_S) \times 100$$

C_U = glycerin concentration in the *Sample solution* from the standard curve (mg/mL)

C_S = concentration of the *Sample solution* (mg/mL)

Acceptance criteria: NMT 7.0%

SPECIFIC TESTS

• **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩ **:** NMT 4

• **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩**:** 90.0%–110.0% of the value indicated in the labeling

• **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩**:** 90.0%–110.0% of the value indicated in the labeling. If the value stated in the labeling is less than 10, the iodine value is NMT 10.

• **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩**:** 90.0%–110.0% of the value indicated in the labeling

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

• **LABELING:** The labeling indicates the monoglyceride content, hydroxyl value, iodine value, saponification value, and name and quantity of any stabilizers.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Glycerin RS

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Monoethanolamine



C_2H_7NO 61.08
Ethanol, 2-amino-;
2-Aminoethanol [141-43-5].

DEFINITION
Monoethanolamine contains NLT 98.0% and NMT 100.5% by weight of monoethanolamine (C_2H_7NO).

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F**▲ (CN 1-May-2020)

ASSAY
• **PROCEDURE**
Sample solution: Weigh a glass-stoppered weighing bottle containing 25 mL of water. Add 1 g of Monoethanolamine, and reweigh.
Blank: 25 mL of water
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.5 N hydrochloric acid VS
Endpoint detection: Visual
Analysis: Transfer the *Sample solution* to a suitable flask, add a mixed indicator of 5 parts bromocresol green TS and 6 parts methyl red TS for a total of approximately 11 parts of solution. Titrate the *Sample solution* with *Titrant*. Perform a blank determination.
Calculate the percentage of monoethanolamine (C_2H_7NO) in the portion of sample taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

V_S = *Titrant* volume consumed by the *Sample solution* (mL)
V_B = *Titrant* volume consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 61.08 mg/mEq
W = sample weight (mg)

Acceptance criteria: 98.0%–100.5%

IMPURITIES
• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%

SPECIFIC TESTS
• **SPECIFIC GRAVITY** ⟨841⟩: 1.013–1.016
• **DISTILLING RANGE,** *Method II* ⟨721⟩: NLT 95% of it distills between 167° and 173°, a correction factor of 0.052° per mm applied as necessary.

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Monoethanolamine RS

Monoglyceride Citrate

Citric acid ester of glyceryl monooleate [36291-32-4].

DEFINITION
Monoglyceride Citrate is a mixture of glyceryl monooleate and its citric acid monoester, manufactured by the reaction of glyceryl monooleate with citric acid under controlled conditions. It contains NLT 14.0% and NMT 17.0% of total citric acid, calculated on the anhydrous basis.

IDENTIFICATION
• **A.**
Sample: 1 g
Analysis: Reflux the *Sample* with 15 mL of 0.5 N potassium hydroxide solution in dehydrated alcohol for 1 h. Add 15 mL of water, and acidify with diluted hydrochloric acid (about 6 mL). Dissolve any oil drops or solid produced in 5 mL of hexane. Remove the hexane layer, extract again with 5 mL of hexane, and again remove the hexane layer. [NOTE—Keep the resulting aqueous layer for *Identification* tests *B* and *C*.]
Acceptance criteria: Oil drops or a white to yellowish-white solid are produced that are soluble in 5 mL of hexane.
• **B. IDENTIFICATION TESTS—GENERAL,** *Citrate* ⟨191⟩
Sample: 1 mL of the aqueous layer resulting from *Identification* test *A*
Analysis: Evaporate the *Sample* in a porcelain dish.
Acceptance criteria: The residue meets the requirements.
• **C.**
Sample: 5 mL of the aqueous layer resulting from *Identification* test *A*
Analysis: Transfer the *Sample* to a test tube. Add excess calcium hydroxide as a powder, place in boiling water for 5 min, shaking several times, cool, and filter. Transfer one drop of the filtrate into a test tube, and add about 50 mg of potassium hydrogen sulfate. On top of the test tube, place a filter paper moistened with a reagent for acrolein consisting of a mixture of 5% nitroprusside solution in water and 20% piperidine solution in water (1:1). Heat the test tube.
Acceptance criteria: The filter paper turns blue (presence of glycerin). The color changes to light red after addition of sodium hydroxide TS.

ASSAY
• **CONTENT OF CITRIC ACID**
Standard solution: 0.23 mg/mL of USP Citric Acid RS
Sample solution: Transfer 150 mg of Monoglyceride Citrate into a saponification flask, add 50 mL of 4% potassium hydroxide solution in dehydrated alcohol, and reflux for 1 h. Acidify the reaction mixture with hydrochloric acid to a pH of 2.8–3.2, transfer into a 400-mL beaker, and evaporate to dryness on a steam bath. Quantitatively transfer the contents of the beaker into a separator, using NMT 50 mL of water, and extract with three 50-mL portions of petroleum ether, discarding the extracts. Transfer the water layer to a 100-mL volumetric flask, and dilute with water to volume.
Blank: Water
Instrumental conditions
Mode: UV-Vis
Analytical wavelength: 450 nm
Cell: 1 cm
Analysis
Samples: *Standard solution*, *Sample solution*, and *Blank*
Pipet 2.0 mL each of the *Standard solution*, *Sample solution*, and *Blank* into separate 40-mL graduated centrifuge tubes. Add 2 mL of a 1 in 2 sulfuric acid solution and 11 mL of water to each tube. Boil for 3 min, cool, and add 5 mL of bromine TS to each tube. Dilute to 20 mL, allow to stand for 10 min, and centrifuge. Transfer 4.0 mL of the supernatant from each tube into separate 19- × 110-mm test tubes, add 1 mL of water, 0.5 mL of a 1 in 2

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sulfuric acid solution, and 0.3 mL of 1 M potassium bromide, and shake. Add 0.3 mL of 1.5 N potassium permanganate, shake, and allow to stand for 2 min. Add 1 mL of a saturated solution of ferrous sulfate, shake, allow to stand for 2 min, and then dilute with water to 10 mL. Add 10.0 mL of *n*-hexane (previously washed with sulfuric acid, followed by a water wash, and then dried over anhydrous sodium sulfate), shake vigorously for 2 min, and centrifuge at low speed for 1 min. Transfer 5.0 mL of the hexane extract into a 20- × 145-mm tube containing 10.0 mL of 4% sodium sulfide solution, and briefly shake vigorously (three oscillations only). Centrifuge the mixture at low speed for 1 min. Immediately determine the absorbance of each aqueous layer from the *Standard solution* and *Sample solution* against the aqueous layer from the *Blank*.

Calculate the percentage of citric acid in the portion of Monoglyceride Citrate taken:

$$\text{Result} = (A_U/A_S) \times (V \times C_S/W) \times 100$$

A_U = absorbance of the *Sample solution*
A_S = absorbance of the *Standard solution*
V = volume of the *Sample solution* (mL)
C_S = concentration of USP Citric Acid RS in the *Standard solution* (mg/mL)
W = weight of Monoglyceride Citrate taken to prepare the *Sample solution* (mg)

Acceptance criteria: 14.0%–17.0% on the anhydrous basis

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.3%, determined on 1 g

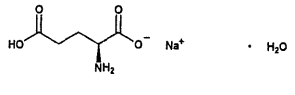
SPECIFIC TESTS

• **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩: 70–100
• **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩: 260–265
• **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 0.2%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Citric Acid RS

Monosodium Glutamate



$C_5H_8NNaO_4 \cdot H_2O$ 187.13

L-Glutamic acid, sodium salt, hydrate;
Monosodium L-glutamate, hydrate [6106-04-3].

DEFINITION

Monosodium Glutamate contains NLT 99.0% and NMT 100.5% of monosodium glutamate ($C_5H_8NNaO_4 \cdot H_2O$).

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197A**▲ (CN 1-May-2020)
• **B. IDENTIFICATION TESTS—GENERAL** ⟨191⟩, *Sodium*: It meets the requirements of test *A*.

ASSAY

• **PROCEDURE**
Sample: 250 mg
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.1 N perchloric acid VS
Blank: 100 mL of glacial acetic acid with a few drops of water
Endpoint detection: Potentiometric
Analysis: Wet the *Sample* with a few drops of water. Dissolve in 100 mL of glacial acetic acid. Titrate with 0.1 N perchloric acid VS. Perform a blank determination.
Calculate the percentage of monosodium glutamate ($C_5H_8NNaO_4 \cdot H_2O$) in the *Sample* taken:

$$\text{Result} = [(V_S - V_B) \times N_A \times F \times 100]/W$$

V_S = *Titrant* volume consumed by the *Sample* (mL)
V_B = *Titrant* volume consumed by the *Blank* (mL)
N_A = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 93.56 mg/mEq
W = *Sample* weight (mg)

Acceptance criteria: 99.0%–100.5%

IMPURITIES

• **CHLORIDE AND SULFATE** ⟨221⟩, *Chloride*: A 280-mg portion shows no more chloride than corresponds to 1.0 mL of 0.020 N hydrochloric acid (0.25%).
• **LEAD** ⟨251⟩: NMT 10 µg/g

SPECIFIC TESTS

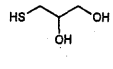
Change to read:

• **CLARITY AND COLOR OF SOLUTION**
Sample solution: 1.0 g in 10 mL of water
Standard solution: To 0.2 mL of a solution of sodium chloride containing 10 µg/mL of chloride ion (Cl), add 20 mL of water and mix. Then add 1 mL of 5 N nitric acid, 0.2 mL of dextrin solution (1 in 50), and 1 mL of silver nitrate TS, and allow to stand for 15 min.
Analysis: Compare the *Sample solution* with the *Standard solution* (see ▲*Visual Comparison* ⟨630⟩▲ (CN 1-May-2019)).
Acceptance criteria: The *Sample solution* is colorless and has no more turbidity than the *Standard solution*.
• **OPTICAL ROTATION** ⟨781S⟩, *Procedures*, *Specific Rotation*
Sample solution: 100 mg/mL in 2 N hydrochloric acid
Acceptance criteria: +24.8° to +25.3°, determined at 20°
• **PH** ⟨791⟩: 6.7–7.2, in a solution (1 in 20)
• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry at 100° for 5 h.
Acceptance criteria: NMT 0.5%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Monosodium Glutamate RS

Monothioglycerol



$C_3H_8O_2S$ 108.16

1,2-Propanediol, 3-mercapto-;

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3-Mercapto-1,2-propanediol [96-27-5].

DEFINITION
Monothioglycerol contains NLT 97.0% and NMT 101.0% of monothioglycerol ($C_3H_8O_2S$), calculated on the anhydrous basis.

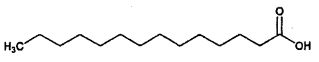
ASSAY
• **PROCEDURE**
Sample: 400 mg
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.1 N iodine VS
Endpoint detection: Visual
Analysis: Dissolve the *Sample* in 50 mL of water. Titrate with *Titrant*, adding 3 mL of starch TS as the endpoint is approached. Each mL of *Titrant* is equivalent to 10.82 mg of monothioglycerol ($C_3H_8O_2S$).
Acceptance criteria: 97.0%–101.0% on the anhydrous basis

IMPURITIES
• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%
• **SELENIUM** ⟨291⟩
Test solution: 200 µL
Acceptance criteria: 30 µg/g

SPECIFIC TESTS
• **SPECIFIC GRAVITY** ⟨841⟩: 1.241–1.250
• **REFRACTIVE INDEX** ⟨831⟩: 1.521–1.526
• **PH** ⟨791⟩
Sample solution: 1 in 10
Acceptance criteria: 3.5–7.0
• **WATER DETERMINATION,** *Method II* ⟨921⟩: NMT 5.0%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight containers.

Myristic Acid



$C_{14}H_{28}O_2$ 228.37
Tetradecanoic acid;
1-Tetradecanoic acid;
1-Tridecanecarboxylic acid [544-63-8].

DEFINITION
Myristic Acid is obtained from coconut oil and other fats. It contains NLT 97.0% of myristic acid ($C_{14}H_{28}O_2$).

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: ⟨197D⟩▲ (CN 1-May-2020) or ⟨197K⟩
Sample: Undried specimen
Acceptance criteria: Meets the requirements

Change to read:

• **B. ▲CHROMATOGRAPHIC IDENTITY:**▲ 2S (NF36) The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained ▲▲ 2S (NF36) in the *Assay*.

ASSAY

Delete the following:

▲• **FATS AND FIXED OILS,** *Fatty Acid Composition* ⟨401⟩
System suitability solution: Prepare as directed in the chapter, except that only stearic acid and palmitic acid are used.
Sample solution: Prepare as directed for the *Test Solution* in the chapter.
Standard solution: Prepare as directed for the *Sample solution*, using 100 mg of USP Myristic Acid RS instead of the substance to be examined.
Chromatographic system: Prepare as directed in the chapter.
Injection size: 1 µL
System suitability
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Sample: *System suitability solution*
Suitability requirements
Resolution: NLT 1.5 between methyl stearate and methyl palmitate
Analysis
Samples: *Standard solution* and *Sample solution*
Identify the methyl myristate peak from the *Sample solution* by comparing the retention times of the peaks with those from the *Standard solution*. Measure the responses for all the peaks from the *Sample solution*, excluding the solvent peak.
Calculate the percentage of myristic acid ($C_{14}H_{28}O_2$) in the portion of Myristic Acid taken:

$$\text{Result} = (A/B) \times 100$$

A = peak response for methyl myristate from the *Sample solution*
B = sum of all the peak responses in the *Sample solution* except the solvent peak

Acceptance criteria: NLT 97.0%▲ 2S (NF36)

Add the following:

▲• **PROCEDURE**
Solution A: Add 1 mL of phosphoric acid to 1 L of water to prepare a 0.1% phosphoric acid solution.
Solution B: Acetonitrile
Diluent: Methanol
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0.0	50	50
20.0	1	99
25.0	1	99
26.0	50	50
30.0	50	50

System suitability solution: 5.0 mg/mL of USP Myristic Acid RS and 0.025 mg/mL of linolenic acid in *Diluent*
Standard solution: 5.0 mg/mL of USP Myristic Acid RS in *Diluent*
Sample solution: 5.0 mg/mL of Myristic Acid in *Diluent*

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Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 210 nm
Column: 4.6-mm × 15-cm; 2.7-µm packing L1
Column temperature: 40°
Flow rate: 1.0 mL/min
Injection volume: 25 µL
Run time: 30 min
System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The relative retention times for linolenic acid and myristic acid are 0.97 and 1.00, respectively.]
Suitability requirements
Resolution: NLT 1.5 between linolenic acid and myristic acid, *System suitability solution*
Tailing factor: 0.8–1.8, *Standard solution*
Relative standard deviation: NMT 0.5%, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of myristic acid ($C_{14}H_{28}O_2$) in the portion of Myristic Acid taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP Myristic Acid RS in the *Standard solution* (mg/mL)
C_U = concentration of Myristic Acid in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 97.0% ▲2S (NF36)

IMPURITIES
• **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 0.1%

Add the following:

▲• **LIMIT OF LAURIC ACID AND PALMITIC ACID**
Solution A, Solution B, Diluent, Mobile phase, System suitability solution, and **Chromatographic system:** Proceed as directed in the *Assay*.
Standard solution: 0.15 mg/mL of USP Lauric Acid RS and 0.15 mg/mL of USP Palmitic Acid RS in *Diluent*
Sample solution: 5.0 mg/mL of Myristic Acid in *Diluent*
System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The relative retention times for lauric acid, linolenic acid, myristic acid, and palmitic acid are 0.73, 0.97, 1.00, and 1.26, respectively.]
Suitability requirements
Resolution: NLT 1.5 between linolenic acid and myristic acid, *System suitability solution*
Relative standard deviation: NMT 5%, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Based on the *Standard solution*, identify the peaks of appropriate fatty acids.
Calculate the percentage of lauric acid ($C_{12}H_{24}O_2$) or palmitic acid ($C_{16}H_{32}O_2$) in the portion of Myristic Acid taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of lauric acid or palmitic acid from the *Sample solution*
r_S = peak response of lauric acid or palmitic acid from the *Standard solution*
C_S = concentration of USP Lauric Acid RS or USP Palmitic Acid RS in the *Standard solution* (mg/mL)
C_U = concentration of Myristic Acid in the *Sample solution* (mg/mL)

Acceptance criteria: Disregard peaks that are less than 0.05% for any individual impurity and any peaks due to solvent.
Sum of lauric acid and palmitic acid: NMT 3.0% ▲2S (NF36)

• **LIMIT OF LEAD**
[NOTE—Select reagents with as low a lead content as practicable, and store all solutions in high-density polyethylene containers. Rinse all plastic and glassware thoroughly with warm 8 N nitric acid followed by deionized water.]
Standard stock solution: Dissolve 160 mg of lead nitrate in 100 mL of water containing 1 mL of nitric acid. Dilute with water to 1000 mL.
Standard solutions: [NOTE—Prepare these solutions on the day of use.] Transfer 10.0 mL of *Standard stock solution* to a 100-mL volumetric flask, and dilute with water to volume. Each milliliter of this solution contains the equivalent of about 10 µg of lead. Dilute accurately measured volumes of the diluted *Standard stock solution* with water to obtain solutions with known concentrations of 1, 2, and 5 µg/mL of lead.
Sample solution: Transfer 5 g of Myristic Acid to an evaporating dish. Add 5 mL of a 25% sulfuric acid solution, and distribute the sulfuric acid uniformly through the sample. Within a hood, place the dish on a steam bath to evaporate most of the water. Place the dish on a burner, and slowly pre-ash the sample by expelling most of the sulfuric acid. Place the dish in a muffle furnace that has been set at 525°, and ash the sample until the residue appears free from carbon. Prepare a blank by ashing 5 mL of a 25% sulfuric acid solution. Cool, and cautiously wash down the inside of each evaporation dish with water. Treat both the sample and the blank as follows. Add 5 mL of 1 N hydrochloric acid. Place each dish on a steam bath, and evaporate to dryness. To each dish add 1.0 mL of 3 N hydrochloric acid and about 5 mL of water, and heat briefly on a steam bath to dissolve any residue. Transfer each solution quantitatively to a 10-mL volumetric flask, and dilute with water to volume.
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Analytical wavelength: 283.3 nm at the lead emission line
Lamp: Lead electrodeless discharge
Flame: Air–acetylene with a suitable burner head
Slit width: 0.7 nm
Blank: Water. [NOTE—Perform a blank determination following the manufacturer's operating instructions.]
Analysis
Samples: *Standard solutions*, *Sample solution*, and *Blank*
Determine the corrected absorbance values by subtracting the absorbance of the *Blank* from the absorbance of each of the *Standard solutions* and from the absorbance of the *Sample solution*. Prepare a standard curve by plotting the corrected absorbance values of the *Standard solutions* versus their corresponding concentrations, in µg/mL. From the calibration curve, determine the lead concentration in the *Sample solution*.

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Calculate the lead content, in ppm, in the portion of Myristic Acid taken:

$$\text{Result} = (C/W_S) \times V$$

C = measured concentration of lead in the *Sample solution* from the standard curve (µg/mL)
W_S = weight of Myristic Acid taken (g)
V = final volume of the *Sample solution*, 10 mL

Acceptance criteria: NMT 2 ppm

• **LIMIT OF MINERAL ACIDS**
Sample: 5 g of melted Myristic Acid
Analysis: Shake the *Sample* with an equal volume of hot water for 2 min, cool, and filter.
Acceptance criteria: The filtrate is not reddened by the addition of 1 drop of methyl orange TS.

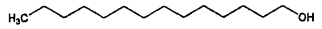
SPECIFIC TESTS
• **CONGEALING TEMPERATURE** ⟨651⟩: 48°–55.5°
• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Acid Value*: 242–249
• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Iodine Value*: NMT 1.0
• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Peroxide Value*: NMT 10.0
• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Unsaponifiable Matter*: NMT 1%
• **WATER DETERMINATION** ⟨921⟩, *Method I*: NMT 0.2%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.

Change to read:

• **USP REFERENCE STANDARDS** ⟨11⟩
▲USP Lauric Acid RS▲ 2S (NF36)
USP Myristic Acid RS
▲USP Palmitic Acid RS▲ 2S (NF36)

Myristyl Alcohol



$C_{14}H_{30}O$ 214.39
n-Tetradecan-1-ol;
1-Tetradecanol;
1-Hydroxytetradecane;
1-Tetradecyl alcohol [112-72-1].

DEFINITION
Myristyl Alcohol contains NLT 90.0% and NMT 102.0% of myristyl alcohol ($C_{14}H_{30}O$), the remainder consisting chiefly of related alcohols. It is obtained from sources of vegetable, animal, or synthetic origin.

IDENTIFICATION
• **A. CHROMATOGRAPHIC IDENTITY**
Analysis: Proceed as directed in the *Assay*.
Acceptance criteria: The retention time of the major peak of the *Sample solution*, excluding the solvent and internal standard peaks, corresponds to the myristyl alcohol peak of the *Standard solution*.

ASSAY
• **PROCEDURE**
Internal standard solution: 1 mg/mL of 1-pentadecanol (internal standard) in ethanol
System suitability solution: Prepare 1 mg/mL of USP Cetyl Alcohol RS, 1 mg/mL of USP Stearyl Alcohol RS, and 1 mg/mL of USP Oleyl Alcohol RS in *Internal standard solution*, and heat the solution in a sealed container in a 50° water bath until all fatty alcohols are dissolved. Allow the solution to cool to room temperature, and mix well.
Standard solution: 1.0 mg/mL of USP Myristyl Alcohol RS in *Internal standard solution*
Sample solution: 1.0 mg/mL of Myristyl Alcohol in *Internal standard solution*
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.25-mm × 30-m fused-silica capillary column, coated with a 0.25-µm layer of phase G7
Temperatures
Injection port: 270°
Detector: 280°
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
60	20	180	—
180	10	220	5

Carrier gas: Hydrogen
Flow rate: 2.0 mL/min, constant flow mode
Injection volume: 1 µL
Injection type: Split injection; split ratio is 100:1
Liner: Single taper, low pressure drop liner with deactivated wool
Run time: 15 min
System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—See *Table 2* for the relative retention times.]

Table 2

Component	Relative Retention Time
Myristyl alcohol	0.92
1-Pentadecanol (internal standard)	1.00
Cetyl alcohol	1.08
Stearyl alcohol	1.25
Oleyl alcohol	1.27

Suitability requirements
Resolution: NLT 30 between the cetyl alcohol and stearyl alcohol peaks; NLT 2.0 between the stearyl alcohol and oleyl alcohol peaks, *System suitability solution*
Tailing factor: 0.8–1.8 for the myristyl alcohol and 1-pentadecanol peaks, *Standard solution*
Relative standard deviation: NMT 1%, using the area ratio of myristyl alcohol to 1-pentadecanol, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of myristyl alcohol ($C_{14}H_{30}O$) in the portion of Myristyl Alcohol taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

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R_U = peak response ratio of myristyl alcohol to the internal standard (peak response of myristyl alcohol/peak response of the internal standard) from the *Sample solution*

R_S = peak response ratio of myristyl alcohol to the internal standard (peak response of myristyl alcohol/peak response of the internal standard) from the *Standard solution*

C_S = concentration of USP Myristyl Alcohol RS in the *Standard solution* (mg/mL)

C_U = concentration of Myristyl Alcohol in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–102.0%

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%, determined on 2 g
- **LIMIT OF RELATED FATTY ALCOHOLS**

Solution A: 1 mg/mL of 1-pentadecanol in ethanol

Resolution solution: Prepare 1 mg/mL of USP Lauryl Alcohol RS, 1 mg/mL of USP Myristyl Alcohol RS, 1 mg/mL of USP Cetyl Alcohol RS, 1 mg/mL of USP Stearyl Alcohol RS, and 1 mg/mL of USP Oleyl Alcohol RS in *Solution A*. Heat the solution in a sealed container in a 50° water bath until all fatty alcohols are dissolved. Allow the solution to cool to room temperature, and mix well. Dilute the solution with ethanol to obtain a solution containing 0.05 mg/mL each of USP Lauryl Alcohol RS, USP Myristyl Alcohol RS, USP Cetyl Alcohol RS, 1-pentadecanol, USP Stearyl Alcohol RS, and USP Oleyl Alcohol RS.

Sample solution: 1 mg/mL of Myristyl Alcohol in ethanol

Chromatographic system: Proceed as directed in the *Assay*, except for the split ratio.

Injection type: Split injection; split ratio is 5:1

System suitability

Sample: *Resolution solution*

[NOTE—See *Table 3* for the relative retention times.]

Table 3

Component	Relative Retention Time
Lauryl alcohol	0.79
Myristyl alcohol	0.92
1-Pentadecanol	1.00
Cetyl alcohol	1.08
Stearyl alcohol	1.25
Oleyl alcohol	1.27

Suitability requirements

Resolution: NLT 15 between myristyl alcohol and 1-pentadecanol peaks; NLT 30 between the cetyl alcohol and stearyl alcohol peaks; NLT 2.0 between the stearyl alcohol and oleyl alcohol peaks

Analysis

Samples: *Resolution solution* and *Sample solution*

Identify each related fatty alcohol peak in the *Sample solution* based on that in the *Resolution solution*.

Calculate the percentage of each related fatty alcohol or any unspecified impurity in the portion of Myristyl Alcohol taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of each related fatty alcohol (or any unspecified impurity) from the *Sample solution*

r_T = sum of all the peak responses excluding peak responses due to solvent from the *Sample solution*

Acceptance criteria: Disregard peaks that are less than 0.05% for any unspecified impurities, and any peaks due to solvent.

Sum of unspecified impurities: NMT 1%

Sum of related fatty alcohols and unspecified impurities: NMT 10.0%

SPECIFIC TESTS

- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩: NMT 2
- **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩: NMT 1
- **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩: 250–267
- **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 0.5%

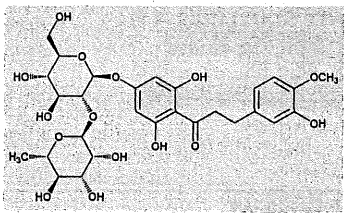
ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **LABELING:** Label it to indicate whether it is derived from vegetable, animal, or synthetic sources.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Cetyl Alcohol RS
USP Lauryl Alcohol RS
USP Myristyl Alcohol RS
USP Oleyl Alcohol RS
USP Stearyl Alcohol RS

Add the following:

▲Neohesperidin Dihydrochalcone



$C_{28}H_{36}O_{15}$ 612.58

1-Propanone, 1-[4-[[2-*O*-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-2, 6-dihydroxyphenyl]-3-(3-hydroxy-4-methoxyphenyl)-;

3,5-Dihydroxy-4-[3-(3-hydroxy-4-methoxyphenyl) propanoyl]phenyl 2-*O*-(α-L-rhamnopyranosyl)-β-D-glucopyranoside [20702-77-6].

DEFINITION

Neohesperidin Dihydrochalcone contains NLT 96.0% and NMT 102.0% of neohesperidin dihydrochalcone ($C_{28}H_{36}O_{15}$), calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

- **A.** **▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197A**▲ (CN 1-May-2020)
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

ASSAY

- **PROCEDURE**

Solution A: 0.5% (v/v) glacial acetic acid in water

Mobile phase: Acetonitrile and *Solution A* (20:80)

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Diluent: Dimethyl sulfoxide
System suitability solution: Suspend 100 mg of USP Neohesperidin Dihydrochalcone RS in 10.0 mL of a 100-g/L solution of sulfuric acid in water. Heat the suspension for 5 min on a boiling water bath. Immediately dilute 1.0 mL of the resulting solution with *Diluent* to 50.0 mL. [NOTE—Neohesperidin dihydrochalcone related compound F and neohesperidin dihydrochalcone related compound G are prepared in situ in this solution.]
Standard solution: 1.0 mg/mL of USP Neohesperidin Dihydrochalcone RS in *Diluent*
Sample solution: 1.0 mg/mL of Neohesperidin Dihydrochalcone in *Diluent*
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 282 nm
Column: 3.9-mm × 15-cm; 4-µm packing L1, with a carbon loading of 7%
Column temperature: 30°
Flow rate: 1.0 mL/min
Injection volume: 10 µL
Run time: 5 times the retention time of neohesperidin dihydrochalcone
System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—For the approximate relative retention times of related substances, see *Table 1*.]
Suitability requirements
Resolution: NLT 2.5 between the neohesperidin dihydrochalcone peak and the neohesperidin dihydrochalcone related compound F peak, *System suitability solution*
Relative standard deviation: NMT 2.0%, determined from the neohesperidin dihydrochalcone peak, *Standard solution*
Chromatogram similarity: The chromatogram obtained from the *Standard solution* is similar to the reference chromatogram provided with the lot of USP Neohesperidin Dihydrochalcone RS being used.
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of neohesperidin dihydrochalcone ($C_{28}H_{36}O_{15}$) in the portion of Neohesperidin Dihydrochalcone taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of neohesperidin dihydrochalcone from the *Sample solution*
r_S = peak area of neohesperidin dihydrochalcone from the *Standard solution*
C_S = concentration of USP Neohesperidin Dihydrochalcone RS in the *Standard solution*
C_U = concentration of Neohesperidin Dihydrochalcone in the *Sample solution*

Acceptance criteria: 96.0%–102.0% on the anhydrous basis

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.2%
- **ORGANIC IMPURITIES**

Mobile phase, Diluent, System suitability solution, and Chromatographic system: Proceed as directed in the *Assay*.
Standard solution A: Use the *Standard solution*, as prepared in the *Assay*.
Standard solution B: 0.04 mg/mL of USP Neodiosmin RS in *Diluent*
Standard solution C: 0.02 mg/mL of USP Neohesperidin Dihydrochalcone RS from *Standard solution A* in *Diluent*
Sensitivity solution: 0.002 mg/mL of USP Neohesperidin Dihydrochalcone RS from *Standard solution C* in *Diluent*
Sample solution: 2.0 mg/mL of Neohesperidin Dihydrochalcone in *Diluent*
System suitability
Samples: *System suitability solution, Standard solution A, Standard solution C,* and *Sensitivity solution*
[NOTE—For the approximate relative retention times of related substances, see *Table 1*.]
Suitability requirements
Resolution: NLT 2.5 between the neohesperidin dihydrochalcone peak and the neohesperidin dihydrochalcone related compound F peak, *System suitability solution*
Relative standard deviation: NMT 5.0%, determined from the neohesperidin dihydrochalcone peak, *Standard solution C*
Signal-to-noise ratio: NLT 10, determined from the neohesperidin dihydrochalcone peak, *Sensitivity solution*
Chromatogram similarity: The chromatogram obtained from the *Standard solution A* is similar to the reference chromatogram provided with the lot of USP Neohesperidin Dihydrochalcone RS being used.
Analysis
Samples: *Standard solution B, Standard solution C,* and *Sample solution*
Calculate the percentage of neodiosmin in the portion of Neohesperidin Dihydrochalcone taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of neodiosmin from the *Sample solution*
r_S = peak area of neodiosmin from *Standard solution B*
C_S = concentration of USP Neodiosmin RS in *Standard solution B* (mg/mL)
C_U = concentration of Neohesperidin Dihydrochalcone in the *Sample solution* (mg/mL)

Calculate the percentage of each impurity except neodiosmin in the portion of Neohesperidin Dihydrochalcone taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of each individual impurity except neodiosmin from the *Sample solution*
r_S = peak area of neohesperidin dihydrochalcone from *Standard solution C*
C_S = concentration of USP Neohesperidin Dihydrochalcone RS in *Standard solution C* (mg/mL)
C_U = concentration of Neohesperidin Dihydrochalcone in the *Sample solution* (mg/mL)

Acceptance criteria: See *Table 1*.

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Table 1

Name	Relative Retention Time	Acceptance Criteria, NMT (%)
Neohesperidin dihydrochalcone related compound A[a]	0.2	0.5
Neodiosmin[b]	0.38	2.0
Neohesperidin dihydrochalcone related compound C[c]	0.44	0.5
Neohesperidin dihydrochalcone related compound D[d]	0.7	2.0
Neohesperidin dihydrochalcone related compound E[e]	0.8	0.5
Neohesperidin dihydrochalcone	1.0	—
Neohesperidin dihydrochalcone related compound F[f]	1.1	0.5
Neohesperidin dihydrochalcone related compound G[g]	3.4	0.5
Any other individual impurity	—	0.5
Total impurities excluding neodiosmin	—	2.5

[a] 1-[4-[[2-*O*-(6-Deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-2,6-dihydroxyphenyl]ethanone (phloroacetophenone neohesperidoside).
[b] 7-[[2-*O*-(6-Deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4*H*-1-benzopyran-4-one; 5-Hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-oxo-4*H*-chromen-7-yl 2-*O*-(α-L-rhamnopyranosyl)-β-D-glucopyranoside.
[c] (2*RS*)-7-[[2-*O*-(6-Deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-2,3-dihydro-4*H*-1-benzopyran-4-one (neohesperidin).
[d] 1-[4-[[2-*O*-(6-Deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-2,6-dihydroxyphenyl]-3-(4-hydroxyphenyl)propan-1-one (naringin dihydrochalcone).
[e] 1-[4-[[6-*O*-(6-Deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-2,6-dihydroxyphenyl]-3-(3-hydroxy-4-methoxyphenyl)propan-1-one (hesperidin dihydrochalcone).
[f] 1-[4-(β-D-Glucopyranosyloxy)-2,6-dihydroxyphenyl]-3-(3-hydroxy-4-methoxyphenyl)propan-1-one (hesperetin dihydrochalcone 7′-glucoside).
[g] 3-(3-Hydroxy-4-methoxyphenyl)-1-(2,4,6-trihydroxyphenyl)propan-1-one (hesperetin dihydrochalcone).

SPECIFIC TESTS

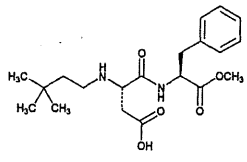
- **WATER DETERMINATION** ⟨921⟩, *Method I*: NMT 12.0%
- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩: The total aerobic microbial count does not exceed 10^3 cfu/g and the total combined molds and yeasts count does not exceed 10^2 cfu/g.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Neodiosmin RS
  5-Hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-oxo-4*H*-chromen-7-yl 2-*O*-(α-L-rhamnopyranosyl)-β-D-glucopyranoside.
  $C_{28}H_{32}O_{15}$ 608.55
  USP Neohesperidin Dihydrochalcone RS▲ (NF 1-Dec-2019)

Neotame



$C_{20}H_{30}N_2O_5$ 378.46
L-Phenylalanine, *N*-[*N*-(3,3-dimethylbutyl)-L-α-aspartyl]-1-methyl ester;
N-[*N*-(3,3-Dimethylbutyl)-L-α-aspartyl]-L-phenylalanine 1-methyl ester [165450-17-9].

DEFINITION

Neotame contains NLT 97.0% and NMT 102.0% of neotame ($C_{20}H_{30}N_2O_5$), calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

ASSAY

- **PROCEDURE**

Mobile phase: Dissolve 3.0 g of sodium 1-heptanesulfonate in 740 mL of water in a suitable 1000-mL vessel, and add 3.8 mL of triethylamine. Adjust the resulting solution with phosphoric acid to a pH of 3.5, and dilute with water to 750 mL. Add 250 mL of acetonitrile, and adjust with phosphoric acid to an apparent pH of 3.7.

Standard solution: 1.0 mg/mL of USP Neotame RS in *Mobile phase*

Sample solution: 1.0 mg/mL of Neotame in *Mobile phase*. [NOTE—This solution is stable for up to 32 h when stored at a temperature of 0°–10°.]

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 210 nm
Column: 4.6-mm × 10-cm; packing L1
Column temperature: 45°
Flow rate: 1.5 mL/min
Injection volume: 25 µL

System suitability
Sample: *Standard solution*
Suitability requirements
Tailing factor: NMT 2.0
Relative standard deviation: NMT 2.0%

Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of neotame ($C_{20}H_{30}N_2O_5$) in the portion of Neotame taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

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r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP Neotame RS in the *Standard solution* (mg/mL)
C_U = concentration of Neotame in the *Sample solution* (mg/mL)

Acceptance criteria: 97.0%–102.0% on the anhydrous basis

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 0.2%

• **LEAD**

[NOTE—Use acid-cleaned (mixture of 5% nitric acid and 5% hydrochloric acid followed by rinsing with water) autosampler cups and volumetric glassware to avoid contamination. For the preparation of all aqueous solutions and for the rinsing of glassware before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin. Select all reagents to have as low a content of lead as practicable. Store standards and samples in acid-cleaned polyethylene containers.]

Diluent: Transfer 2 mL of lead-free nitric acid into a 1000-mL volumetric flask, dilute with water to volume, and mix.

Standard stock solution: 79.9 mg of lead nitrate in 100 mL of *Diluent* in a 500-mL volumetric flask, and dilute with *Diluent* to volume. Transfer 10.0 mL of the resulting solution into a 100-mL volumetric flask, and dilute with *Diluent* to volume. Each mL of the *Standard stock solution* contains the equivalent of 10 µg of lead.

Standard solution A: Dilute an aliquot of the *Standard stock solution* with *Diluent* to obtain a solution having a concentration of 0.03 µg/mL.

Standard solution B: Dilute an aliquot of the *Standard stock solution* with *Diluent* to obtain a solution having a concentration of 0.015 µg/mL.

Sample solution: Transfer 160 mg of Neotame to a 10-mL volumetric flask. Dissolve in and dilute with *Diluent* to volume.

Blank: *Diluent*

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

[NOTE—Optimize the instrument program as recommended by the manufacturer for lead, using a char temperature of 500° and an atomization temperature of 2000°.]

Mode: Atomic absorption spectrophotometer with a graphite furnace, pyrolytically coated graphite tubes, a solid pyrolytic graphite platform, and a background compensation system

Analytical wavelength: 283.3 nm

Lamp: Lead hollow-cathode

Purge gas: Argon

Alternate gas: Breathing-quality air

Volume: 15 µL

Analysis

Samples: *Standard solution A, Standard solution B, Sample solution,* and *Blank*

Correct the peak areas of the *Sample solution, Standard solution A,* and *Standard solution B* for the *Blank* peak area. Generate the appropriate lead calibration algorithm, and determine the lead concentration in the *Sample solution,* in µg/mL.

Calculate the amount of lead, in µg/g, in the portion of Neotame taken:

$$\text{Result} = (C \times V)/(W \times F)$$

C = blank-corrected lead concentration in the *Sample solution* (µg/mL)
V = volume of the *Sample solution,* 10 mL
W = weight of Neotame taken to prepare the *Sample solution* (mg)
F = conversion factor, mg/g

Acceptance criteria: NMT 2 µg/g

• **RELATED COMPOUNDS**

Mobile phase and **Chromatographic system:** Proceed as directed in the *Assay.*

Standard solution A: 0.03 mg/mL of USP Neotame Related Compound A RS in *Mobile phase*

Standard solution B: Prepare as directed for the *Standard solution* in the *Assay.*

Detector sensitivity solution: Transfer 2 mL of *Standard solution A* to a 50-mL volumetric flask, and dilute with *Mobile phase* to volume.

Sample solution: 2 mg/mL of Neotame in *Mobile phase.* [NOTE—This solution is stable for up to 32 h when stored at a temperature of 0°–10°.]

System suitability

Samples: *Standard solution A* and *Detector sensitivity solution*

Suitability requirements

Relative standard deviation: NMT 5.0%, *Standard solution A*

Signal-to-noise ratio: NLT 10, *Detector sensitivity solution*

Analysis

Samples: *Standard solution A, Standard solution B,* and *Sample solution*

Calculate the percentage of neotame related compound A in the portion of Neotame taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of neotame related compound A from the *Sample solution*
r_S = peak response of neotame related compound A from *Standard solution A*
C_S = concentration of USP Neotame Related Compound A RS in *Standard solution A* (mg/mL)
C_U = concentration of Neotame in the *Sample solution* (mg/mL)

Calculate the percentage of other impurities in the portion of Neotame taken:

$$\text{Result} = (r_T/r_S) \times (C_S/C_U) \times 100$$

r_T = sum of the peak responses of all impurities (except those of neotame related compound A and the solvent, if observed) from the *Sample solution*
r_S = peak response of neotame from *Standard solution B*
C_S = concentration of USP Neotame RS in *Standard solution B* (mg/mL)
C_U = concentration of Neotame in the *Sample solution* (mg/mL)

Acceptance criteria

Neotame related compound A: NMT 1.5%

Other impurities: NMT 2.0%

SPECIFIC TESTS

• **OPTICAL ROTATION** ⟨781S⟩, *Procedures, Specific Rotation*

Sample solution: 5 mg/mL in water

Acceptance criteria: –40.0° to –43.4°, at 20°

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• **WATER DETERMINATION** ⟨921⟩, *Method I, Method Ia*
Sample: 0.50 g
Acceptance criteria: NMT 5.0%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers, store in a dry place, and avoid exposure to excessive heat.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Neotame RS
USP Neotame Related Compound A RS
N-[*N*-(3,3-Dimethylbutyl)-L-α-aspartyl]-L-phenylalanine.

Nitric Acid

HNO_3 63.01
Nitric acid [7697-37-2].

DEFINITION
Nitric Acid contains NLT 69.0% and NMT 71.0%, by weight, of nitric acid (HNO_3). [**CAUTION**—Avoid contact, because Nitric Acid rapidly destroys tissues.]

IDENTIFICATION
• **A. IDENTIFICATION TESTS—GENERAL,** *Nitrate* ⟨191⟩**:** Meets the requirements

ASSAY
• **PROCEDURE**
Sample solution: Weigh 2 mL of Nitric Acid in a glass-stoppered conical flask, and add 25 mL of water. Add methyl red TS.
Analysis: Titrate the *Sample solution* with 1 N sodium hydroxide VS. Each mL of 1 N sodium hydroxide is equivalent to 63.01 mg of HNO_3 (see *Titrimetry* ⟨541⟩).
Acceptance criteria: 69.0%–71.0%

IMPURITIES
• **RESIDUE ON IGNITION** ⟨281⟩
Sample: 70 mL (100 g)
Analysis: Place the *Sample* in a tared crucible, add 2 drops of sulfuric acid, and evaporate to dryness. Ignite for 15 min.
Acceptance criteria: NMT 0.5 mg (5 ppm)
• **CHLORIDE AND SULFATE,** *Chloride* ⟨221⟩
Sample: 35 mL (50 g)
Control: 35 µL of 0.020 N hydrochloric acid
Acceptance criteria: NMT 0.5 ppm; the *Sample* shows no more chloride than corresponds to the *Control.*
• **CHLORIDE AND SULFATE,** *Sulfate* ⟨221⟩
Sample: 28 mL
Control: 40 µL of 0.020 N sulfuric acid in an equal volume of solution containing the quantities of reagents used in the analysis
Analysis: Add 10 mg of sodium carbonate to the *Sample.* Evaporate to dryness, dissolve in a mixture of 4 mL of water and 1 mL of dilute hydrochloric acid (50 mg/mL), and filter if necessary. Wash with two 2-mL portions of water, dilute with water to 10 mL, and add 1 mL of barium chloride TS. Observe 10 min after adding the barium chloride.
Acceptance criteria: 1 ppm; any turbidity produced by the *Sample* is not greater than that produced by the *Control.*
• **IRON** ⟨241⟩
Sample: 35 mL (50 g)
Analysis: Evaporate the *Sample* to dryness, dissolve the residue in 2 mL of hydrochloric acid, and dilute with water to 47 mL.
Acceptance criteria: NMT 0.2 µg/g

SPECIFIC TESTS
• **CLARITY AND COLOR OF SOLUTION**
Analysis: Mix it in its original container, and transfer 10 mL to a 20- × 150-mm test tube. Compare with water in a similar test tube.
Acceptance criteria: The liquids are equally clear and free from suspended matter, and when viewed transversely by transmitted light, exhibit no apparent difference in color.

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight containers.

Nitrogen

N_2 28.01
Nitrogen [7727-37-9].

DEFINITION
Nitrogen contains NLT 99.0%, by volume, of nitrogen (N_2).

IDENTIFICATION
• **A.** The flame of a burning wood splinter is extinguished when inserted into a test tube filled with Nitrogen. [NOTE—Exercise caution.]

ASSAY
• **PROCEDURE**
Sample: Nitrogen
Standard: Oxygen–helium certified standard (see *Reagents, Indicators, and Solutions*)
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability.*)
Mode: GC
Detector: Thermal conductivity
Column: 3-m length × 4-mm inside diameter: molecular sieve prepared from a synthetic alkali-metal aluminosilicate capable of absorbing molecules having diameters of up to 0.5 nm and completely separating oxygen from nitrogen
Carrier gas: Helium (99.99%)
Temperature: Thermostatically controlled
Analysis
Samples: *Standard* and *Sample*
Introduce the *Samples* separately into the gas chromatograph by means of a gas sampling valve.
Acceptance criteria: The peak response produced by the *Sample* exhibits a retention time corresponding to that produced by the *Standard* and is equivalent to NMT 1.0% of oxygen when compared to the peak response of the *Standard,* indicating NLT 99.0%, by volume, of N_2.

SPECIFIC TESTS
[NOTE—Reduce the container pressure by means of a regulator. Measure the gases with a gas volume meter downstream from the detector tube to minimize contamination or change of the specimens.]
• **CARBON MONOXIDE**
Sample: 1000 ± 50 mL
Analysis: Pass the *Sample* through a carbon monoxide detector tube (see *Reagents, Indicators, and Solutions*) at the rate specified for the tube.
Acceptance criteria: NMT 10 ppm
• **LIMIT OF OXYGEN**
Analysis: Determined as directed in the *Assay*
Acceptance criteria: NMT 1.0%
• **ODOR**
Analysis: Carefully open the container valve to produce a moderate flow of gas. Do not direct the gas stream toward

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the face, but deflect a portion of the stream toward the nose.
Acceptance criteria: No appreciable odor is discernible.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in cylinders.

Nitrogen 97 Percent

DEFINITION
Nitrogen 97 Percent is Nitrogen produced from air by physical separation methods. It contains NLT 97.0%, by volume, of nitrogen (N_2).

IDENTIFICATION
- **A.** The flame of a burning wood splinter is extinguished when inserted into a test tube filled with Nitrogen 97 Percent. [NOTE—Exercise caution.]

ASSAY
- **PROCEDURE**

Standard: Oxygen–helium certified standard (see *Reagents, Indicators, and Solutions*)
Sample: Nitrogen 97 Percent
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Thermal conductivity
Column: 3-m length × 4-mm inside diameter: molecular sieve prepared from a synthetic alkali-metal aluminosilicate capable of absorbing molecules having diameters of up to 0.5 nm and completely separating oxygen from nitrogen
Carrier gas: Helium (99.99%)
Temperature: Thermostatically controlled
Analysis
Samples: *Standard* and *Sample*
Introduce the *Samples* separately into the gas chromatograph by means of a gas sampling valve.
Acceptance criteria: The peak response produced by the *Sample* exhibits a retention time corresponding to that produced by the *Standard* and is equivalent to NMT 3.0% of oxygen when compared to the peak response of the *Standard*, indicating NLT 97.0%, by volume, of nitrogen (N_2).

IMPURITIES
[NOTE—Reduce the container pressure by means of a regulator. Measure the gases with a gas volume meter downstream from the detector tube to minimize contamination or change of the specimens.]
- **CARBON DIOXIDE**

Sample: 1000 ± 50 mL
Analysis: Pass the *Sample* through a carbon dioxide detector tube (see *Reagents, Indicators, and Solutions*) at the rate specified for the tube.
Acceptance criteria: The indicator change corresponds to NMT 300 ppm
- **CARBON MONOXIDE**

Sample: 1000 ± 50 mL
Analysis: Pass the *Sample* through a carbon monoxide detector tube (see *Reagents, Indicators, and Solutions*) at the rate specified for the tube.
Acceptance criteria: NMT 10 ppm
- **SULFUR DIOXIDE**

Sample: 1000 ± 50 mL
Analysis: Pass the *Sample* through a sulfur dioxide detector tube (see *Reagents, Indicators, and Solutions*) at the rate specified for the tube.
Acceptance criteria: NMT 5 ppm
- **LIMIT OF NITRIC OXIDE AND NITROGEN DIOXIDE**

Sample: 500 ± 50 mL
Analysis: Pass the *Sample* through a nitric oxide–nitrogen dioxide detector tube (see *Reagents, Indicators, and Solutions*) at the rate specified for the tube.
Acceptance criteria: NMT 2.5 ppm
- **LIMIT OF OXYGEN**

Analysis: Determined as directed in the *Assay*
Acceptance criteria: NMT 3.0%

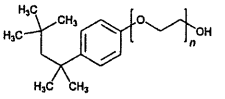
SPECIFIC TESTS
- **ODOR**

Analysis: Carefully open the container valve to produce a moderate flow of gas. Do not direct the gas stream toward the face, but deflect a portion of the stream toward the nose.
Acceptance criteria: No appreciable odor is discernible.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in cylinders or in a low-pressure collecting tank.
- **LABELING:** Where it is piped directly from the collecting tank to the point of use, label each outlet "Nitrogen 97 Percent".

Nonoxynol 9—*see Nonoxynol 9 General Monographs*

Octoxynol 9



Poly(oxy-1,2-ethanediyl), α-[4-(1,1,3,3-tetramethylbutyl) phenyl]-ω-hydroxy-;
α-[4-(1,1,3,3,-Tetramethylbutyl)phenyl]-ω-hydroxypoly(oxy-1,2-ethanediyl);
Polyethylene glycol mono[*p*-(1,1,3,3-tetramethylbutyl) phenyl] ether;
Polyethylene glycol mono(4-*tert*-octylphenyl) ether [9002-93-1].

DEFINITION
Octoxynol 9 is an anhydrous liquid mixture consisting chiefly of mono[*p*-(1,1,3,3-tetramethylbutyl)] phenyl ethers of polyethylene glycols, corresponding to:

$$(CH_3)_3C(CH_2)C(CH_3)_2C_6H_4(OCH_2CH_2)_n OH$$

in which the average value of *n* is about 9. It contains NLT 90.0% and NMT 110.0% of Octoxynol 9.

IDENTIFICATION

Change to read:

- **A.** ▲**SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F.**▲ (CN 1-May-2020) On undried specimen
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

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ASSAY

• **PROCEDURE**

Mobile phase: Methanol:water (4:1)

Standard solution: 25 mg/mL of USP Octoxynol 9 RS in *Mobile phase*

System suitability solution: 25 mg/mL of USP Octoxynol 9 RS and 25 mg/mL of USP Nonoxynol 9 RS in *Mobile phase*

Sample solution: 25 mg/mL of Octoxynol 9 in *Mobile phase*

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 4.6-mm × 25-cm, 5-µm packing L1

Column temperature: Ambient

Flow rate: 1.0 mL/min

Injection size: 10 µL

System suitability

Samples: *System suitability solution* and *Standard solution*

[NOTE—The relative retention times for octoxynol 9 and nonoxynol 9 are 1.0 and 1.4, respectively.]

Suitability requirements

Resolution: NLT 2.0 between octoxynol 9 and nonoxynol 9, *System suitability solution*

Relative standard deviation: NMT 2.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Record the chromatograms, and measure the responses for octoxynol 9, including any shoulders and bumps.

Calculate the percentage of octoxynol 9 in the portion of test specimen taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of octoxynol 9 from the *Sample solution*

r_S = peak response of octoxynol 9 from the *Standard solution*

C_S = concentration of USP Octoxynol 9 RS in the *Standard solution* (mg/mL)

C_U = concentration of Octoxynol 9 in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–110.0%

• **CONTENT OF FREE POLYETHYLENE GLYCOLS**

Sample: 10 g

Analysis: Transfer the *Sample* to a 250-mL beaker. Add 100 mL of ethyl acetate, and stir on a magnetic stirrer to make a solution. Transfer, with the aid of 100 mL of 5 N sodium chloride, to a pear-shaped, 500-mL separator fitted with a glass stopper. Insert the stopper, and shake vigorously for 1 min. Remove the stopper carefully to release the pressure. Immerse a thermometer in the mixture, and support the separator so that it is partially immersed in a water bath maintained at 50°. Swirl the separator gently while letting the internal temperature rise to 40°–45°. Immediately remove the separator from the bath, dry the outside surface, and drain the salt (lower) layer into another pear-shaped, 500-mL separator. In the same manner, extract the ethyl acetate layer a second time with 100 mL of fresh 5 N sodium chloride, combining the two aqueous extracts. Discard the ethyl acetate layer.

Wash the combined aqueous layers with 100 mL of ethyl acetate, using the same technique, and drain the salt (lower) layer into a clean pear-shaped, 500-mL separator. Discard the ethyl acetate layer.

Extract the aqueous layer with two successive 100-mL portions of chloroform, draining the chloroform (lower) layers through Whatman folded filter paper 2V, and combining them into a 250-mL beaker.

Evaporate on a steam bath or with a rotary evaporator to dryness, and continue heating to remove chloroform. Allow the beaker to cool. Add 25 mL of acetone, and dissolve the residue on a magnetic stirrer. Pass through Whatman folded filter paper 2V into a tared 250-mL beaker, rinsing with two 25-mL portions of acetone. Evaporate on a steam bath or with a rotary evaporator to dryness. Dry in vacuum at 60° for 1 h. Allow the beaker to cool, and weigh.

Acceptance criteria: NMT 1.0% of polyethylene glycol

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.4%

• **LIMIT OF FREE ETHYLENE OXIDE**

Stripped octoxynol 9: Maintain Octoxynol 9 at a temperature of 150° with constant stirring in an open vessel until it no longer displays a peak for ethylene oxide when chromatographed as directed below.

Standard stock solution: [NOTE—Ethylene oxide is toxic and flammable. Prepare these solutions in a well-ventilated hood, using great care.] Chill all apparatus and reagents used in the preparation of standards in a refrigerator or freezer before use. Fill a chilled pressure bottle with liquid ethylene oxide from a lecture bottle, and store in a freezer when not in use. Use a small piece of polyethylene film to protect the liquid from contact with the rubber gasket. Transfer about 100 mL of chilled isopropyl alcohol to a 500-mL volumetric flask. Using a chilled graduated cylinder, transfer 25 mL of ethylene oxide to the isopropyl alcohol, and swirl gently to mix. Dilute with additional chilled isopropyl alcohol to volume, replace the stopper, and swirl gently to mix. This stock solution contains about 43.6 mg/mL of ethylene oxide.

Standard solutions: Pipet 25 mL of 0.5 N alcoholic hydrochloric acid, prepared by mixing 45 mL of hydrochloric acid with 1 L of alcohol, into a 500-mL conical flask containing 40 g of magnesium chloride hexahydrate. Shake the mixture to effect saturation. Pipet 10 mL of the *Standard stock solution* into the flask, and add 20 drops of bromocresol green TS. If the solution is not yellow (acid), add an additional volume, accurately measured, of 0.5 N alcoholic hydrochloric acid to give an excess of about 10 mL. Record the total volume of 0.5 N alcoholic hydrochloric acid added. Insert the stopper into the flask, and allow to stand for 30 min. Titrate the excess acid with 0.5 N alcoholic potassium hydroxide VS. Perform a blank titration, using 10.0 mL of isopropyl alcohol instead of *Standard stock solution*, adding the same total volume of 0.5 N alcoholic hydrochloric acid, and note the difference in volumes required. Each mL of the difference in volumes of 0.5 N alcoholic potassium hydroxide consumed is equivalent to 22.02 mg of ethylene oxide. Calculate the concentration, in mg/mL, of ethylene oxide in the *Standard stock solution*. Standardize daily. Store in a refrigerator. Prepare a 1000-ppm standard by pipeting into a container the calculated volume (about 2 mL) of cold *Standard stock solution* that on the basis of the standardization contains 88.6 mg of ethylene oxide, and adding 87.0 g of *Stripped octoxynol 9*. Prepare 10-, 5-, and 0.5-ppm standards by quantitatively diluting the 1000-ppm standard with additional *Stripped octoxynol 9*.

Standard solution 0.5 ppm: Transfer 5 ± 0.01 g of the *Standard solution* containing 0.5 ppm ethylene oxide to suitable serum vials equipped with pressure-tight septum closures designed to relieve any excessive pressure, and seal them.

Standard solution 5 ppm: Transfer 5 ± 0.01 g of the *Standard solution* containing 5 ppm ethylene oxide to

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suitable serum vials equipped with pressure-tight septum closures designed to relieve any excessive pressure, and seal them.

Standard solution 10 ppm: Transfer 5 ± 0.01 g of the *Standard solution* containing 10 ppm ethylene oxide to suitable serum vials equipped with pressure-tight septum closures designed to relieve any excessive pressure, and seal them.

System suitability solution: 10 µg/mL of ethylene oxide and 10 µg/mL of acetaldehyde in *Stripped octoxynol 9*

Sample solution: Transfer 5 ± 0.01 g of Octoxynol 9 to a serum vial of the same kind as the vials used for *Standard solution A*.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 2.1-mm × 6.4-m nickel; 60- to 80-mesh support S9 (under typical conditions)

Temperature

Column: 100°

Injector: 160°

Detector: 200°

Carrier gas: Helium

Flow rate: 30 mL/min

System suitability

Samples: *System suitability solution, Standard solution 0.5 ppm, Standard solution 5 ppm,* and *Standard solution 10 ppm*

Suitability requirements

Resolution: NLT 1.5 between ethylene oxide and acetaldehyde, *System suitability solution*

Calibration: None of the points used for constructing the straight line *Calibration* curve deviates from the line by more than 10%, *Standard solution 0.5 ppm, Standard solution 5 ppm, Standard solution 10 ppm.*

Analysis

Samples: *System suitability solution, Standard solution 0.5 ppm, Standard solution 5 ppm, Standard solution 10 ppm,* and *Sample solution*

Calibration: Place the vial containing *Standard solution 10 ppm* in an oven, and heat at 90° for 30 min. Remove the vial from the oven. Using a gas-tight syringe, immediately inject a 100-µL aliquot of the headspace gas into the gas chromatograph. Obtain the area for the ethylene oxide peak (retention time approximately 8 min). Raise the temperature of the column to 200° after ethylene oxide elutes to volatilize heavy components. Re-equilibrate the column at 100°. Repeat the foregoing steps, using the vials containing *Standard solution 0.5 ppm* and *Standard solution 5 ppm*. On linear graph paper, plot area units versus ppm ethylene oxide for the standards, and draw the best straight line through the points.

Place the vial containing the *Sample solution* in an oven, and heat at 90° for 30 min. Remove the vial from the oven. Immediately inject a 100-µL aliquot of the headspace gas into the gas chromatograph, and obtain the area for the ethylene oxide peak.

Calculate the concentration of ethylene oxide in the sample, in ppm:

$$\text{Result} = r_U \times S$$

r_U = peak area from the *Sample solution*

S = slope of the standard curve (ppm/peak area unit)

Acceptance criteria: NMT 5 ppm

• **LIMIT OF DIOXANE**

Apparatus: Assemble a closed-system vacuum distillation apparatus, using glass vacuum stopcocks (*A, B,* and *C*), as shown in *Figure 1*. The concentrator tube (*D*)[1] is made of borosilicate or quartz (not flint) glass, graduated precisely enough to measure the 0.9 mL or more of distillate collected and marked so that the analyst can dilute accurately to 2.0 mL.

Standard solution: 100 µg/mL of dioxane in water. Use a freshly prepared solution.

Sample solution: Transfer 20.0 g to a 50-mL round-bottom flask (*E*) having a 24/40 ground-glass neck joint. Add 1.0 mL of water. Place a small polytef-covered stirring bar in the flask, insert the stopper, and stir to mix. Immerse the flask in an ice bath, and chill for 1 min. Wrap heating tape around the tube connecting the concentrator tube (*D*) and the round-bottom flask, and apply 10 V to the tape. Apply a light coating of high-vacuum silicone grease to the ground-glass joints, and connect the concentrator tube to the 10/30 joint and the round-bottom flask to the 24/40 joint. Immerse the vacuum trap in a Dewar flask filled with liquid nitrogen, close stopcocks *A* and *B*, open stopcock *C*, and begin evacuating the system with a vacuum pump. Prepare a slurry bath from powdered dry ice and methanol, and raise the bath to the neck of the round-bottom flask. After freezing the contents of the flask for 10 min, and when the vacuum system is operating at a 0.05-mm pressure or lower, open stopcock *A* for 20 s, then close it. Remove the

[1] A suitable tube is available as Chromaflex concentrator tube, Kontes Glass Co., Vineland, NJ (Catalog No. K42560-0000).

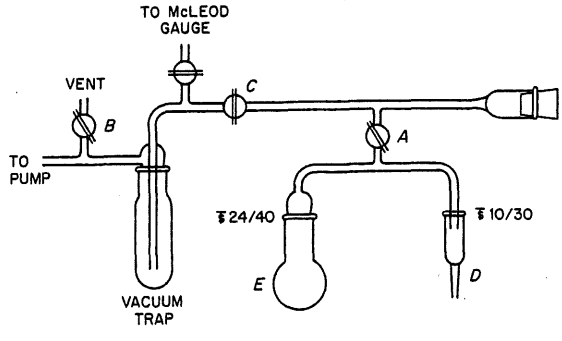


Figure 1. Closed-system vacuum distillation apparatus for dioxane. (Octoxynol 9)

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slurry bath, and allow the flask to warm in air for 1 min. Immerse the flask in a water bath maintained at a temperature of 20°–25°, and after about 5 min warm the water bath to 35°–40° (sufficient to liquefy most specimens) while stirring slowly but constantly with the magnetic bar. Cool the water in the bath by adding ice, and chill for about 2 min. Replace the water bath with the slurry bath, freeze the contents of the round-bottom flask for 10 min, open stopcock *A* for 20 s, and then close it. Remove the slurry bath, and repeat the heating steps as before, this time reaching a final temperature of 45°–50° or a temperature necessary to melt the specimen completely. If there is any condensation in the tube connecting the round-bottom flask to the concentrator tube, slowly increase the voltage to the heating tape, and heat until the condensation disappears.

Stir with the magnetic stirrer throughout the following steps. Very slowly immerse the concentrator tube in a Dewar flask containing liquid nitrogen.

[**CAUTION**—When there is liquid distillate in the concentrator tube, immerse the tube in the liquid nitrogen very slowly, or the tube will break.]

Water will begin to distill into the concentrator tube. As ice forms in the concentrator tube, raise the Dewar flask to keep the liquid nitrogen level only slightly below the level of ice in the tube. When water begins to freeze in the neck of the 10/30 joint, or when liquid nitrogen reaches the 2.0-mL graduation mark on the concentrator tube, remove the Dewar flask, and allow the ice to melt without heating. After the ice has melted, check the volume of water that has distilled, and repeat the sequence of chilling and thawing until NLT 0.9 mL of water has been collected. Freeze the tube once again for about 2 min, and release the vacuum first by opening stopcock *B*, followed by opening stopcock *A*. Remove the concentrator tube from the apparatus, close it with a greased stopper, and allow the ice to melt without heating. Mix the contents of the concentrator tube by swirling, note the volume of distillate, and dilute with water to 2.0 mL, if necessary.

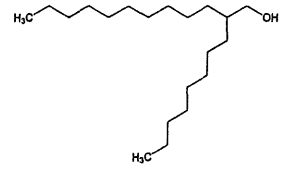
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 2-mm × 1.8-m glass; support S10 (under typical conditions)
Temperature
Column: 140°
Injector: 200°
Detector: 250°
Carrier gas: Nitrogen or helium
Flow rate: 35 mL/min
Install an oxygen scrubber between the carrier gas line and the column. Condition the column for 72 h at 230° with 30–40 mL/min carrier flow. [NOTE—Support S10 is oxygen-sensitive. Each time a column is installed, flush with carrier gas for 30–60 min before heating.]
Injection size: 2–4 µL

Analysis
Samples: *Standard solution* and *Sample solution*
Acceptance criteria: The height of the peak from the *Sample solution* is NMT that from the *Standard solution:* NMT 10 µg/g (ppm).

SPECIFIC TESTS
- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩: NMT 0.2
- **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩: 85–101
- **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩: NMT 10.0
- **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 0.5%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers. Store at room temperature.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Nonoxynol 9 RS
USP Octoxynol 9 RS

Octyldodecanol



$C_{20}H_{42}O$ 298.55
1-Dodecanol, 2-octyl-;
2-Octyldodecan-1-ol;
2-Octyldodecanol;
2-Octyldodecyl alcohol [5333-42-6].

DEFINITION
Octyldodecanol contains NLT 90.0% and NMT 102.0% of 2-octyldodecan-1-ol ($C_{20}H_{42}O$), the remainder consisting chiefly of related alcohols. It is obtained from sources of vegetable, animal, or synthetic origin.

IDENTIFICATION
- **A. CHROMATOGRAPHIC IDENTITY**
Analysis: Proceed as directed in the *Assay*.
Acceptance criteria: The retention time of the major peak of the *Sample solution*, excluding the solvent and internal standard peaks, corresponds to the octyldodecanol peak of the *Standard solution*.
- **B. FATS AND FIXED OILS** ⟨401⟩, *Procedures, Hydroxyl Value*: 175–190

ASSAY
- **PROCEDURE**
Internal standard solution: 1 mg/mL of 1-pentadecanol (internal standard) in ethanol
System suitability solution: Prepare 1 mg/mL each of USP Cetyl Alcohol RS, USP Stearyl Alcohol RS, and USP Oleyl Alcohol RS in *Internal standard solution*, and heat the solution in a sealed container in a 50° water bath until all fatty alcohols are dissolved. Allow the solution to cool to room temperature, and mix well.
Standard solution: Prepare 1.0 mg/mL of USP Octyldodecanol RS in *Internal standard solution*.
Sample solution: Prepare 1.0 mg/mL of Octyldodecanol in *Internal standard solution*.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.25-mm × 30-m fused-silica capillary, coated with a 0.25-µm layer of phase G7
Temperatures
Injection port: 270°
Detector: 280°
Column: See *Table 1*.

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Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
60	20	180	—
180	10	220	5

Carrier gas: Hydrogen
Flow rate: 2.0 mL/min, constant flow mode
Injection volume: 1 µL
Injection type: Split ratio, 100:1
Liner: Single taper, low-pressure drop liner with deactivated wool
Run time: 15 min

System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—See *Table 2* for the relative retention times.]

Table 2

Component	Relative Retention Time
1-Pentadecanol (internal standard)	1.00
Cetyl alcohol	1.08
Stearyl alcohol	1.25
Oleyl alcohol	1.27
Octyldodecanol	1.32

Suitability requirements
Resolution: NLT 10 between the cetyl alcohol and stearyl alcohol peaks; NLT 2.0 between the stearyl alcohol and oleyl alcohol peaks, *System suitability solution*
Tailing factor: 0.8–1.8 for the octyldodecanol and 1-pentadecanol peaks, *Standard solution*
Relative standard deviation: NMT 1%, using the area ratio of octyldodecanol to 1-pentadecanol, *Standard solution*

Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of octyldodecanol ($C_{20}H_{42}O$) in the portion of Octyldodecanol taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of octyldodecanol to the internal standard (peak response of octyldodecanol/peak response of the internal standard) from the *Sample solution*
R_S = peak response ratio of octyldodecanol to the internal standard (peak response of octyldodecanol/peak response of the internal standard) from the *Standard solution*
C_S = concentration of USP Octyldodecanol RS in the *Standard solution* (mg/mL)
C_U = concentration of Octyldodecanol in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–102.0%

IMPURITIES

[NOTE—On the basis of the manufacturing route, perform either *Organic Impurity Test 1* or *Organic Impurity Test 2*.]

• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%, determined on 2 g

• **ORGANIC IMPURITY TEST 1: LIMIT OF RELATED FATTY ALCOHOLS AND ALKANES**
System suitability solution: Prepare 1 mg/mL each of USP Stearyl Alcohol RS, USP Oleyl Alcohol RS, USP Linoleyl Alcohol RS, and USP Octyldodecanol RS in ethanol, and heat the solution in a sealed container in a 50° water bath until all fatty alcohols are dissolved. Allow the solution to cool to room temperature, and mix well. Dilute the solution with ethanol to obtain a solution containing 0.05 mg/mL each of USP Stearyl Alcohol RS, USP Oleyl Alcohol RS, USP Linoleyl Alcohol RS, and USP Octyldodecanol RS.
Sample solution: 1 mg/mL of Octyldodecanol in ethanol
Chromatographic system: Proceed as directed in the *Assay*, except use split injection with a split ratio of 5:1.
System suitability
Samples: *System suitability solution* and *Sample solution*
[NOTE—See *Table 3* for the relative retention times.]

Table 3

Component	Relative Retention Time
n-Nonadecane[a]	0.63
9-Methyl nonadecane[a]	0.65
2-Octyl-1-decanol or 2-hexyl-1-dodecanol[b]	0.87
Stearyl alcohol[c]	0.95
Oleyl alcohol[c]	0.96
Linoleyl alcohol[c]	0.99
Octyldodecanol[d]	1.00
2-Octyl-1-tetradecanol or 2-decyl-1-dodecanol[b]	1.17
Any other unspecified related fatty alcohol or impurity	—

[a] Alkane.
[b] Related branched chain fatty alcohol.
[c] Related linear chain fatty alcohol.
[d] Sample.

Suitability requirements
Resolution: NLT 2.0 between the stearyl alcohol and oleyl alcohol peaks; NLT 2.0 between the linoleyl alcohol and octyldodecanol peaks, *System suitability solution*

Analysis
Samples: *System suitability solution* and *Sample solution*
Identify *n*-nonadecane, 9-methyl nonadecane, and each of the linear chain fatty alcohols and branched chain fatty alcohols in the *Sample solution* according to *Table 3*.
Calculate the percentage of *n*-nonadecane (9-methyl nonadecane, each of the linear chain fatty alcohols and branched chain fatty alcohols, or any other unspecified related fatty alcohol or impurity) in the portion of Octyldodecanol taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of *n*-nonadecane (9-methyl nonadecane, each of the linear chain fatty alcohols and branched chain fatty alcohols, or any other unspecified related fatty alcohol or impurity) from the *Sample solution*
r_T = sum of all the peak responses, excluding peak responses due to solvent, from the *Sample solution*

Acceptance criteria: Disregard any unspecified peaks that are less than 0.05%, and any peaks due to solvent.

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Sum of unspecified related fatty alcohols and impurities: NMT 1%

Sum of *n*-nonadecane, 9-methyl nonadecane, 2-octyl-1-decanol, 2-hexyl-1-dodecanol, 2-octyl-1-tetradecanol, and 2-decyl-1-dodecanol: NMT 1.5%

• **ORGANIC IMPURITY TEST 2: LIMIT OF BRANCHED CHAIN FATTY ALCOHOLS AND BRANCHED CHAIN ALDEHYDE**

System suitability solution: Prepare 1 mg/mL each of USP Stearyl Alcohol RS, USP Oleyl Alcohol RS, USP Linoleyl Alcohol RS, and USP Octyldodecanol RS in ethanol, and heat the solution in a sealed container in a 50° water bath until all fatty alcohols are dissolved. Allow the solution to cool to room temperature, and mix well. Dilute the solution with ethanol to obtain a solution containing 0.05 mg/mL each of USP Stearyl Alcohol RS, USP Oleyl Alcohol RS, USP Linoleyl Alcohol RS, and USP Octyldodecanol RS.

Sample solution: 1 mg/mL of Octyldodecanol in ethanol

Chromatographic system: Proceed as directed in the *Assay*, except use split injection with a split ratio of 5:1 and run time of 30 min.

System suitability

Samples: *System suitability solution* and *Sample solution*

[NOTE—See *Table 4* for the relative retention times for branched chain fatty alcohols and branched chain aldehyde.]

Table 4

Component	Relative Retention Time
2-Octyl-1-decanol or 2-hexyl-1-dodecanol[a]	0.87
2-Octyldodecanal[b]	0.93
Stearyl alcohol[c]	0.95
Oleyl alcohol[c]	0.96
Linoleyl alcohol[c]	0.99
Octyldodecanol[d]	1.00
2-Octyl-1-tetradecanol or 2-decyl-1-dodecanol[a]	1.17
Any other unspecified fatty alcohol or impurity	—

[a] Related branched chain fatty alcohol.
[b] Branched aldehyde.
[c] Related linear chain fatty alcohol.
[d] Sample.

Suitability requirements

Resolution: NLT 2.0 between the stearyl alcohol and oleyl alcohol peaks; NLT 2.0 between the linoleyl alcohol and octyldodecanol peaks, *System suitability solution*

Analysis

Samples: *System suitability solution* and *Sample solution*

Identify each branched chain fatty alcohol peak and branched chain aldehyde peak in the *Sample solution* according to *Table 4*.

Calculate the percentage of each branched chain fatty alcohol (2-octyl-1-decanol, 2-hexyl-1-dodecanol, 2-octyl-1-tetradecanol, or 2-decyl-1-dodecanol), branched chain aldehyde (2-octyldodecanal), or any unspecified fatty alcohol or impurity in the portion of Octyldodecanol taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of each branched chain fatty alcohol (2-octyl-1-decanol, 2-hexyl-1-dodecanol, 2-octyl-1-tetradecanol, or 2-decyl-1-dodecanol), branched chain aldehyde (2-octyldodecanal), or any unspecified fatty alcohol or impurity from the *Sample solution*

r_T = sum of all the peak responses, excluding peak responses due to solvent, from the *Sample solution*

Acceptance criteria: Disregard any unspecified peaks that are less than 0.05%, and any peaks due to solvent.

Sum of unspecified fatty alcohols and impurities: NMT 5%

Branched chain fatty alcohols (2-octyl-1-decanol, 2-hexyl-1-dodecanol, 2-octyl-1-tetradecanol, and 2-decyl-1-dodecanol): NMT 5%

Branched chain aldehyde (2-octyldodecanal): NMT 2%

SPECIFIC TESTS

• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Acid Value*: NMT 0.5
• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Iodine Value*: NMT 8
• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Peroxide Value*: NMT 5.0
• **WATER DETERMINATION** ⟨921⟩, *Method I*: NMT 0.5%

ADDITIONAL REQUIREMENTS

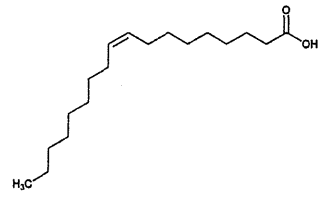
• **PACKAGING AND STORAGE:** Preserve in tight containers.
• **LABELING:** If a test for *Impurities* other than *Organic Impurity Test 1* is used, the labeling states the test with which the article complies. Label it to indicate whether it is derived from vegetable, animal, or synthetic sources.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Cetyl Alcohol RS
USP Linoleyl Alcohol RS
USP Octyldodecanol RS
USP Oleyl Alcohol RS
USP Stearyl Alcohol RS

Ointment, Hydrophilic—*see Hydrophilic Ointment General Monographs*

Ointment, White—*see White Ointment General Monographs*

Ointment, Yellow—*see Yellow Ointment General Monographs*

Oleic Acid



$C_{18}H_{34}O_2$ 282.46
9-Octadecenoic acid, (*Z*)-;
Oleic acid [112-80-1].

NF Monographs

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DEFINITION

Oleic Acid is manufactured from fats and oils derived from edible sources, animal or vegetable, and consists chiefly of (*Z*)-9-octadecenoic acid [$CH_3(CH_2)_7CH{:}CH(CH_2)_7COOH$]. It contains NLT 65.0% of (*Z*)-9-octadecenoic acid [$CH_3(CH_2)_7CH{:}CH(CH_2)_7COOH$]. It may contain suitable stabilizers.

[NOTE—Oleic Acid labeled solely for external use is exempt from the requirement that it be prepared from edible sources.]

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F▲** (CN 1-May-2020)

Sample: Undried specimen

Acceptance criteria: Meets the requirements

- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

ASSAY

- **PROCEDURE**

Standard solution: 1.7 mg/mL of USP Oleic Acid RS in tetrahydrofuran

Sample solution: 1.7 mg/mL of Oleic Acid in tetrahydrofuran

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.53-mm × 30-m capillary; 0.25-µm layer of phase G25 (or G35)

Temperature

Detector: 280°

Injection port: 280°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
120	—	120	5
120	10	250	20

Carrier gas: Helium

Flow rate: 7.0 mL/min

Injection volume: 1.0 µL

Injection type: Splitless

System suitability

Sample: *Standard solution*

[NOTE—The retention time for oleic acid is about 19.2 min.]

System suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of oleic acid in the portion of the sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response for oleic acid from the *Sample solution*

r_S = peak response for oleic acid from the *Standard solution*

C_S = concentration of USP Oleic Acid RS in the *Standard solution* (mg/mL)

C_U = concentration of Oleic Acid in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 65.0%

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩

Sample: 10 mL

Acceptance criteria: NMT 1 mg (about 0.01%)

SPECIFIC TESTS

- **CONTENT OF FATTY ACIDS**

Oleic Acid exhibits the composition profiles of fatty acids shown in *Table 2* below, as determined in *Fats and Fixed Oils* ⟨401⟩, *Fatty Acid Composition*.

Table 2

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
14	0	≤5.0
16	0	≤16.0
16	1	≤8.0
18	0	≤6.0
18	1	≥65.0
18	2	≤18.0
18	3	≤4.0
20, 22[a]	0	≤4.0

[a] The sum of these fatty acids should be NMT 4.0%.

Test solution: Prepare as directed in the chapter, but omitting the initial hydrolysis.

- **CONGEALING TEMPERATURE** ⟨651⟩**:** 3°–10° for Oleic Acid derived from animal sources; 10°–16° for Oleic Acid derived from vegetable sources
- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩**:** 196–204, 2 g being used
- **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩**:** 85–105
- **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩**:** NMT 10.0
- **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 0.4%
- **MINERAL ACIDS**

Sample: 5 mL

Analysis: Shake the *Sample* with an equal volume of water at a temperature of about 25° for 2 min, allow the liquids to separate, and pass the water layer through a paper filter previously moistened with water.

Acceptance criteria: The filtrate is not reddened by the addition of 1 drop of methyl orange TS.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light. Store at room temperature, and avoid exposure to excessive heat.
- **LABELING:** If it is for external use only, the labeling so indicates. Label it to indicate whether it is derived from animal or vegetable sources. Indicate the names and quantity of any added stabilizers.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Oleic Acid RS

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Oleoyl Polyoxylglycerides

DEFINITION

Oleoyl Polyoxylglycerides is a mixture of monoesters, diesters, and triesters of glycerol and monoesters and diesters of polyethylene glycols. Polyethylene glycols used have a mean molecular weight between 300 and 400. The article is produced by partial alcoholysis of unsaturated oils, mainly containing triglycerides of oleic acid with polyethylene glycol, by esterification of glycerol and polyethylene glycol with fatty acids, or as a mixture of glycerol esters and ethylene oxide condensate with the fatty acids of the unsaturated oils. It may contain free polyethylene glycols.

IDENTIFICATION

Change to read:

- **A. ▲Spectroscopic Identification Tests** ⟨197⟩, *Infrared Spectroscopy*: **197F** ▲ (CN 1-May-2020)
- **B. Thin-Layer Chromatographic Identification Test** ⟨201⟩

Standard solution: 50 mg/mL of USP Oleoyl Polyoxylglycerides RS in methylene chloride
Sample solution: 50 mg/mL of Oleoyl Polyoxylglycerides in methylene chloride
Application volume: 10 µL
Developing solvent system: Ether and hexanes (70:30)
Spray reagent: 0.1 mg/mL of rhodamine B in alcohol
Analysis
Samples: *Standard solution* and *Sample solution*
Proceed as directed in the chapter. Then spray the plate with *Spray reagent*, and locate the spots on the plate by examination under UV light at a wavelength of 365 nm.
Acceptance criteria: The R_F values of the principal spots of the *Sample solution* correspond to those of the *Standard solution*.

- **C.** It meets the requirements in *Specific Tests* (see *Table 1*) for *Fats and Fixed Oils, Fatty Acid Composition* ⟨401⟩.

IMPURITIES

- **Alkaline Impurities**

Sample: 5.0 g
Analysis: To the *Sample* add 10 mL of alcohol and 0.05 mL of bromophenol blue TS, and mix well. Titrate with 0.01 N hydrochloric acid VS to change the color to yellow.
Acceptance criteria: NMT 1.0 mL of 0.01 N hydrochloric acid is required.

- **Limit of Free Ethylene Oxide and Dioxane**

Analysis: Proceed as directed in *Ethylene Oxide and Dioxane* ⟨228⟩, *Method I*.
Acceptance criteria
Ethylene oxide: NMT 1 µg/g
Dioxane: NMT 10 µg/g

- **Limit of Free Glycerol**

Sample: 1.2 g
Periodic acetic acid solution: Dissolve 0.446 g of sodium periodate in 2.5 mL of a 25% (v/v) solution of sulfuric acid, and dilute with glacial acetic acid to 100.0 mL.
Potassium iodide solution: 75 mg/mL of potassium iodide
Blank: 25 mL of methylene chloride
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Residual titration
Titrant: 0.1 M sodium thiosulfate VS
Endpoint detection: Visual
Analysis: Dissolve the *Sample* in 25 mL of methylene chloride, heating if necessary. Cool, and add 100 mL of water and 25.0 mL of *Periodic acetic acid solution*. Shake, and allow to stand for 30 min. Add 40 mL of *Potassium iodide solution*, and allow to stand for 1 min. Add 1 mL of starch TS, and titrate the liberated iodine with 0.1 M sodium thiosulfate VS. Perform a blank determination, and make any necessary correction.
Calculate the percentage of glycerol in the sample taken:

$$\text{Result} = \{[(V_B - V_S) \times N \times F]/W\} \times 100$$

V_B = *Titrant* volume consumed by the *Blank* (mL)
V_S = *Titrant* volume consumed by the *Sample* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 23.0 mg/mEq
W = *Sample* weight (mg)

Acceptance criteria: NMT 5.0%

SPECIFIC TESTS

- **Articles of Botanical Origin,** *Total Ash* ⟨561⟩: NMT 0.1%
- **Fats and Fixed Oils,** *Acid Value* ⟨401⟩

Sample: 2.0 g
Acceptance criteria: NMT 2.0

- **Fats and Fixed Oils,** *Fatty Acid Composition* ⟨401⟩: Oleoyl Polyoxylglycerides exhibits the composition profile of fatty acids shown in *Table 1*.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
16	0	4.0–9.0
18	0	≤6.0
18	1	58.0–80.0
18	2	15.0–35.0
18	3	≤2.0
20	0	≤2.0
20	1	≤2.0

- **Fats and Fixed Oils,** *Hydroxyl Value* ⟨401⟩

Sample: 1.0 g
Acceptance criteria: 45–65

- **Fats and Fixed Oils,** *Iodine Value* ⟨401⟩: 75–95
- **Fats and Fixed Oils,** *Peroxide Value* ⟨401⟩

Sample: 2.0 g
Acceptance criteria: NMT 12.0

- **Fats and Fixed Oils,** *Saponification Value* ⟨401⟩

Sample: 2.0 g
Acceptance criteria: 150–170

- **Water Determination,** *Method I* ⟨921⟩

Sample: 1.0 g
Analysis: Instead of using methanol as the solvent, one of two solvent systems can be used: a mixture of methylene chloride and anhydrous methanol (70:30 v/v), or anhydrous pyridine.
Acceptance criteria: NMT 1.0%

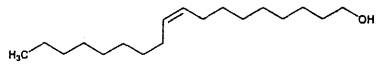
ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in tight containers, protected from light and moisture. Store at controlled room temperature.
- **Labeling:** Label it to indicate the type and the average nominal molecular weight of polyethylene glycol used as part of the official title.
- **USP Reference Standards** ⟨11⟩
USP Oleoyl Polyoxylglycerides RS

Supplement N

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Oleyl Alcohol



$C_{18}H_{36}O$ 268.48
9-Octadecen-1-ol, (*Z*)-;
(*Z*)-9-Octadecen-1-ol [143-28-2].

DEFINITION
Oleyl Alcohol is a mixture of unsaturated and saturated high molecular weight fatty alcohols consisting of 75.0%–102.0% of oleyl alcohol ($C_{18}H_{36}O$) and its isomers. It is obtained from sources of vegetable, animal, or synthetic origin. It may contain suitable stabilizers.

IDENTIFICATION
• **A. Chromatographic Identity**
Analysis: Proceed as directed in the *Assay*.
Acceptance criteria: The retention time of the major peak of the *Sample solution*, excluding the solvent and internal standard peaks, corresponds to the oleyl alcohol peak of the *System suitability solution*.

ASSAY
• **Procedure**
Internal standard solution: 1 mg/mL of 1-pentadecanol (internal standard) in ethanol
System suitability solution: Prepare 1 mg/mL each of USP Cetyl Alcohol RS, USP Stearyl Alcohol RS, and USP Oleyl Alcohol RS in *Internal standard solution*, and heat the solution in a sealed container in a 50° water bath until all fatty alcohols are dissolved. Allow the solution to cool to room temperature, and mix well.
Standard solution: 1.0 mg/mL of USP Oleyl Alcohol RS in *Internal standard solution*
Sample solution: 1.0 mg/mL of Oleyl Alcohol in *Internal standard solution*
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.25-mm × 30-m fused silica capillary; coated with a 0.25-µm layer of phase G7
Temperatures
Detector: 280°
Injection port: 270°
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
60	20	180	—
180	10	220	5

Carrier gas: Hydrogen
Flow rate: 2.0 mL/min, constant flow mode
Injection volume: 1 µL
Injection type: Split injection; split ratio is 100:1
Liner: Single taper, low pressure drop liner with deactivated wool
Run time: 15 min
System suitability
Samples: *System suitability solution* and *Standard solution*
[Note—See *Table 2* for the relative retention times.]

Table 2

Component	Relative Retention Time
1-Pentadecanol (internal standard)	1.00
Cetyl alcohol	1.08
Stearyl alcohol	1.25
Oleyl alcohol	1.27

Suitability requirements
Resolution: NLT 30 between the cetyl alcohol and stearyl alcohol peaks; NLT 2.0 between the stearyl and oleyl alcohol peaks, *System suitability solution*
Tailing factor: 0.8–1.8 for the oleyl alcohol and 1-pentadecanol peaks, *Standard solution*
Relative standard deviation: NMT 1%, using the area ratio of oleyl alcohol to 1-pentadecanol, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
The peak of elaidyl alcohol, which is an isomer of oleyl alcohol, can be resolved from the oleyl alcohol peak with a resolution of about 1, and with a relative retention time with reference to oleyl alcohol of 0.995. An additional small peak that may be observed on the peak tail of oleyl alcohol can be assigned to another oleyl alcohol isomer. If elaidyl alcohol is observed, a combination of both peaks of elaidyl alcohol and oleyl alcohol is used to determine oleyl alcohol content.
Calculate the percentage of oleyl alcohol ($C_{18}H_{36}O$) or its isomers in the portion of Oleyl Alcohol taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of oleyl alcohol (or elaidyl alcohol) to the internal standard [peak response of oleyl alcohol (or elaidyl alcohol)/peak response of the internal standard] from the *Sample solution*
R_S = peak response ratio of oleyl alcohol to the internal standard (peak response of oleyl alcohol/peak response of the internal standard) from the *Standard solution*
C_S = concentration of USP Oleyl Alcohol RS in the *Standard solution* (mg/mL)
C_U = concentration of Oleyl Alcohol in the *Sample solution* (mg/mL)

Acceptance criteria: 75.0%–102.0% for oleyl alcohol and its isomers

IMPURITIES
• **Residue on Ignition** ⟨281⟩: NMT 0.1%, determined on 2 g
• **Limit of Related Fatty Alcohols**
Resolution solution: Prepare 1 mg/mL each of USP Cetyl Alcohol RS, USP Stearyl Alcohol RS, USP Oleyl Alcohol RS, USP Linoleyl Alcohol RS, USP Linolenyl Alcohol RS, and USP Arachidyl Alcohol RS in ethanol. Heat the solution in a sealed container in a 50° water bath until all fatty alcohols are dissolved. Allow the solution to cool to room temperature, and mix well. Dilute the solution with ethanol to obtain a solution containing 0.05 mg/mL each of USP Cetyl Alcohol RS, USP Stearyl Alcohol RS, USP Oleyl Alcohol RS, USP Linoleyl Alcohol RS, USP Linolenyl Alcohol RS, and USP Arachidyl Alcohol RS.
Sample solution: 1 mg/mL of Oleyl Alcohol in ethanol
Chromatographic system: Proceed as directed in the *Assay*, except use split injection with a split ratio of 5:1.

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System suitability
Sample: *Resolution solution*
[NOTE—See *Table 3* for the relative retention times.]

Table 3

Component	Relative Retention Time
Cetyl alcohol	0.85
Stearyl alcohol	0.99
Oleyl alcohol	1.00
Linoleyl alcohol	1.03
Linolenyl alcohol	1.06
Arachidyl alcohol	1.14

Suitability requirements
Resolution: NLT 30 between the cetyl alcohol and stearyl alcohol peaks; NLT 2.0 between the stearyl and oleyl alcohol peaks; NLT 6.0 between the oleyl alcohol and linoleyl alcohol peaks

Analysis
Samples: *Resolution solution* and *Sample solution*
Identify each related fatty alcohol peak in the *Sample solution* based on that in the *Resolution solution.*
Calculate the percentage of each related fatty alcohol in the portion of Oleyl Alcohol taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of each related fatty alcohol from the *Sample solution*
r_T = sum of all the peak responses excluding peak responses due to solvent from the *Sample solution*

Acceptance criteria: See *Table 4*. Disregard peaks that are less than 0.05% for any unspecified impurities, and any peaks due to solvent.

Table 4

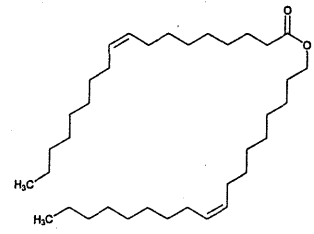
Component	Acceptance Criteria, NMT (%)
Cetyl alcohol	8.0
Stearyl alcohol	5.0
Linoleyl alcohol	7.0
Linolenyl alcohol	1.0
Arachidyl alcohol	1.0

SPECIFIC TESTS
- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩: NMT 1
- **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩: 205–215
- **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩: NMT 10.0
- **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 0.5%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-filled, tight containers, and store at controlled room temperature.
- **LABELING:** Label it to indicate whether it is derived from vegetable, animal, or synthetic sources. Indicate the names and amounts of any added stabilizers.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Arachidyl Alcohol RS
  USP Cetyl Alcohol RS
  USP Linolenyl Alcohol RS
  USP Linoleyl Alcohol RS
  USP Oleyl Alcohol RS
  USP Stearyl Alcohol RS

Oleyl Oleate

Change to read:



$C_{36}H_{68}O_2$ ▲532.94▲ (ERR 1-Jan-2019)
9-Octadecenoic acid, (*Z*)-, oleyl ester;
Oleyl oleate [3687-45-4].

DEFINITION
Oleyl Oleate consists of esters of oleyl alcohol and high molecular weight fatty acids, principally oleic acid.

IDENTIFICATION

Change to read:

- **A.** ▲**SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F**▲ (CN 1-May-2020)

IMPURITIES
- **RESIDUE ON IGNITION** ⟨281⟩
  Sample: 2 g
  Acceptance criteria: NMT 0.1%
- **ARSENIC,** *Method II* ⟨211⟩: 2 µg/g

SPECIFIC TESTS
- **CLARITY OF SOLUTION**
  Sample solution: 200 mg/mL in ether
  Acceptance criteria: The resulting solution is clear.
- **SPECIFIC GRAVITY** ⟨841⟩: 0.860–0.884 at 20°
- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩: NMT 3.0
- **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩: NMT 10
- **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩: 70–120
- **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩: 90–125
- **REFRACTIVE INDEX** ⟨831⟩: 1.464–1.468 at 20°

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers. No storage conditions specified.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Oleyl Oleate RS

Olive Oil

[8001-25-0].

DEFINITION
Olive Oil is the refined fixed oil obtained from the ripe fruit of *Olea europaea* L. (Fam. Oleaceae). It may contain suitable antioxidants.

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IDENTIFICATION

• **A. Identity by Fatty Acid Composition**

Analysis: Proceed as directed in the test for *Fats and Fixed Oils* ⟨401⟩, *Fatty Acid Composition.*

Acceptance criteria: Meets the composition profile of fatty acids in *Table 1*

• **B. Identity by Triglyceride Profile**

Analysis: Proceed as directed in the test for *Identification of Fixed Oils by Thin-Layer Chromatography* ⟨202⟩.

Acceptance criteria: Meets the requirements in the chapter

IMPURITIES

• **Alkaline Impurities**

Sample: 10 mL of Olive Oil

Analysis: Mix 10 mL of freshly opened acetone and 0.3 mL of water, and add 0.05 mL of bromophenol blue TS. Add the *Sample*, shake, and allow to stand. Titrate with 0.01 N hydrochloric acid VS to change the color of the upper layer to yellow.

Acceptance criteria: NMT 0.1 mL of 0.01 N hydrochloric acid is required.

SPECIFIC TESTS

• **Fats and Fixed Oils,** *Acid Value (Free Fatty Acids)* ⟨401⟩**:** NMT 0.3. [Note—Petroleum ether with a 100°–120° boiling range can be used to replace ether in the test.]

• **Fats and Fixed Oils,** *Peroxide Value* ⟨401⟩**:** NMT 10.0

• **Fats and Fixed Oils,** *Fatty Acid Composition* ⟨401⟩**:** Olive Oil exhibits the composition profile of fatty acids shown in *Table 1*, as determined in the chapter.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
<16	0	≤0.1
16	0	7.5–20.0
16	1	≤3.5
18	0	0.5–5.0
18	1	56.0–85.0
18	2	3.5–20.0
18	3	≤1.2
20	0	≤0.7
20	1	≤0.4
22	0	≤0.2
24	0	≤0.2

• **Absence of Sesame Oil**

Sample: 10 mL of Olive Oil

Analysis: Mix the *Sample* with a mixture of 0.5 mL of a 0.35% (v/v) solution of furfural in acetic anhydride and 4.5 mL of acetic anhydride, and shake the mixture for about 1 min. Pass through a filter paper previously wetted with acetic anhydride. Add 0.2 mL of sulfuric acid to the filtrate.

Acceptance criteria: No bluish-green color develops.

• **Fats and Fixed Oils,** *Unsaponifiable Matter* ⟨401⟩**:** NMT 1.5%. [Note—Petroleum ether with a 40°–60° boiling range can be used to replace ether in the test.]

• **Ultraviolet Absorbance**

Sample solution: Dissolve 1.0 g of Olive Oil in cyclohexane, and dilute with cyclohexane to 100 mL.

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Mode: UV-Vis

Wavelength: 270 nm

Path length of the cell: 1 cm

Analysis: Determine the UV-Vis absorbance using the *Instrumental conditions* described above.

Acceptance criteria: The absorbance is NMT 1.20.

• **Water Determination,** *Method Ic* ⟨921⟩**:** NMT 0.1%

• **Sterol Composition**

2 M Alcoholic potassium hydroxide solution: Dissolve 12 g of potassium hydroxide in 10 mL of water, and dilute with alcohol (ethanol) to 100 mL.

Sample A: Accurately weigh 5 g of Olive Oil into a 150-mL flask fitted with a reflux condenser. Add 50 mL of *2 M Alcoholic potassium hydroxide solution*, and heat on a water bath for 1 h, shaking frequently. Add 50 mL of water through the top of the condenser, shake, and allow to cool. Transfer the contents of the flask to a separating funnel. Rinse the flask with several portions totaling 50 mL of petroleum ether with a 40°–60° boiling range, and add the rinsings to the separating funnel. Shake vigorously for 1 min. Allow to separate, and transfer the aqueous layer to a second separating funnel. If an emulsion forms, add small quantities of alcohol or a concentrated solution of potassium hydroxide. Shake the aqueous layer with two 50-mL quantities of petroleum ether with a 40°–60° boiling range. Combine the petroleum ether layers in a third separating funnel and wash with three 50-mL quantities of 50% alcohol. Transfer the petroleum ether layer to a tared 250-mL flask. Rinse the separating funnel with small quantities of petroleum ether with a 40°–60° boiling range, and add to the flask. Evaporate the petroleum ether on a water bath and dry the residue at 100°–105° for 15 min, keeping the flask horizontal. Allow to cool in a desiccator and weigh.

Reference A: Accurately weigh 5 g of sunflower oil into a 150-mL flask fitted with a reflux condenser. Proceed as directed for *Sample A*, beginning with "Add 50 mL of *2 M Alcoholic potassium hydroxide solution*".

Separation of the sterol fraction by LC

Mobile phase: Isopropyl alcohol and *n*-hexane (1:99)

Sample solution A: Transfer *Sample A* with three 4-mL quantities of petroleum ether with a 40°–60° boiling range to a 15-mL test tube. [Note—Ether can be used to replace petroleum ether if *Sample A* is not well soluble in petroleum ether.] Evaporate to dryness under a stream of nitrogen. Dissolve *Sample A* in *Mobile phase* to obtain a solution with an approximate concentration of 40 mg/mL. Add a few drops of isopropyl alcohol to improve the solubility. [Note—3 drops are normally sufficient to ensure complete solubilization.] Pass through a membrane filter (nominal 0.45-µm pore size).

Reference solution A: Prepare as directed for *Sample solution A*, except use *Reference A* instead of *Sample A*.

Chromatographic system

(See *Chromatography* ⟨621⟩ .)

Mode: LC

Detector: UV 210 nm

Columns

Guard: 4.6-mm × 0.5-cm (or 4.6-mm × 1.0-cm); 5-µm packing L3, with a 6-nm pore size

Analytical: 4.6-mm × 25-cm; 5-µm packing L3, with a 6-nm pore size

Flow rate: 1.0 mL/min

Injection volume: 50 µL

Identification of the peaks due to sterols

Samples: *Sample solution A* and *Reference solution A*

Sterol identification: The sterol fraction elutes at the end of the chromatogram. Locate the fraction to be collected by using the chromatogram from *Reference solution A*. The chromatogram from *Reference solution A* shows two or three principal peaks, which elute at approximately

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21–35 min depending on the column used. The chromatogram from *Sample solution A* may have one principal peak.

Sterol collection: Collect the fraction at the detector outlet in a 15-mL tube with a screw cap. Evaporate the solvent under a stream of nitrogen. [NOTE—If necessary, to increase the sample amount for later analysis, make the second injection of 50 µL on the HPLC column and collect the fraction at the detector outlet in the same 15-mL test tube with a screw cap. Evaporate the solvent under a stream of nitrogen.]

Determination of sterols by GC

Sample solution B: Dissolve the residue of the sterol fraction obtained from *Sample solution A* in the previous LC step in 0.2 mL of anhydrous pyridine and 0.2 mL of a mixture of 1 volume of chlorotrimethylsilane and 99 volumes of bis(trimethylsilyl)trifluoroacetamide. Insert the stopper into the test tube tightly, and heat at 80° for 20 min. Allow to cool and use the liquid phase.

Reference solution B: Dissolve 9 parts of the residue of the sterol fraction obtained from *Reference solution A* in the previous LC step and 1 part of cholesterol in 0.2 mL of anhydrous pyridine and 0.2 mL of a mixture of 1 volume of chlorotrimethylsilane and 99 volumes of bis(trimethylsilyl)trifluoroacetamide. Insert the stopper into the test tube tightly and heat at 80° for 20 min. Allow to cool and use the liquid phase.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.25-mm × 30-m fused-silica capillary; 0.25-µm layer of phase G27

Temperatures

Injection port: 290°

Detector: 290°

Column: See *Table 2*.

Table 2

Initial Temperature (°)	Tempera-ture Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
260	—	260	38
260	5	290	5

Carrier gas: Helium

Flow rate: 2.6 mL/min

Injection volume: 1–3 µL (depending on the expected amount of sterols in the test sample)

Injection type: Split injection; split ratio is 25:1

System suitability

Sample: *Reference solution B*

The chromatogram from *Reference solution B* shows five principal peaks corresponding to cholesterol, campesterol, stigmasterol, β-sitosterol, and Δ7-stigmastenol.

[NOTE—The retention times of the sterols with reference to β-sitosterol are given in *Table 3*.]

Table 3

Identification	Relative Retention Time
Cholesterol	0.65
Brassicasterol	0.71
24-Methylene-cholesterol	0.80
Campesterol	0.82
Campestanol	0.83
Stigmasterol	0.87
Δ7-Campesterol	0.92
Δ5,23-Stigmastadienol	0.95
Clerosterol	0.96
β-Sitosterol	1.00
Sitostanol	1.01
Δ5-Avenasterol	1.03
Δ5,24-Stigmastadienol	1.09
Δ7-Stigmastenol	1.13
Δ7-Avenasterol	1.17

Suitability requirements

Resolution: NLT 3.0 between the campesterol and stigmasterol peaks

Analysis

Samples: *Sample solution B* and *Reference solution B*

Use the chromatogram from *Reference solution B* to identify the peaks due to cholesterol, campesterol, stigmasterol, β-sitosterol, and Δ7-stigmastenol. Identify the peaks due to the sterols in the chromatogram from *Sample solution B* using the chromatograms from *Reference solution B* and the relative retention times with reference to β-sitosterol (main peak) given in *Table 3*.

Calculate the percentage content of each sterol in the sterol fraction of Olive Oil taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = area of the peak due to the sterol component to be determined

r_T = sum of the areas of the peaks due to the components indicated in *Table 3*

Acceptance criteria: Olive Oil exhibits the composition profiles of sterols shown in *Table 4*.

Table 4

Component	Percentage (%)
Cholesterol	≤0.5
Campesterol	≤4.0
Δ7-Stigmastenol	≤0.5
Sum of the contents of Δ5,23-stig-mastadienol, clerosterol, β-sitosterol, sitostanol, Δ5-avenasterol, and Δ5,24-stigmastadienol	≥93.0

The content of stigmasterol is NMT that of campesterol.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant, well-filled containers, and prevent exposure to excessive heat.
- **LABELING:** Label it to indicate the name and quantity of any suitable antioxidants.

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Vehicle for Oral Solution

DEFINITION
Prepare Vehicle for Oral Solution as follows (see *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩).

Sucrose	80 g
Glycerin	5 g
Sorbitol	5 g
Sodium Phosphate, Dibasic	120 mg
Citric Acid	200 mg
Potassium Sorbate	100 mg
Methylparaben	100 mg
Purified Water, a sufficient quantity to make	100 mL

Calculate the quantity of each ingredient required for the total amount to be prepared. Accurately weigh/measure each ingredient. Heat about 30 mL of *Purified Water* to 70°–75°. Add the *Glycerin* and *Methylparaben*, and stir until the *Methylparaben* is dissolved. Add the *Dibasic Sodium Phosphate*, *Citric Acid*, *Potassium Sorbate*, and *Sorbitol*, and mix well. Add the *Sucrose*, and mix until dissolved; remove from the heat, and allow to cool. Add sufficient *Purified Water* to volume, and mix well. Adjust the pH if necessary. Package, and label.

SPECIFIC TESTS
- **pH** ⟨791⟩: An apparent pH between 4.0 and 5.0

ADDITIONAL REQUIREMENTS
- **Packaging and Storage:** Package in a tight, light-resistant container, and store at controlled room temperature.
- **Labeling:** Label it to indicate that it is for use in compounding oral solutions and suspensions.

Change to read:

- **Beyond-Use Date:** NMT 6 months after preparation. A beyond-use date of more than 6 months may be assigned if supporting stability data exist. (See ▲*10. Establishing Beyond-Use Dates, Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩▲ (CN 1-May-2020).)

Vehicle for Oral Solution, Sugar Free

DEFINITION
Prepare Vehicle for Oral Solution, Sugar Free as follows (see *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩).

Xanthan Gum	50 mg
Glycerin	10 mL
Sorbitol Solution	25 mL
Saccharin Sodium	100 mg
Citric Acid Monohydrate	1.5 g
Sodium Citrate	2 g
Methylparaben	100 mg
Potassium Sorbate	100 mg
Purified Water, a sufficient quantity to make	100 mL

Calculate the quantity of each ingredient required for the total amount to be prepared. Accurately weigh/measure each ingredient. Heat about 60 mL of *Purified Water* to about 70°–75°. Add the *Methylparaben*, and stir until dissolved. Remove from the heat, and add the *Glycerin*, *Sorbitol Solution*, *Saccharin Sodium*, *Citric Acid Monohydrate*, *Sodium Citrate*, *Potassium Sorbate*, and *Xanthan Gum*. Add sufficient *Purified Water* to volume, and mix well. Adjust the pH if necessary. Package, and label.

SPECIFIC TESTS
- **pH** ⟨791⟩: An apparent pH between 4.0 and 5.0

ADDITIONAL REQUIREMENTS
- **Packaging and Storage:** Package in a tight, light-resistant container, and store at controlled room temperature.
- **Labeling:** Label it to indicate that it is for use in compounding sugar-free oral solutions and suspensions.

Change to read:

- **Beyond-Use Date:** NMT 6 months after preparation. A beyond-use date of more than 6 months may be assigned if supporting stability data exist. (See ▲*10. Establishing Beyond-Use Dates, Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩▲ (CN 1-May-2020).)

Vehicle for Oral Suspension

DEFINITION
Prepare Vehicle for Oral Suspension as follows (see *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩).

Cellulose, Microcrystalline	800 mg
Xanthan Gum	200 mg
Carrageenan	150 mg
Carboxymethylcellulose Sodium (High Viscosity)	25 mg
Citric Acid	250 mg
Sodium Phosphate, Dibasic	120 mg
Simethicone	0.1 mL
Potassium Sorbate	100 mg
Methylparaben	100 mg
Purified Water, a sufficient quantity to make	100 mL

Calculate the quantity of each ingredient required for the total amount to be prepared. Accurately weigh/measure each ingredient. Heat about 90 mL of the *Purified Water* to 70°–75°. Dissolve the *Methylparaben*, followed by the *Citric Acid*, *Dibasic Sodium Phosphate*, and *Potassium Sorbate* in the heated water. Remove from the heat. With constant mixing, slowly sprinkle the *Microcrystalline Cellulose*, *Xanthan Gum*, *Carrageenan*, and *Carboxymethylcellulose Sodium* into the mixture. Continue to stir until fully hydrated, add the *Simethicone*, and mix well. Add sufficient *Purified Water* to volume, and mix well. Adjust the pH if necessary. Package, and label.

SPECIFIC TESTS
- **pH** ⟨791⟩: An apparent pH between 4.0 and 5.0

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ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Package in a tight, light-resistant container, and store at controlled room temperature.
- **LABELING:** Label it to indicate that it is for use in compounding oral solutions and suspensions.

Change to read:

- **BEYOND-USE DATE:** NMT 6 months after preparation. A beyond-use date of more than 6 months may be assigned if supporting stability data exist. (See *▲10. Establishing Beyond-Use Dates, Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩▲ (CN 1-May-2020).)

Orange Oil

DEFINITION

Orange Oil is the volatile oil obtained by expression from the fresh peel of the ripe fruit of *Citrus sinensis* (L.) Osbeck (Fam. Rutaceae). The total aldehyde content, calculated as decanal ($C_{10}H_{20}O$), is NLT 1.2% and NMT 2.5%. It may contain a suitable antioxidant.

[NOTE—Do not use Orange Oil that has a terebinthine odor.]

ASSAY

- **TOTAL ALDEHYDE CONTENT**

Reagent solution: Dissolve 4.5 g of hydroxylamine hydrochloride in 13 mL of water. Add 85 mL of tertiary butyl alcohol, mix, and adjust with 0.5 N potassium hydroxide to a pH of 3.4.

Sample: 5 mL of Orange Oil, accurately weighed

Analysis: Pipet 50 mL of the *Reagent solution* into a conical flask containing the *Sample*. Insert the stopper in the flask, and allow to stand at room temperature for 30 min, with occasional shaking. Titrate the liberated hydrochloric acid with 0.5 N alcoholic potassium hydroxide VS to a pH of 3.4. Each mL of 0.5 N alcoholic potassium hydroxide consumed in the titration is equivalent to 78.13 mg of total aldehydes, calculated as decanal ($C_{10}H_{20}O$).

Acceptance criteria: 1.2%–2.5%

SPECIFIC TESTS

- **SPECIFIC GRAVITY** ⟨841⟩: 0.842–0.846
- **OPTICAL ROTATION,** *Angular Rotation* ⟨781A⟩: +94° to +99°
- **REFRACTIVE INDEX** ⟨831⟩: 1.472–1.474 at 20°
- **ULTRAVIOLET ABSORBANCE**

Sample solution: 250 mg of Oil in 100 mL of alcohol

Blank: Alcohol

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Mode: UV-Vis

Wavelength range: 260–400 nm

Analysis: Record the spectrum in 1-cm cell. Determine the absorbance at the wavelength of maximum absorbance at 330 nm, using the line drawn tangent to the curves appearing as minima in the spectrum in wavelength regions above and below the maximum wavelength as the baseline.

Acceptance criteria: The absorbance, calculated on the basis of a 250-mg specimen, is NLT 0.130 for California-type Orange Oil or NLT 0.240 for Florida-type Orange Oil.

- **FOREIGN OILS**

Analysis: Place 50 mL of Oil in a four-bulb Ladenburg flask having the following dimensions: the lower or main bulb is about 6 cm in diameter, and the smaller condensing bulbs are about 3.5, 3.0, and 2.5 cm in diameter; the distance from the bottom of the flask to the side-arm is about 20 cm. Distill Oil at a rate of 1 drop/s until the distillate measures 5 mL.

Acceptance criteria: The angular rotation of the distillate does not differ from that of the original Oil by more than 2°, and the refractive index at 20° is 0.001–0.002 lower than that of the original Oil.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-filled, tight containers, and avoid exposure to excessive heat.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant source from which the article was derived. Label it also to indicate whether it is California-type or Florida-type Orange Oil. The label indicates that Oil is not to be used if it has a terebinthine odor.

Compound Orange Spirit

DEFINITION

Compound Orange Spirit contains NLT 25 mL and NMT 30 mL of the mixed oils in 100 mL of Spirit.

Prepare Compound Orange Spirit as follows (see *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩).

Orange Oil	200 mL
Lemon Oil	50 mL
Coriander Oil	20 mL
Anise Oil	5 mL
Alcohol, a sufficient quantity to make	1000 mL

Mix the oils with sufficient *Alcohol* to make the product measure 1000 mL.

ASSAY

- **PROCEDURE**

Sample solution: Transfer 2.0 mL of Compound Orange Spirit and 1.0 mL of kerosene to a Babcock bottle, graduated to 8%, and mix.

Analysis: To the *Sample solution* add sufficient saturated calcium chloride solution, acidified with hydrochloric acid, to almost fill the bulb of the bottle. Rotate the bottle vigorously to ensure mixing, then add a sufficient quantity of the calcium chloride solution to bring the separated oil into the neck of the bottle. Centrifuge for 5 min at 1500 rpm, and read the volume of oil in the stem. Subtract five divisions on the volumetric flask for the kerosene added, and multiply the remaining number of divisions by 10.5 to obtain the volume, in mL, of mixed oils in 100 mL of the Compound Orange Spirit.

Acceptance criteria: 25–30 mL

OTHER COMPONENTS

- **ALCOHOL DETERMINATION,** *Method I* ⟨611⟩: 65.0%–70.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Package in tight containers, protected from light, and store in a cold place.

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Sweet Orange Peel Tincture

DEFINITION

Sweet Orange Peel Tincture is prepared from sweet orange peel, which is the outer rind of the non-artifically colored, fresh, ripe fruit of *Citrus sinesis* (L.) Osbeck (Fam. Rutaceae). Prepare Sweet Orange Peel Tincture as follows.

Sweet Orange Peel	500 g
Alcohol	900 mL
Alcohol, a sufficient quantity to make	1000 mL

Macerate the *Sweet Orange Peel* in 900 mL of *Alcohol* in a container that can be closed, and put in a warm place. Agitate it frequently during 3 days or until the soluble matter is dissolved. Transfer the mixture to a filter, using Talc as the filtering medium, and when most of the liquid has drained away, wash the residue on the filter with a sufficient quantity of *Alcohol*, combining the filtrates, to produce 1000 mL, and mix. [NOTE—Exclude the inner, white portion of the rind.]

OTHER COMPONENTS

- **ALCOHOL DETERMINATION,** *Method I* ⟨611⟩: 62.0%–72.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Package in tight, light-resistant containers. Avoid exposure to direct sunlight and excessive heat.
- **LABELING:** The label states the Latin binomial and the official name.

Orange Syrup

DEFINITION

Orange Syrup contains NLT 0.45% and NMT 0.55% of citric acid ($C_6H_8O_7$).

Prepare Orange Syrup as follows (see *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩).

Sweet Orange Peel Tincture	50 mL
Anhydrous Citric Acid	5 g
Talc	15 g
Sucrose	820 g
Purified Water, a sufficient quantity to make	1000 mL

Triturate *Talc* with *Sweet Orange Peel Tincture* and *Anhydrous Citric Acid*, and gradually add 400 mL of *Purified Water*. Filter, returning the first portions of the filtrate until it becomes clear, and wash the mortar and the filter with sufficient *Purified Water* to make the filtrate measure 450 mL. Dissolve *Sucrose* in this filtrate by agitation, without heating, and add *Purified Water* to make the product measure 1000 mL. Mix, and strain. [NOTE—Do not use Orange Syrup that has a terebinthine odor or taste or shows other indications of deterioration.]

ASSAY

- **CITRIC ACID**

Sample: 20 mL

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Direct titration

Titrant: 0.1 N sodium hydroxide VS

Endpoint detection: Visual

Analysis: To the *Sample* add 20 mL of water and add phenolphthalein TS. Titrate with *Titrant*. Each mL of *Titrant* is equivalent to 6.404 mg of citric acid ($C_6H_8O_7$).

Acceptance criteria: 0.45%–0.55%

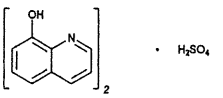
OTHER COMPONENTS

- **ALCOHOL DETERMINATION,** *Method I* ⟨611⟩: 2.0%–5.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Package in tight containers, and store in a cold place.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant source from which the article was derived. The label indicates that Syrup is not to be used if it has a terebinthine odor or taste or shows other indications of deterioration.

Oxyquinoline Sulfate



$(C_9H_7NO)_2 \cdot H_2SO_4$ 388.39

8-Quinolinol sulfate (2:1) (salt) [134-31-6].

DEFINITION

Oxyquinoline Sulfate is 8-hydroxyquinoline sulfate. It contains NLT 97.0% and NMT 101.0% of oxyquinoline sulfate $[(C_9H_7NO)_2 \cdot H_2SO_4]$, calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197M**▲ (CN 1-May-2020)

Analysis: On the undried specimen

Acceptance criteria: Meets the requirements

- **B. IDENTIFICATION TESTS—GENERAL,** *Sulfate* ⟨191⟩

Analysis: 100 mg/mL

Acceptance criteria: Meets the requirements

ASSAY

- **PROCEDURE**

Sample: 100 mg

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Residual titration

Titrant: 0.1 N bromine VS

Back-titrant: 0.1 N sodium thiosulfate VS

Endpoint detection: Visual

Analysis: Transfer the *Sample* to an iodine flask, add 30 mL of glacial acetic acid, 25.0 mL of *Titrant*, 10 mL of potassium bromide solution (150 mg/mL), and 10 mL of hydrochloric acid. Immediately insert the stopper, mix, and allow to stand for 15 min, protected from light. Quickly add 10 mL of potassium iodide solution (100 mg/mL) and 100 mL of water, taking precautions against the escape of bromine vapor. At once insert the stopper, and shake the mixture thoroughly. Remove the stopper, and rinse it and the neck of the flask with a small quantity of water so that the washing flows into the flask. Shake the mixture thoroughly. Titrate the liberated iodine with *Back-titrant*, adding 3 mL of starch TS as the endpoint is approached. Perform a blank

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determination. Each mL of *Titrant* is equivalent to 4.855 mg of oxyquinoline sulfate $[(C_9H_7NO)_2 \cdot H_2SO_4]$.
Acceptance criteria: 97.0%–101.0% on the anhydrous basis

IMPURITIES
- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.3%

SPECIFIC TESTS
- **WATER DETERMINATION,** *Method I* ⟨921⟩: 4.0%–6.0%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Oxyquinoline Sulfate RS

Palm Oil

Palm oil [8002-75-3].

DEFINITION
Palm Oil is the refined fixed oil obtained from the pulp of the fruit of the oil palm *Elaeis guineensis* Jacq. (Fam. Arecaceae). It may contain suitable antioxidants.

IDENTIFICATION
- **A. IDENTITY BY FATTY ACID COMPOSITION**
  Analysis: Proceed as directed in the test for *Fats and Fixed Oils* ⟨401⟩, *Procedures, Fatty Acid Composition.*
  Acceptance criteria: Meets the composition profile of fatty acids in *Table 1*
- **B.** It meets the requirements of the test for *Melting Range or Temperature* ⟨741⟩.

Add the following:

▲• **C. IDENTITY BY TRIGLYCERIDE PROFILE**
Analysis: Proceed as directed in *Identification of Fixed Oils by Thin-Layer Chromatography* ⟨202⟩, *Identification, Method I* or *Method II.*
Acceptance criteria: Meets the requirements in the chapter ▲ 2S (NF36)

IMPURITIES
- **RESIDUE ON IGNITION** ⟨281⟩
  Sample: 5 g of Palm Oil
  Acceptance criteria: NMT 0.1%

Delete the following:

▲• **HEAVY METALS,** *Method II* ⟨231⟩: NMT 10 µg/g ▲ (Official 1-Jan-2018)
- **ALKALINE IMPURITIES**
  Sample: 10 mL of Palm Oil
  Analysis: Mix 10 mL of acetone and 0.3 mL of water, and add 0.05 mL of bromophenol blue TS. If necessary, neutralize the solution to a green color with 0.01 N hydrochloric acid or 0.01 N sodium hydroxide. Add the *Sample*, shake, and allow to stand. Titrate with 0.01 N hydrochloric acid VS until the color of the upper layer changes to yellow.
  Acceptance criteria: NMT 0.1 mL of 0.01 N hydrochloric acid is required.

SPECIFIC TESTS
- **MELTING RANGE OR TEMPERATURE** ⟨741⟩: 30°–40°
- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Acid Value, Method II*: NMT 2.0
- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Fatty Acid Composition*: Palm Oil exhibits the composition profile of fatty acids as shown in *Table 1*.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage
≤12	0	≤2.5
14	0	0.5–5.9
16	0	39.0–47.0
18	0	2.0–8.0
16	1	≤0.5
18	1	36.0–44.0
18	2	7.0–12.0
18	3	≤0.5
≥20	0 or 1	≤1.0

- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Peroxide Value*: NMT 5.0
- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Unsaponifiable Matter*: NMT 1.0%
- **WATER DETERMINATION** ⟨921⟩, *Method I, Method Ic*: NMT 0.1%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers. Do not store above 55°.
- **LABELING:** Label it to indicate the name and quantity of any added antioxidants.

Hydrogenated Palm Oil

R^1COOCH_2–$CH(OOCR^2)$–CH_2OOCR^3, where R^1, R^2, and R^3 are mainly C_{15} and C_{17};
Hydrogenated palm oil [68514-74-9].

DEFINITION
Hydrogenated Palm Oil is the product obtained by refining and hydrogenating the oil obtained from the pulp of the fruit of the oil palm *Elaeis guineensis* Jacq. (Fam. Aracaceae). The product consists mainly of triglycerides of palmitic and stearic acids.

IDENTIFICATION
- **A.** It meets the requirements of the test for *Fats and Fixed Oils, Fatty Acid Composition* ⟨401⟩.
- **B.** It meets the requirements of the test for *Melting Range or Temperature* ⟨741⟩.

IMPURITIES
- **RESIDUE ON IGNITION** ⟨281⟩
  Sample: 5 g
  Acceptance criteria: NMT 0.1%
- **LIMIT OF NICKEL**
  Sample solution: Weigh 5.0 g of Hydrogenated Palm Oil into a previously tared platinum or silica crucible. Cautiously heat the substance, and introduce into it a wick formed from twisted, ashless filter paper. Ignite the wick. When the substance ignites, stop heating. After combustion, ignite in a muffle furnace at about 600°. Continue the incineration until a white ash is obtained. After cooling, with the aid of two 2-mL portions of diluted hydrochloric acid, transfer the residue to a 25-mL

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volumetric flask, add 0.3 mL of nitric acid, and dilute with water to volume.

Nickel standard solution: Immediately before use, dilute 10 mL of nickel standard solution TS with water to 500 mL. This solution contains the equivalent of 0.2 µg/mL of nickel.

Standard solutions: Into four separate identical 10-mL volumetric flasks introduce respectively 0, 1.0, 2.0, and 4.0 mL of *Nickel standard solution*. To each flask add a 2.0-mL portion of the *Sample solution*, and dilute with water to volume to obtain four *Standard solutions* containing added quantities of 0, 0.2, 0.4, and 0.8 µg of nickel, respectively.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometer equipped with a graphite furnace

Analytical wavelength: 232.0 nm

Lamp: Nickel hollow-cathode

Analysis

Samples: *Standard solutions*

Concomitantly determine the absorbances of the *Standard solutions* at least three times each. Record the average of the steady readings for each of the *Standard solutions*. Plot the absorbances of the *Standard solutions* versus the added quantity, in µg, of nickel. Extrapolate the line joining the points on the graph until it meets the quantity axis. The distance between this point and the intersection of the axes represents the quantity of nickel in the 2-mL portion of the *Sample solution* added to the *Standard solutions*.

Calculate the content of nickel in the portion of Hydrogenated Palm Oil taken:

$$\text{Result} = [V \times (A/V_A)]/W$$

V = volume of the *Sample solution*, 25 mL
A = quantity of nickel (µg)
V_A = volume of the *Sample solution* added to the *Standard solutions*, 2 mL
W = weight of Hydrogenated Palm Oil taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 1 µg/g

- **ALKALINE IMPURITIES**

Sample: A mixture of 2.0 g of Hydrogenated Palm Oil, 1.5 mL of alcohol, and 3.0 mL of toluene

Analysis: Dissolve the *Sample* by gentle heating. Add 0.05 mL of bromophenol blue TS, and titrate with 0.01 N hydrochloric acid VS until the mixture turns yellow.

Acceptance criteria: NMT 0.4 mL of 0.01 N hydrochloric acid is required.

SPECIFIC TESTS

- **MELTING RANGE OR TEMPERATURE** ⟨741⟩**:** 58°–62°
- **FATS AND FIXED OILS,** *Acid Value, Method II* ⟨401⟩**:** NMT 2.0
- **FATS AND FIXED OILS,** *Fatty Acid Composition* ⟨401⟩**:** Hydrogenated Palm Oil exhibits the composition profile of fatty acids as shown in *Table 1*.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage
≤12	0	≤2.5
14	0	0.5–5.9
16	0	32.0–47.0
18	0	49.0–57.0
20	0	≤1.0
22	0	≤1.0
16	1	≤2.5
18	1	≤2.5
18	2	≤0.5
18	3	≤0.5

- **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩**:** NMT 5.0
- **FATS AND FIXED OILS,** *Unsaponifiable Matter* ⟨401⟩**:** NMT 0.8%
- **LOSS ON DRYING** ⟨731⟩

Analysis: Dry a sample at 105° for 4 h.

Acceptance criteria: NMT 0.1%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers. No storage requirement specified.

Palm Kernel Oil

Elaeis guineensis seed oil [8023-79-8].

DEFINITION

Palm Kernel Oil is the refined fixed oil obtained from the kernel of the fruit of the oil palm *Elaeis guineensis* Jacq. (Fam. Arecaceae). It may contain suitable antioxidants.

IDENTIFICATION

- **A.** It meets the requirements in *Specific Tests* for *Fats and Fixed Oils, Fatty Acid Composition* ⟨401⟩.
- **B.** It meets the requirements in *Specific Tests* for *Melting Range or Temperature* ⟨741⟩.

IMPURITIES

- **LIMIT OF LEAD**

[NOTE—For this test, use reagent-grade chemicals with as low a lead content as is practicable, as well as high-purity water and gases. Before use in this analysis, rinse all glassware and plasticware twice with diluted nitric acid and twice with diluted hydrochloric acid, and then rinse them thoroughly with Purified Water.]

Hydrogen peroxide–nitric acid solution: 10% hydrogen peroxide and diluted nitric acid (1:1). [NOTE—Use caution.]

Lead nitrate stock solution: Dissolve 159.8 mg of lead nitrate in 100 mL of *Hydrogen peroxide–nitric acid solution*. Dilute with *Hydrogen peroxide–nitric acid solution* to 1000 mL, and mix. Prepare and store this solution in glass containers that are free from lead salts. Each mL of this solution contains the equivalent of 100 µg of lead.

Standard lead solution: On the day of use, dilute 10.0 mL of *Lead nitrate stock solution* with *Hydrogen peroxide–nitric acid solution* to 100.0 mL, and mix. Each mL of *Standard lead solution* contains the equivalent of 10 µg of lead.

Butanol–nitric acid solution: Slowly add 50 mL of nitric acid to approximately 500 mL of butanol in a 1000-mL volumetric flask. Dilute with butanol to volume.

Standard solutions: Into five separate 100-mL volumetric flasks pipet 0.2, 0.5, 1, 2, and 5 mL, respectively, of *Standard lead solution*, and dilute with *Butanol–nitric acid solution* to volume. The *Standard solutions* contain 0.02, 0.05, 0.1, 0.2, and 0.5 µg/mL of lead, respectively.

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Sample solution: [**CAUTION**—Prepare this solution in a fume hood, and wear safety glasses.] Transfer 1.0 g of Oil into a large test tube. Add 1 mL of nitric acid. Place the test tube in a rack in a boiling water bath. As soon as the rusty tint is gone, add 1 mL of 30% hydrogen peroxide dropwise to avoid a vigorous reaction, and wait for bubbles to form. Stir with an acid-washed plastic spatula if necessary. Remove the test tube from the water bath, and allow it to cool. Transfer the solution to a 10-mL volumetric flask, and dilute with *Butanol–nitric acid solution* to volume.

Tungsten solution: Transfer 0.1 g of tungstic acid and 5 g of sodium hydroxide pellets to a 50-mL plastic bottle. Add 5.0 mL of water, and mix. Heat the mixture in a hot water bath until complete solution is achieved. Cool, and store at room temperature.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Graphite furnace atomic absorption spectrophotometry

Analytical wavelength: 283.3 nm lead emission line

Injection size: 20 µL

Lamp: Lead hollow-cathode

Furnace conditioning: Place the graphite tube in the furnace. Inject the *Tungsten solution* into the graphite tube, using an argon flow rate of 300 mL/min. Maintain the drying temperature at 110° for 20 s, the ashing temperature at 700°–900° for 20 s, and with the argon flow stopped, the atomization temperature at 2700° for 10 s; repeat this process once more using a second 20-µL aliquot of the *Tungsten solution*. Clean the quartz windows.

Analysis

Samples: *Standard solutions* and *Sample solution*

[NOTE—The sample injection technique is the most crucial step in controlling the precision of the analysis; the volume of each of the *Standard solutions* and the *Sample solution* must remain constant. Rinse the µL-pipet tip three times with either the *Standard solutions* or the *Sample solution* before injection. Use a fresh pipet tip for each injection, and start the atomization process immediately after injecting the *Samples*. Between injections, flush the graphite tube of any residual lead by purging at a high temperature recommended by the manufacturer.]

Concomitantly determine the absorbances of the *Samples*.

Atomize equal volumes of the *Standard solutions* and the *Sample solution* with an argon flow rate of 300 mL/min.

Maintain the drying temperature of the furnace at 110° for 30 s after a 20-s ramp time and a 10-s hold time; the ashing temperature at 700° for 42 s after a 20-s ramp time and a 22-s hold time; and the atomization temperature at 2300° for 7 s with the argon flow stopped.

Plot the absorbance of each of the *Standard solutions*, compensated for background correction, versus its content of lead, in µg/mL, and draw the best straight line fitting the five points. From this plot, determine the concentration, *C*, in µg/mL, of lead in the *Sample solution*.

Calculate the quantity, in µg/g, of lead in the portion of Oil taken:

$$\text{Result} = (C/W) \times V$$

C = measured concentration of lead in the *Sample solution* (µg/mL)

W = weight of the Oil taken to prepare the *Sample solution* (g)

V = final volume of the *Sample solution*, 10 mL

Acceptance criteria: NMT 0.1 µg/g of lead

SPECIFIC TESTS

- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩: NMT 2.0
- **FATS AND FIXED OILS,** *Fatty Acid Composition* ⟨401⟩: Palm Kernel Oil exhibits the composition profile of fatty acids shown in *Table 1*.

Table 1

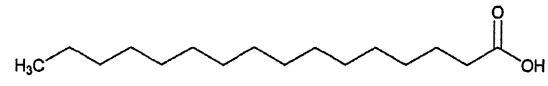
Carbon-Chain Length	Number of Double Bonds	Percentage (%)
6	0	≤1.5
8	0	3–5
10	0	2.5–6
12	0	40–52
14	0	14–18
16	0	7–10
18	0	1–3
20	0	≤1
16	1	≤1
18	1	11–19
18	2	0.5–4

- **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩: NMT 10.0
- **FATS AND FIXED OILS,** *Unsaponifiable Matter* ⟨401⟩: NMT 1.5%
- **MELTING RANGE OR TEMPERATURE** ⟨741⟩: 27°–29°
- **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 0.1%, 50 mL of chloroform being used instead of 35–40 mL of methanol as the solvent.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. Do not store above 45°.
- **LABELING:** Label it to indicate the name and quantity of any added antioxidants.

Palmitic Acid



$C_{16}H_{32}O_2$ 256.42

n-Hexadecanoic acid;
1-Pentadecanecarboxylic acid [57-10-3].

DEFINITION

Change to read:

Palmitic Acid is a mixture of ▲fatty▲ 2S (NF36) acids obtained from fats or oils of animal or vegetable origin. It contains NLT 92.0% of palmitic acid ($C_{16}H_{32}O_2$) and NMT 6.0% of stearic acid ($C_{18}H_{36}O_2$).

IDENTIFICATION

Change to read:

- **A.** ▲**CHROMATOGRAPHIC IDENTITY:**▲ 2S (NF36) The retention time of the major peak for palmitic acid of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

NF Monographs

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Add the following:

▲• **B. Acid Value**
Sample: 1 g of Palmitic Acid
Analysis: Proceed as directed in *Fats and Fixed Oils* ⟨401⟩, *Procedures, Acid Value.*
Acceptance criteria: 216–220 ▲2S (NF36)

ASSAY

Change to read:

• **Procedure**
▲**Solution A:** Add 1 mL of phosphoric acid to 1 L of water. This is a 0.1% phosphoric acid solution.
Solution B: Acetonitrile
Diluent: Methanol
Mobile phase: See *Table 1.*

Table 1

Time (min)	Solution A (%)	Solution B (%)
0.0	50	50
20.0	1	99
25.0	1	99
26.0	50	50
30.0	50	50

System suitability solution: 5.0 mg/mL of USP Myristic Acid RS and 0.025 mg/mL of linolenic acid in *Diluent*
Standard solution: 5.0 mg/mL of USP Palmitic Acid RS in *Diluent*
Sample solution: 5.0 mg/mL of Palmitic Acid in *Diluent*
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability.*)
Mode: LC
Detector: UV 210 nm
Column: 4.6-mm × 15-cm; 2.7-µm packing L1
Column temperature: 40°
Flow rate: 1.0 mL/min
Injection volume: 25 µL
Run time: 30 min
System suitability
Samples: *System suitability solution* and *Standard solution*
[Note—The relative retention times for linolenic acid, myristic acid, and palmitic acid are 0.77, 0.80, and 1.00, respectively.]
Suitability requirements
Resolution: NLT 1.5 between linolenic acid and myristic acid, *System suitability solution*
Tailing factor: 0.8–1.5, *Standard solution*
Relative standard deviation: NMT 0.5%, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of palmitic acid ($C_{16}H_{32}O_2$) in the portion of Palmitic Acid taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP Palmitic Acid RS in the *Standard solution* (mg/mL)
C_U = concentration of Palmitic Acid in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 92.0% of palmitic acid ($C_{16}H_{32}O_2$) ▲2S (NF36)

IMPURITIES

Delete the following:

▲• **Heavy Metals,** *Method II* ⟨231⟩**:** NMT 10 ppm ▲(Official 1-Jan-2018)

Add the following:

▲• **Limit of Stearic Acid, Myristic Acid, and Other Related Fatty Acids**
Solution A, Solution B, Diluent, Mobile phase, System suitability solution, and **Chromatographic system:** Proceed as directed in the *Assay.*
Standard solution: 0.3 mg/mL of USP Stearic Acid RS, 0.1 mg/mL of USP Myristic Acid RS, and 0.05 mg/mL of USP Palmitic Acid RS in *Diluent*
Sample solution: 5.0 mg/mL of Palmitic Acid in *Diluent*
System suitability
Samples: *System suitability solution* and *Standard solution*
[Note—The relative retention times for linolenic acid, myristic acid, palmitic acid, and stearic acid are 0.77, 0.80, 1.00, and 1.17, respectively.]
Suitability requirements
Resolution: NLT 1.5 between linolenic acid and myristic acid, *System suitability solution*
Relative standard deviation: NMT 5%, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Based on the *Standard solution* and *Table 2,* identify the peaks of appropriate fatty acids.
[Note—The relative retention times for several fatty acids are provided in *Table 2.*]

Table 2

Fatty Acid	Relative Retention Time	Relative Response Factor
Caprylic acid	0.20	1.85
Capric acid	0.36	1.54
Lauric acid	0.57	1.34
Myristic acid	0.80	—
Palmitic acid	1.00	1.00
Stearic acid	1.17	—

Calculate the percentage of myristic acid ($C_{14}H_{28}O$) or stearic acid ($C_{18}H_{36}O_2$) in the portion of Palmitic Acid taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of myristic acid or stearic acid from the *Sample solution*
r_S = peak response of myristic acid or stearic acid from the *Standard solution*
C_S = concentration of USP Myristic Acid RS or USP Stearic Acid RS in the *Standard solution* (mg/mL)

NF Monographs

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C_U = concentration of Palmitic Acid in the *Sample solution* (mg/mL)

Calculate the percentage of any other single fatty acid (caprylic acid, capric acid, or lauric acid, if present) in the portion of Palmitic Acid taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (1/F) \times 100$$

r_U = peak response of any other single fatty acid (caprylic acid, capric acid, or lauric acid, if present) from the *Sample solution*

r_S = peak response of palmitic acid from the *Standard solution*

C_S = concentration of USP Palmitic Acid RS in the *Standard solution* (mg/mL)

C_U = concentration of Palmitic Acid in the *Sample solution* (mg/mL)

F = relative response factor for any other single fatty acid (caprylic acid, capric acid, or lauric acid, if present) (see *Table 2*)

Acceptance criteria: Disregard peaks that are less than 0.05% for any individual impurity, and any peaks due to solvents.
Stearic acid: NMT 6.0%
Myristic acid: NMT 2.0%
Any other single fatty acid (caprylic acid, capric acid, or lauric acid): NMT 1.0%▲ 2S (NF36)

SPECIFIC TESTS
- **COLOR:** Heat a sample of Palmitic Acid to 75°. The resulting liquid is not more intensely colored than a solution prepared by mixing 1.2 mL of ferric chloride CS and 0.3 mL of cobaltous chloride CS with 0.3 N hydrochloric acid to make 10 mL, and diluting 5 mL of this solution with 0.3 N hydrochloric acid to make 100 mL. Make the comparison by viewing the solutions downward in matched color-comparison tubes against a white surface (see *Color and Achromicity* ⟨631⟩).
- **CONGEALING TEMPERATURE** ⟨651⟩: 60°–66°

Delete the following:

- ▲• **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩: 216–220, using 1 g▲ 2S (NF36)
- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Iodine Value, Method I*
  Analysis: Proceed as directed in the chapter, except use 35 mL of chloroform.
  Acceptance criteria: NMT 1
- **MINERAL ACID**
  Analysis: Shake 5 g of melted Palmitic Acid with an equal volume of hot water for 2 min. Cool, and filter.
  Acceptance criteria: The filtrate is not reddened by the addition of 1 drop of methyl orange TS.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at room temperature.
- **LABELING:** Label it to indicate whether it is derived from animal or vegetable sources.

Change to read:

- **USP REFERENCE STANDARDS** ⟨11⟩
  ▲USP Myristic Acid RS▲ 2S (NF36)
  USP Palmitic Acid RS
  USP Stearic Acid RS

Paraffin

[8002-74-2].

DEFINITION
Paraffin is a purified mixture of solid saturated hydrocarbons obtained from petroleum. It may contain suitable antioxidants.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F**▲ (CN 1-May-2020)
  Sample: Use a thin film of melted specimen.
  Analysis: Ensure complete melting to avoid doublet peaks that may be observed at wavenumbers at about 1460 and 730 cm^{-1}.
  Acceptance criteria: Meets the requirements
- **B.** It meets the requirements in *Specific Tests* for *Congealing Temperature* ⟨651⟩.

IMPURITIES
- **LIMIT OF SULFUR COMPOUNDS**
  Sample: 4.0 g
  Analysis: To the *Sample* add 2 mL of dehydrated alcohol, and add 2 drops of a clear saturated solution of lead(II) oxide in sodium hydroxide solution (200 mg/mL). Heat the mixture at 70° for 10 min with frequent shaking, and cool.
  Acceptance criteria: No dark brown color develops.
- **LIMIT OF POLYCYCLIC AROMATIC HYDROCARBONS**
  Dimethyl sulfoxide: Use spectrophotometric grade dimethyl sulfoxide.
  Standard solution: 7.0 µg/mL of USP Naphthalene RS in *Dimethyl sulfoxide*. Determine the absorbance of this solution at 278 nm using *Dimethyl sulfoxide* as the blank.
  Sample: 0.50 g
  Instrumental conditions
  (See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
  Mode: UV
  Wavelength range: 260–350 nm
  Cell: 1 cm
  Analysis: Dissolve the *Sample* in 25 mL of *n*-heptane, place in a 125-mL separator with unlubricated ground-glass parts (stopper, stopcock), and mix. Add 5.0 mL of *Dimethyl sulfoxide*, and shake the mixture vigorously for 1 min. Allow to stand until two clear layers are formed. Transfer the lower layer to another 125-mL separator, add 2 mL of *n*-heptane, and shake the mixture vigorously. Allow to stand until two clear layers are formed. Separate the lower layer, and determine its absorbance using as the blank *Dimethyl sulfoxide* that previously has been shaken vigorously for 1 min with *n*-heptane in the ratio of 5 mL of *Dimethyl sulfoxide* to 25 mL of *n*-heptane.
  Acceptance criteria: The absorbance at any wavelength in the specified range is not greater than one-third of the absorbance, at 278 nm, of the *Standard solution*.

SPECIFIC TESTS
- **CONGEALING TEMPERATURE** ⟨651⟩: 47°–65°
- **ACIDITY**
  Sample: 15 g
  Analysis: Introduce the *Sample* into a suitable separator, add 30 mL of boiling water, and shake vigorously for about 1 min. Allow to cool, and draw off the separated water. To 10 mL of the filtrated aqueous layer add 0.1 mL of phenolphthalein TS.
  Acceptance criteria: The solution does not produce a pink color. NMT 1.0 mL of 0.01 M sodium hydroxide is

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subsequently required to change the color of the indicator to pink.

• **ALKALINITY**
Sample: 10 mL of the filtrated aqueous layer obtained from the test for *Acidity*
Analysis: To the *Sample* add 0.1 mL of methyl red TS2.
Acceptance criteria: The solution produces a yellow color. NMT 0.5 mL of 0.01 M hydrochloric acid is subsequently required to change the color of the indicator to red.

• **READILY CARBONIZABLE SUBSTANCES** ⟨271⟩
Standard solution: A mix of 3 mL of ferric chloride CS, 1.5 mL of cobaltous chloride CS, and 0.50 mL of cupric sulfate CS, overlaid with 5 mL of mineral oil
Sample: 5 mL, at a temperature just above the melting point
Analysis: Use a clean, dry, heat-resistant, glass-stoppered test tube, 140 ± 2 mm in length with an outside diameter between 14.5 and 15.0 mm, and calibrated at the 5- and 10-mL liquid levels. The capacity of the tube with the stopper inserted is between 13.6 and 15.6 mL.[1] Place the *Sample* in the test tube, add 5 mL of sulfuric acid (94.5%–94.9% of H_2SO_4), and heat in a water bath at 70° for 10 min. When 5 min have elapsed, and at each successive min thereafter, remove the tube from the bath, place a finger over the stopper, and give the tube three vigorous vertical shakes over an amplitude of about 12 cm, returning the tube to the bath within 3 s after the time when it was removed therefrom.
Acceptance criteria: At the end of 10 min from the time the tube was placed in the bath, the acid (lower layer) has no more color than the *Standard solution*. If the sulfuric acid remains dispersed in the molten paraffin, the color of the emulsion is not darker than that of the *Standard solution* when shaken vigorously.

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in light resistant, well-closed containers, and avoid exposure to excessive heat.
• **LABELING:** Label it to indicate the name and quantity of any antioxidants.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Naphthalene RS
USP Paraffin RS

Synthetic Paraffin

DEFINITION
Synthetic Paraffin is synthesized by the Fischer-Tropsch process from carbon monoxide and hydrogen, which are catalytically converted to a mixture of paraffin hydrocarbons; the lower molecular weight fractions are removed by distillation, and the residue is hydrogenated and further treated by percolation through activated charcoal. This mixture may be fractionated into its components by a solvent separation method, using a suitable synthetic isoparaffinic petroleum hydrocarbon solvent. It may contain NMT 0.005% of a suitable antioxidant.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F.▲** (CN 1-May-2020) A thin film of it, cast from a melt onto a cesium bromide plate, exhibits a pair of very strong IR absorption peaks between 2840 cm^{-1} and 3000 cm^{-1}, a pair of moderately strong peaks between 1430 cm^{-1} and 1490 cm^{-1}, a pair of medium peaks between 720 cm^{-1} and 750 cm^{-1}

IMPURITIES
• **LIMIT OF OIL CONTENT**
Analysis: Follow ASTM Method D721-68, "Standard Test Method for Oil Content of Petroleum Waxes" (Reapproved 1987).[1]
Acceptance criteria: NMT 0.5%

SPECIFIC TESTS
• **ABSORPTIVITY**
Sample solution: Transfer 50–100 mg to a 100-mL volumetric flask. Dissolve in decahydronaphthalene at 88°, dilute with the same solvent at this temperature to volume, and mix.
Blank: Decahydronaphthalene
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: UV
Analytical wavelength: 290 nm
Cell: 10 cm (jacketed cells maintained at 88°)
Analysis
Samples: *Sample solution* and *Blank*
Determine the absorbance of the *Sample solution*, and calculate the absorptivity.
Acceptance criteria: NMT 0.01

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers.
• **LABELING:** The labeling indicates its congealing temperature, viscosity, and needle penetration range under the specified conditions.

Peanut Oil

[8002-03-7].

DEFINITION
Peanut Oil is the fully-refined (alkali-refined, bleached, and deodorized at 230°–260°) oil obtained from the seed kernels of one or more of the cultivated varieties of *Arachis hypogaea* L. (Fam. Leguminosae). It may contain suitable antioxidants.

IDENTIFICATION
• **A. IDENTITY BY FATTY ACID COMPOSITION**
Analysis: Proceed as directed in the test for *Fats and Fixed Oils* ⟨401⟩, *Procedures, Fatty Acid Composition*.
Acceptance criteria: Meets the composition profile of fatty acids in *Table 1*
• **B. IDENTITY BY TRIGLYCERIDE PROFILE**
Analysis: Proceed as directed in *Identification of Fixed Oils by Thin-Layer Chromatography* ⟨202⟩, *Identification, Method I* or *Method II*.
Acceptance criteria: Meets the requirements in the chapter

IMPURITIES
• **ALKALINE IMPURITIES**
Sample: 10 mL of Peanut Oil
Analysis: Mix 10 mL of freshly opened acetone and 0.3 mL of water, and add 0.05 mL of bromophenol blue TS. Add

[1] A suitable test tube is available from Kimble Kontes. Item number: 34-19426. Description: Nessler Tube. Contact: phone 800-682-6644, fax 856-692-6644, e-mail customglass@kimkon.com.

[1] Available from the American Society for Testing and Materials, 1916 Race St., Philadelphia, PA 19103.

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the *Sample*, shake, and allow to stand. Titrate with 0.01 N hydrochloric acid VS to change the color of the upper layer to yellow.

Acceptance criteria: NMT 0.1 mL of 0.01 N hydrochloric acid is required.

SPECIFIC TESTS

- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Fatty Acid Composition*: Peanut Oil exhibits the composition profile of fatty acids in *Table 1*.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
<14	0	≤0.1
14	0	≤0.2
16	0	7.0–16.0
16	1	≤1.0
18	0	1.3–6.5
18	1	35.0–72.0
18	2	13.0–43.0
18	3	≤0.6
20	0	0.5–3.0
20	1	0.5–2.1
22	0	1.0–5.0
22	1	≤0.5
24	0	0.5–3.0

- **RANCIDITY**

  Sample: 1 mL of a solution (1 in 10) of Peanut Oil in ether

  Analysis: Shake the *Sample* with 1 mL of hydrochloric acid, and add 1 mL of a solution (1 in 1000) of phloroglucinol in ether.

  Acceptance criteria: No red or pink color develops.
- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Acid Value*: NMT 0.2
- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Peroxide Value*: NMT 5.0
- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Unsaponifiable Matter*: NMT 1.5%
- **WATER DETERMINATION** ⟨921⟩, *Method I, Method Ic*: NMT 0.1%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and prevent exposure to excessive heat.
- **LABELING:** Label it to indicate the name and quantity of any added antioxidant. Where Peanut Oil is intended for use in the manufacture of injectable dosage forms, it is so labeled.
- **OTHER REQUIREMENTS:** For Peanut Oil intended for use in injectable dosage forms, which is specified in *Labeling*, the requirements under *Injections and Implanted Drug Products* ⟨1⟩, *Product Quality Tests Common to Parenteral Dosage Forms, Specific Tests, Vehicles and Added Substances, Nonaqueous Vehicles*, must be met.

Pectin—*see Pectin General Monographs*

Peppermint

DEFINITION

Peppermint consists of the dried leaf and flowering top of *Mentha piperita* L. (Fam. Labiatae).

SPECIFIC TESTS

- **ARTICLES OF BOTANICAL ORIGIN,** *Foreign Organic Matter* ⟨561⟩: NMT 2.0% of stems more than 3 mm in diameter and other foreign organic matter
- **BOTANIC CHARACTERISTICS**

  Unground peppermint: Leaves, slender stems, and flowering tops. The leaves are opposite, usually more or less crumpled, and frequently detached from the stem. The petiole is 4–15 mm in length, slightly pubescent; the blade, when entire, is ovate-oblong to oblong-lanceolate, 1.5–9 cm in length with an acute apex, a narrowed or rounded base, and a sharply serrate margin; light green to dark green in color; its upper surface is nearly glabrous, its lower surface has a few hairs on the veins and many amber-colored glandular hairs. The stem is quadrangular, 1–3 mm in diameter, glabrous except for a few scattered deflexed hairs, green to dark purple. The flowers occur as a compact, oblong or oval spike of verticillasters, 1–1.5 cm in breadth, rounded at the summit, and in fruit attaining a length of 3–7 cm. The bracts are oblong-lanceolate, 4–7 mm in length; the calyx, tubular-campanulate, equally five-toothed, pubescent, and glandular-punctate, green to dark purple; the corolla is glabrous, light purple, tubular-campanulate, four-cleft, 3 mm in length; stamens, 4, short and equal; style two- or rarely three-cleft at the summit. The nutlets are ellipsoidal, 500 µm in diameter. Peppermint has an aromatic, characteristic odor and a pungent taste, and produces a cooling sensation in the mouth.

  Histology

  Leaf: The lamina is dorsiventral. Both the upper and the lower epidermis consist of epidermal cells with wavy, anticlinal walls and stomata, the latter enclosed by a pair of subsidiary cells with a common wall at right angles to the guard cells. Many of the epidermal cells, especially over the veins and midrib, bear nonglandular and glandular hairs. The nonglandular hairs, also numerous along the margin, are uniseriate with longitudinally striate and papillose cuticle, up to 8 cells in length and tapered to a pointed apex. The glandular hairs occur in two types. The larger of these are sunken in depressions of the epidermis and consist of a one- to two-celled stalk and a glandular head of 8 radiating cells beneath the raised cuticle of which volatile oil is secreted. The smaller type of glandular hair consists of a one- to two-celled stalk and a one-celled glandular head containing volatile oil. Beneath the upper epidermis occurs a single layer of palisade parenchyma up to 80 µm in length and, directly underneath it, spongy parenchyma of 3 or 4 layers of chloroplastid-containing cells, through which zone course the fibrovascular tissues of the veins.

  Stem: The stem is quadrangular. It shows a layer of epidermis bearing hairs similar to those of the leaf and possessing cuticularized outer convex walls, a narrow cortex of chlorenchyma, a clear endodermis of tangentially elongated, thin-walled cells with colorless contents, a narrow phloem, a cambium, and a xylem broadest in the regions beneath the stem angles and containing narrow wood-wedges separated by xylem rays one cell in width. The wood-wedges consist chiefly of simple pitted and spiral vessels, tracheids, and wood-fibers. Beneath each of the four angles of the stem occurs an elliptic to ovate zone of collenchyma. A large

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pith composed of thin-walled parenchyma occupies the center.

Powdered peppermint: Green to light olive green. Shows fragments of leaf epidermis with wavy vertical walls and, if from the lower surface of the leaf, with numerous stomata and glandular and nonglandular hairs, the latter especially numerous along the veins; glandular hairs with a one- to two-celled stalk and one- to eight-celled head, usually set in a depression in the leaf and containing volatile oil and frequently yellowish or brownish crystals that are birefringent; nonglandular hairs with thin, papillose walls and frequently with short, longitudinal striations of 1–8 cells and up to 1.4 mm in length, the terminal cell pointed or sometimes globular; fragments of chlorenchyma with vascular tissue, the vessels spiral or with simple pits and but slightly lignified; fragments of collenchyma and of thin-walled, nonlignified fibers associated with parenchyma. The pollen grains are spheroidal and smooth.

Peppermint Oil

DEFINITION

Peppermint Oil is the volatile oil distilled with steam from the fresh overground parts of the flowering plant of *Mentha piperita* L. (Fam. Labiatae), rectified by distillation and neither partially nor wholly dementholized. It yields NLT 5.0% of esters, calculated as menthyl acetate ($C_{12}H_{22}O_2$), and NLT 50.0% of total menthol ($C_{10}H_{20}O$), free and as esters.

IDENTIFICATION

• **A.**

Sample: 6 drops of Oil

Analysis: Place the *Sample* in a dry test tube and mix with 5 mL of a 1-in-300 solution of nitric acid in glacial acetic acid, and place the tube in a beaker of boiling water.

Acceptance criteria: Within 5 min the liquid develops a blue color that, on continued heating, deepens and shows a copper-colored fluorescence and then fades, leaving a golden-yellow solution.

ASSAY

• **TOTAL ESTERS**

Sample: 10 g of Oil

Analysis: Place the *Sample* in a 250-mL conical flask, add 10 mL of neutralized alcohol and 2 drops of phenolphthalein TS, then add, dropwise, 0.1 N sodium hydroxide until a faint pink color appears. Add 25.0 mL of 0.5 N alcoholic potassium hydroxide VS, connect the flask to a reflux condenser, and heat on a boiling water bath for 1 h. Allow the mixture to cool, add 20 mL of water, and add phenolphthalein TS.

Titrate the excess alkali with 0.5 N hydrochloric acid VS. Perform a blank determination, disregarding the 0.1 N sodium hydroxide (see *Titrimetry* ⟨541⟩, *Residual Titrations*). Each mL of 0.5 N alcoholic potassium hydroxide consumed in the saponification is equivalent to 99.15 mg of total esters calculated as $C_{12}H_{22}O_2$.

Acceptance criteria: NLT 5.0% of esters, calculated as $C_{12}H_{22}O_2$

• **TOTAL MENTHOL**

Sample: 10 mL of Oil

Analysis: Place the *Sample* in an acetylation flask of 100-mL capacity, and add 10 mL of acetic anhydride and 1 g of anhydrous sodium acetate. Boil the mixture gently for 1 h, accurately timed, cool, disconnect the flask from the condenser, transfer the mixture to a small separator, rinsing the acetylation flask with three 5-mL portions of warm water, and add the rinsings to the separator. When the liquids have completely separated, discard the water layer, and wash the remaining oil with successive portions of sodium carbonate TS, diluted with an equal volume of water, until the last washing is alkaline to phenolphthalein TS. Dry the resulting oil with anhydrous sodium sulfate, and filter. Transfer 5 mL of the dry acetylated oil to a tared, 100-mL conical flask, and weigh. Add 50.0 mL of 0.5 N alcoholic potassium hydroxide VS, connect the flask to a reflux condenser, and boil the mixture on a steam bath for 1 h, accurately timed. Allow the mixture to cool, and add 10 drops of phenolphthalein TS.

Titrate the excess alkali with 0.5 N hydrochloric acid VS. Perform a blank determination (see *Titrimetry* ⟨541⟩, *Residual Titrations*).

Calculate the percentage of total menthol in the Oil tested:

$$\text{Result} = 7.813 \times A \times (1 - 0.0021 \times E)/(W - 0.021 \times A)$$

A = result obtained by subtracting the number of mL of 0.5 N hydrochloric acid required in the above titration from the number of mL of 0.5 N hydrochloric acid required in the residual titration blank

E = percentage of esters calculated as menthyl acetate ($C_{12}H_{22}O_2$)

W = weight of acetylated Oil taken (g)

Acceptance criteria: NLT 50.0% of $C_{10}H_{20}O$, free and as esters

IMPURITIES

• **LIMIT OF DIMETHYL SULFIDE**

Analysis: Distill 1 mL from 25 mL of Oil, and carefully superimpose the distillate on 5 mL of mercuric chloride TS in a test tube.

Acceptance criteria: A white film does not form at the zone of contact within 1 min.

SPECIFIC TESTS

• **SPECIFIC GRAVITY** ⟨841⟩: 0.896–0.908

• **OPTICAL ROTATION,** *Angular Rotation* ⟨781A⟩: −18° to −32°

• **REFRACTIVE INDEX** ⟨831⟩: 1.459–1.465 at 20°

• **SOLUBILITY IN 70% ALCOHOL:** One volume dissolves in 3 volumes of 70% alcohol, with NMT slight opalescence.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers, and prevent exposure to excessive heat.

Peppermint Spirit—*see Peppermint Spirit General Monographs*

Peppermint Water

DEFINITION

Peppermint Water is a clear, saturated solution of Peppermint Oil in Purified Water, prepared by one of the processes described in *Pharmaceutical Dosage Forms* ⟨1151⟩, *Solutions, Waters, Aromatic.*

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers.

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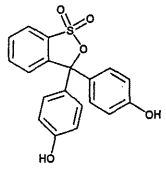
Petrolatum—*see Petrolatum General Monographs*

Petrolatum, Hydrophilic—*see Petrolatum, Hydrophilic General Monographs*

Petrolatum, White—*see Petrolatum, White General Monographs*

Phenol—*see Phenol General Monographs*

Phenolsulfonphthalein



$C_{19}H_{14}O_5S$ 354.38

Phenol red;
Phenol, 4,4′-(3*H*-2,1-benzoxathiol-3-ylidene)bis-,(*S,S*-dioxide);
3,3-Bis(4-hydroxyphenyl)-3*H*-2,1-benzoxathiole 1,1-dioxide [143-74-8].

DEFINITION

Phenolsulfonphthalein contains NLT 98.0% and NMT 102.0% of 4,4′-(3*H*-2,1-benzoxathiol-3-ylidene)bis-,(*S,S*-dioxide) phenol ($C_{19}H_{14}O_5S$), calculated on the dried basis.

IDENTIFICATION

• **A.**

Sample: 5 mg of Phenolsulfonphthalein

Analysis: Transfer the *Sample* to a 100-mL volumetric flask, dissolve in and dilute with sodium carbonate solution (1 in 100) to volume, and mix. Dilute 5.0 mL of the solution so obtained to 100.0 mL with sodium carbonate solution (1 in 100). Examine between 400 and 630 nm.

Acceptance criteria: The solution exhibits an absorption maximum at 558 nm, and the specific absorbance at the maximum is between 1900 and 2100.

• **B.**

Sample: 10 mg of Phenolsulfonphthalein

Analysis: Dissolve the *Sample* in 2 mL of 1 N sodium hydroxide, and add 8 mL of water. To 5 mL of the solution so obtained add 1 mL of 0.1 N potassium bromide–bromate and 1 mL of diluted hydrochloric acid, shake, and allow to stand for 15 min. Render the solution alkaline with 1 N sodium hydroxide.

Acceptance criteria: An intense violet-blue color is produced.

ASSAY

• **PROCEDURE**

Sample: 0.9 g

Titrimetric system
(See *Titrimetry* ⟨541⟩.)

Mode: Residual titration

Titrant: 0.1 N sodium thiosulfate VS

Endpoint detection: Visual

Analysis: Transfer the *Sample* to a 250-mL volumetric flask, dissolve in 15 mL of 1 N sodium hydroxide, dilute with water to volume, and mix. Transfer 10.0 mL of the solution so obtained to a glass-stoppered flask. Add 25 mL of glacial acetic acid, 20.0 mL of 0.1 N potassium bromate VS, 5 mL of potassium bromide solution (1 in 10), and 5 mL of hydrochloric acid, and immediately insert the stopper into the flask. Allow to stand protected from light for 15 min. Quickly add 10 mL of potassium iodide solution (1 in 10), taking care to avoid the escape of bromine vapor. Immediately insert the stopper into the flask, and shake vigorously. Rinse the stopper and the neck of the flask with a small quantity of water. Titrate the liberated iodine with 0.1 N sodium thiosulfate VS, using starch TS as the indicator. Perform a blank determination, and note the difference in volumes required. Each mL of the difference in volumes of 0.1 N sodium thiosulfate is equivalent to 4.43 mg of phenolsulfonphthalein ($C_{19}H_{14}O_5S$).

Acceptance criteria: 98.0%–102.0% on the dried basis

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩

Sample: 0.5 g

Acceptance criteria: NMT 0.2%

• **CHROMATOGRAPHIC PURITY**

Sample solution A: 20 mg/mL of Phenolsulfonphthalein in 0.1 N sodium hydroxide

Sample solution B: Transfer 0.5 mL of *Sample solution A* to a 100-mL volumetric flask, dilute with 0.1 N sodium hydroxide to volume, and mix.

Chromatographic system
(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Mode: TLC

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture

Application volume: 10 µL

Developing solvent system: *tert*-Amyl alcohol, glacial acetic acid, and water (4:1:1)

Analysis

Samples: *Sample solution A* and *Sample solution B*

Allow the plate to air-dry until the solvent has evaporated, and expose the plate to ammonia vapor. Examine the plate under short-wavelength UV light.

Acceptance criteria: NMT 0.5%; NMT one spot, apart from the principal spot, appears in the chromatogram from *Sample solution A*. This spot is not more intense than the spot in the chromatogram from *Sample solution B*.

• **INSOLUBLE SUBSTANCES**

Sample: 1 g of finely powdered Phenolsulfonphthalein

Analysis: To the *Sample* add a solution of 0.5 g of sodium bicarbonate in 12 mL of water. Allow to stand for 1 h, shaking frequently. Dilute with sufficient water to make 100 mL, and allow to stand for 15 h. Centrifuge at 2000–3000 *g* for 30 min, and decant the supernatant. Wash the residue first with 25 mL of sodium bicarbonate solution (1 in 100), then with 25 mL of water, and dry at 105°.

Acceptance criteria: The weight of the insoluble residue does not exceed 0.5% of the weight of the Phenolsulfonphthalein taken.

SPECIFIC TESTS

• **VISUAL TRANSITION INTERVAL**

Potassium chloride solution: Dissolve 1.0 g of potassium chloride in 100 mL of water, and adjust with 0.01 N hydrochloric acid or sodium hydroxide to a pH of 6.8.

Sample solution: Dissolve 0.1 g of Phenolsulfonphthalein in 100 mL of alcohol.

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Analysis 1: Add 0.15 mL of the *Sample solution* to the *Potassium chloride solution*.
Acceptance criteria 1: The color is yellow with NMT a faint trace of green color.
Analysis 2: Titrate the solution from *Analysis 1* with 0.01 N sodium hydroxide to a pH of 7.0.
Acceptance criteria 2: The color of the solution becomes orange.
Analysis 3: Continue the titration of the solution from *Analysis 2* with 0.01 N sodium hydroxide to a pH of 8.2.
Acceptance criteria 3: The color of the solution becomes red. NMT 0.20 mL of 0.01 N sodium hydroxide is consumed in the entire titration.

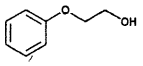
• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.

• **LOSS ON DRYING** ⟨731⟩
Sample: 1 g of powdered Phenolsulfonphthalein
Analysis: Dry the *Sample* at 105° to constant weight.
Acceptance criteria: NMT 1.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.

Phenoxyethanol



$C_8H_{10}O_2$ 138.16
2-Phenoxyethanol;
2-Phenoxyethyl alcohol;
Ethylene glycol, 2-monophenyl ether [122-99-6].

DEFINITION

Phenoxyethanol contains NLT 98.0% and NMT 102.0% of phenoxyethanol ($C_8H_{10}O_2$).

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F.▲** (CN 1-May-2020) On an undried specimen

ASSAY

• **PROCEDURE**
Phenol solution, Standard solution, and **Chromatographic system:** Prepare as directed in the test for *Organic Impurities*.
Sample stock solution: 5 mg/mL of Phenoxyethanol in isopropyl alcohol
Sample solution: Transfer 500 µL of the *Sample stock solution* to a vial, add 1000 µL of isopropyl alcohol, crimp the vial, and mix on a vortex mixer for 15 s.
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of phenoxyethanol ($C_8H_{10}O_2$) in the portion of Phenoxyethanol taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP Phenoxyethanol RS in the *Standard solution* (mg/mL)
C_U = concentration of Phenoxyethanol in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–102.0%

IMPURITIES

• **ORGANIC IMPURITIES**
Phenol solution: 0.25 mg/mL of phenol in isopropyl alcohol
Standard stock solution: 5 mg/mL of USP Phenoxyethanol RS in the *Phenol solution*
Standard solution: Transfer 500 µL of the *Standard stock solution* to a vial, add 1000 µL of isopropyl alcohol, crimp the vial, and mix on a vortex mixer for 15 s.
Sample solution: Transfer 500 µL of Phenoxyethanol to a tared vial, and determine the weight of Phenoxyethanol taken. Add 1000 µL of isopropyl alcohol, crimp the vial, and mix on a vortex mixer for 15 s.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.32-mm × 10-m capillary coated with a 5-µm film of stationary phase G27
Temperatures
Injection port: 300°
Detector: 300°
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
80	8	260	10

Carrier gas: Helium
Injection volume: 1 µL
Injection type: Split injection mode
Split flow rate: 44 mL/min
System suitability
Sample: *Standard solution*
Suitability requirements
Resolution: NLT 10 between the phenol and phenoxyethanol peaks
Relative standard deviation: NMT 2.0% for the phenoxyethanol peak
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of total impurities in the portion of Phenoxyethanol taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of all additional peak areas in the *Sample solution*, excluding the main peak, the solvent peak, and the phenol peak
r_S = peak response of phenoxyethanol from the *Standard solution*
C_S = concentration of phenoxyethanol in the *Standard solution* (mg/mL)
C_U = concentration of the *Sample solution* (mg/mL)

Acceptance criteria: NMT 1.0%

• **LIMIT OF PHENOL**
Phenol solution, Standard solution, Sample solution, and **Chromatographic system:** Proceed as directed in the test for *Organic Impurities*.

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Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of phenol in the portion of Phenoxyethanol taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of phenol from the *Sample solution*
r_S = peak response of phenol from the *Standard solution*
C_S = concentration of phenol in the *Standard solution* (mg/mL)
C_U = concentration of the *Sample solution* (mg/mL)

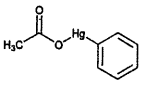
Acceptance criteria: NMT 0.1%

SPECIFIC TESTS
• **SPECIFIC GRAVITY** ⟨841⟩**:** 1.105–1.110 at 20°

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight containers, and store in a cool, dry place, protected from light.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Phenoxyethanol RS

Phenylethyl Alcohol—*see Phenylethyl Alcohol General Monographs*

Phenylmercuric Acetate



$C_8H_8HgO_2$ 336.74
Mercury, (acetato-*O*)phenyl-;
(Acetato)phenylmercury [62-38-4].

DEFINITION
Phenylmercuric Acetate contains NLT 98.0% and NMT 100.5% of phenylmercuric acetate ($C_8H_8HgO_2$).

IDENTIFICATION
• **A.**
Sample: 0.1 g
Analysis: To the *Sample* add 0.5 mL of nitric acid. Warm gently until a dark brown color is produced, and dilute with water to 10 mL.
Acceptance criteria: The characteristic odor of nitrobenzene is evolved.
• **B.**
Sample: 0.1 g
Analysis: To the *Sample* add 0.5 mL of sulfuric acid and 1 mL of alcohol, and warm.
Acceptance criteria: The characteristic odor of ethyl acetate is evolved.
• **C.**
Sample solution: Saturated solution in water
Analysis: To 5 mL of the *Sample solution* add a few drops of sodium sulfide TS.
Acceptance criteria: A white precipitate is formed, which turns black when the mixture is boiled and then allowed to stand.

ASSAY
• **PROCEDURE**
Sample solution: Transfer 500 mg of Phenylmercuric Acetate to a 100-mL flask. Add 15 mL of water, 5 mL of formic acid, and 1 g of zinc dust, and reflux for 30 min. Cool, filter, and wash the filter paper and the amalgam with water until the washings are no longer acid to litmus. Dissolve the amalgam in 40 mL of 8 N nitric acid. Heat on a steam bath for 3 min, and then add 500 mg of urea and enough potassium permanganate TS to produce a permanent pink color. Cool, decolorize the solution with hydrogen peroxide TS, and add 1 mL of ferric ammonium sulfate TS.
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.1 N ammonium thiocyanate VS
Endpoint detection: Visual
Analysis: Titrate with *Titrant*, and each mL of 0.1 N ammonium thiocyanate is equivalent to 16.84 mg of phenylmercuric acetate ($C_8H_8HgO_2$).
Acceptance criteria: 98.0%–100.5%

IMPURITIES
• **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 0.2%
• **POLYMERCURATED BENZENE COMPOUNDS**
Sample solution: Shake 2.0 g of Phenylmercuric Acetate with 100 mL of acetone, and filter.
Analysis: Wash the residue with successive portions of acetone until a total of 50 mL is used, then dry the residue at 105° for 1 h. Weigh the residue.
Acceptance criteria: NMT 1.5%; the weight of residue is NMT 30 mg.

SPECIFIC TESTS
• **MELTING RANGE OR TEMPERATURE** ⟨741⟩**:** 149°–153°

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

Phenylmercuric Nitrate

Mercury, (nitrato-*O*)phenyl-;
Nitratophenylmercury [55-68-5].

DEFINITION
Phenylmercuric Nitrate is a mixture of phenylmercuric nitrate and phenylmercuric hydroxide containing NLT 87.0% and NMT 87.9% of phenylmercuric ion ($C_6H_5Hg^+$), and NLT 62.75% and NMT 63.50% of mercury (Hg).

IDENTIFICATION
• **A.**
Sample: 0.1 g
Analysis: To the *Sample* add 3 mL of sulfuric acid.
Acceptance criteria: The mixture becomes yellow, and the characteristic odor of nitrobenzene is evolved.
• **B.**
Sample solution: Saturated solution in water
Analysis: To 5 mL of the *Sample solution* add 1 mL of 3 N hydrochloric acid.
Acceptance criteria: A white precipitate is formed.
• **C.**
Sample solution: Saturated solution in water
Analysis: To 5 mL of the *Sample solution* add 5 mL of ammonium sulfide TS.

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Acceptance criteria: There is no reaction in the cold, but upon heating in a boiling water bath for 10 min, a black precipitate is formed.

ASSAY

• **PHENYLMERCURIC IONS**

Sample: 200 mg

Analysis: Dissolve the *Sample* in 90 mL of water and 10 mL of nitric acid. Add 2 mL of ferric ammonium sulfate TS. Titrate with 0.05 N ammonium thiocyanate VS. Each mL of 0.05 N ammonium thiocyanate is equivalent to 13.88 mg of phenylmercuric ion ($C_6H_5Hg^+$).

Acceptance criteria: 87.0%–87.9% of phenylmercuric ion

• **MERCURY**

Sample solution: Transfer 400 mg of Phenylmercuric Nitrate to a 100-mL flask. Add 15 mL of water, 5 mL of formic acid, and 1 g of zinc dust, and reflux for 30 min. Cool. Filter, and wash the filter paper and the amalgam with water until the washings are no longer acid to litmus. Dissolve the amalgam in 40 mL of 8 N nitric acid. Heat on a steam bath for 3 min, then add 0.5 g of urea and enough potassium permanganate TS to produce a permanent pink color. Cool. Decolorize the solution with hydrogen peroxide TS, and add 1 mL of ferric ammonium sulfate TS.

Analysis: Titrate with 0.1 N ammonium thiocyanate VS. Each mL of 0.1 N ammonium thiocyanate is equivalent to 10.03 mg of Hg.

Acceptance criteria: 62.75%–63.50% of mercury

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%

SPECIFIC TESTS

• **MERCURY IONS**

Sample solution: Saturated solution in water

Analysis: To 5 mL of the *Sample solution* add 5 mL of 1 N sodium hydroxide.

Acceptance criteria: No yellow precipitate is formed (mercuric ions), and the solution does not darken (mercurous ions).

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

Phosphoric Acid

H_3PO_4 98.00

Phosphoric acid [7664-38-2].

DEFINITION

Phosphoric Acid contains NLT 85.0% and NMT 88.0%, by weight, of H_3PO_4.

[**CAUTION**—Avoid contact, because Phosphoric Acid rapidly destroys tissues.]

IDENTIFICATION

• **A. IDENTIFICATION TESTS—GENERAL,** *Phosphate* ⟨191⟩: When carefully neutralized with 1 N sodium hydroxide, phenolphthalein TS being used as the indicator, it meets the requirements.

ASSAY

• **PROCEDURE**

Sample: 1 g

Blank: 120 mL of water

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Direct titration

Titrant: 1 N sodium hydroxide VS

Endpoint detection: Visual

Analysis: Place the *Sample* in a tared, glass-stoppered flask, and dilute it with water to 120 mL. Add 0.5 mL of thymolphthalein TS. Titrate with 1 N sodium hydroxide VS to the first appearance of a blue color. Perform a blank determination.

Calculate the percentage of phosphoric acid (H_3PO_4) in the *Sample* taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

V_S = volume of *Titrant* consumed by the *Sample* (mL)
V_B = volume of *Titrant* consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 49.00 mg/mEq
W = weight of the *Sample* (mg)

Acceptance criteria: 85.0%–88.0% by weight

IMPURITIES

• **LIMIT OF NITRATE**

Sample solution: Dilute 6 mL of Phosphoric Acid with 14 mL of water.

Analysis: Mix 5 mL of the *Sample solution* with about 0.1 mL of indigo carmine TS, then add 5 mL of sulfuric acid.

Acceptance criteria: The blue color is not discharged within 1 min.

SPECIFIC TESTS

• **CHLORIDE AND SULFATE,** *Sulfate* ⟨221⟩

Sample solution: Dilute 6 mL of Phosphoric Acid with 90 mL of water.

Analysis: To the *Sample solution* add 1 mL of barium chloride TS.

Acceptance criteria: No precipitate is formed immediately.

• **ALKALI PHOSPHATES**

Sample: 1 mL

Analysis: Transfer the *Sample* to a graduated cylinder, and add 6 mL of ether and 2 mL of alcohol.

Acceptance criteria: No turbidity is produced.

• **PHOSPHOROUS OR HYPOPHOSPHOROUS ACID**

Sample solution: Dilute 6 mL of Phosphoric Acid with 14 mL of water.

Analysis: Gently warm 5 mL of the *Sample solution*, and add 2 mL of silver nitrate TS.

Acceptance criteria: The mixture does not become brownish.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers.

Diluted Phosphoric Acid

DEFINITION

Diluted Phosphoric Acid contains, in each 100 mL, NLT 9.5 g and NMT 10.5 g of phosphoric acid (H_3PO_4).

Diluted Phosphoric Acid may be prepared as follows.

Phosphoric Acid	69 mL
Purified Water, a sufficient quantity to make	1000 mL

Mix the ingredients.

IDENTIFICATION

• **A. IDENTIFICATION TESTS—GENERAL,** *Phosphate* ⟨191⟩

Sample: 100 mL

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Analysis: Carefully neutralize the *Sample* with 1 N sodium hydroxide. Use phenolphthalein TS as the indicator.
Acceptance criteria: Meets the requirements

ASSAY

• **PROCEDURE**
Sample: 10 mL
Blank: 50 mL of water
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 1 N sodium hydroxide VS
Endpoint detection: Visual
Analysis: Dilute the *Sample* with water to 50 mL. Add 0.5 mL of thymolphthalein TS. Titrate with *Titrant* to the first appearance of a blue color. Perform a blank determination. Calculate the amount of phosphoric acid (H_3PO_4) in the portion of the sample taken:

$$\text{Result} = (V_S - V_B) \times N \times F$$

V_S = *Titrant* volume consumed by the *Sample* (mL)
V_B = *Titrant* volume consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 4.9×10^{-2} g/mEq

Acceptance criteria: 9.5–10.5 g per 100 mL

IMPURITIES

• **LIMIT OF NITRATE**
Sample: 100 mL
Analysis: To the *Sample* add 0.1 mL of indigo carmine TS, then 5 mL of sulfuric acid.
Acceptance criteria: The blue color is not discharged within 1 min.

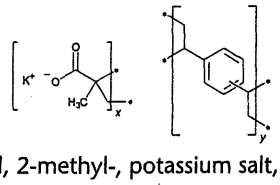
SPECIFIC TESTS

• **ALKALI PHOSPHATES**
Sample: 20 mL
Analysis: Evaporate the *Sample* on a steam bath to a weight of 5 g. Cool, transfer 2 mL to a graduated cylinder, and add 6 mL of ether and 2 mL of alcohol.
Acceptance criteria: No turbidity is produced
• **PHOSPHOROUS OR HYPOPHOSPHOROUS ACID**
Sample: 100 mL
Analysis: Gently warm the *Sample*, and add 2 mL of silver nitrate TS.
Acceptance criteria: The mixture does not become brownish.
• **CHLORIDE AND SULFATE,** *Sulfate* ⟨221⟩
Sample: 100 mL
Analysis: To the *Sample* add 1 mL of barium chloride TS.
Acceptance criteria: No precipitate is formed immediately.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers.

Polacrilin Potassium



2-Propenoic acid, 2-methyl-, potassium salt, polymer with diethenylbenzene;

Potassium methacrylate–divinylbenzene, copolymer [65405-55-2].

DEFINITION

Polacrilin Potassium is the potassium salt of a unifunctional low-cross-linked carboxylic cation-exchange resin prepared from methacrylic acid and divinylbenzene. When previously dried at 105° for 6 h, it contains NLT 20.6% and NMT 25.1% of potassium (K).

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
• **B. IDENTIFICATION TESTS—GENERAL,** *Potassium* ⟨191⟩
Sample solution A: Shake 1 g with 10 mL of water.
Sample solution B: Shake 1 g with 10 mL of 0.1 N hydrochloric acid.
Analysis: Use the aqueous phase from *Sample solution A* and *Sample solution B*.
Acceptance criteria
Sample solution A: Does not meet the requirements
Sample solution B: Meets the requirements

ASSAY

• **CONTENT OF POTASSIUM**
Sodium stock solution: 14.612 mg/mL of sodium chloride, previously dried at 125° for 30 min. This solution contains 5.76 mg/mL of sodium (Na).
Potassium stock solution: 745.5 µg/mL of potassium chloride, previously dried at 125° for 30 min. This solution contains 391 µg/mL of potassium (K).
Surfactant solution: Transfer 5.0 g of a suitable nonionic surfactant to a 250-mL beaker, add 200 mL of water, and stir to dissolve. Transfer this solution to a 500-mL volumetric flask, dilute with water to volume, and mix. [NOTE—To prevent foaming when using this solution, gently run the solution down the sides of the vessel, and use gentle action when mixing.]
Diluted sodium solution: Transfer 50.0 mL of *Sodium stock solution* and 10.0 mL of *Surfactant solution* to a 100-mL volumetric flask, dilute with water to volume, and mix gently to prevent foaming.
Standard solutions: To three separate 500-mL volumetric flasks transfer, respectively, 3.0-, 4.0-, and 5.0-mL portions of *Potassium stock solution*. To each flask add 50.0 mL of *Sodium stock solution* and 10.0 mL of *Surfactant solution*, dilute with water to volume, and mix gently to prevent foaming. Each mL of these solutions contains 2.346, 3.128, and 3.910 µg of K, respectively.
Sample solution: Transfer 1.4 g of Polacrilin Potassium, previously dried, to a 50-mL silica crucible, moisten with 4 mL of sulfuric acid, heat over a small flame until the acid has fumed off, moisten the residue with a few drops of sulfuric acid, and ignite strongly. Allow to cool, transfer, with the aid of water, to a 1000-mL volumetric flask, dilute with water to volume, and mix. Transfer 1.00 mL of this solution to a 100-mL volumetric flask, add 20.0 mL of *Diluted sodium solution*, dilute with water to volume, and mix gently to prevent foaming.
Instrumental conditions
Mode: Flame photometry
Analytical wavelength: 766 nm
Analysis
Samples: *Standard solutions* and *Sample solution*
Concomitantly determine the emittances of the *Standard solutions* and *Sample solution*, adjusting the instrument so that the most concentrated *Standard solution* gives a reading near 100%.

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Prepare a standard curve by plotting the readings from the *Standard solutions* versus the square root of the potassium concentrations. From the curve, determine the concentration of potassium in the *Sample solution*.
Calculate the percentage of potassium in the portion of sample taken:

$$\text{Result} = (C_O/C_U) \times 100$$

C_O = concentration of the *Sample solution* determined from the standard curve (µg/mL)
C_U = concentration of Polacrilin Potassium in the *Sample solution* (µg/mL)

Acceptance criteria: 20.6%–25.1%

IMPURITIES

• **IRON** ⟨241⟩
Sample: 0.10 g
Analysis: Transfer the *Sample* to a suitable crucible, and ignite at a low heat until thoroughly ashed. Add to the carbonized mass 2 mL of nitric acid and 5 drops of sulfuric acid, and heat cautiously until white fumes are no longer evolved. Ignite, preferably in a muffle furnace, at 500°–600°, until the carbon is completely burned off. Cool, add 4 mL of 6 N hydrochloric acid, cover, digest on a steam bath for 15 min, uncover, and slowly evaporate on a steam bath to dryness. Moisten the residue with 1 drop of hydrochloric acid, add 10 mL of hot water, and digest for 2 min. Dilute with water to 25 mL. Filter, if necessary. Rinse the crucible and the filter with 10 mL of water, combining the filtrate and rinsing in a 50-mL color-comparison tube, add 2 mL of hydrochloric acid, dilute with water to 45 mL, and mix.
Acceptance criteria: NMT 0.01%

• **LIMIT OF SODIUM**
Sample solution: Transfer 2 g to a 400-mL borosilicate beaker, add 20 mL of sulfuric acid, cover with a borosilicate watch glass, and heat until charring is complete. While continuing to heat the beaker, add 20 mL of nitric acid dropwise. Continue to heat, and add nitric acid until all of the organic material has been destroyed as indicated by the contents of the beaker turning from brown to a very pale straw-colored or colorless solution. Continue to evaporate the solution, and if it turns brown during the evaporation, add nitric acid dropwise until the brown color disappears. Evaporate just to dryness, cool, and dissolve the residue in 40 mL of water and 10 mL of 6 N hydrochloric acid. Heat to boiling, cool, transfer to a 100-mL volumetric flask, dilute with water to volume, and mix.
Standard solutions: To three separate 100-mL volumetric flasks add, respectively, 1.00, 2.00, and 3.00 mL of a solution containing 254.2 mg of sodium chloride in 1000 mL of water. Add water to volume, and mix to obtain sodium chloride solutions having concentrations equivalent to 1, 2, and 3 µg/mL of Na, respectively.
Instrumental conditions
Mode: Flame photometry
Analytical wavelength: 589 nm
Analysis
Samples: *Standard solutions* and *Sample solution*
Adjust the instrument so that the emission of the *Standard solution* with a concentration of 3 µg/mL reads close to 100% at 589 nm.
Determine the readings of the three *Standard solutions* at 589 nm. Readjust the wavelength setting to 580 nm, and determine the background emission reading for one of these *Standard solutions*.
Pipet 5 mL of the *Sample solution* into a 100-mL volumetric flask, add water to volume, and mix. Observe the emission reading of this solution at 589 nm, using the same instrument settings, then readjust the wavelength setting to 580 nm, and observe the background emission reading.
Subtract the corresponding background readings from the readings of *Standard solutions* and *Sample solution*.
Prepare a standard curve by plotting the corrected *Standard solution* readings versus the square root of the sodium concentration. From this standard curve, determine the sodium content in the sample taken.
Acceptance criteria: NMT 0.20%

SPECIFIC TESTS

• **POWDER FINENESS** ⟨811⟩
Sample: 4 g
Analysis: Transfer the *Sample* to a No. 100 standard sieve placed on top of a No. 200 standard sieve and pan. Using a soft 2-cm brush, brush the sample lightly across the No. 100 sieve until no more particles pass through. By brushing and tapping, dust off the particles on the underside of the No. 100 sieve into the No. 200 sieve. Obtain the weight of the material retained on the No. 100 sieve. Similarly, determine the weight of material retained by the No. 200 sieve.
Acceptance criteria: NMT 1.0% is retained on the No. 100 sieve, and NMT 30.0% is retained on the No. 200 sieve.

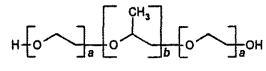
• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry at 105° for 6 h.
Acceptance criteria: NMT 10.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

• **USP REFERENCE STANDARDS** ⟨11⟩
USP Polacrilin Potassium RS

Poloxamer



$HO(C_2H_4O)_a(C_3H_6O)_b(C_2H_4O)_aH$
Oxirane, methyl-, polymer with oxirane;
α-Hydro-ω-hydroxypoly(oxyethylene)$_a$-poly(oxypropylene)$_b$-poly(oxyethylene)$_a$ block copolymer, in which a and b have the values shown in the following table:

Poloxamer	a	b
124	12	20
188	80	27
237	64	37
338	141	44
407	101	56

Polyethylene-polypropylene glycol [9003-11-6].

DEFINITION
Poloxamer is a synthetic block copolymer of ethylene oxide and propylene oxide. It is available in several types, conforming to the requirements shown in the following table.

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Poloxamer	Physical Form	Average Molecular Weight	Weight (% Oxyethylene)	Unsaturation (mEq/g)
124	Liquid	2090–2360	46.7 ± 1.9	0.020 ± 0.008
188	Solid	7680–9510	81.8 ± 1.9	0.026 ± 0.008
237	Solid	6840–8830	72.4 ± 1.9	0.034 ± 0.008
338	Solid	12,700–17,400	83.1 ± 1.7	0.031 ± 0.008
407	Solid	9840–14,600	73.2 ± 1.7	0.048 ± 0.017

It may contain a suitable antioxidant.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F.▲** (CN 1-May-2020) USP Poloxamer Liquid RS for Poloxamer 124, and use USP Poloxamer Solid RS for Poloxamer 188, 237, 338, and 407. Because of the differences in the ratios of copolymer composition, the intensity of some absorption bands may vary.

ASSAY

• **AVERAGE MOLECULAR WEIGHT**

Phthalic anhydride–pyridine solution: Dissolve 144 g of phthalic anhydride in freshly opened or freshly distilled pyridine containing less than 0.1% of water, and dilute with pyridine to 1000 mL. Protect from light, and allow to stand overnight. To verify that the *Phthalic anhydride–pyridine solution* has adequate strength, pipet 10 mL into a 250-mL conical flask, add 25 mL of pyridine and 50 mL of water, and after 15 min add 0.5 mL of a solution of phenolphthalein in pyridine (1 in 100), then titrate with 0.5 N sodium hydroxide VS: it consumes between 37.6 and 40.0 mL of 0.5 N sodium hydroxide.

Analysis: Weigh a suitable quantity, not exceeding 15 g, of Poloxamer, calculated by multiplying the average molecular weight by 0.004, into a glass-stoppered, 250-mL boiling flask. Carefully pipet 25 mL of *Phthalic anhydride–pyridine* solution into the flask, touching the tip of the drained pipet to the protrusion in the flask. Add a few glass beads, and swirl to dissolve the specimen. Pipet 25 mL of *Phthalic anhydride–pyridine solution* into a second, glass-stoppered, conical flask, add a few glass beads, and use as the reagent blank. (An additional 25-mL portion of pyridine may be added to both the test specimen and reagent blank, before refluxing, if necessary to ensure fluidity.) Heat both flasks, fitted with suitable reflux condensers, and allow to reflux for 1 h. Allow to cool, and pour two 10-mL portions of pyridine through each condenser. Remove the flasks from the condensers, add 10 mL of water to each, insert the stoppers, swirl, and allow to stand for 10 min. To each flask add 50.0 mL of 0.66 N sodium hydroxide and 0.5 mL of a solution (1 in 100) of phenolphthalein in pyridine. Titrate with 0.5 N sodium hydroxide VS to a light pink endpoint that persists for NLT 15 s.

Calculate the average molecular weight:

$$\text{Result} = 2000 \times W/[(V_B - V_S) \times N]$$

W = weight of the sample taken (g)
V_B = volume of 0.5 N sodium hydroxide VS consumed by the blank (mL)
V_S = volume of 0.5 N sodium hydroxide VS consumed by the residual acid in the test solution (mL)
N = actual normality of the 0.5 N sodium hydroxide VS

Acceptance criteria: See the table in the *Definition*.

• **WEIGHT PERCENT OXYETHYLENE**

Solvent: Use deuterated water or deuterochloroform.

NMR reference: Use sodium 2,2-dimethyl-2-silapentane-5-sulfonate (for deuterated water) or tetramethylsilane (for deuterochloroform).

Sample solution: Dissolve 0.1–0.2 g of Poloxamer in deuterated water containing 1% of sodium 2,2-dimethyl-2-silapentane-5-sulfonate to obtain 1 mL of solution, or, if the Poloxamer does not dissolve in water, use deuterochloroform containing 1% of tetramethylsilane as the solvent.

Instrumental conditions

(See *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩, *Relative Method of Quantitation*.)

Mode: Nuclear magnetic spectrometry

Sample size: 0.5–1.0 mL of the *Sample solution*

Analysis:

Sample: *Sample solution*

Transfer the *Sample solution* to a standard 5-mm NMR spinning tube, and if deuterochloroform is the solvent, add 1 drop of deuterated water, and shake the tube. Scan the region at 0–5 ppm, and use the calculation formulas specified below. Record as A_1 the average area of the doublet appearing at about 1.08 ppm, representing the methyl groups of the oxypropylene units, and record as A_2 the average area of the composite band at a range of 3.2–3.8 ppm, due to the CH_2O groups of both the oxyethylene and oxypropylene units and also the CHO groups of the oxypropylene units, with reference to the sodium 2,2-dimethyl-2-silapentane-5-sulfonate or tetramethylsilane singlet at 0 ppm.

Calculate the percentage of oxyethylene, by weight, in the Poloxamer taken:

$$\alpha = (A_2/A_1) - 1$$

A_2 = average area of the composite band at a range of 3.2–3.8 ppm
A_1 = average area of the doublet appearing at about 1.08 ppm

$$\text{Result} = 3300 \times \alpha/(33 \times \alpha + 58)$$

Acceptance criteria: See the table in the *Definition*.

• **UNSATURATION**

Solution A: Place 50 g of mercuric acetate in a 1000-mL volumetric flask, and dissolve with 900 mL of methanol to which 0.5 mL of glacial acetic acid has been added. Dilute with methanol to volume, and mix. Discard the solution if it is yellow. If it is turbid, filter it. Discard it if it is still turbid. Use fresh reagents if it is necessary to repeat the preparation of the solution. Protect the solution from light by storing it in an amber bottle in the dark.

Sample: 15.0 g

Analysis: Transfer the *Sample* to a 250-mL conical flask. Pipet 50 mL of *Solution A* into the flask, and mix on a magnetic stirrer until solution is complete. Allow to stand for 30 min with occasional swirling. Add 10 g of sodium bromide crystals, and stir on a magnetic stirrer for 2 min. Without delay, add 1 mL of phenolphthalein TS, and titrate the liberated acetic acid with 0.1 N methanolic potassium hydroxide VS. Perform a blank determination. Determine also the initial acidity as follows. Dissolve 15.0 g of Poloxamer in 75 mL of methanol that has been neutralized with methanolic potassium hydroxide to the

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phenolphthalein endpoint. Add 1 mL of phenolphthalein TS, and titrate with the same 0.1 N methanolic potassium hydroxide VS under a nitrogen sweep.
Calculate the unsaturation, in mEq/g:

$$\text{Result} = (V_U - V_B - V_A) \times N/15$$

V_U = volume of 0.1 N methanolic potassium hydroxide used for titrating the test specimen (mL)
V_B = volume of 0.1 N methanolic potassium hydroxide used for titrating the blank (mL)
V_A = volume of 0.1 N methanolic potassium hydroxide used for titrating the initial acidity (mL)
N = normality of the titrant

Acceptance criteria: See the table in the *Definition*.

IMPURITIES

• **LIMIT OF FREE ETHYLENE OXIDE, PROPYLENE OXIDE, AND 1,4-DIOXANE**

Stripped poloxamer: Place 500 g of Poloxamer 124 into a suitable 3-neck, round-bottom flask equipped with a stirrer, a thermometer, a vacuum outlet, and a heating mantle. Evacuate the flask carefully at room temperature to a pressure of less than 10 mm of mercury, applying the vacuum slowly to avoid excessive foaming due to entrapped gases. After any foaming has subsided, heat the flask to 80° and continue to apply vacuum for 2 h; then cool to room temperature. Shut off the vacuum pump, and introduce nitrogen to bring the flask pressure back to atmospheric pressure. Transfer the *Stripped poloxamer* to a suitable nitrogen-filled container.

Standard solution

[**CAUTION**—Ethylene oxide, propylene oxide, and 1,4-dioxane are toxic and flammable. Prepare these solutions in a well-ventilated fume hood.]

To a tared vial that can be sealed add 50.0 g of *Stripped poloxamer*. Add 60 µL of 1,4-dioxane and 75 µL of propylene oxide from a chilled syringe. Add ethylene oxide, using the following special handling procedure. Ethylene oxide, which is a gas at room temperature, is usually stored in a lecture-type gas cylinder or a small, metal pressure-bomb. Chill the cylinder in a refrigerator before use. Transfer 5 mL of the liquid ethylene oxide to a 100-mL beaker chilled in wet ice. Using a gas-tight syringe that has been chilled in a refrigerator, transfer 15 µL of the liquid ethylene oxide to the mixture. Immediately seal the vial, and shake on a vortex mixer for at least 30 s. Transfer 0.20 g of this solution to a tared vial that can be sealed, and add *Stripped poloxamer* to obtain a *Standard solution* having a final weight of 50.0 g. Each g of this *Standard solution* contains 1 µg of ethylene oxide, 5 µg of propylene oxide, and 5 µg of 1,4-dioxane. Transfer 1.00 ± 0.01 g of this solution to a 22-mL pressure headspace vial, and add about 0.01 g of butylated hydroxytoluene. Seal with a silicone septum with or without a pressure-relief star spring and with a pressure-relief, aluminum, safety sealing-cap, and crimp the cap closed with a cap-sealing tool.

Sample solution: Transfer 1.00 ± 0.01 g of Poloxamer to a 22-mL pressure headspace vial, and add 0.01 g of butylated hydroxytoluene. Seal, cap, and crimp as directed for the *Standard solution*.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC (equipped with a balanced-pressure automated headspace sampler)
Detector: Flame ionization
Column: 0.32-mm × 50-m fused-silica capillary; 5-µm layer of stationary phase G27 coating
Temperature
Detector: 250°
Injector: 250°
Transfer line: 140°
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	—	70	10
70	10	240	10

Carrier gas: Helium
Flow rate: 1.6 mL/min
Injection size: Separately place the vials containing the *Standard solution* and the *Sample solution* in the automated sampler, and start the sequence so that the vial is heated at a temperature of 110° for 30 min before a suitable portion of its headspace is injected into the chromatograph.
Autosampler
Needle-withdrawal time: 0.3 min
Pressurization time: 1 min
Injection time: 0.08 min
Vial pressure: 22 psig with the vial vent off

System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for ethylene oxide, propylene oxide, and 1,4-dioxane are about 1.0, 1.3, and 3.8, respectively.]
Suitability requirements
Resolution: NLT 2.0 between ethylene oxide and propylene oxide
Relative standard deviation: NMT 15%

Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the concentrations, in µg/g, of ethylene oxide, propylene oxide, and 1,4-dioxane in the portion of Poloxamer taken:

$$\text{Result} = (r_U/r_S) \times C$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C = concentration of ethylene oxide, propylene oxide, or 1,4-dioxane in the *Standard solution* (µg/g)

Acceptance criteria
Ethylene oxide: NMT 1 µg/g
Propylene oxide: NMT 5 µg/g
1,4-Dioxane: NMT 5 µg/g

SPECIFIC TESTS

• **pH** ⟨791⟩: 5.0–7.5, in a solution (1 in 40)

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.
• **LABELING:** Label it to state, as part of the official title, the Poloxamer number. Label it to indicate the name and quantity of any antioxidant.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Poloxamer Liquid RS
USP Poloxamer Solid RS

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Hydrogenated Polydecene

$C_{30 \le n \le 70}H_{2n+2}$
1-Decene, homopolymer, hydrogenated [68037-01-4].

DEFINITION

Hydrogenated Polydecene is a mixture of saturated, synthetic hydrocarbons in the range $C_{30}H_{62}$ through $C_{70}H_{142}$ made from direct oligomerization of 1-decene (C_{10} alpha olefin). The oligomer mixture may be distilled to fractions of a suitable calculated viscosity and hydrogenated to reach saturation, or it may be hydrogenated to reach saturation and then distilled to the desired viscosity. The requirements for specific gravity, viscosity, and content of decene oligomer differ for the various types of Hydrogenated Polydecene, as set forth in the two tables below. Hydrogenated Polydecene may contain a suitable stabilizer.

Specific Gravity and Viscosity

Type	Specific Gravity	Kinematic Viscosity Range, Centistokes (mm^2/s)
I	0.814-0.819	16.0-20.0
II	0.823-0.827	28.0-34.0
III	0.828-0.832	40.0-52.0

Content of Decene Oligomers

Type	$C_{30}H_{62}$	$C_{40}H_{82}$	$C_{50}H_{102}$	$C_{60}H_{122}$	$C_{70}H_{142}$
I	70-93	5-25	0-5	0-1	0-1
II	13-40	35-70	9-25	0-7	0-2
III	3-15	25-55	25-40	13-28	0-10

IDENTIFICATION

• **A.** The chromatogram of the *Sample solution* from the test for *Content of Decene Oligomer* exhibits major peaks for trimers, tetramers, pentamers, hexamers, and possibly heptamers. The decene oligomer content is within the range given in the table *Content of Decene Oligomers* in the *Definition* for the labeled type of Hydrogenated Polydecene.

ASSAY

• **CONTENT OF DECENE OLIGOMER**

System suitability solution: 10 mg/mL of hexadecane, 10 mg/mL of squalane, and 1 mg/mL of tetradecane in pentane

Sample solution: Dissolve 0.1 mL of Hydrogenated Polydecene in 10 mL of pentane.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.52-mm × 16-m fused-silica capillary; coated with 0.1-mm stationary phase G2

Carrier gas: Helium

Flow rate: 10 mL/min

Injection volume: 2 µL

Temperatures

Injection port: 310°

Detector: 320°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
35	5	50	—
50	12	170	—
170	10	310	18

System suitability

Sample: *System suitability solution*

[NOTE—The retention time for squalane is about 18 min; the relative retention times for tetradecane, hexadecane, and squalane are about 0.5, 0.6, and 1.0, respectively.]

Suitability requirements

Resolution: NLT 2.0 between tetradecane and hexadecane

Relative standard deviation: NMT 2.0% for each peak

Analysis

Sample: *Sample solution*

Record the chromatogram, and measure the areas for the major peaks.

[NOTE—The tetramer oligomer has a retention time of about 23 min. The trimer, pentamer, hexamer, and heptamer oligomers, if present, have relative retention times of about 0.8, 1.1, 1.3, and 1.4, respectively, relative to the tetramer.]

Calculate the percentage of each oligomer present:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = response of each oligomer

r_T = sum of the responses of all the peaks, excluding the solvent peak

Acceptance criteria: The decene oligomer content is within the limits specified in the table *Content of Decene Oligomers* in the *Definition*.

IMPURITIES

• **LIMIT OF NICKEL**

Nickel stock solution: Immediately before use, dilute an appropriate quantity of organometallic standard[1] with kerosene to prepare a solution containing the equivalent of 1.0 µg/mL of nickel.

Standard solutions: Transfer 0.5, 1.0, 2.0, and 4.0 mL of *Nickel stock solution*, respectively, to four identical 10-mL volumetric flasks, dilute the contents of each flask with kerosene to volume, and mix. These *Standard solutions* contain, respectively, 0.05, 0.1, 0.2, and 0.40 µg/mL of nickel. [NOTE—The calibration range, especially the upper limit, can be adjusted for certain instruments, provided that instrument validation and calibration linearity are achieved.]

Sample solution: 0.3 g/mL of Hydrogenated Polydecene in kerosene. [NOTE—If necessary, dilute with an appropriate quantity of kerosene to obtain a reading within the calibrated absorbance range.]

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Graphite furnace atomic absorption spectrophotometer equipped with a deuterium background corrector and a pyrolytically coated tube with platform

[1] Suitable organometallic standards are available from, e.g., Continental Oil Co., Ponca City, OK (Conostan, 100 ppm), or Merck, D-6100 Darmstadt, Germany (metal in standard oil, 1000 ppm).

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Analytical wavelength: 232.0 nm (nickel emission line)
Injection volume: 20 µL
Lamp: Nickel hollow-cathode
Blank: Kerosene
Temperature: See *Table 2*.
[NOTE—The temperature program may be modified to obtain optimum furnace temperatures.]

Table 2

Step	Temperature (°)	Hold Time (s)
Drying	80	1
Drying	120	10
Drying	300	20
Ashing	600	20
Ashing	1000	20
Atomization	2500	3
Cleaning	2600	5

Analysis
Samples: *Standard solutions* and *Sample solution*
Place the *Standard solutions* and the *Sample solution* in an oven, setting the temperature at about 60° during the period of determination, and shake these solutions vigorously before analysis. Use micropipettor and pipettor tips to make all injections. [NOTE—Positive displacement pipets can be used when viscosity may become a problem.]
Pretreat the pipettor tip by pipetting and then discarding 20 µL of heptane. The tip must be pretreated before each injection. [NOTE—The film of heptane remaining on the wall of the tip facilitates a reproducible transfer of the oil sample.]
Separately inject the *Standard solutions* and the *Sample solution* into a graphite furnace, and concomitantly determine the integrated absorbances of the *Standard solutions* and the *Sample solution*.
Plot the integrated absorbances of the *Standard solutions* versus concentration, in µg/mL, of nickel, and draw the straight line best fitting the four plotted points. From the graph so obtained, determine the concentration of nickel, *C*, in µg/mL, in the *Sample solution*.
Calculate the content of nickel in the Hydrogenated Polydecene taken:

$$\text{Result} = C/C_U$$

C = concentration of nickel obtained from the graph (µg/mL)
C_U = concentration of nickel in the *Sample solution* (g/mL)

Acceptance criteria: NMT 1 µg/g

• **LIMIT OF SHORT-CHAIN HYDROCARBONS**
System suitability solution, Sample solution, Chromatographic system, System suitability, and Analysis: Proceed as directed in the test for *Content of Decene Oligomer*.
Calculate the percentage of each of the short-chain hydrocarbons present:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of any peak eluting before the trimer but different from the solvent peak
r_T = sum of the responses of all the peaks in the chromatogram, excluding the solvent peak

Acceptance criteria: NMT 2.5% of total short-chain hydrocarbons is found.

SPECIFIC TESTS
• **SPECIFIC GRAVITY** ⟨841⟩
Analysis: Determine at 20°.
Acceptance criteria: Meets the requirements of the specific gravity range specified in the table *Specific Gravity and Viscosity* in the *Definition* for the labeled type
• **VISCOSITY—CAPILLARY METHODS** ⟨911⟩
Analysis: Determine using a capillary viscometer, in a liquid bath maintained at 40.0 ± 0.1°.
Acceptance criteria: Meets the requirements of the viscosity range specified in the table *Specific Gravity and Viscosity* in the *Definition* for the labeled type.
• **READILY CARBONIZABLE SUBSTANCES TEST** ⟨271⟩
Standard solution: 3 mL of ferric chloride CS, 1.5 mL of cobaltous chloride CS, and 0.5 mL of cupric sulfate CS in a glass-stoppered test tube previously treated to remove organic matter (see *Cleaning Glass Apparatus* ⟨1051⟩).
Sample: 5 mL
Analysis: Transfer the *Sample* to a glass-stoppered test tube previously treated to remove organic matter (see *Cleaning Glass Apparatus* ⟨1051⟩), add 5 mL of sulfuric acid, and heat in a boiling water bath for 30 s. Quickly remove the test tube, and, while holding the stopper in place, shake three times in a vertically reciprocating cycle with an amplitude of about 13 cm. Repeat this procedure every 30 s for 10 min. Do not keep the test tube out of the water bath any longer than 3 s for each shaking cycle. Remove the test tube from the water bath, and let it cool for about 20 min to room temperature.
Acceptance criteria: The oil phase of the *Sample* may turn hazy but remains colorless; the interface between the two layers is free from solids; and the acid layer does not become darker than the standard color produced by the *Standard solution*, the *Standard solution* being overlaid with 5 mL of Hydrogenated Polydecene.

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements are specified.
• **LABELING:** Label it to indicate, as part of the official title, the Hydrogenated Polydecene type (Type I, Type II, or Type III), and label it to indicate the name and concentration of any added stabilizer.

Polydextrose

[68424-04-4].

DEFINITION
Polydextrose is a randomly branched polymer prepared by melting and subsequent condensation of the ingredients, which consist of approximately 90 parts dextrose, 10 parts sorbitol, and up to 1 part citric acid or 0.1 part phosphoric acid. The 1,6-glycosidic linkage predominates in the polymer but other linkages are present. It contains NLT 90.0% of dextrose polymer units, calculated on the anhydrous and ash-free basis. It contains small quantities of free dextrose, sorbitol, and 1,6-anhydro-D-glucose (levoglucosan), with traces of citric acid or phosphoric acid.

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IDENTIFICATION

- **A.** To 1 drop of a solution (1 in 10), add 4 drops of 5% phenol solution, then rapidly add 15 drops of sulfuric acid TS: a deep yellow to orange color is produced.
- **B.** With vigorous swirling, add 1 mL of acetone to 1 mL of a solution (1 in 10): the solution remains clear.
- **C.** With vigorous swirling, add 2 mL of acetone to the solution obtained in *Identification B*: a heavy, milky turbidity develops immediately.
- **D.** To 1 mL of a solution (1 in 50), add 4 mL of alkaline cupric citrate TS. Boil vigorously for 2–4 min. Remove from heat, and allow the precipitate (if any) to settle: the supernatant is blue or blue-green.

Add the following:

▲• **E. CHROMATOGRAPHIC IDENTITY**

Analysis: Proceed as directed in the *Assay*.

Acceptance criteria: The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.▲ 1S (NF36)

ASSAY

Change to read:

• **PROCEDURE**

Mobile phase: 0.001 N sulfuric acid. Pass this solution through a filter of 0.5-µm pore size, and degas.

Standard solution: 4.0 mg/mL of USP Polydextrose RS ▲▲ 1S (NF36) in *Mobile phase*

Sample solution: 4.0 mg/mL of ▲Polydextrose in *Mobile phase*▲ (ERR 1-Jul-2018)

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Refractive index

Detector temperature: 35 ± 0.1°

Columns

Guard: 4.6-mm × 3.0-cm; packing L17

Analytical: 7.8-mm × 30-cm; packing L17

Flow rate: 0.6 mL/min

Injection volume: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of dextrose polymer units in the portion of Polydextrose taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of dextrose polymer units from the *Sample solution*

r_S = peak response of dextrose polymer units from the *Standard solution*

C_S = concentration of USP Polydextrose RS in the *Standard solution* (mg/mL)

C_U = concentration of Polydextrose in the *Sample solution* (mg/mL)

Acceptance criteria: ▲NLT 90.0% on the anhydrous and ash-free basis▲ (ERR 1-Jul-2018)

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.3%

• **LIMIT OF LEAD**

[NOTE—Use reagent-grade chemicals with as low a lead content as is practicable, as well as high-purity water and gases. Before use in this analysis, rinse all glassware and plasticware twice with 10% nitric acid and twice with 10% hydrochloric acid, and then rinse them thoroughly with Purified Water.]

Matrix modifier solution: Prepare a solution in water containing 100.0 mg of dibasic ammonium phosphate per 10 mL of solution.

Lead nitrate stock solution: Dissolve 159.8 mg of lead nitrate in 100 mL of water to which has been added 1 mL of nitric acid, then dilute with water to 1000 mL. Prepare and store this solution in glass containers free from soluble lead salts.

Standard lead solution: On the day of use, dilute 10.0 mL of *Lead nitrate stock solution* with water to 100.0 mL. Each milliliter of *Standard lead solution* contains the equivalent of 10 µg of lead.

Standard solution A: 0.02 µg/mL of lead, from *Standard lead solution* in water

Standard solution B: 0.05 µg/mL of lead, from *Standard lead solution* in water

Standard solution C: 0.1 µg/mL of lead, from *Standard lead solution* in water

Standard solution D: 0.2 µg/mL of lead, from *Standard lead solution* in water

Standard solution E: 0.5 µg/mL of lead, from *Standard lead solution* in water

Sample solution: Transfer 1.0 g of Polydextrose, weighed and calculated on the anhydrous and ash-free basis, into a 10-mL volumetric flask, and dissolve in and dilute with water to volume.

Spiked sample solution: Transfer 1.0 g of Polydextrose, weighed and calculated on the anhydrous and ash-free basis, into a 10-mL volumetric flask, and dissolve in water. Add 100 µL of the *Standard lead solution*, and dilute with water to volume. This solution contains 0.1 µg/mL of added lead.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Graphite furnace atomic absorption spectrophotometer, equipped with a pyrolytic tube with a platform

Analytical wavelength: Lead emission line of 283.3 nm

Lamp: A lead hollow-cathode lamp, using a slit width of 0.7 mm (set low) and a deuterium arc lamp for background correction

Autosampler

Sample volume: 10 µL

Alternative volume: 10 µL of *Matrix modifier solution*

Furnace program: For the temperature program, see *Table 1*.

Table 1

Step	Dry	Char	Atomize	Clean	Recharge
Temperature (°)	130	800	2400	2600	20
Ramp time (s)	20	20	0	1	2
Hold time (s)	40	40	6	5	20
Argon flow rate (mL/min)	300	300	50	300	300

Analysis

Samples: 10 µL of the *Matrix modifier solution* was added into each 10-µL aliquot of the five *Standard solutions*, a

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mixture of 10 µL of the *Matrix modifier solution* and 10 µL of the *Sample solution*, and a mixture of 10 µL of the *Matrix modifier solution* and 10 µL of the *Spiked sample solution*

Concomitantly determine the absorbances of the *Samples* using the *Instrumental conditions* described above. Plot the absorbance of each *Standard solution*, compensated for background correction, versus its content of lead, in µg/mL, and draw the best straight line fitting the five points. From this plot, determine the concentrations, C_T and C_{ST}, in µg/mL, of lead in the *Sample solution* and the *Spiked sample solution*, respectively.

Calculate the percentage recovery taken:

$$\text{Result} = [(C_{ST} - C_T)/A] \times 100$$

A = quantity of lead added to the *Spiked sample solution*, 0.1 µg/mL

Calculate the content, in µg/g, of lead in the portion of Polydextrose taken:

$$\text{Result} = (C_T/W) \times V$$

W = weight of Polydextrose taken to prepare the *Sample solution* (g)

V = volume of the *Sample solution*, 10 mL

Acceptance criteria: NMT 0.5 µg/g. The recovery is 80%–120%.

• **ORGANIC IMPURITIES, PROCEDURE 1: LIMIT OF 5-HYDROXYMETHYLFURFURAL AND RELATED COMPOUNDS**

Sample solution: 1.0 g of Polydextrose, weighed and calculated on the anhydrous and ash-free basis, diluted with water to 100 mL

Analysis: Determine the absorbance of the *Sample solution* in a 1-cm quartz cell at 283 nm, with a suitable spectrophotometer, using water as the blank.

Calculate the percentage of 5-hydroxymethylfurfural and related compounds in the Polydextrose taken:

$$\text{Result} = 100 \times (V \times M_r \times A)/(M \times L \times W)$$

V = volume of the *Sample solution*, 0.1 L

M_r = molecular weight of 5-hydroxymethylfurfural, 126 g/mol

A = absorbance of the *Sample solution*

M = molar extinction coefficient of 5-hydroxymethylfurfural at a wavelength of 283 nm, 16,830 L/mol cm

L = path length of the spectrophotometer cell (cm)

W = weight of Polydextrose taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1%

• **ORGANIC IMPURITIES, PROCEDURE 2: LIMIT OF MONOMERS**

Mobile phase, Sample solution, and Chromatographic system: Proceed as directed in the *Assay*.

Standard solution: 0.08 mg/mL each of USP 1,6-Anhydro-D-glucose RS and USP Sorbitol RS, and 0.16 mg/mL of USP Dextrose RS, in *Mobile phase*

System suitability

Sample: *Standard solution*

[NOTE—For relative retention times, see *Table 2*.]

Table 2

Name	Relative Retention Time
Dextrose (glucose)	0.7
Sorbitol	0.8
An isomer of 1,6-anhydro-D-glucose (D-anhydroglucose furanose form)	0.9
1,6-Anhydro-D-glucose (D-anhydro-glucose pyranose form)	1.0

Suitability requirements

Resolution: NLT 1.0

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Use peak response of USP 1,6-Anhydro-D-glucose RS in the *Standard solution* for calculation of percentage of the isomer of 1,6-anhydro-D-glucose in the *Sample solution*.

Calculate the percentage of each monomer in the portion of Polydextrose taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of the respective monomer from the *Sample solution*

r_S = peak response of the respective monomer from the *Standard solution*

C_S = concentration of the respective standard monomer in the *Standard solution* (mg/mL)

C_U = concentration of Polydextrose in the *Sample solution* (mg/mL)

Acceptance criteria: NMT 4.0% for 1,6-anhydro-D-glucose, NMT 4.0% for dextrose, and NMT 2.0% for sorbitol. [NOTE—In the case of 1,6-anhydro-D-glucose, the peak areas for the pyranose and furanose forms are combined.]

SPECIFIC TESTS

• **MOLECULAR WEIGHT LIMIT**

Mobile phase: Dissolve 35.0 g of sodium nitrate and 1.0 g of sodium azide in 100 mL of water. Dilute with water to 4 L. Pass through a filter of 0.45-µm pore size, and degas by applying an aspirator vacuum for 30 min. The resulting *Mobile phase* is 0.1 N sodium nitrate containing 0.025% sodium azide.

Standard solution: Transfer 20 mg each of USP Dextrose RS, stachyose, and 5800-, 23,700-, and 100,000-molecular weight (MW) pullulan standards into a 10-mL volumetric flask. Dissolve in and dilute with *Mobile phase* to volume. Pass through a syringe filter of 0.45-µm pore size into a suitable autosampler vial, and seal.

Sample solution: Transfer 50 mg of Polydextrose into a 10-mL volumetric flask. Dissolve in and dilute with *Mobile phase* to volume. Pass through a syringe filter of 0.45-µm pore size into a suitable autosampler vial, and seal.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Refractive index set at a sensitivity of 4×10^{-6} refractive index units full scale and maintained at a temperature of 35 ± 0.1°

Column: 7.8-mm × 30-cm; packing L39

Column temperature: 45°

Flow rate: 0.8 mL/min

[NOTE—After installation of a new column, pump *Mobile phase* through the column overnight at a rate of 0.3 mL/min. Before calibration or analysis, increase the flow slowly over a 1-min period to 0.8 mL/min. Continue to pump *Mobile phase* through the column

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at this flow rate for at least 1 h before the first injection. Check the flow gravimetrically, and adjust it if necessary. Reduce the flow rate to about 0.1 mL/min when the system is not in use.]

Injection volume: 50 µL

System suitability

Sample: *Standard solution*

[NOTE—The retention times for each component determined on replicate injections agree within ±2 s.]

Chromatograph five replicate injections of the *Standard solution*, allowing 15 min between injections, and record the retention times of the components of the *Standard solution*.

Insert the average retention time along with the molecular weight of each component in the *Standard solution* into the calibration table of the molecular weight distribution software. Check the regression results for a cubic fit of the calibration points, and obtain a *Correlation coefficient R*, for the line.

Suitability requirements

Resolution: Dextrose and stachyose are baseline resolved from one another and from the 5800-MW pullulan standard.

[NOTE—Prominent negative baseline valleys are usually observed between the peaks for the 5800-; 23,700-; and 100,000-MW pullulan standards.]

Correlation coefficient R: NLT 0.9999

Analysis

Samples: *Standard solution* and *Sample solution*

Use the molecular weight distribution software of the data reduction system to generate a molecular weight distribution plot of Polydextrose.

Acceptance criteria: No measurable peak above a molecular weight of 22,000 is found.

- **pH** ⟨791⟩: 2.5–5.0, in a solution (1 in 10)
- **WATER DETERMINATION** ⟨921⟩, *Method I*: NMT 4.0%. Use a mixture of Hydranal solvent and Hydranal formamide dry (2:1) as a solvent. Perform the titration at 50° in a jacketed beaker.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers. Store in a cool and dry place.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP 1,6-Anhydro-D-glucose RS
USP Dextrose RS
USP Polydextrose RS
USP Sorbitol RS

Hydrogenated Polydextrose

DEFINITION

Hydrogenated Polydextrose is obtained by transition metal catalytic hydrogenation of Polydextrose in aqueous solution. It contains NLT 90.0% of dextrose polymer units, calculated on the anhydrous and ash-free basis. The polymer chain end groups are mainly sorbitol-terminated.

IDENTIFICATION

- **A.** To 1 drop of a solution (1 in 10), add 4 drops of 5% phenol solution, then rapidly add 15 drops of sulfuric acid TS: a deep yellow to orange color is produced.
- **B.** With vigorous swirling, add 1 mL of acetone to 1 mL of a solution (1 in 10): the solution remains clear.
- **C.** With vigorous swirling, add 2 mL of acetone to the solution obtained in *Identification* test *B*: a heavy, milky turbidity develops immediately.
- **D.** To 1 mL of a solution (1 in 50), add 4 mL of alkaline cupric citrate TS. Boil vigorously for 2–4 min. Remove from heat, and allow the precipitate (if any) to settle: the supernatant is blue or blue-green.
- **E.** Meets the requirements for dextrose in *Procedure 2, Limit of Monomers*

ASSAY

Change to read:

- **PROCEDURE**

Mobile phase: 0.001 N sulfuric acid. Pass through a filter of 0.5-µm or finer pore size, and degas.

Standard solution: 4.0 mg/mL of ▲USP Polydextrose RS in *Mobile phase*▲ (ERR 1-Jul-2018)

Sample solution: 4.0 mg/mL of ▲Hydrogenated Polydextrose in *Mobile phase*▲ (ERR 1-Jul-2018)

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Refractive index

Detector temperature: 35 ± 0.1°

Guard column: 4.6-mm × 3.0-cm; packing L17

Analytical column: 7.8-mm × 30-cm; packing L17

Flow rate: 0.6 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of dextrose polymer units in the Hydrogenated Polydextrose taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response for dextrose polymer units from the *Sample solution*

r_S = peak response for dextrose polymer units from the *Standard solution*

C_S = concentration of USP Polydextrose RS in the *Standard solution* (mg/mL)

C_U = concentration of Hydrogenated Polydextrose in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 90.0% on the anhydrous and ash-free basis

IMPURITIES

INORGANIC IMPURITIES

- **Residue on Ignition** ⟨281⟩: NMT 0.3%
- **Limit of Lead**

[NOTE—Use reagent-grade chemicals with a lead content of as low as possible, as well as high-purity water and gases. Before use in this analysis, rinse all glassware and plasticware twice with 10% nitric acid and twice with 10% hydrochloric acid, and then rinse them thoroughly with Purified Water.]

Matrix modifier solution: 10.0 mg/mL of dibasic ammonium phosphate

Lead nitrate stock solution: Dissolve 159.8 mg of lead nitrate in 100 mL of water to which has been added 1 mL of nitric acid, then dilute with water to 1000 mL. Prepare and store this solution in glass containers free from soluble lead salts.

Standard lead solution: On the day of use, dilute 10.0 mL of *Lead nitrate stock solution* with water to 100.0 mL. Each mL

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of *Standard lead solution* contains the equivalent of 10 µg of lead.
Standard solution A: 0.02 µg/mL of lead, from *Standard lead solution* in water
Standard solution B: 0.05 µg/mL of lead, from *Standard lead solution* in water
Standard solution C: 0.1 µg/mL of lead, from *Standard lead solution* in water
Standard solution D: 0.2 µg/mL of lead, from *Standard lead solution* in water
Standard solution E: 0.5 µg/mL of lead, from *Standard lead solution* in water
Sample solution: Transfer 1.0 g of Hydrogenated Polydextrose, weighed and calculated on the anhydrous and ash-free basis, into a 10-mL volumetric flask, dissolve in and dilute with water to volume.
Spiked sample solution: Transfer 1.0 g of Hydrogenated Polydextrose, weighed and calculated on the anhydrous and ash-free basis, into a 10-mL volumetric flask, and dissolve in water. Add 100 µL of *Standard lead solution*, and dilute with water to volume. This solution contains 0.1 µg/mL of added lead.
Spectrometric conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Graphite furnace atomic absorption spectrophotometer, equipped with a pyrolytic tube with a platform
Lamp: A lead hollow-cathode lamp, using a slit width of 0.7 mm (set low) and a deuterium arc lamp for background correction
Analytical wavelength: Lead emission line of 283.3 nm
Autosampler
Sample volume: 10 µL
Alternative volume: 10 µL of *Matrix modifier solution*
Furnace program: See the temperature program table below.

Step	Dry	Char	Atomize	Clean	Recharge
Temperature (°)	130	800	2400	2600	20
Ramp time (s)	20	20	0	1	2
Hold time (s)	40	40	6	5	20
Argon flow rate (mL/min)	300	300	50	300	300

Analysis
Samples: 10 µL of the *Matrix modifier solution* was added into each of the 10-µL aliquots of the five *Standard solutions*, a mixture of 10 µL of the *Matrix modifier solution* and 10 µL of the *Sample solution*, and a mixture of 10 µL of the *Matrix modifier solution* and 10 µL of the *Spiked sample solution*
Concomitantly determine the absorbances of the *Samples* using the *Spectrometric conditions* described above. Plot the absorbance of each *Standard solution*, compensated for background correction, versus its content of lead, in µg/mL, and draw the best straight line fitting the five points. From this plot, determine the concentrations, C_T and C_{ST}, in µg/mL, of lead in the *Sample solution* and the *Spiked sample solution*, respectively.
Calculate the percentage recovery taken:

$$\text{Result} = [(C_{ST} - C_T)/A] \times 100$$

A = quantity of lead added to the *Spiked sample solution*, 0.1 µg/mL

Calculate the content, in µg/g, of lead in Hydrogenated Polydextrose taken:

$$\text{Result} = (C_T/W) \times V$$

W = weight of Hydrogenated Polydextrose taken to prepare the *Sample solution* (g)
V = volume of the *Sample solution*, 10 mL

Acceptance criteria: NMT 0.5 µg/g; recovery is 80%–120%
• **Limit of Nickel**
[NOTE—All glassware used must be soaked in *1% Nitric acid* for at least 2 h and then rinsed with water.]
1% Nitric acid: Cautiously add 10 mL of nitric acid to a 1000-mL volumetric flask containing about 500 mL of water. Mix, and dilute with water to volume.
Blank solution: Use *1% Nitric acid*.
Nickel stock standard solution: Immediately before use, dilute an appropriate amount of nickel standard* with *1% Nitric acid* to prepare a solution containing the equivalent of 10 µg/mL of nickel.
Standard solutions: Into four identical 100-mL volumetric flasks, introduce respectively 1.0, 2.0, 5.0, and 10.0 mL of *Nickel stock standard solution*. Dilute with *1% Nitric acid* to volume, and mix. These standards contain 0.1, 0.2, 0.5, and 1.0 µg/mL of nickel.
Sample solution: Weigh 5 g of Hydrogenated Polydextrose into a 100-mL volumetric flask. Dissolve in and dilute with *1% Nitric acid* to volume, and mix.
Spectrometric conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometer equipped with an air–acetylene flame
Lamp: Nickel hollow-cathode
Analytical wavelength: 352.0 nm
System suitability
Sample: *Standard solution* of 0.2 µg/mL of nickel
Suitability requirements
Relative standard deviation: NMT 20%
Analysis
Samples: *Standard solutions* and *Sample solution*
Use the *Blank solution* to zero the instrument. Concomitantly determine the absorbances of the *Samples* at least three times each. Record the average of the steady readings for each of the *Samples*. Clear the nebulizer using the *Blank solution*, and aspirate each of the *Samples* in turn. The standard chosen for reslope should be run every four to five samples. If there is a significant change in its response, reslope and repeat the previous samples.
Plot the absorbances of the *Standard solutions* versus concentration, in µg/mL, of nickel, and draw the straight line best fitting the four plotted points. From the graph so obtained, determine the concentration, C, in µg/mL, of nickel in the *Sample solution*. Calculate the quantity, in µg, of nickel in each g of Hydrogenated Polydextrose taken:

$$\text{Result} = (V \times C)/W$$

V = volume of the *Sample solution*, 100 mL
W = weight of Hydrogenated Polydextrose taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 2 µg/g

* Suitable nickel standards are available from e.g., Fisher Scientific, Fair Lawn, NJ (nickel, reference standard solution, 1000 ppm ±1%, certified, application for atomic absorption) or RICCA Chemical Company, Arlington, TX (nickel standard, 1000 ppm Ni, for atomic absorption).

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ORGANIC IMPURITIES

• Procedure 1: Limit of 5-Hydroxymethylfurfural and Related Compounds

Sample solution: 1.0 g of Hydrogenated Polydextrose, weighed and calculated on the anhydrous and ash-free basis, diluted with water to 100 mL

Analysis: Determine the absorbance of the *Sample solution* in a 1-cm quartz cell at 283 nm, with a suitable spectrophotometer, using water as the blank.

Calculate the percentage of 5-hydroxymethylfurfural and related compounds in the Hydrogenated Polydextrose taken:

$$\text{Result} = (V \times M_r \times A)/(\varepsilon_{283} \times L \times W) \times 100$$

V = volume of the *Sample solution*, 0.1 L
M_r = molecular weight of 5-hydroxymethylfurfural, 126 g/mol
A = absorbance of the *Sample solution*
ε_{283} = molar extinction coefficient of 5-hydroxy methylfurfural at a wavelength of 283 nm, 16,830 $L \cdot mol^{-1} \cdot cm^{-1}$
L = path length of the spectrophotometer cell (cm)
W = weight of Hydrogenated Polydextrose taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1%

• Procedure 2: Limit of Monomers

Mobile phase, Sample solution, and Chromatographic system: Prepare as directed in the *Assay*.

Standard solution: 0.08 mg/mL of each for USP 1,6-Anhydro-D-glucose RS and USP Sorbitol RS, and 0.04 mg/mL of USP Dextrose RS, in *Mobile phase*

System suitability

Sample: *Standard solution*

[NOTE—See the relative retention times table below.]

Name	Relative Retention Time
Dextrose (glucose)	0.7
Sorbitol	0.8
An isomer of 1,6-anhydro-D-glucose (D-anhydroglucose furanose form)	0.9
1,6-Anhydro-D-glucose (levoglucosan) (D-anhydroglucose pyranose form)	1.0

Suitability requirements

Resolution: NLT 1.0

Relative standard derivation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Use the peak response of USP 1,6-Anhydro-D-glucose RS in the *Standard solution* for calculation of the percentage of the isomer of 1,6-anhydro-D-glucose in the *Sample solution*. Calculate the percentage of each monomer in the portion of Hydrogenated Polydextrose taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response for the respective monomer from the *Sample solution*
r_S = peak response for the respective monomer from the *Standard solution*
C_S = concentration of the respective standard monomer in the *Standard solution* (mg/mL)
C_U = concentration of Hydrogenated Polydextrose in the *Sample solution* (mg/mL)

Acceptance criteria: NMT 4.0% of 1,6-anhydro-D-glucose, NMT 5.75% for sorbitol and NMT 0.25% for dextrose

[NOTE—In the case of 1,6-anhydro-D-glucose, the peak areas for the pyranose and furanose forms are combined.]

SPECIFIC TESTS

• MOLECULAR WEIGHT LIMIT

Mobile phase: Dissolve 35.0 g of sodium nitrate and 1.0 g of sodium azide in 100 mL of water. Dilute with water to 4 L. Pass through a filter of 0.45-µm or finer pore size, and degas by applying an aspirator vacuum for 30 min. The resulting *Mobile phase* is 0.1 N sodium nitrate containing 0.025% sodium azide.

Standard solution: Transfer 20 mg each of USP Dextrose RS, stachyose, and 5800-, 23,700-, and 100,000-molecular weight (MW) pullulan standards into a 10-mL volumetric flask. Dissolve in and dilute with *Mobile phase* to volume. Pass through a syringe filter of 0.45-µm or finer pore size into a suitable autosampler vial, and seal.

Sample solution: Transfer 50 mg of Hydrogenated Polydextrose into a 10-mL volumetric flask. Dissolve in and dilute with *Mobile phase* to volume. Pass through a syringe filter of 0.45-µm or finer pore size into a suitable autosampler vial, and seal.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Refractive index set at a sensitivity of 4×10^{-6} refractive index units full scale and maintained at a temperature of 35 ± 0.1°

Column: 7.8-mm × 30-cm; packing L39

Column temperature: 45°

Flow rate: 0.8 mL/min

[NOTE—After installation of a new column, pump *Mobile phase* through the column overnight at a rate of 0.3 mL/min. Before calibration or analysis, increase the flow slowly over a 1-min period to 0.8 mL/min. Continue to pump *Mobile phase* through the column at this flow rate for at least 1 h before the first injection. Check the flow gravimetrically, and adjust it if necessary. Reduce the flow rate to about 0.1 mL/min when the system is not in use.]

Injection size: 50 µL

System suitability

Sample: *Standard solution*

Chromatograph five replicate injections of the *Standard solution*, allowing 15 min between injections, and record the retention times of the components of the *Standard solution*.

Insert the average retention time along with the molecular weight of each component in the *Standard solution* into the calibration table of the molecular weight distribution software. Check the regression results for a cubic fit of the calibration points, and obtain a correlation coefficient, R, for the line.

Suitability requirements

Retention time: The retention times for each component determined on replicate injections agree within ±2 s.

Resolution: Dextrose and stachyose are baseline resolved from one another and from the 5800-MW pullulan standard.

[NOTE—Prominent negative baseline valleys are usually observed between the peaks for the 5800-, 23,700-, and 100,000-MW pullulan standards.]

Correlation coefficient R: NLT 0.9999

Analysis

Samples: *Standard solution* and *Sample solution*

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Use the molecular weight distribution software of the data reduction system to generate a molecular weight distribution plot of Hydrogenated Polydextrose.

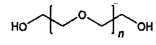
Acceptance criteria: No measurable peak above a molecular weight of 22,000 is found.

- **pH** ⟨791⟩**:** 5.0–7.0, in a solution (1 in 10)
- **Water Determination,** *Method I* ⟨921⟩**:** NMT 4.0%. Use a mixture of Hydranal Solvent and Hydranal Formamide dry (2:1) as a solvent. Perform the titration at 50° in a jacketed beaker.

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in tight, light-resistant containers. Store in a cool and dry place.
- **USP Reference Standards** ⟨11⟩
  USP 1,6-Anhydro-D-glucose RS
  USP Dextrose RS
  USP Polydextrose RS
  USP Sorbitol RS

Polyethylene Glycol



Poly(oxy-1,2-ethanediyl), α-hydro-ω-hydroxy-; Polyethylene glycol [25322-68-3].

DEFINITION

Polyethylene Glycol is an addition polymer of ethylene oxide and water, represented by the formula $H(OCH_2CH_2)_nOH$, in which *n* represents the average number of oxyethylene groups. The average molecular weight is NLT 95.0% and NMT 105.0% of the labeled nominal value if the labeled nominal value is less than 1000; it is NLT 90.0% and NMT 110.0% of the labeled nominal value if the labeled nominal value is between 1000 and 7000; and it is NLT 87.5% and NMT 112.5% of the labeled nominal value if the labeled nominal value is more than 7000. It may contain a suitable antioxidant.

ASSAY

- **Average Molecular Weight**

Phthalic anhydride solution: Place 49.0 g of phthalic anhydride into an amber bottle, and dissolve in 300 mL of pyridine from a freshly opened bottle or pyridine that has been freshly distilled over phthalic anhydride. Shake vigorously until completely dissolved. Add 7 g of imidazole, swirl carefully to dissolve, and allow to stand for 16 h before using.

Sample solution for liquid polyethylene glycols: Carefully introduce 25.0 mL of the *Phthalic anhydride solution* into a dry, heat-resistant pressure bottle. Add an amount of the specimen equivalent to its expected average molecular weight divided by 160. Insert the stopper in the bottle, and wrap it securely in a cloth bag.

Sample solution for solid polyethylene glycols: Carefully introduce 25.0 mL of *Phthalic anhydride solution* into a dry, heat-resistant pressure bottle. Add an amount of the specimen equivalent to its expected molecular weight divided by 160; however, because of limited solubility, do not use more than 25 g. Add 25 mL of pyridine, from a freshly opened bottle or pyridine that has been freshly distilled over phthalic anhydride. Swirl to dissolve, insert the stopper in the bottle, and wrap it securely in a cloth bag.

Blank: 25.0 mL of *Phthalic anhydride solution* plus any additional pyridine added to the bottle

Analysis: Immerse the bottle in a water bath maintained at a temperature between 96° and 100°, to the same depth as that of the mixture in the bottle. Remove the bottles from the bath after 5 min and, without unwrapping, swirl for 30 s to homogenize. Heat in the water bath for 30 min (60 min for polyethylene glycols having molecular weights of 3000 or more), then remove from the bath, and allow to cool to room temperature. Uncap the bottle carefully to release any pressure, remove from the bag, add 10 mL of water, and swirl thoroughly. Wait 2 min, add 0.5 mL of a solution of phenolphthalein in pyridine (1 in 100), and titrate with 0.5 N sodium hydroxide VS to the first pink color that persists for 15 s. Perform a blank determination. Calculate the average molecular weight taken:

$$\text{Result} = (2000 \times W)/[(V_B - V_S) \times N]$$

W = weight of Polyethylene Glycol taken for the *Sample solution* (g)
V_B = volume of 0.5 N sodium hydroxide consumed by the *Blank* (mL)
V_S = volume of 0.5 N sodium hydroxide consumed by the specimen (mL)
N = normality of the sodium hydroxide solution

Acceptance criteria: See *Table 1*.

Table 1

Label Claim (Nominal Value)	Acceptance Criteria (%)
<1000	95.0–105.0
1000–7000	90.0–110.0
>7000	87.5–112.5

IMPURITIES

- **Residue on Ignition** ⟨281⟩

Sample: 25 g

Analysis: Proceed as directed, using a platinum dish, and moistening the residue with 2 mL of sulfuric acid.

Acceptance criteria: NMT 0.1%

- **Limit of Free Ethylene Oxide and 1,4-Dioxane**

Stripped polyethylene glycol 400: Into a 5000-mL, 3-neck, round-bottom flask equipped with a stirrer, a gas dispersion tube, and a vacuum outlet, place 3000 g of polyethylene glycol 400. At room temperature, evacuate the flask carefully to a pressure of less than 1 mm of mercury, applying the vacuum slowly while observing for excessive foaming due to entrapped gases. After any foaming has subsided and while stirring continuously, sparge with nitrogen, allowing the pressure to rise to 10 mm of mercury. Continue stripping for a minimum of 1 h. Completeness of the stripping procedure should be verified by making a headspace injection of the *Stripped polyethylene glycol 400*. [NOTE—The 10-mm value is a guideline. Deviations from this value affect only the total time required to strip polyethylene glycol 400.]

Shut off the vacuum pump, and bring the flask pressure back to atmospheric pressure while maintaining nitrogen sparging. Remove the gas dispersion tube with the gas still flowing, then turn off the gas flow. Transfer *Stripped polyethylene glycol 400* to a suitable nitrogen-filled container.

Standard solution: Transfer 4.90 g of *Stripped polyethylene glycol 400* to a tared 22-mL pressure headspace vial that can be sealed. Add 48 µL of 1,4-dioxane, equivalent to 50 mg of 1,4-dioxane, from a syringe; seal, and cap the vial. [**CAUTION**—Ethylene oxide and 1,4-dioxane are toxic and flammable. Prepare these solutions in a well-ventilated fume hood.]

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Using the special handling described in the following, complete the preparation. Ethylene oxide is a gas at room temperature. It is usually stored in a lecture-type gas cylinder or small metal pressure bomb. Chill the cylinder in a refrigerator before use. Transfer 5 mL of the liquid ethylene oxide to a 100-mL beaker chilled in wet ice. Using a gas-tight syringe that has been chilled in a refrigerator, transfer 57 µL of the liquid ethylene oxide, equivalent to 50 mg of ethylene oxide, to the mixture contained in the headspace vial, and mix. With the aid of a syringe, transfer 2 mL of this solution to a 5-mL beaker. Transfer 1.0 mL of this solution to a 100-mL volumetric flask, and dilute with *Stripped polyethylene glycol 400* to volume. Transfer 10 mL of this solution to a 100-mL volumetric flask, dilute with *Stripped polyethylene glycol 400* to volume, and mix to obtain a *Standard solution* having known concentrations of 10 µg/g for both ethylene oxide and 1,4-dioxane. Transfer 1.0 mL of the *Standard solution* to a 22-mL pressure headspace vial, seal with a silicone septum with or without a pressure relief star spring and a pressure relief safety aluminum sealing cap, and crimp the cap closed with a cap-sealing tool.

System suitability solution: Transfer 4.90 g of *Stripped polyethylene glycol 400* to a 22-mL pressure headspace vial. Pipet 50 µL of acetaldehyde into the vial. Using the special handling described in the *Standard solution*, transfer 50.0 µL of liquid ethylene oxide into the vial. Immediately seal the vial, and shake. Transfer 1.0 mL of this solution to a 100-mL volumetric flask, and dilute with *Stripped polyethylene glycol 400* to volume. Transfer 10.0 mL of this solution to a 100-mL volumetric flask, and dilute with *Stripped polyethylene glycol 400* to volume. Transfer 1.0 mL of the *System suitability solution* to a 22-mL pressure headspace vial. Seal, cap, and crimp as directed for the *Standard solution*.

Sample solution: Transfer 1.0 g of Polyethylene Glycol to a 22-mL pressure headspace vial. Seal, cap, and crimp as directed in the *Standard solution*.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: Headspace GC (balanced pressure automatic headspace sampler)

Detector: Flame ionization

Column: 0.32-mm × 50-m fused-silica capillary; bonded with 5-µm film of phase G27

Temperatures

Injection port: 85°

Detector: 250°

Column: See *Table 2*.

Table 2

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	10	250	—

Carrier gas: Helium

Flow rate: 2.9 mL/min

Injection volume: 1.0 mL of headspace using a 2-mL gas syringe preheated in an oven at 90°

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for acetaldehyde and ethylene oxide are about 0.9 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 1.3 between the acetaldehyde and ethylene oxide peaks

Analysis

Samples: *Standard solution* and *Sample solution*

[NOTE—The relative retention times for ethylene oxide and 1,4-dioxane are about 1.0 and 3.4, respectively.]

Place the vials containing the *Standard solution* and the *Sample solution* into the automated sampler, and heat the vials at a temperature of 80° for 30 min.

[NOTE—A headspace apparatus that automatically transfers the measured amount of headspace may be used to perform the injection.]

Acceptance criteria: The peak areas for ethylene oxide and 1,4-dioxane of the *Sample solution* are NMT those of the corresponding peaks of the *Standard solution* corresponding to NMT 10 µg/g of ethylene oxide and NMT 10 µg/g of 1,4-dioxane.

• **LIMIT OF ETHYLENE GLYCOL AND DIETHYLENE GLYCOL** (for Polyethylene Glycol having a nominal molecular weight less than 450)

Standard solution: 0.5 mg/mL each of ethylene glycol and diethylene glycol in water

Sample solution: 400 mg/mL of Polyethylene Glycol in water

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 3-mm × 1.5-m stainless steel; packing of 12% G13 on support S1NS

Temperatures

Injection port: 250°

Detector: 280°

Column: 140°

Carrier gas: Nitrogen or another suitable inert gas

Flow rate: 50 mL/min

Injection volume: 2.0 µL

Analysis

Samples: *Standard solution* and *Sample solution*

[NOTE—The elution order is ethylene glycol followed by diethylene glycol.]

Calculate the percentage of ethylene glycol in the portion of Polyethylene Glycol taken:

$$\text{Result} = (r_{U1}/r_{S1}) \times (C_{S1}/C_U) \times 100$$

r_{U1} = peak height of ethylene glycol from the *Sample solution*

r_{S1} = peak height of ethylene glycol from the *Standard solution*

C_{S1} = concentration of ethylene glycol in the *Standard solution* (mg/mL)

C_U = concentration of Polyethylene Glycol in the *Sample solution* (mg/mL)

Calculate the percentage of diethylene glycol in the portion of Polyethylene Glycol taken:

$$\text{Result} = (r_{U2}/r_{S2}) \times (C_{S2}/C_U) \times 100$$

r_{U2} = peak height of diethylene glycol from the *Sample solution*

r_{S2} = peak height of diethylene glycol from the *Standard solution*

C_{S2} = concentration of diethylene glycol in the *Standard solution* (mg/mL)

C_U = concentration of Polyethylene Glycol in the *Sample solution* (mg/mL)

Acceptance criteria: NMT 0.25% of the sum of ethylene glycol and diethylene glycol

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• **LIMIT OF ETHYLENE GLYCOL AND DIETHYLENE GLYCOL** (for Polyethylene Glycol having a nominal molecular weight of 450 or more but NMT 1000)

Ceric ammonium nitrate solution: 6.25 g of ceric ammonium nitrate in 100 mL of 0.25 N nitric acid. Use within 3 days.

Standard stock solution: 2.5 mg/mL of diethylene glycol in freshly distilled acetonitrile and water (1:1)

Standard solution: Add 10.0 mL of the *Standard stock solution* to 15.0 mL of *Ceric ammonium nitrate solution*. Within 2–5 min, determine the absorbance of the *Standard solution*.

Sample stock solution: Dissolve 50.0 g of Polyethylene Glycol in 75 mL of diphenyl ether, previously warmed if necessary, just to melt the crystals, in a 250-mL distilling flask. Slowly distill at a pressure of 1–2 mm of mercury into a receiver graduated to 100 mL in 1-mL subdivisions, until 25 mL of distillate has been collected. Add 20.0 mL of water to the distillate, shake vigorously, and allow the layers to separate. Cool in an ice bath to solidify the diphenyl ether and facilitate its removal. Filter the separated aqueous layer, wash the diphenyl ether with 5.0 mL of ice-cold water, pass the washings through the filter, and collect the filtrate and washings in a 25-mL volumetric flask. Warm to room temperature, and dilute with water to volume, if necessary. Mix this solution with 25.0 mL of freshly distilled acetonitrile in a glass-stoppered, 125-mL conical flask.

Sample solution: Add 10.0 mL of the *Sample stock solution* to 15.0 mL of *Ceric ammonium nitrate solution*. Within 2–5 min, determine the absorbance of the *Sample solution*.

Blank: Mixture of 15.0 mL of *Ceric ammonium nitrate solution* and 10.0 mL of freshly distilled acetonitrile and water (1:1)

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)

Mode: UV-Vis

Cell: 1 cm

Analytical wavelength: 450 nm

Analysis

Samples: *Standard solution* and *Sample solution*

Acceptance criteria: The absorbance of the *Sample solution* does not exceed that of the *Standard solution*, corresponding to NMT 0.25% of combined ethylene glycol and diethylene glycol.

SPECIFIC TESTS

• **PH** ⟨791⟩

Sample solution: 5.0 g of Polyethylene Glycol in 100 mL of carbon dioxide-free water. Add 0.30 mL of saturated potassium chloride solution.

Acceptance criteria: 4.5–7.5

• **COMPLETENESS AND COLOR OF SOLUTION:** A solution of 5 g of Polyethylene Glycol in 50 mL of water is colorless; it is clear for liquid grades and NMT slightly hazy for solid grades.

• **VISCOSITY—CAPILLARY METHODS** ⟨911⟩**:** Determine the viscosity by using a capillary viscometer giving a flow time of NLT 200 s and using a liquid bath maintained at 98.9 ± 0.3° (210° F). The viscosity is within the limits specified in *Table 3*. For a polyethylene glycol not listed in the table, calculate the limits by interpolation.

Table 3

Nominal Average Molecular Weight	Viscosity Range, Centistokes	Nominal Average Molecular Weight	Viscosity Range, Centistokes
200	3.9–4.8	2400	49–65
300	5.4–6.4	2500	51–70
400	6.8–8.0	2600	54–74
500	8.3–9.6	2700	57–78
600	9.9–11.3	2800	60–83
700	11.5–13.0	2900	64–88
800	12.5–14.5	3000	67–93
900	15.0–17.0	3250	73–105
1000	16.0–19.0	—	—
1100	18.0–22.0	3500	87–123
1200	20.0–24.5	3750	99–140
1300	22.0–27.5	4000	110–158
1400	24–30	4250	123–177
1450	25–32	4500	140–200
1500	26–33	4750	155–228
1600	28–36	5000	170–250
1700	31–39	5500	206–315
1800	33–42	6000	250–390
1900	35–45	6500	295–480
2000	38–49	7000	350–590
2100	40–53	7500	405–735
2200	43–56	8000	470–900
2300	46–60	—	—

[NOTE—*Polyethlyene Glycol 3350* is listed in the *USP* official monographs section to reflect its use as both an active and inactive ingredient.]

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers.

• **LABELING:** Label it to state, as part of the official title, the average nominal molecular weight of the Polyethylene Glycol. Label it to indicate the name and quantity of any added antioxidant.

Polyethylene Glycol 3350—*see Polyethylene Glycol 3350 General Monographs*

Polyethylene Glycol Ointment

DEFINITION

Prepare Polyethylene Glycol Ointment as follows.

Polyethylene Glycol 3350	400 g
Polyethylene Glycol 400	600 g
To make	1000 g

Heat the two ingredients on a water bath to 65°. Allow to cool, and stir until congealed. If a firmer preparation is desired, replace up to 100 g of the *Polyethylene Glycol 400* with an

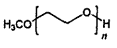
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equal amount of *Polyethylene Glycol 3350*. [NOTE—If 6%–25% of an aqueous solution is to be incorporated in the Ointment, replace 50 g of the *Polyethylene Glycol 3350* with an equal amount of stearyl alcohol.]

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Package in well-closed containers.

Polyethylene Glycol Monomethyl Ether



Poly(oxy-1,2-ethanediyl), α-methyl-ω-hydroxy-; Methoxy polyethylene glycol [9004-74-4].

DEFINITION

Polyethylene Glycol Monomethyl Ether is an addition polymer of ethylene oxide and methanol, represented as:

$$CH_3(OCH_2CH_2)_n OH$$

in which *n* represents the average number of oxyethylene groups. The average molecular weight is NLT 95.0% and NMT 105.0% of the labeled nominal value if the labeled nominal value is below 1000; it is NLT 90.0% and NMT 110.0% of the labeled nominal value if the labeled nominal value is between 1000 and 4750; and it is NLT 87.5% and NMT 112.5% of the labeled nominal value if the labeled nominal value is above 4750.

ASSAY

- **AVERAGE MOLECULAR WEIGHT**

Phthalic anhydride solution: Place 49.0 g of phthalic anhydride into an amber bottle, and dissolve in 300 mL of pyridine, either from a freshly opened bottle or freshly distilled over phthalic anhydride. Shake vigorously until completely dissolved. Add 7 g of imidazole, swirl carefully to dissolve, and allow to stand for 16 h before using.

Sample solution for liquid Polyethylene Glycol Monomethyl Ethers: Carefully introduce 25.0 mL of the *Phthalic anhydride solution* into a dry, heat-resistant pressure bottle. Add a weighed amount of the sample, equivalent to its expected average molecular weight divided by 80. Insert the stopper in the bottle, and wrap it securely in a cloth bag.

Sample solution for solid Polyethylene Glycol Monomethyl Ethers: Carefully introduce 25.0 mL of *Phthalic anhydride solution* into a dry, heat-resistant pressure bottle. Add an amount of the sample, equivalent to its expected molecular weight divided by 80; however, because of limited solubility, do not use more than 25 g. Add 25 mL of pyridine, either from a freshly opened bottle or freshly distilled over phthalic anhydride, swirl to dissolve, insert the stopper in the bottle, and wrap it securely in a cloth bag.

Analysis: Immerse the bottle in a water bath maintained at 96°–100° to the same depth as that of the mixture in the bottle. Remove the bottle from the bath after 5 min, and without unwrapping, swirl for 30 s to homogenize. Heat in the water bath for 30 min (60 min for Polyethylene Glycol Monomethyl Ethers having molecular weights of 3000 or higher), then remove from the bath, and allow to cool to room temperature. Uncap the bottle carefully to release any pressure, remove from the bag, add 10 mL of water, and swirl thoroughly. Wait for 2 min, add 0.5 mL of a solution of phenolphthalein in pyridine (1 in 100). Titrate with 0.5 N sodium hydroxide VS to the first pink color that persists for 15 s, recording the volume, in mL, of 0.5 N sodium hydroxide required as V_S. Perform a blank determination on 25.0 mL of *Phthalic anhydride solution* plus any additional pyridine added to the bottle, and record the volume, in mL, of 0.5 N sodium hydroxide required as V_B. Calculate the average molecular weight:

$$\text{Result} = (1000 \times W)/[(V_B - V_S) \times N]$$

W = weight of Polyethylene Glycol Monomethyl Ether taken for the *Sample solution* (g)
V_B = volume of 0.5 N sodium hydroxide consumed by the blank (mL)
V_S = volume of 0.5 N sodium hydroxide consumed by the sample (mL)
N = normality of the sodium hydroxide solution

Acceptance criteria: See *Table 1*.

Table 1

Label Claim (nominal value)	Acceptance Criteria (%)
<1000	95.0–105.0
1000–4750	90.0–110.0
>4750	87.5–112.5

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩

Sample: 25 g of Polyethylene Glycol Monomethyl Ether, moistened with 2 mL of sulfuric acid in a platinum dish
Acceptance criteria: NMT 0.1%

- **LIMIT OF ETHYLENE GLYCOL AND DIETHYLENE GLYCOL** (for Polyethylene Glycol Monomethyl Ether having a nominal molecular weight of less than 600)

Standard solution: 500 µg/mL of ethylene glycol and 500 µg/mL of diethylene glycol in water
Sample solution: 400 mg/mL of Polyethylene Glycol Monomethyl Ether in water
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 3-mm × 1.0-m; 60- to 80-mesh support S2
Temperatures
Column: 200°
Injection port: 260°
Carrier gas: Nitrogen or another suitable inert gas
Flow rate: 20 mL/min
Injection volume: 1.0 µL
Analysis
Samples: *Standard solution* and *Sample solution*
[NOTE—The elution order is ethylene glycol, diethylene glycol, and polyethylene glycol monomethyl ether.]
Calculate the percentage of ethylene glycol and diethylene glycol in the portion of Polyethylene Glycol Monomethyl Ether taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak height of ethylene glycol or diethylene glycol from the *Sample solution*
r_S = peak height of ethylene glycol or diethylene glycol from the *Standard solution*
C_S = concentration of ethylene glycol or diethylene glycol in the *Standard solution*
C_U = concentration of Polyethylene Glycol Monomethyl Ether in the *Sample solution*

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Acceptance criteria: NMT 0.25% of combined ethylene glycol and diethylene glycol

• **LIMIT OF ETHYLENE GLYCOL AND DIETHYLENE GLYCOL** (for Polyethylene Glycol Monomethyl Ether having a nominal molecular weight of 600–1500)

Solution A: 62.5 mg/mL of ceric ammonium nitrate in 0.25 N nitric acid. Use within 3 days.

Solution B: Freshly distilled acetonitrile and water (50:50)

Standard solution: 2.5 mg/mL of diethylene glycol in *Solution B*

Sample solution: Dissolve 50.0 g of Polyethylene Glycol Monomethyl Ether in 75 mL of diphenyl ether, previously warmed if necessary, to melt the crystals, in a 250-mL distilling flask. Slowly distill at a pressure of 1–2 mm of mercury into a receiver graduated to 100 mL in 1-mL subdivisions until 25 mL of distillate has been collected. Add 20.0 mL of water to the distillate, shake vigorously, and allow the layers to separate. Cool in an ice bath to solidify the diphenyl ether and facilitate its removal. Filter the separated aqueous layer, wash the diphenyl ether with 5.0 mL of ice-cold water, pass the washings through the filter, and collect the filtrate and washings in a 25-mL volumetric flask. Warm to room temperature, and dilute with water to volume, if necessary. Mix this solution with 25.0 mL of freshly distilled acetonitrile in a glass-stoppered, 125-mL conical flask.

Instrumental conditions

Mode: Vis

Analytical wavelength: About 450 nm

Cell: 1 cm

Blank: *Solution A* and *Solution B* (60:40)

Analysis

Samples: *Standard solution*, *Sample solution*, and *Blank*

Transfer 10.0 mL each of the *Standard solution* and the *Sample solution* to separate 50-mL flasks, each containing 15.0 mL of *Solution A*. Within 2–5 min, determine the absorbances of the *Samples*.

Acceptance criteria: The absorbance of the *Sample solution* does not exceed that of the *Standard solution*, corresponding to NMT 0.25% of combined ethylene glycol and diethylene glycol.

• **FREE ETHYLENE OXIDE AND 1,4-DIOXANE**

Stripped MPEG 350: Into a 5000-mL 4-neck, round-bottom flask, equipped with a stirrer, a thermometer, a gas dispersion tube, a dry ice trap, a vacuum outlet, and a heating mantle, place 3000 g of Polyethylene Glycol Monomethyl Ether 350. At room temperature, evacuate the flask carefully to a pressure of less than 1 mm of mercury, applying the vacuum slowly while observing for excessive foaming due to entrapped gases. After any foaming has subsided, sparge with nitrogen, allowing the pressure to rise to 10 mm of mercury. Heat the flask to 130° while increasing the pressure to 60 mm of mercury. Continue stripping for 4 h, then cool to room temperature. Shut off the vacuum pump, and bring the flask pressure back to atmospheric pressure while maintaining nitrogen sparging. Remove the sparging tube with the gas still flowing, then turn off the gas flow. Transfer the *Stripped MPEG 350* to a suitable nitrogen-filled container.

Standard solutions: [**CAUTION**—Ethylene oxide and 1,4-dioxane are toxic and flammable. Prepare these solutions in a well-ventilated fume hood.] To a known weight of *Stripped MPEG 350* in a vial that can be sealed add a suitable quantity of 1,4-dioxane. Determine the amount added by weight difference. Using the special handling described in the following, complete the preparation. Ethylene oxide is a gas at room temperature. It is usually stored in a lecture-type gas cylinder or small metal pressure bomb. Chill the cylinder in a refrigerator before use. Transfer 5 mL of the liquid ethylene oxide to a 100-mL beaker chilled in wet ice. Using a gas-tight gas chromatographic syringe that has been chilled in a refrigerator, transfer a suitable amount of the liquid ethylene oxide into the mixture. Immediately seal the vial, and shake. Determine the amount added by weight difference. By appropriate dilution with *Stripped MPEG 350*, prepare four solutions, covering a range of 5–20 ppm for the two components added to the matrix (e.g., 5, 10, 15, and 20 ppm). Transfer 1.0 mL of each of these solutions to separate 22-mL pressure headspace vials. Seal each with a silicone septum, star spring, and pressure relief safety aluminum sealing cap. Crimp the cap closed with a cap-sealing tool.

Sample solution: Transfer 1 ± 0.01 g of Polyethylene Glycol Monomethyl Ether to a 22-mL pressure headspace vial. Seal, cap, and crimp as directed for the *Standard solutions*.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC (equipped with a balanced pressure automatic headspace sampler)

Detector: Flame ionization

Column: 50-m × 0.32-mm fused silica; bonded phase G27 in a 5-µm film thickness

Temperatures

Detector: 250°

Transfer line: 140°

Column: See *Table 2*.

Table 2

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	10	250	—

Carrier gas: Helium

Flow rate: 0.8 mL/min

Calibration: Place the vials containing the *Standard solutions* in the automated sampler, and start the sequence so that each vial is heated at 110° for 30 min before a suitable portion of its headspace is injected into the chromatograph. Set the automatic sampler for a needle withdrawal time of 0.3 min, a pressurization time of 1 min, an injection time of 0.08 min, and a vial pressure of 22 psig with the vial vent off. Obtain the peak areas for ethylene oxide and 1,4-dioxane, which have relative retention times of 1.0 and 3.1, respectively. Plot the area versus parts per million on linear graph paper, and draw the best straight line through the points. On the two *Calibration* plots, no point digresses from its line by more than 10%.

Analysis: Place the vial containing the *Sample solution* in the automatic sampler, and chromatograph its headspace as done for the *Standard solutions*. Obtain the peak areas of each of the components, and read the concentrations directly from the *Calibration* plots.

Acceptance criteria: NMT 10 ppm of ethylene oxide or 1,4-dioxane

• **LIMIT OF 2-METHOXYETHANOL**

Stripped MPEG 350 and **Sample solution:** Prepare as directed in the test for *Free Ethylene Oxide and 1,4-Dioxane*.

Standard solutions: [**CAUTION**—2-Methoxyethanol is toxic and flammable. Prepare these solutions in a well-ventilated fume hood.] To a known weight of *Stripped MPEG 350* in a vial that can be sealed add a suitable quantity of 2-methoxyethanol. Determine the amount added by weight difference. By appropriate dilution with *Stripped MPEG 350*, prepare four solutions, covering a range of 5–20 ppm (e.g., 5, 10, 15, and 20 ppm). Transfer 1.0 mL of

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each of these solutions to separate 22-mL pressure headspace vials. Seal each with a silicone septum, star spring, and pressure relief safety aluminum sealing cap. Crimp the cap closed with a cap-sealing tool.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC (equipped with a balanced pressure automatic headspace sampler)

Detector: Flame ionization

Column: 15-m × 0.53-mm fused silica capillary; bonded phase G16 in a 1-µm film thickness

Temperatures

Detector: 275°

Transfer line: 140°

Column: See *Table 3*.

Table 3

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
50	—	50	2
70	10	250	—

Carrier gas: Helium

Flow rate: 15 mL/min

Calibration: Place the vials containing the *Standard solutions* in the automated sampler, and start the sequence so that each vial is heated at 100° for 20 min before a suitable portion of its headspace is injected into the chromatograph. Set the automatic sampler for a needle withdrawal time of 0.3 min, a pressurization time of 1 min, an injection time of 0.08 min, and a vial pressure of 22 psig with the vial vent off. Obtain the peak area for 2-methoxyethanol. Plot the area versus ppm on linear graph paper, and draw the best straight line through the points. On the *Calibration* plot, no point digresses from its line by more than 10%.

Analysis: Place the vial containing the *Sample solution* in the automatic sampler, and chromatograph its headspace as done for the *Standard solutions*. Obtain the peak area, and read the concentration directly from the *Calibration* plot.

Acceptance criteria: NMT 10 ppm

SPECIFIC TESTS

- **pH** ⟨791⟩

Sample solution: 5.0 g of Polyethylene Glycol Monomethyl Ether in 100 mL of carbon dioxide-free water. Add 0.30 mL of saturated potassium chloride solution.

Acceptance criteria: 4.5–7.5

- **Completeness and Color of Solution**

Sample solution: 5 g of Polyethylene Glycol Monomethyl Ether in 50 mL of water

Acceptance criteria: The resulting solution is colorless, and is clear for liquid grades and NMT slightly hazy for solid grades.

- **Viscosity—Capillary Methods** ⟨911⟩: Determine its viscosity, using a capillary viscometer giving a flow time of NLT 200 s and a liquid bath maintained at 98.9 ± 0.3°. The viscosity is within the limits specified in *Table 4*. For a Polyethylene Glycol Monomethyl Ether not listed in *Table 4*, calculate the limits by interpolation.

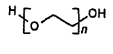
Table 4

Nominal Average Molecular Weight	Viscosity Range (centistokes)	Nominal Average Molecular Weight	Viscosity Range (centistokes)
350	3.5–4.5	2750	50–78
450	4.9–6.0	3000	60–95
550	6.1–7.3	3250	72–113
650	7.9–9.2	3500	85–133
750	9.7–11.1	3750	99–155
850	11.5–13.1	4000	114–178
950	13.3–15.2	4250	130–204
1000	13.3–17.3	4500	148–231
1100	15.0–19.7	4750	167–260
1200	16.9–22.1	5000	175–305
1300	18.8–24.6	5500	215–375
1400	20.7–27.1	6000	260–455
1500	23–30	6500	310–545
1600	25–33	7000	365–640
1700	27–35	7500	425–745
1800	29–38	8000	490–860
1900	31–41	8500	560–980
2000	33–44	9000	640–1110
2250	36–54	9500	715–1250
2500	40–64	10000	775–1475

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in tight containers.
- **Labeling:** Label it to state, as part of the official title, the average nominal molecular weight of the Polyethylene Glycol Monomethyl Ether.

Polyethylene Oxide



DEFINITION

Polyethylene Oxide is a nonionic homopolymer of ethylene oxide, represented:

$$H(OCH_2CH_2)_nOH$$

in which n represents the average number of oxyethylene groups. The number n varies from about 2000 to 200,000. It is a white to off-white powder obtainable in several grades, varying in viscosity profile in an aqueous isopropyl alcohol solution. It may contain a suitable antioxidant.

IDENTIFICATION

Change to read:

- **A. ▲Spectroscopic Identification Tests** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

Sample: Previously dried in a vacuum at room temperature to constant weight

NF Monographs

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• **B. Procedure**

Viscosity

[Note—Based on *Labeling* information, perform the following test accordingly.]

Analysis: Pass Polyethylene Oxide through a 20-mesh screen. Then transfer Polyethylene Oxide to a 800-mL low-form beaker the amount which is specified in *Table 1* to provide the solution concentration.

Table 1

	Sample solution		
Polyethylene oxide weight	1% Solution	2% Solution	5% Solution
	6 g	12 g	30 g

Add 125 mL of dehydrated isopropyl alcohol to the beaker. Place the stirrer into the beaker with an appropriate glass cover. Stir the polyethylene oxide-isopropanol mixture at a rate to ensure that a slurry is formed. Add the prescribed amount of water to the polyethylene oxide-isopropanol slurry, refer to *Table 2*. All solution concentrations are based on the water content of the aqueous isopropyl alcohol solution. Be careful to avoid splashing of the water. Adjust the temperature to near 25° to assist the final solution coming to temperature.

Table 2

	Sample solution		
Water weight	1% Solution	2% Solution	5% Solution
	594 g	588 g	570 g

Ensure that the stirring is very effective at the beginning for about 1 min. Then allow to gently stir for at least 3 h. Ensure that Polyethylene Oxide dissolves in solution, take care to avoid mixing in excess air, and stop the stirring. [Note—Ensure a colloidal dispersion by stirring for at least 3 h if added antioxidant or silicon dioxide is not soluble in the system.]

Place a watchglass over the beaker and place in a bath for at least 30 min. When the solution reaches 25 ± 0.1°, determine the viscosity of the *Sample solution* using the viscometer, spindle, and speed indicated on the *Labeling*. [Note—A guard may be required as indicated on the *Labeling*.] Follow the instrument manufacturer's directions to measure the apparent viscosity.

Acceptance criteria: Viscosity falls within the viscosity range indicated by the *Labeling*.

IMPURITIES

Inorganic Impurities

• **Silicon Dioxide and Nonsilicon Dioxide Residue on Ignition**

Sample: 1 g

Analysis: Weigh the *Sample* into a previously ignited, tared 50-mL platinum crucible. Add 4 drops of sulfuric acid. Heat carefully on a hot plate until the specimen is thoroughly charred and fumes no longer are evolved. Ignite the crucible at 700 ± 25° (see *Residue on Ignition* ⟨281⟩) to constant weight. Wet the residue carefully with 1 mL of water, and slowly add 20 drops of hydrofluoric acid. [Caution—Hydrofluoric acid is an extremely hazardous chemical. When handling it, wear a face shield, arm protection, and rubber gloves, and perform the operation in a hood.] Evaporate slowly on a hot plate to dryness, then ignite at 700 ± 25° for 10 min, cool to room temperature in a desiccator, and weigh. Repeat the addition of hydrofluoric acid, evaporation, and ignition, to constant weight.

Calculate the percentage of silicon dioxide residue on ignition from the difference between the net weights before and after the hydrofluoric acid treatment.

Calculate the percentage of nonsilicon dioxide residue on ignition from the final net weight.

Acceptance criteria

Silicon dioxide residue on ignition: NMT 3%

Nonsilicon dioxide residue on ignition: NMT 2%

Organic Impurities

• **Procedure: Limit of Free Ethylene Oxide**

Standard stock solution: [Caution—Ethylene oxide is toxic and flammable. Prepare solutions of it in a well-ventilated fume hood.] Prepare the solution using the special handling described below. Ethylene oxide is a gas at room temperature. It is usually stored in a lecture-type gas cylinder or small metal pressure bomb. Chill the cylinder in a refrigerator before use. Transfer 5 mL of the liquid ethylene oxide to a cold, 10-mL serum vial. Seal the vial, and store in a refrigerator. Transfer 40 g of acetone to a tared 50-mL serum vial that is capable of being tightly sealed with a polytef-lined septum and a metallic crimp cap. Seal the vial, and weigh it. Using a gas-tight gas chromatographic syringe that has been chilled in a refrigerator, transfer 60 µL of the liquefied ethylene oxide to the same vial. Weigh the vial, and determine the amount added by weight difference. The solution contains about 1 µg/µL of ethylene oxide. [Note—This solution may be kept for 1 week in the crimp-sealed serum vial, stored in a freezer.]

[Note—*Standard solutions A–D* and the *Sample solution* should be prepared in vials designed for use in the headspace sampling system specified in the *Chromatographic system*.]

Standard solution A: To a tared vial add 1.0 g of Polyethylene Oxide, and seal the vial. Through the septum add 2.0 µL of *Standard stock solution*, heat the vial at 100° for 30 min, and cool to room temperature.

Standard solution B: To a tared vial add 1.0 g of Polyethylene Oxide, and seal the vial. Through the septum add 4.0 µL of *Standard stock solution*, heat the vial at 100° for 30 min, and cool to room temperature.

Standard solution C: To a tared vial add 1.0 g of Polyethylene Oxide, and seal the vial. Through the septum add 6.0 µL of *Standard stock solution*, heat the vial at 100° for 30 min, and cool to room temperature.

Standard solution D: To a tared vial add 1.0 g of Polyethylene Oxide, and seal the vial. Through the septum add 8.0 µL of *Standard stock solution*, heat the vial at 100° for 30 min, and cool to room temperature.

Sample solution: To a tared vial add 1.0 g of Polyethylene Oxide, and seal the vial. Heat the vial at 100° for 30 min, and cool to room temperature.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.53-mm × 10-m capillary column bonded with a 20-µm layer of phase G45

Temperature

Injector: 200°

Detector: 250°

Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	—	70	5

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Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	10	200	5

Flow rate: 15 mL/min
Injection size: 300 µL of headspace gas
Injection type: Split
Carrier gas: Helium
[NOTE—The makeup gas is also helium, with a split flow rate of 15 mL/min.]

System suitability
Sample: *Standard solution C*
Suitability requirements
Relative standard deviation: NMT 5%. [NOTE—Multiple vials are prepared for replicate injections.]

Analysis
Samples: *Standard solutions A–D* and *Sample solution*
[NOTE—A headspace apparatus that automatically transfers the measured amount of gaseous headspace may be used to perform the injections.]
Using a gas-tight syringe, separately inject equal volumes (about 300 µL) of the gaseous headspace of each of the *Standard solutions* and the *Sample solution* into the gas chromatograph, record the chromatograms, and measure the areas of the peak responses. Determine by a retention time comparison whether ethylene oxide is detected in the *Sample solution*. Plot the responses of the *Sample solution* and the *Standard solutions* versus the content, in µg, of ethylene oxide in each vial, as furnished by the *Standard stock solution*. Draw the straight line best fitting the five points, and calculate the correlation coefficient for the line.
[NOTE—The content of ethylene oxide, as furnished by the *Standard stock solution*, is 0 µg in the *Sample solution*.]
A suitable system is one that yields a line having a correlation coefficient of NLT 0.99. Extrapolate the line until it intercepts the content axis on the negative side. From the intercept, determine the total amount, T_U, in µg, of ethylene oxide in the *Sample solution*.
Calculate the percentage of ethylene oxide in the portion of Polyethylene Oxide taken:

$$\text{Result} = (T_U/W) \times 100$$

T_U = total amount of ethylene oxide in the *Sample solution* (µg)
W = weight of Polyethylene Oxide taken to prepare the *Sample solution* (µg)

Acceptance criteria: NMT 0.001%

SPECIFIC TESTS
- **LOSS ON DRYING** ⟨731⟩**:** Dry 4 g at 105° for 45 min: it loses NMT 1.0% of its weight.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers. No storage requirements specified.
- **LABELING:** Label it to indicate the viscosity and acceptable limits, giving the viscosity measurement parameters, concentration of the solution, and the type of equipment used. Label it to indicate the name and quantity of any added antioxidant.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Polyethylene Oxide RS

Polyglyceryl Dioleate

$R–O–(CH_2–CH(OR)–CH_2–O)_3–R$

R = H, or $CO–C_{17}H_{33}$
1,2,3-Propanetriol, homotrimer, di[(9*Z*)-9-octadecenoate];
Triglyceril dioleate;
Polyglyceryl 3 dioleate [9007-48-1].

$R–O–(CH_2–CH(OR)–CH_2–O)_6–R$

R = H, or $CO–C_{17}H_{33}$
1,2,3-Propanetriol, homohexamer, di[(9*Z*)-9-octadecenoate];
Hexaglyceril dioleate;
Polyglyceryl 6 dioleate [76009-37-5].

DEFINITION
Polyglyceryl Dioleate is a mixture of polyglyceryl diesters of mainly oleic acid, obtained by esterification of poly glycerin and oleic acid. The polyglycerin consists mainly of triglycerin or hexaglycerin.

IDENTIFICATION

Change to read:

- **A.** ▲**SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F** ▲ (CN 1-May-2020)
- **B.** Meets the requirements of the test for *Content of Fatty Acids*
- **C.** Meets the requirements of the test for *Fats and Fixed Oils* ⟨401⟩, *Hydroxyl Value*. [NOTE—This test will differentiate for Polyglyceryl 3 Dioleate and Polyglyceryl 6 Dioleate.]

ASSAY
- **CONTENT OF FATTY ACIDS**

0.5 N methanolic sodium hydroxide solution: Dissolve 20 g of sodium hydroxide in 50 mL of water, and mix. Cool to room temperature, and add 950 mL of methanol.
Boron trifluoride methanol solution: Dissolve 14 g of boron trifluoride in methanol to make 100 mL, and mix well.[1]
Saturated sodium chloride solution: Dissolve about 375 g of sodium chloride in water to make 1000 mL.
Standard solution: Prepare the calibration ester mixture by mixing up each individual ester component (see *Table 1* for the component's composition). Dissolve 500 mg of the calibration ester mixture in *n*-heptane, and dilute with *n*-heptane to 50 mL. [NOTE—Commercially available mixtures of fatty acid methyl esters may also be used.]

Table 1

Component in the Calibration Ester Mixture	Composition (%)
USP Methyl Myristate RS (C14:0)	5
USP Methyl Palmitate RS (C16:0)	15
USP Methyl Palmitoleate RS (C16:1)	10
USP Methyl Stearate RS (C18:0)	10
USP Methyl Oleate RS (C18:1)	20
USP Methyl Linoleate RS (C18:2)	15
USP Methyl Linolenate RS (C18:3)	10
Methyl arachidate (C20:0)	10
Methyl gadoleate (C20:1)	5

[1] Boron trifluoride–methanol solution (14% in methanol) is also commercially available from Sigma, B-1252, or equivalent quality.

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Sample solution: Introduce 100 mg of Polyglyceryl Dioleate into a 25-mL conical flask fitted with a suitable water-cooled reflux condenser and a magnetic stir bar. Add 2 mL of *0.5 N methanolic sodium hydroxide solution*, mix, and reflux for about 30 min. Add 2 mL of *Boron trifluoride methanol solution* through the condenser and reflux for about 30 min. Add 4 mL of *n*-heptane through the condenser, and reflux for 5 min. Cool, remove the condenser, add about 10 mL of *Saturated sodium chloride solution*, shake, add a quantity of *Saturated sodium chloride solution* to bring the upper layer up to the neck of the flask, and allow the layers to separate. Collect 2 mL of the *n*-heptane layer (upper layer), wash with three quantities, each at 2 mL of water, and dry the *n*-heptane phase over anhydrous sodium sulfate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.32-mm × 30-m fused-silica capillary; 0.25-µm layer of phase G16
Temperature
Detector: 250°
Injection port: 240°
Column: See temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
150	6	250	6

Carrier gas: Nitrogen
Flow rate: 1.0–1.2 mL/min
Injection size: 1 µL
Injection type: Split injection. The split ratio is about 1:80.

System suitability
Sample: *Standard solution*
[NOTE—See the relative retention time table below.]

Component	Relative Retention Time
Methyl myristate	0.74
Methyl palmitate	1.00
Methyl palmitoleate	1.03
Methyl stearate	1.29
Methyl oleate	1.33
Methyl linoleate	1.37[a]
Methyl linolenate	1.46[b]
Methyl arachidate	1.55
Methyl gadoleate	1.58

[a] There could be an isomer eluting at a relative retention time of 1.39.
[b] There could be two isomers eluting at relative retention times of 1.45 and 1.48.

Suitability requirements
Resolution: NLT 1.5 between the peaks due to methyl stearate and methyl oleate
Relative standard deviation: NMT 6.0% for the palmitate and stearate peak areas

Analysis
Samples: *Standard solution* and *Sample solution*
Identify the fatty acid ester peaks of the *Sample solution* by comparing the retention times of these peaks with those of the *Standard solution*, and measure the peak areas for all of the fatty acid esters in the *Sample solution*.
Calculate the percentage of each fatty acid ester component in the test specimen:

$$\text{Result} = (A/B) \times 100$$

A = peak area of each individual fatty acid ester component
B = sum of the peak areas, excluding the solvent peak, of the *Sample solution*

Acceptance criteria: Polyglyceryl Dioleate exhibits the following composition profile of fatty acids.

Carbon-Chain Length	Number of Double Bonds	Percentage
14	0	≤5.0
16	0	2.0–16.0
16	1	≤8.0
18	0	≤6.0
18	1	65.0–88.0
18	2	5.0–18.0[a]
18	3	≤4.0[a]
Sum of fatty acids with C >18	0	≤4.0

[a] The content of C18:2 or C18:3 is the content of each fatty acid with its respective isomers.

IMPURITIES

INORGANIC IMPURITIES

• **Residue on Ignition**
Analysis: Heat a silica crucible to redness for 30 min, allow to cool in a desiccator, and weigh. Evenly distribute about 1.0 g of Polyglyceryl Dioleate in the crucible, and weigh. Dry at 100°–105° for 1 h, and ignite in a muffle furnace at 600 ± 25°, until the test substance is thoroughly charred. Perform the test for *Residue on Ignition* ⟨281⟩ on the residue obtained, starting with "Moisten the sample with a small amount (usually 1 mL) of sulfuric acid."
Acceptance criteria: NMT 1%

SPECIFIC TESTS

• **ACID VALUE**
Analysis: Accurately weigh (to within 0.1 mg) 5–10 g of Polyglyceryl Dioleate, add 10 mL of alcohol and 3 drops of phenolphthalein TS, and titrate with 0.1 N potassium hydroxide VS or 0.1 N sodium hydroxide VS until the solution remains faintly pink after shaking for 30 s. Proceed as directed in *Fats and Fixed Oils* ⟨401⟩, *Acid Value* to perform the calculation.
Acceptance criteria
Polyglyceryl 3 dioleate: NMT 6
Polyglyceryl 6 dioleate: NMT 6

• **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩
Acceptance criteria
Polyglyceryl 3 dioleate: 195–245, determined on a 0.7-g to 1.0-g specimen
Polyglyceryl 6 dioleate: 270–320, determined on 0.5-g to 0.7-g specimen

• **IODINE VALUE**
Analysis: Accurately weigh 0.15 g of Polyglyceryl Dioleate, transfer to a dry 250-mL flask with a ground-glass stopper, and add 25 mL of methylene chloride. Add 20 mL of the

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Wijs' solution.[2] Close the flask, and keep it in the dark for 1 h while shaking frequently. Perform the test in *Fats and Fixed Oils* ⟨401⟩, *Iodine Value*, starting with "Then add, in the order named, 30 mL of potassium iodide TS and 100 mL of water."

Acceptance criteria
Polyglyceryl 3 dioleate: 60–80
Polyglyceryl 6 dioleate: 50–70

- **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩**:** Use 30 mL of a mixture of glacial acetic acid and methylene chloride (3:2) to replace 30 mL of a mixture of glacial acetic acid and chloroform (3:2).

Acceptance criteria
Polyglyceryl 3 dioleate: NMT 12.5
Polyglyceryl 6 dioleate: NMT 12.5

- **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩**:** Determined on 1-g specimen

Acceptance criteria
Polyglyceryl 3 dioleate: 135–155
Polyglyceryl 6 dioleate: 110–140

- **WATER DETERMINATION,** *Method I* ⟨921⟩ **:** NMT 1%, determined on a 2.0-g specimen

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, protected from heat and moisture.
- **LABELING:** Label to indicate whether it is Polyglyceryl 3 Dioleate or Polyglyceryl 6 Dioleate.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Methyl Linoleate RS
  USP Methyl Linolenate RS
  USP Methyl Myristate RS
  USP Methyl Oleate RS
  USP Methyl Palmitate RS
  USP Methyl Palmitoleate RS
  USP Methyl Stearate RS
  USP Polyglyceryl 3 Dioleate RS
  USP Polyglyceryl 6 Dioleate RS

Polyglyceryl 3 Diisostearate

$R–O–(CH_2–CH(OR)–CH_2–O)_3–R$

R = H, or $CO–C_{17}H_{35}$-iso
1,2,3-Propanetriol, homopolymer, diisooctadecanoate; Triglyceryl diisostearate [63705-03-3].

DEFINITION

Polyglyceryl 3 Diisostearate is a mixture of polyglyceryl diesters of mainly isostearic acid, obtained by esterification of polyglycerin and isostearic acid. The polyglycerin consists mainly of triglycerin.

IDENTIFICATION

Change to read:

- **A.** ▲**SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F**▲ (CN 1-May-2020)
- **B.** It meets the requirements of the test for *Content of Fatty Acids*.

ASSAY

- **CONTENT OF FATTY ACIDS**

0.5 N methanolic sodium hydroxide solution: Dissolve 20 g of sodium hydroxide in 50 mL of water, and mix. Cool to room temperature, and add 950 mL of methanol.
Boron trifluoride–methanol solution: Dissolve 14 g of boron trifluoride in methanol to make 100 mL, and mix well.[1]
Saturated sodium chloride solution: Dissolve about 375 g of sodium chloride in water to make 1000 mL.
Standard solution: Prepare the calibration ester mixture by mixing up each individual ester component (see *Table 1*). Dissolve 500 mg of the calibration ester mixture in *n*-heptane, and dilute with *n*-heptane to 50 mL.

Table 1

Component in the Calibration Ester Mixture	Composition (%)[a]
USP Methyl Myristate RS (C14:0)	7
USP Methyl Palmitate RS (C16:0)	70
USP Methyl Stearate RS (C18:0)	23

[a] Composition is proposed according to the relative composition of these three fatty acid groups in Polyglyceryl 3 Diisostearate.

Sample solution: Introduce 100 mg of Polyglyceryl 3 Diisostearate into a 25-mL conical flask, fitted with a suitable water-cooled reflux condenser and a magnetic stir bar. Add 2 mL of *0.5 N methanolic sodium hydroxide solution*, mix, and reflux for about 30 min. Add 2 mL of *Boron trifluoride–methanol solution* through the condenser, and reflux for about 30 min. Add 4 mL of *n*-heptane through the condenser, and reflux for 5 min. Cool, remove the condenser, add about 10 mL of *Saturated sodium chloride solution*, shake, add a quantity of *Saturated sodium chloride solution* to bring the upper layer up to the neck of the flask, and allow the layers to separate. Collect 2 mL of *n*-heptane layer (upper layer), wash with three quantities, each of 2 mL of water, and dry the *n*-heptane phase over anhydrous sodium sulfate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.32-mm × 30-m fused-silica capillary column, 0.25-µm layer of phase G16
Temperature
Detector: 250°
Injection port: 240°
Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
150	6	250	6

Carrier gas: Nitrogen
Flow rate: 1.0–1.2 mL/min
Injection size: 1 µL
Injection type: Split injection. Split ratio is about 1:80.
System suitability
Sample: *Standard solution*
[NOTE—See the relative retention time table below.]

[2] Wijs' reagent RPE for analysis from Carlo Erba Reference 491902; Wijs' solution from www.sigmaaldrich.com, or equivalent quality.

[1] Boron trifluoride–methanol solution (14% in methanol) is also commercially available from Sigma, B-1252, or equivalent quality.

Supplement 1

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Name	Relative Retention Time
Methyl myristate	1.0
Methyl palmitate	1.4
Methyl stearate	1.8

Suitability requirements
Resolution: NLT 10 between the peaks due to methyl palmitate and methyl stearate
Relative standard deviation: NMT 6.0% for the peak responses for palmitate and stearate

Analysis
Samples: *Standard solution* and *Sample solution*
Identify the fatty acid ester peaks in the chromatogram of the *Sample solution* by comparing the retention times of these peaks with those obtained in the chromatogram of the *Standard solution*, and measure the peak areas for all of the fatty acid ester peaks in the chromatogram obtained from the *Sample solution*.
Calculate the percentage of each fatty acid ester component in the test specimen:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response for each individual fatty acid ester component
r_T = sum of the peak responses, excluding the solvent peak, in the chromatogram obtained from the *Sample solution*

Acceptance criteria
Sum of the contents of the fatty acids eluting between palmitic acid and stearic acid (excluding palmitic acid and stearic acid): NLT 60.0%
Sum of the contents of myristic acid, palmitic acid, and stearic acid: NMT 11.0%

IMPURITIES

INORGANIC IMPURITIES

- **Residue on Ignition**

Analysis: Heat a silica crucible to redness for 30 min, allow to cool in a desiccator, and weigh. Evenly distribute about 1.0 g of Polyglyceryl 3 Diisostearate in the crucible and weigh. Dry at 100°–105° for 1 h, and ignite in a muffle furnace at 600 ± 25°, until the test substance is thoroughly charred. Perform the test for *Residue on Ignition* ⟨281⟩ on the residue obtained, starting with "Moisten the sample with a small amount (usually 1 mL) of sulfuric acid".
Acceptance criteria: NMT 0.5%

SPECIFIC TESTS

- **ACID VALUE**

Analysis: Accurately weigh (to within 0.1 mg) 5–10 g of Polyglyceryl 3 Diisostearate, add 10 mL of alcohol and 3 drops of phenolphthalein TS, and titrate with 0.1 N potassium hydroxide VS or 0.1 N sodium hydroxide VS until the solution remains faintly pink after shaking for 30 s. Follow the procedures for *Fats and Fixed Oils, Acid Value* ⟨401⟩ to perform the calculation.
Acceptance criteria: NMT 3.0

- **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩**:** 180–230, determined on a 0.25-g specimen
- **IODINE VALUE**

Analysis: Accurately weigh 3 g of Polyglyceryl 3 Diisostearate, transfer to a dry 250-mL flask with a ground-glass stopper, and add 25 mL of methylene chloride. Add 20 mL of the Wijs' solution.[2] Close the flask, and keep it in the dark for 1 h while shaking frequently. Perform the test in *Fats and Fixed Oils* ⟨401⟩ *Iodine Value*, starting with "Then add, in the order named, 30 mL of potassium iodide TS and 100 mL of water".
Acceptance criteria: NMT 3.0

- **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩**:** NMT 6.0. Use 30 mL of a mixture of glacial acetic acid and methylene chloride (3:2) to replace the 30 mL of a mixture of glacial acetic acid and chloroform (3:2).
- **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩**:** 128–160
- **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 0.5%, determined on a 2.0-g specimen

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, protected from heat and moisture.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Methyl Myristate RS
USP Methyl Palmitate RS
USP Methyl Stearate RS
USP Polyglyceryl 3 Diisostearate RS

Polyisobutylene



[9003-27-4].

DEFINITION

Polyisobutylene is a synthetic polymer produced by the low-temperature polymerization of isobutylene in liquid ethylene, methylene chloride, or hexane, using an aluminum-chloride or boron-trifluoride catalyst. It may contain a suitable stabilizer.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F** ▲ (CN 1-May-2020)

Analysis: Prepare the sample by dissolving it in hot toluene and evaporating on a salt plate.
Acceptance criteria: Meets the requirements

IMPURITIES

- **LEAD** ⟨251⟩

Sample: 3.3 g
Control: 10 mL of *Diluted Standard Lead Solution* (10 µg of lead)
Analysis: Transfer the *Sample* to a porcelain dish, and heat on a hot plate until completely charred. Then heat in a muffle furnace at 480° for 8 h, and cool. Cautiously add 5 mL of nitric acid, evaporate to dryness on a hot plate, then heat again in the muffle furnace for exactly 15 min, and cool. Extract the ash with two 10-mL portions of water, filtering the extract into a separator. Leach any insoluble material on the filter with 6 mL of *Ammonium Citrate Solution*, 2 mL of *Hydroxylamine Hydrochloride Solution*, and 5 mL of water, adding the filtered washings to the separator. To the resulting solution and *Control* continue as directed in the chapter for *Procedure*, beginning with "Add 2 drops of phenol red TS".

[2] Wijs' reagent RPE for analysis from Carlo Erba Reference 491902, Wijs' solution from www.sigmaaldrich.com, or equivalent quality.

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Acceptance criteria: NMT 3 µg/g; the color generated by the *Sample* does not exceed that generated by the *Control*.

SPECIFIC TESTS

• **VISCOSITY—CAPILLARY METHODS** ⟨911⟩

Solvent: Use isooctane.

Sample solution: Prepare a solution of Polyisobutylene in the *Solvent* having a known concentration as indicated in *Table 1*. The solution must be homogenous before testing. For the Polyisobutylene having a Staudinger Index of 100 and higher, add the *Solvent* to the weighed material, and allow it to stand in an oven at 80° for 12–24 h. [NOTE—A heated mechanical shaker may be used to shorten the dissolution time; it is recommended that a gentle shaker be used to avoid shearing the polymers. Take adequate precautions to prevent evaporation of the *Solvent*.]

Table 1

Staudinger Index[a]	Concentration (g/cm³)
25–60	0.01
60–100	0.005
100–350	0.002
350–700	0.001

[a] The Staudinger Index is equal to 100 times the intrinsic viscosity.

Analysis: Before each measurement, let the solutions be temperature equilibrated for 10 min. Using a suitable Ubbelohde capillary viscometer having dimensions such that the flow time is NLT 200 s, immersed in a controlled temperature bath, measure the flow of the *Solvent* and of the *Sample solution* at 20°. Repeat the *Analysis* three times, and calculate the average.

Calculate the reduced viscosity:

$$J = (t/t_0 - 1)/C$$

t = average flow time of the *Sample solution* (s)
t_0 = average flow time of the *Solvent* (s)
C = concentration of the *Sample solution* (g/cm³)

Calculate the Staudinger Index:

$$J_0 = J/[1 + 0.31(t/t_0 - 1)]$$

Acceptance criteria: It is within the limits specified on the label.

• **LOSS ON DRYING** ⟨731⟩

Sample: 5 g

Analysis: Dry for 2 h at 105°.

Acceptance criteria: NMT 0.3%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.

• **LABELING:** Label it to indicate the range for intrinsic viscosity or the range for the Staudinger Index, and the name and quantity of any added stabilizer. [NOTE—The Staudinger Index is equal to 100 times the intrinsic viscosity.]

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Polyisobutylene RS

Polyoxyl 10 Oleyl Ether

Polyoxy-1,2-ethanediyl, α-(*Z*)-9-octadecenyl-ω-hydroxy-; Polyethylene glycol monooleyl ether [9004-98-2].

DEFINITION

Polyoxyl 10 Oleyl Ether is a mixture of the mono-oleyl ethers of mixed polyoxyethylene diols, the average polymer length being equivalent to NLT 9.1 and NMT 10.9 oxyethylene units. It may contain suitable stabilizers.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F** ▲ (CN 1-May-2020)

Sample: Use undried specimen.

Acceptance criteria: Meets the requirements

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩

Sample: 25 g

Analysis: Weigh the *Sample* into a tared 40-mL porcelain crucible, and heat in contact with air until it ignites spontaneously or can be ignited with a glowing splint. Allow the flame to go out, and place the crucible in a muffle furnace with the door partly open until the carbon is consumed. Close the door, and heat at 700 ± 100° for 1 h. Cool in a desiccator, weigh, and calculate the percentage of residue. If it exceeds 0.4%, again heat until constant weight is attained.

Acceptance criteria: NMT 0.4%

• **FREE POLYETHYLENE GLYCOLS**

Sample solution: Transfer 12 g to a 500-mL separator containing 50 mL of ethyl acetate. Add 50 mL of sodium chloride solution (0.29 g/mL), shake vigorously for 2 min, and allow to separate for 15 min. Drain the lower, aqueous phase into a second 500-mL separator, and extract the upper layer with a second 50-mL portion of sodium chloride solution (0.29 g/mL). To the combined aqueous layers add 50 mL of ethyl acetate, shake vigorously for 2 min, and allow to separate as before. Drain the lower, aqueous phase into a third 500-mL separator, and extract with two 50-mL portions of chloroform by shaking for 2 min each time.

Analysis: Evaporate the combined chloroform extracts in a 150-mL beaker on a steam bath, with the aid of a stream of nitrogen, to apparent dryness. Redissolve in 15 mL of chloroform, and transfer to a filter, collecting the filtrate in a 150-mL beaker. Rinse the funnel with several small portions of chloroform, and evaporate the combined filtrate and rinsings, as described above, until no odor of chloroform or ethyl acetate is perceptible. Cool in a desiccator, and weigh.

Acceptance criteria: NMT 7.5%

• **FREE ETHYLENE OXIDE**

Internal standard solution: 100 mg/mL of *n*-butyl chloride in chlorobenzene. Store in a tightly closed container. Prepare fresh weekly.

Standard stock solution

[**CAUTION**—Ethylene oxide is toxic and flammable. Prepare this solution in a well-ventilated hood, using great care.]

Place 250 mL of chlorobenzene in a glass-stoppered 500-mL conical flask. Bubble ethylene oxide through the chlorobenzene at a moderate rate for 30 min, insert the stopper, and store with protection from heat. Pipet 25 mL of 0.5 N alcoholic hydrochloric acid solution, prepared by mixing 45 mL of hydrochloric acid with 1 L of alcohol, into a 500-mL conical flask containing 40 g of magnesium chloride hexahydrate. Shake the mixture to effect saturation. Pipet 10 mL of the ethylene oxide solution into the flask, and add 20 drops of bromocresol green TS. If the solution is not yellow (acid) at this point, add an

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additional volume of 0.5 N alcoholic hydrochloric acid to give an excess of 10 mL. Record the total volume of 0.5 N alcoholic hydrochloric acid added. Insert the stopper in the flask, and allow to stand for 30 min. Titrate the excess acid with 0.5 N alcoholic potassium hydroxide VS. Perform a blank titration, using 10.0 mL of chlorobenzene instead of ethylene oxide solution, adding the same total volume of 0.5 N alcoholic hydrochloric acid, and note the difference in volumes required. Each mL of the difference in volumes of 0.5 N alcoholic potassium hydroxide consumed is equivalent to 22.02 mg of ethylene oxide. Calculate the concentration, in mg/mL, of ethylene oxide in the *Standard stock solution*. Standardize daily.

Standard solution: Transfer 5 g of Polyoxyl 10 Oleyl Ether to a suitable glass bottle of 60-mL capacity, and add 10 mL of chlorobenzene, exactly 50 µL of *Internal standard solution*, and a volume of *Standard stock solution* containing 0.5 mg of ethylene oxide. Insert a magnetic stirring bar, cap the bottle tightly, and stir until homogeneity is attained.

Sample solution: Transfer 5 g of Polyoxyl 10 Oleyl Ether to a suitable glass bottle of 60-mL capacity, and add 10 mL of chlorobenzene and 50 µL of *Internal standard solution*. Add a volume of chlorobenzene equal to the volume of the *Standard stock solution* added to prepare the *Standard solution*. Insert a magnetic stirring bar, cap the bottle tightly, and stir until homogeneity is attained.

Interference check solution: Transfer 5 g of Polyoxyl 10 Oleyl Ether to a suitable glass bottle of 60-mL capacity, and add 10 mL of chlorobenzene. Add a volume of chlorobenzene equal to the volume of the *Standard stock solution* used to prepare the *Standard solution*. Insert a magnetic stirring bar, cap the bottle tightly, and stir until homogeneity is attained.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 3-mm (OD) × 1.8-m stainless steel packed with S3

Temperatures

Injection port: 210°

Detector: 230°

Column: 160°

Carrier gas: Helium

Flow rate: 66 mL/min

Injection volume: 2 µL

System suitability

Samples: Chlorobenzene, *Internal standard solution*, *Standard stock solution*, and *Interference check solution*

Interference check: Inject a suitable volume of chlorobenzene into the gas chromatograph, and allow the chromatogram to run until the solvent has eluted. Similarly inject and chromatograph the *Internal standard solution*, the *Standard stock solution*, and the *Interference check solution*. No interfering peaks are observed.

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the weight of ethylene oxide in the portion of sample taken:

$$W_T = (W_E \times W_U \times R_U)/[(W_U \times R_S) - (W_S \times R_U)] \times F$$

W_E = weight of ethylene oxide added to the *Standard solution* (mg)

W_U = weight of Polyoxyl 10 Oleyl Ether used to prepare the *Sample solution* (g)

R_U = peak area ratio of ethylene oxide to the internal standard for the *Sample solution*

R_S = peak area ratio of ethylene oxide to the internal standard for the *Standard solution*

W_S = weight of Polyoxyl 10 Oleyl Ether used to prepare the *Standard solution* (g)

F = unit conversion, mg to g (10^{-3})

Calculate the percentage of ethylene oxide in the portion of Polyoxyl 10 Oleyl Ether taken:

$$\text{Result} = (W_T/W_U) \times 100$$

W_T and W_U are as defined above.

Acceptance criteria: NMT 0.01%

SPECIFIC TESTS

- **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 3.0%
- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩**:** NMT 1.0.
- **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩**:** 75–95
- **FATS AND FIXED OILS,** *Iodine Value, Method I* ⟨401⟩

Sample: 550 mg

Analysis: Proceed as directed in the chapter, with the reaction time being extended to 60 min.

Acceptance criteria: 23–40

- **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩**:** NMT 3
- **AVERAGE POLYMER LENGTH**

Sample solution: If solid material is present, place the Polyoxyl 10 Oleyl Ether in a 60° water bath overnight. Shake vigorously to eliminate any possibility of molecular weight gradients within it. Add 1 mL of the melt to 1 mL of deuterated chloroform in a test tube, and shake the test tube until dissolution is complete. Transfer 0.5 mL to an NMR tube, and add a small amount of tetramethylsilane as an internal reference standard. Cap the tube tightly, and shake thoroughly.

Analysis: Place the tube in an NMR spectrometer that is capable of performing quantitative analysis, and record the NMR spectrum (see *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩, *Quantitative Applications*). Integrate the areas from 0.4 to 2.35 ppm (A_1), and from 2.35 to 4.9 ppm (A_2).

Calculate the number of oxyethylene units per molecule taken:

$$\text{Result} = [(31 \times A_2/A_1) - 3]/4$$

31 = total number of protons in the molecule not activated by either oxygen or a double bond

3 = number of oxygen-activated protons not included in the oxyethylene unit count

4 = number of protons in each oxyethylene unit

Acceptance criteria: 9.1–10.9

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and store in a cool place.
- **LABELING:** Label to indicate the names and proportions of any added stabilizers.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Polyoxyl 10 Oleyl Ether RS

Polyoxyl 15 Hydroxystearate

12-Hydroxyoctadecanoic acid polymer with α-hydro-ω-hydroxypoly(oxy-1,2-ethanediyl);
Polyethylene glycol 15 hydroxystearate [70142-34-6].

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DEFINITION

Polyoxyl 15 Hydroxystearate results from the reaction of about 15 moles of ethylene oxide with 1 mole of 12-hydroxystearic acid. The product consists mainly of 12-hydroxystearic acid polyethoxylated at both the carboxyl and the hydroxyl groups with polyethylene glycol. It contains free polyethylene glycols.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F.▲** (CN 1-May-2020) If the sample is solid or too viscous for thin film formation, the sample should be gently warmed to achieve a mobile liquid, which may then be used to prepare the thin film.
- **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩

Sample solution: To 1.0 g of Polyoxyl 15 Hydroxystearate add 100 mL of a 100-mg/mL solution of potassium hydroxide, and boil under a reflux condenser for 30 min. Acidify the warm solution with 20 mL of hydrochloric acid, and cool to room temperature. Shake the mixture with 50 mL of ether, and allow to stand until a separation of the layers is visible. Separate the clear upper layer, add 5 g of anhydrous sodium sulfate, wait for 30 min, filter, and evaporate to dryness on a water bath. Dissolve 50 mg of the residue in 25 mL of ether.
Standard solution: 2 mg/mL of USP 12-Hydroxystearic Acid RS in methylene chloride
Plate: Octadecylsilyl silica gel for chromatography as the coating substance
Application volume: 2 µL
Developing solvent system: Acetone, methylene chloride, and glacial acetic acid (50:10:40)
Spray reagent: Prepare a solution of 80 mg/mL of phosphomolybdic acid in 2-propanol.
Analysis: Proceed as directed in the chapter. Develop over two-thirds of the plate, and dry in a current of cold air. Then spray the plate with *Spray reagent*, heat the plate at 120° for 1–2 min, and locate the spots on the plate.
Acceptance criteria: The R_F value and color of the principal spot of the *Sample solution* correspond to those of the *Standard solution*.

- **C.** It meets the requirements of the test for *Free Polyethylene Glycols*.

COMPOSITION

- **FREE POLYETHYLENE GLYCOLS**

Mobile phase: Methanol and water (8:2)
Standard solution A: 1.6 mg/mL of USP Polyethylene Glycol 1000 RS in *Mobile phase*
Standard solution B: 0.8 mg/mL of USP Polyethylene Glycol 1000 RS in *Mobile phase*, diluted from *Standard solution A* in *Mobile phase*
Sample solution: 4.8 mg/mL of Polyoxyl 15 Hydroxy stearate in *Mobile phase*
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Refractive index
Columns: 7.8-mm × 30-cm analytical column; 6-µm packing L39 and a 12-nm pore size; two 4-mm × 12.5-cm precolumns; 5-µm packing L1 and a 10-nm pore size. Connect both precolumns to the analytical column using a 3-way valve, and switch the *Mobile phase* flow according to the following program. [NOTE—Shown in *Figure 1*, the analysis is started with precolumn 2 and an analytical column in series. After about 114 s, the valves, controlled by the detector program, switch over such that the eluent flows past precolumn 2, and direct to precolumn 1 and the analytical column. The columns are switched when the components to be determined, but not the interfering matrix, are ready to reach the analytical column. Simultaneously, precolumn 2 is washed out in the reverse direction by a second pump to remove the unwanted matrix components.]

Time (s)	Program
0–114	Precolumn 2 and analytical column
115–end	Precolumn 1 and analytical column
115–420	Reverse flow of precolumn 2

Temperature
Column: Room temperature
Detector: Room temperature
Flow rate: 1.1 mL/min
Injection size: 50 µL
System suitability
Sample: *Standard solution A*
Suitability requirements
Relative standard deviation: NMT 3.0%
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*
Calculate the percentage of polyethylene glycols in the portion of Polyoxyl 15 Hydroxystearate taken:

$$\text{Result} = 2 \times (C_S/C_U)[r_U/(r_{S1} + 2r_{S2})] \times 100$$

C_S = concentration of USP Polyethylene Glycol 1000 RS in *Standard solution A* (mg/mL)
C_U = concentration of Polyoxyl 15 Hydroxystearate in the *Sample solution* (mg/mL)
r_U = peak response of polyethylene glycol from the *Sample solution*
r_{S1} = peak response of polyethylene glycol 1000 from *Standard solution A*
r_{S2} = peak response of polyethylene glycol 1000 from *Standard solution B*

Acceptance criteria: 27.0%–39.0% of free polyethylene glycols

IMPURITIES

INORGANIC IMPURITIES

- **Articles of Botanical Origin,** *Total Ash* ⟨561⟩: NMT 0.3%, determined on 1.0 g
- **Limit of Nickel**

[**CAUTION**—When using closed high-pressure digestion vessels and microwave laboratory equipment, the safety precautions and operating instructions given by the manufacturer must be followed.]
[NOTE—If an alternative apparatus is used, adjustment of the instrument parameters may be necessary.]
Nickel standard stock solution: Dilute nickel standard solution TS two-fold with water. This solution contains the equivalent of 5 µg/mL of nickel.
Standard solutions: Transfer 25, 50, 75, and 100 µL of *Nickel standard stock solution* to four identical 25-mL volumetric flasks. To each flask add 0.5 mL of a 10-mg/mL solution of magnesium nitrate, 0.5 mL of a 100-mg/mL solution of monobasic ammonium phosphate, and 6.0 mL of nickel-free nitric acid, dilute with water to volume, and mix well. [NOTE—Content of nickel in the nickel-free nitric acid is NMT 0.005 ppm.] The *Standard solutions* contain 0.005, 0.01, 0.015, and 0.02 µg/mL of nickel, respectively.

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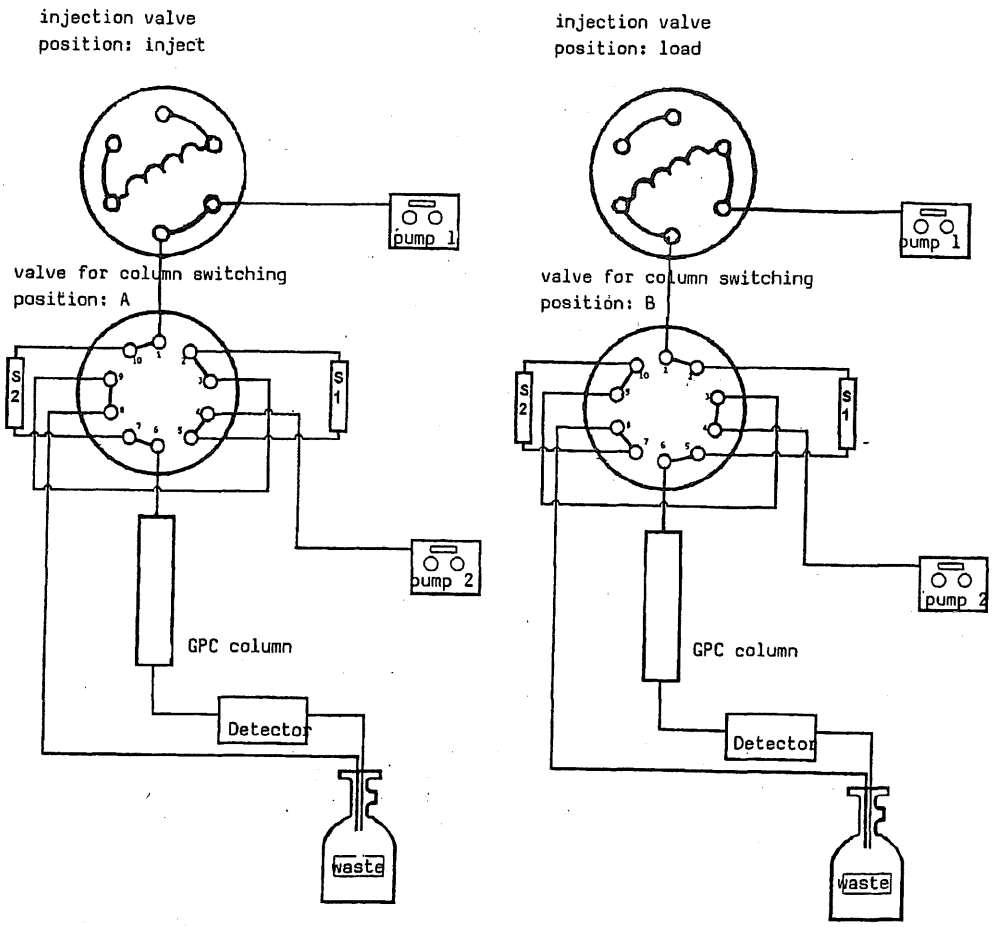


Figure 1. Apparatus (Polyoxyl 15 Hydroxystearate)

Sample solution: Transfer about 250 mg of Polyoxyl 15 Hydroxystearate to a suitable high-pressure-resistant digestion vessel (fluoropolymer or quartz glass), and add 6.0 mL of nickel-free nitric acid and 2.0 mL of 30% hydrogen peroxide. Place the closed vessel in a laboratory microwave oven, and digest using an appropriate program, e.g., 1000 W for 40 min. Allow the digestion vessel to cool down before opening. Add 2.0 mL of 30% hydrogen peroxide, and repeat the digestion step. Allow the digestion vessel to cool down before opening. Quantitatively transfer to a 25-mL volumetric flask, add 0.5 mL of a 10-mg/mL solution of magnesium nitrate and 0.5 mL of a 100-mg/mL solution of monobasic ammonium phosphate, dilute with water to volume, and mix well.

Blank solution: Place 6.0 mL of nickel-free nitric acid and 2.0 mL of 30% hydrogen peroxide in a suitable high-pressure-resistant digestion vessel. Proceed as directed under *Sample solution*, beginning with "Place the closed vessel in a laboratory microwave oven, and digest using an appropriate program, e.g., 1000 W for 40 min."

Zero solution: In a 50-mL volumetric flask, introduce 1.0 mL of a 10-mg/mL solution of magnesium nitrate, 1.0 mL of a 100-mg/mL solution of monobasic ammonium phosphate, and 12.0 mL of nickel-free nitric acid. Dilute with water to volume, and mix well.

Spectrometric conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Graphite furnace atomic absorption spectrophotometer equipped with a background compensation system, a coated tube resistant to pyrolysis, and a nickel hollow-cathode lamp.

Analytical wavelength: Nickel emission line of 232.0 nm

Temperature: Maintain the drying temperature of the furnace at 120° for 35 s after a 5-s ramp; maintain the ashing temperature at 1100° for 10 s after a 30-s ramp; maintain the cooling temperature at 800° for 5 s after a 5-s decrease; and maintain the atomization temperature at 2600° for 7 s. [NOTE—The temperature program may be modified to obtain optimum furnace temperatures.]

Analysis

Samples: *Standard solutions*, *Sample solution*, and *Blank solution*

Concomitantly determine the absorbances of the *Samples* using the *Spectrometric conditions* described above. Use the *Zero solution* to set the instrument to zero. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of nickel, and draw the straight line best fitting the plotted points. From the graph so obtained, determine the concentration, C_T, in µg/mL, of nickel in the *Sample solution*, and determine the concentration, C_B, in µg/mL, of nickel in the *Blank solution*. If necessary, dilute with the *Zero solution* to obtain a reading within the calibrated absorbance range.

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Calculate the quantity, in µg, of nickel in each g of Polyoxyl 15 Hydroxystearate taken:

$$\text{Result} = V \times (C_T - C_B)/W$$

V = volume of the *Sample solution* and the *Blank solution*, 25 mL
W = weight of Polyoxyl 15 Hydroxystearate taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 1 µg/g of nickel

ORGANIC IMPURITIES

• **Procedure: Limit of Free Ethylene Oxide and Dioxane**

[**CAUTION**—Ethylene oxide is toxic and flammable. Prepare these solutions in a well-ventilated fume hood, using great care. Protect both hands and face by wearing polyethylene protective gloves and an appropriate face mask. Store all solutions in hermetic containers, and refrigerate at a temperature between 4° and 8°.]

[NOTE—Before using the polyethylene glycol 200 in this test, remove any volatile components from it by placing 500 mL of polyethylene glycol 200 in a 1000-mL round-bottom flask, attaching the flask to a rotary evaporator maintained at a temperature of 60° and under a vacuum of 10–20 mm Hg for 6 h.]

Acetaldehyde solution: 10 µg/mL of acetaldehyde. [NOTE—Prepare the *Acetaldehyde solution* immediately prior to use.]

Ethylene oxide stock solution: Fill a chilled pressure bottle with liquid ethylene oxide, and store in a freezer when not in use. Use a small piece of polyethylene film to protect the liquid from contact with the rubber gasket. Tare a glass-stoppered conical flask, add about 50 mL of polyethylene glycol 200, and reweigh the flask. Transfer about 5 mL of the liquid ethylene oxide to a 100-mL beaker chilled in a mixture of sodium chloride and ice (1:3). Using a gas-tight syringe that has been previously cooled to −10°, transfer about 300 µL (corresponding to about 250 mg) of liquid ethylene oxide to the polyethylene glycol 200, and swirl gently to mix. Replace the stopper, reweigh the flask, and determine the amount of ethylene oxide absorbed by weight difference. Adjust the weight of the mixture with polyethylene glycol 200 to 100.0 g, replace the stopper, and swirl gently to mix. This stock solution contains about 2.5 mg/g of ethylene oxide. [NOTE—Prepare this stock solution immediately prior to use, and store in a refrigerator.]

Ethylene oxide solution: Tare a glass-stoppered conical flask, and chill it in a refrigerator. Add about 35 mL of polyethylene glycol 200, and reweigh the flask. Using a gas-tight gas chromatographic syringe that has been chilled in a refrigerator, transfer about 1 g of the chilled *Ethylene oxide stock solution*, weighed, to the tared, conical flask. Adjust the weight of the solution with polyethylene glycol 200 to 50.0 g, replace the stopper, and swirl gently to mix. Transfer about 10 g of this solution, weighed, to a 50-mL volumetric flask. Add 30 mL of water, and mix. Dilute with water to volume, and mix to obtain a solution containing about 10 µg/mL of ethylene oxide. [NOTE—Prepare this solution immediately prior to use, and use directly after preparation.]

Dioxane solution: 500 µg/mL of dioxane

Standard solution A: Transfer 0.1 mL of *Ethylene oxide solution* to a 10-mL pressure headspace vial. [NOTE—Other sizes may be used depending on the operating conditions, however, the same size must be used for *Standard solution A*, *Standard solution B*, and the *Sample solution*.] Add 0.1 mL of *Acetaldehyde solution* and 0.1 mL of *Dioxane solution*, seal the vial, and mix.

Standard solution B: Transfer about 1.0 g of Polyoxyl 15 Hydroxystearate to another 10-mL pressure headspace vial, add 0.1 mL of *Ethylene oxide solution*, 0.1 mL of *Dioxane solution*, and 1.0 mL of *N,N*-dimethylacetamide. Seal the vial, and mix.

Sample solution: Transfer about 1.0 g of Polyoxyl 15 Hydroxystearate to a 10-mL pressure headspace vial, add 1.0 mL of *N,N*-dimethylacetamide and 0.2 mL of water, seal the vial, and mix.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

[NOTE—The use of a headspace apparatus that automatically transfers a measured amount of headspace is allowed.]

Mode: GC

Detector: Flame ionization

Column: 0.32-mm × 30-m glass or quartz capillary; 1.0-µm layer of phase G1

Temperature

Injector port: 150°

Detector: 250°

Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
50	—	50	5
50	5	180	—
180	30	230	—
230	—	230	5

Carrier gas: Helium

Linear velocity: 20 cm/s

Injection size: 1 mL (the gaseous headspace)

Injection type: Split ratio 20:1

[NOTE—If the headspace apparatus is used, then an injection time of 12 s and a transfer line temperature of 150° are recommended.]

Headspace sampler: Each vial is heated at a temperature of 90° for 45 min, before a suitable portion of its headspace is injected.

System suitability

Sample: *Standard solution A*

[NOTE—The relative retention times for acetaldehyde and ethylene oxide are 0.94 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 2.0 between acetaldehyde and ethylene oxide

Signal-to-noise ratio: NLT 5, determined from the dioxane peak

Relative standard deviation: NMT 15%

Analysis

Samples:

Standard solution B and *Sample solution*

Using a heated, gas-tight, gas chromatographic syringe, separately inject equal volumes of the gaseous headspace of the *Samples* into the chromatograph, record the chromatograms, and measure the areas for the major peaks: the mean areas of the ethylene oxide and dioxane peaks from the *Sample solution* are not greater than half the mean areas of the corresponding peaks from *Standard solution B*.

Calculate the content of ethylene oxide, in ppm, in the portion of Polyoxyl 15 Hydroxystearate taken:

$$\text{Result} = (A_E \times r_U)/[(r_S \times W_U) - (r_U \times W_S)]$$

A_E = quantity of ethylene oxide added to *Standard solution B* (µg)

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r_U = ethylene oxide peak response from the *Sample solution*
r_S = ethylene oxide peak response from *Standard solution B*
W_U = weight of test substance taken to prepare the *Sample solution* (g)
W_S = weight of test substance taken to prepare *Standard solution B* (g)

Calculate the content of dioxane, in ppm, in the portion of Polyoxyl 15 Hydroxystearate taken:

$$\text{Result} = (A_D \times r_U)/[(r_S \times W_U) - (r_U \times W_S)]$$

A_D = quantity of dioxane added to *Standard solution B* (µg)
r_U = dioxane peak response from the *Sample solution*
r_S = dioxane peak response from *Standard solution B*
W_U = weight of test substance taken to prepare the *Sample solution* (g)
W_S = weight of test substance taken to prepare *Standard solution B* (g)

Acceptance criteria
Ethylene oxide: NMT 1 ppm
Dioxane: NMT 50 ppm

SPECIFIC TESTS
- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩: NMT 1.0, determined on 2.0 g
- **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩: 90–110
- **FATS AND FIXED OILS,** *Iodine Value, Method I* ⟨401⟩: NMT 2.0
- **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩: NMT 5.0
- **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩: 53–63
- **WATER DETERMINATION,** *Method Ia* ⟨921⟩: NMT 1.0%, determined on 2.0 g

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers at a temperature below 25°.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP 12-Hydroxystearic Acid RS
  USP Polyethylene Glycol 1000 RS
  USP Polyoxyl 15 Hydroxystearate RS

Polyoxyl 20 Cetostearyl Ether

DEFINITION
Polyoxyl 20 Cetostearyl Ether is a mixture of mono-cetostearyl (mixed hexadecyl and octadecyl) ethers of mixed polyoxyethylene diols, the average polymer length being equivalent to NLT 17.2 and NMT 25.0 oxyethylene units.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F.▲** (CN 1-May-2020) Use undried specimen.

IMPURITIES
- **RESIDUE ON IGNITION** ⟨281⟩
  Sample: 25 g
  Analysis: Weigh the *Sample* into a tared 40-mL porcelain crucible, and heat in contact with air until it ignites spontaneously or can be ignited with a glowing splint. Allow the flame to go out, place the crucible in a muffle furnace with the door partly open until the carbon is consumed, close the door, and heat at 700 ± 100° for 1 h. Cool in a desiccator, weigh, and calculate the percentage of residue. If the amount so obtained exceeds 0.4%, heat again until constant weight is attained.
  Acceptance criteria: NMT 0.4%
- **FREE POLYETHYLENE GLYCOLS**
  Sample solution: Transfer 12 g to a 500-mL separator containing 50 mL of ethyl acetate. Add 50 mL of sodium chloride solution (0.29 g/mL), shake vigorously for 2 min, and allow to separate for 15 min. Drain the lower, aqueous phase into a second 500-mL separator, and extract the upper layer with a second 50-mL portion of sodium chloride solution (0.29 g/mL). To the combined aqueous layers, add 50 mL of ethyl acetate, shake vigorously for 2 min, and allow to separate as before. Drain the lower, aqueous phase into a third 500-mL separator, and extract with two 50-mL portions of chloroform by shaking for 2 min each time.
  Analysis: Evaporate the combined chloroform extracts in a 150-mL beaker on a steam bath, with the aid of a stream of nitrogen, to apparent dryness. Redissolve in 15 mL of chloroform, and transfer to a filter, collecting the filtrate in a 150-mL beaker. Rinse the funnel with several small portions of chloroform, and evaporate the combined filtrate and rinsings, as described above, until no odor of chloroform or ethyl acetate is perceptible. Cool in a desiccator, and weigh.
  Acceptance criteria: NMT 7.5%
- **FREE ETHYLENE OXIDE**
  Internal standard solution: 100 mg/mL of *n*-butyl chloride in chlorobenzene. Store in a tightly closed container. Prepare fresh weekly.
  Standard stock solution
  [**CAUTION**—Ethylene oxide is toxic and flammable. Prepare this solution in a well-ventilated hood, using great care.]
  Place 250 mL of chlorobenzene in a glass-stoppered, 500-mL conical flask. Bubble ethylene oxide through the chlorobenzene at a moderate rate for 30 min, insert the stopper, and store with protection from heat. Pipet 25 mL of a 0.5 N alcoholic hydrochloric acid solution, prepared by mixing 45 mL of hydrochloric acid with 1 L of alcohol, into a 500-mL conical flask containing 40 g of magnesium chloride hexahydrate. Shake the mixture to effect saturation. Pipet 10 mL of the ethylene oxide solution into the flask, and add 20 drops of bromocresol green TS. If the solution is not yellow (acid) at this point, add an additional volume of 0.5 N alcoholic hydrochloric acid to give an excess of 10 mL. Record the total volume of 0.5 N alcoholic hydrochloric acid added. Insert the stopper in the flask, and allow to stand for 30 min. Titrate the excess acid with 0.5 N alcoholic potassium hydroxide VS.
  Perform a blank titration, using 10.0 mL of chlorobenzene instead of ethylene oxide solution, adding the same total volume of 0.5 N alcoholic hydrochloric acid, and note the difference in volumes required. Each mL of the difference in volumes of 0.5 N alcoholic potassium hydroxide consumed is equivalent to 22.02 mg of ethylene oxide. Calculate the concentration, in mg/mL, of ethylene oxide in the *Standard stock solution*. Standardize daily.
  Standard solution: Transfer 5 g of Polyoxyl 20 Cetostearyl Ether to a suitable glass bottle of 60-mL capacity. Add 10 mL of chlorobenzene, exactly 50 µL of *Internal standard solution*, and a volume of *Standard stock solution* containing 0.5 mg of ethylene oxide. Insert a magnetic stirring bar, cap the bottle tightly, and stir until homogeneity is attained.
  Sample solution: Transfer 5 g of Polyoxyl 20 Cetostearyl Ether to a suitable glass bottle of 60-mL capacity. Add 10 mL of chlorobenzene and 50 µL of *Internal standard*

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solution. Add a volume of chlorobenzene equal to the volume of the *Standard stock solution* added to prepare the *Standard solution*. Insert a magnetic stirring bar, cap the bottle tightly, and stir until homogeneity is attained.

Interference check solution: Transfer 5 g of Polyoxyl 20 Cetostearyl Ether to a suitable glass bottle of 60-mL capacity, and add 10 mL of chlorobenzene. Add an additional volume of chlorobenzene equal to the volume of the *Standard stock solution* used to prepare the *Standard solution*. Insert a magnetic stirring bar, cap the bottle tightly, and stir until homogeneity is attained.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 3-mm (OD) × 1.8-m stainless steel packed with S3

Temperatures

Injection port: 210°

Detector: 230°

Column: 160°

Carrier gas: Helium

Flow rate: 66 mL/min

Injection volume: 2 µL

System suitability

Samples: Chlorobenzene, *Internal standard solution*, *Standard stock solution*, and *Interference check solution*

Interference check: Inject a suitable volume of chlorobenzene, and allow the chromatogram to run until the solvent has eluted. Similarly inject the *Internal standard solution*, *Standard stock solution*, and *Interference check solution*. No interfering peaks are observed.

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the weight of ethylene oxide in the portion of the sample taken:

$$W_T = (W_E \times W_U \times R_U)/[(W_U \times R_S) - (W_S \times R_U)] \times F$$

W_E = weight of ethylene oxide added to the *Standard solution* (mg)

W_U = weight of Polyoxyl 20 Cetostearyl Ether used to prepare the *Sample solution* (g)

R_U = peak area ratio of ethylene oxide to the internal standard from the *Sample solution*

R_S = peak area ratio of ethylene oxide to the internal standard from the *Standard solution*

W_S = weight of Polyoxyl 20 Cetostearyl Ether used to prepare the *Standard solution* (g)

F = unit conversion, mg to g (10^{-3})

Calculate the percentage of ethylene oxide in the portion of Polyoxyl 20 Cetostearyl Ether taken:

$$\text{Result} = (W_T/W_U) \times 100$$

W_T and W_U are as defined above.

Acceptance criteria: NMT 0.01%

SPECIFIC TESTS

- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩: NMT 0.5
- **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩: 42–60
- **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩: NMT 2
- **pH** ⟨791⟩

Sample solution: 100 mg/mL

Acceptance criteria: 4.5–7.5

- **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 1.0%
- **AVERAGE POLYMER LENGTH**

Sample solution: Place the Polyoxyl 20 Cetostearyl Ether in a 50° water bath overnight to melt it completely. Shake vigorously to eliminate any possibility of molecular weight gradients within it, and transfer 200 µL to a 5- × 180-mm high-resolution NMR sample tube. Add 200 µL of deuterated chloroform by means of a separate microsyringe. Add 5 drops of tetramethylsilane as an internal reference standard. Cap the tube tightly, and shake thoroughly.

Analysis: Place the tube in the NMR spectrometer, and record the NMR spectrum at an appropriate RF power level and a sweep time of 250 s/500 Hz (see *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩, *Qualitative Applications*). Adjust the spectrum amplitude so that the signal at 1.1 ppm is at least 80% of full scale. Record the integral areas, from 0.4 to 2.35 ppm (A_1), and from 2.35 to 4.9 ppm (A_2), at a sweep time of 50 s/500 Hz at an integral power level such that the integral of the ethylene oxide peak at 3.5 ppm is at least 80% of full chart height. Do not change the power level during the sweep. Record the integral of each peak several times, and calculate the average integral area. Calculate the number of oxyethylene units per molecule taken:

$$\text{Result} = [(32 \times A_2/A_1) - 3]/4$$

32 = total number of protons in the molecule not activated by oxygen, averaged for the cetyl and stearyl radicals

3 = number of oxygen-activated protons not included in the oxyethylene unit count

4 = number of protons in each oxyethylene unit

Acceptance criteria: 17.2–25.0

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, in a cool place.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Polyoxyl 20 Cetostearyl Ether RS

Polyoxyl 35 Castor Oil

Polyethylene glycol 35 castor oil;
Polyoxyethylene 35 castor oil [61791-12-6].

DEFINITION

Polyoxyl 35 Castor Oil contains mainly the triricinoleate ester of ethoxylated glycerol with smaller amounts of polyethylene glycol ricinoleate and the corresponding free glycols. It results from the reaction of glycerol ricinoleate with 35 mol of ethylene oxide.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F** ▲ (CN 1-May-2020)
- **B. CONSTITUTING FATTY ACIDS**

Sample: 0.1 g

Analysis: Dissolve the *Sample* in 10 mL of potassium hydroxide TS, alcoholic; boil for 3 min; and evaporate to dryness. Mix the residue with 5 mL of water.

Acceptance criteria: The residue dissolves, yielding a clear solution. Add a few drops of glacial acetic acid. A white precipitate is formed.

- **C. IDENTITY BY FATTY ACID COMPOSITION**

Diluent: *n*-Heptane

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Standard solution 1: 0.2 mg/mL each of methyl palmitate, methyl stearate, methyl oleate, methyl linoleate, methyl *cis*-11-eicosenoate, and methyl ricinoleate, from USP Methyl Palmitate RS, USP Methyl Stearate RS, USP Methyl Oleate RS, USP Methyl Linoleate RS, methyl *cis*-11-eicosenoate, and USP Methyl Ricinoleate RS, in *Diluent*

Standard solution 2: 4 mg/mL each of methyl stearate and methyl ricinoleate from USP Methyl Stearate RS and USP Methyl Ricinoleate RS in *Diluent*

Sample solution: Transfer 140 mg of Polyoxyl 35 Castor Oil to a 10-mL screw-cap test tube, add 3.0 mL of *Diluent*, and mix well. Add 0.5 mL of 0.5 M sodium methoxide in methanol,[1] and mix with the sample. Allow the reaction to proceed at room temperature for 2 h. After 2 h, add 5 mL of water, and mix. Centrifuge the test tube at 1000 × g for 10–15 min until a clear upper layer forms. Separate the organic layer (the upper layer), and remove the lower layer. Place an aliquot of the organic layer into an autosampler vial.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.25-mm × 15-m fused silica capillary; bonded with a 0.25-µm layer of phase G7

Temperatures

Injection port: 240°

Detector: 250°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)	Total Time (min)
80	0	80	1	1
80	30	140	0	3
140	3	150	0	6.3
150	1	155	0	11.3
155	2	165	0	16.3
165	3	220	10	45

Column mode: See *Table 2* for the pressure program.

Table 2

Pressure (psi)	Pressure Ramp (psi/min)	Hold Time (min)	Total Time (min)
10	0	16	16
4	5	9 or 0[a]	26.2 or 17.2
3	10	19 or 28	45

[a] If considerable discrimination of late eluting compounds is observed, the hold time can be adjusted from 9 to 0 min. Thus the total time should be 17.2 min. Then the next step of the hold time should be 28 min.

Carrier gas: Hydrogen

Injection volume: 0.5 µL

Injection type: Split ratio, 60:1

Liner: Single taper, low-pressure drop liner with deactivated wool

[1] 0.5 M sodium methoxide in methanol is available from Sigma-Aldrich (www.sigmaaldrich.com), product #403067. Any other equivalent reagent can be used as well.

Run time: 45 min

System suitability

Sample: *Standard solution 1*

[NOTE—See *Table 3* for relative retention times.]

Table 3

Component	Relative Retention Time
Methyl palmitate (C16:0)	0.61
Methyl stearate (C18:0)	0.98
Methyl oleate (C18:1)	1.00
Methyl linoleate (C18:2)	1.02
Methyl *cis*-11-eicosenoate (C20:1)	1.70
Methyl ricinoleate	2.30

Suitability requirements

Resolution: NLT 1.5 between methyl stearate and methyl oleate peaks

Relative standard deviation: NMT 2.0% for the peak area ratio of methyl ricinoleate to methyl linoeate

Analysis

Samples: *Standard solution 1*, *Standard solution 2*, and *Sample solution*

The peak of methyl *cis*-11-octadecenoate, which is an isomer of methyl oleate, can be resolved from the methyl oleate peak with a resolution of about 1 and a relative retention time of 1.01 with respect to methyl oleate.

Calculate the relative response factor, *F*, for methyl ricinoleate:

$$F = (r_S/r_R) \times (C_R/C_S)$$

r_S = peak area of methyl stearate from *Standard solution 2*

r_R = peak area of methyl ricinoleate from *Standard solution 2*

C_R = concentration of USP Methyl Ricinoleate RS in *Standard solution 2* (mg/mL)

C_S = concentration of USP Methyl Stearate RS in *Standard solution 2* (mg/mL)

Correct the peak area of methyl ricinoleate in the *Sample solution* by multiplying by *F*.

Calculate the percentage of each fatty acid component in the portion of Polyoxyl 35 Castor Oil taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak area of each individual fatty acid methyl ester, except for the uncorrected peak area of methyl ricinoleate (or the corrected peak area of methyl ricinoleate), from the *Sample solution*

r_T = sum of all the peak areas, excluding the solvent and methyl ricinoleate peaks and including the corrected peak area of methyl ricinoleate, from the *Sample solution*

Acceptance criteria: Polyoxyl 35 Castor Oil exhibits the composition profile of fatty acids shown in *Table 4*.

Table 4

Component	Percentage (%)
Palmitic acid (C16:0)	≤4.0

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Table 4 *(continued)*

Component	Percentage (%)
Stearic acid (C18:0)	≤5.0
Oleic acid (C18:1)	4.0–10.0
Linoleic acid (C18:2)	≤5.0
cis-11-Eicosenoic acid (C20:1)	≤1.0
Ricinoleic acid	45.0–75.0

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.3%
- **ETHYLENE OXIDE AND DIOXANE** ⟨228⟩, *Method I*
  Acceptance criteria
  Ethylene oxide: NMT 1 µg/g
  Dioxane: NMT 10 µg/g
- **ETHYLENE GLYCOL, DIETHYLENE GLYCOL, AND TRIETHYLENE GLYCOL IN ETHOXYLATED SUBSTANCES** ⟨469⟩
  Acceptance criteria
  Ethylene glycol: NMT 620 µg/g
  Sum of diethylene glycol and ethylene glycol: NMT 2500 µg/g

SPECIFIC TESTS

- **SPECIFIC GRAVITY** ⟨841⟩: 1.05–1.06
- **VISCOSITY—CAPILLARY METHODS** ⟨911⟩: 600–850 mPa · s at 25°, using a capillary viscometer
- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Acid Value*: NMT 2.0
- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Hydroxyl Value*: 65–80
- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Iodine Value*: 25–35
- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Saponification Value*: 60–75
- **WATER DETERMINATION** ⟨921⟩, *Method I*: NMT 3.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light and moisture. Store at room temperature, and avoid exposure to excessive heat.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Methyl Linoleate RS
  USP Methyl Oleate RS
  USP Methyl Palmitate RS
  USP Methyl Ricinoleate RS
  USP Methyl Stearate RS
  USP Polyoxyl 35 Castor Oil RS

Polyoxyl 40 Hydrogenated Castor Oil

Polyethylene glycol 40 hydrogenated castor oil; Polyoxyethylene 40 hydrogenated castor oil [61788-85-0].

DEFINITION

Polyoxyl 40 Hydrogenated Castor Oil contains mainly the trihydroxystearate ester of ethoxylated glycerol, with smaller amounts of polyethylene glycol hydroxystearate and the corresponding free glycols. It results from the reaction of glycerol tri-hydroxystearate with 40–45 mol of ethylene oxide.

IDENTIFICATION

- **A. IDENTITY BY FATTY ACID COMPOSITION**
  Diluent: Chloroform
  Standard solution 1: 0.4 mg/mL each of methyl palmitate, methyl arachidate, methyl 12-ketostearate, methyl stearate, and methyl 12-hydroxystearate, from USP Methyl Palmitate RS, methyl arachidate, methyl 12-ketostearate, USP Methyl Stearate RS, and USP Methyl 12-Hydroxystearate RS, in *Diluent*
  Standard solution 2: 4 mg/mL each of methyl stearate and methyl 12-hydroxystearate from USP Methyl Stearate RS and USP Methyl 12-Hydroxystearate RS in *Diluent*
  Sample solution: Transfer 140 mg of Polyoxyl 40 Hydrogenated Castor Oil to a 10-mL screw-cap test tube, add 3.0 mL of *Diluent*, and mix well. Add 0.5 mL of 0.5 M sodium methoxide in methanol,[1] and mix with the sample. Allow the reaction to proceed at room temperature for 2 h. After 2 h, add 5 mL of water, and mix. Centrifuge the test tube at 2000 × g for 10 min at 4° until a clear organic layer forms. Separate the organic layer, and remove the aqueouslayer. Place an aliquot of the organic layer into an autosampler vial.
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: GC
  Detector: Flame ionization
  Column: 0.32-mm × 30-m fused silica capillary; bonded with a 0.25-µm layer of phase G16
  Temperatures
  Injection port: 240°
  Detector: 250°
  Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)	Total Time (min)
80	0	80	1	1
80	30	140	0	3
140	20	180	5	10
180	2	250	10	55

Carrier gas: Hydrogen
Flow rate: 5.0 mL/min, constant flow mode
Injection volume: 1.0 µL
Injection type: Split ratio, 120:1 or 60:1
Liner: Single gooseneck liner with deactivated glass wool
Run time: 55 min
System suitability
Sample: *Standard solution 1*
[NOTE—For relative retention times, see *Table 2*.]

Table 2

Component	Relative Retention Time
Methyl palmitate (C16:0)	0.27
Methyl stearate (C18:0)	0.37
Methyl arachidate (C20:0)	0.54
Methyl 12-ketostearate	0.85
Methyl 12-hydroxystearate	1.00

Suitability requirements
Resolution: NLT 5 between any two adjacent peaks

[1] 0.5 M sodium methoxide in methanol is available from Sigma-Aldrich (www.sigmaaldrich.com), product #403067. Any other equivalent reagent can be used as well.

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Relative standard deviation: NMT 2.0% for the peak area ratio of methyl 12-hydroxystearate to methyl 12-ketostearate

Analysis

Samples: *Standard solution 1*, *Standard solution 2*, and *Sample solution*

Calculate the relative response factor, *F*, for methyl 12-hydroxystearate:

$$F = (r_S/r_R) \times (C_R/C_S)$$

r_S = peak area of methyl stearate from *Standard solution 2*

r_R = peak area of methyl 12-hydroxystearate from *Standard solution 2*

C_R = concentration of USP Methyl 12-Hydroxystearate RS in *Standard solution 2* (mg/mL)

C_S = concentration of USP Methyl Stearate RS in *Standard solution 2* (mg/mL)

Correct the peak area of methyl 12-hydroxystearate in the *Sample solution* by multiplying by *F*.

Calculate the percentage of each fatty acid component in the portion of Polyoxyl 40 Hydrogenated Castor Oil taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak area of each individual fatty acid methyl ester except for the uncorrected peak area of methyl 12-hydroxystearate (or the corrected peak area of methyl 12-hydroxystearate), from the *Sample solution*

r_T = sum of all the peak areas, excluding the solvent and methyl 12-hydroxystearate peaks and including the corrected peak area of methyl 12-hydroxystearate, from the *Sample solution*

Acceptance criteria: Polyoxyl 40 Hydrogenated Castor Oil exhibits the composition profile of fatty acids shown in *Table 3*.

Table 3

Component	Percentage (%)
Palmitic acid (C16:0)	≤4.0
Stearic acid (C18:0)	15.0–25.0
Arachidic acid (C20:0)	≤2.0
12-Ketostearic acid (or 12-oxostearic acid)	≤5.0
12-Hydroxystearic acid	50.0–70.0

• **B. Constituting Fatty Acids**

Sample: 0.1 g

Analysis: Dissolve the *Sample* in 10 mL of alcoholic potassium hydroxide TS, boil for 3 min, and evaporate to dryness. Mix the residue with 5 mL of water.

Acceptance criteria: The residue dissolves, yielding a clear solution. Add a few drops of glacial acetic acid. A white precipitate is formed.

• **C. Indication of Saturation**

Analysis: Proceed as directed in *Fats and Fixed Oils* ⟨401⟩, *Procedures, Iodine Value*.

Acceptance criteria: NMT 2.0

IMPURITIES

• **Residue on Ignition** ⟨281⟩: NMT 0.3%

• **Ethylene Oxide and Dioxane** ⟨228⟩, *Method I*

Acceptance criteria

Ethylene oxide: NMT 1 µg/g

Dioxane: NMT 10 µg/g

• **Limit of Nickel**

[Caution—When using closed high-pressure digestion vessels and laboratory microwave equipment, the safety precautions and operating instructions given by the manufacturer must be followed.]

[Note—If an alternative apparatus is used, adjustment of the instrument parameters may be necessary.]

Nickel standard stock solution: Dilute nickel standard solution TS two-fold with water. This solution contains the equivalent of 5 µg/mL of nickel.

Standard solutions: Transfer 25, 50, 75, and 100 µL of *Nickel standard stock solution* to four identical 25-mL volumetric flasks. To each flask add 0.5 mL of a 10-mg/mL solution of magnesium nitrate, 0.5 mL of a 100-mg/mL solution of monobasic ammonium phosphate, and 6.0 mL of nickel-free nitric acid. Dilute with water to volume, and mix well. [Note—Content of nickel in the nickel-free nitric acid is NMT 0.005 ppm. The *Standard solutions* contain 0.005, 0.01, 0.015, and 0.02 µg/mL of nickel, respectively.]

Sample solution: Transfer about 250 mg of Polyoxyl 40 Hydrogenated Castor Oil to a suitable high-pressure-resistant digestion vessel (fluoropolymer or quartz glass), and add 6.0 mL of nickel-free nitric acid and 2.0 mL of 30% hydrogen peroxide. Place the closed vessel in a laboratory microwave oven, and digest using an appropriate program (e.g., 1000 W for 40 min). Allow the digestion vessel to cool before opening. Add 2.0 mL of 30% hydrogen peroxide, and repeat the digestion step. Allow the digestion vessel to cool before opening. Quantitatively transfer to a 25-mL volumetric flask, add 0.5 mL of a 10-mg/mL solution of magnesium nitrate and 0.5 mL of a 100-mg/mL solution of monobasic ammonium phosphate. Dilute with water to volume, and mix well.

Blank solution: Place 6.0 mL of nickel-free nitric acid and 2.0 mL of 30% hydrogen peroxide in a suitable high-pressure-resistant digestion vessel. Prepare as directed in the *Sample solution*, beginning with "Place the closed vessel in a laboratory microwave oven, and digest using an appropriate program (e.g., 1000 W for 40 min)."

Zero solution: In a 50-mL volumetric flask, introduce 1.0 mL of a 10-mg/mL solution of magnesium nitrate, 1.0 mL of a 100-mg/mL solution of monobasic ammonium phosphate, and 12.0 mL of nickel-free nitric acid. Dilute with water to volume, and mix well.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption, equipped with a graphite furnace, a background compensation system, and a coated tube resistant to pyrolysis

Analytical wavelength: 232.0 nm

Lamp: Nickel hollow-cathode

Temperature: Maintain the drying temperature of the furnace at 120° for 35 s after a 5-s ramp; maintain the ashing temperature at 1100° for 10 s after a 30-s ramp; maintain the cooling temperature at 800° for 5 s after a 5-s decrease; and maintain the atomization temperature at 2600° for 7 s. [Note—The temperature program may be modified to obtain optimum furnace temperatures.]

Analysis

Samples: *Standard solutions*, *Sample solution*, and *Blank solution*

Concomitantly determine the absorbances of the *Samples* using the *Instrumental conditions* described above. Use the *Zero solution* to set the instrument to zero. Plot the absorbances of the *Standard solutions* versus the

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concentration, in µg/mL, of nickel, and draw the straight line best fitting the plotted points. From the graph so obtained, determine the concentration, C_T, in µg/mL, of nickel in the *Sample solution*, and determine the concentration, C_B, in µg/mL, of nickel in the *Blank solution*. If necessary, dilute with the *Zero solution* to obtain a reading within the calibrated absorbance range. Calculate the quantity, in µg, of nickel in each g of Polyoxyl 40 Hydrogenated Castor Oil taken:

$$\text{Result} = V \times [(C_T - C_B)/W]$$

V = volume of the *Sample solution* and the *Blank solution*, 25 mL
C_T = concentration of nickel in the *Sample solution* (µg/mL)
C_B = concentration of nickel in the *Blank solution* (µg/mL)
W = weight of Polyoxyl 40 Hydrogenated Castor Oil taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 20 µg/g

SPECIFIC TESTS
- **CONGEALING TEMPERATURE** ⟨651⟩**:** 16°–26°
- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Acid Value*: NMT 2.0
- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Hydroxyl Value*: 57–80
- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Saponification Value*: 45–69
- **WATER DETERMINATION** ⟨921⟩, *Method I*: NMT 3.0%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light and moisture. Store at room temperature, and avoid exposure to excessive heat.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Methyl 12-Hydroxystearate RS
  USP Methyl Palmitate RS
  USP Methyl Stearate RS

Polyoxyl Lauryl Ether

$CH_3(CH_2)_{11}(OCH_2CH_2)_nOH$, n = 3–23
Polyethylene glycol monolauryl ether [9002-92-0].

DEFINITION
Polyoxyl Lauryl Ether is a mixture of the monolauryl ethers of mixed polyethylene glycols, the average polymer length being equivalent to NLT 3 and NMT 23 oxyethylene units (nominal value). It contains various amounts of free lauryl alcohol, and it may contain some free polyethylene glycols.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F** ▲ (CN 1-May-2020)
  Sample: Use a thin film of melted Polyoxyl Lauryl Ether if the material is a solid.
  Acceptance criteria: Meets the requirements
- **B. PROCEDURE**
  Sample: 0.1 g
  Analysis: Dissolve or disperse the *Sample* in 5 mL of alcohol, and add 10 mL of diluted hydrochloric acid, 5 mL of barium chloride TS, and 10 mL of phosphomolybdic acid solution (1 in 10).
  Acceptance criteria: A precipitate is formed.
- **C.** It meets the requirements of the test for *Fats and Fixed Oil, Hydroxyl Values* ⟨401⟩.

IMPURITIES
ORGANIC IMPURITIES
- **Procedure: Limit of Free Ethylene Oxide and Dioxane**

Analysis: Proceed as directed in *Ethylene Oxide and Dioxane, Method I* ⟨228⟩.
Acceptance criteria
Ethylene oxide: NMT 1 µg/g (ppm)
Dioxane: NMT 10 µg/g (ppm)

SPECIFIC TESTS
- **ALKALINITY**
  Sample: 2.0 g of Polyoxyl Lauryl Ether
  Analysis: Dissolve the *Sample* in a hot mixture of 10 mL of alcohol and 10 mL of water. Add 0.05 mL of bromothymol blue TS, and titrate with 0.1 N hydrochloric acid to a yellow endpoint.
  Acceptance criteria: NMT 0.5 mL of 0.1 N hydrochloric acid is required.
- **APPEARANCE OF SOLUTION:** 5.0 g of Polyoxyl Lauryl Ether in 50.0 mL of alcohol. The solution is not more intensely colored than a solution prepared immediately before use by mixing 12.0 mL of ferric chloride CS, 5.0 mL of cobaltous chloride CS, and 2.0 mL of cupric sulfate CS with dilute hydrochloric acid (10 g/L) to make 50.0 mL, and diluting 12.5 mL of this solution with dilute hydrochloric acid (10 g/L) to make 100.0 mL. Make the comparison by viewing the substance and the solution downward in matched color-comparison tubes against a white surface (see *Color and Achromicity* ⟨631⟩).
- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 0.2%, determined on 2.0 g
- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩**:** NMT 1.0, determined on 5.0 g
- **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩**:** Within the ranges specified in the accompanying table

Oxyethylene Units/Molecule (Nominal Value)	Hydroxyl Value
3	165–185
4	145–165
5	130–140
9	90–100
10	85–95
12	73–83
15	64–74
20–23	40–60

- **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩**:** NMT 2.0
- **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩**:** NMT 3.0, determined on 10.0 g
- **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 3.0%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers, and store in a cool, dry place.
- **LABELING:** Label it to indicate the average nominal number of oxyethylene units.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Polyoxyl 4 Lauryl Ether RS
  USP Polyoxyl 9 Lauryl Ether RS
  USP Polyoxyl 23 Lauryl Ether RS

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Polyoxyl Oleate

Polyethylene glycol monooleate [9004-96-0].

DEFINITION

Polyoxyl Oleate is a mixture of the monoesters and diesters of oleic acid and mixed polyethylene glycols. It may be obtained by ethoxylation of oleic acid or by esterification of polyethylene glycols with oleic acid of animal or vegetable origin. It may contain free polyethylene glycol. The average polymer length is equivalent to either 5–6 or 10 oxyethylene units (nominal values). A suitable antioxidant may be added.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F** ▲ (CN 1-May-2020)

Sample: Undried specimen
Acceptance criteria: Meets the requirements

IMPURITIES

- **LIMIT OF FREE ETHYLENE OXIDE AND DIOXANE**

Analysis: Proceed as directed in *Ethylene Oxide and Dioxane* ⟨228⟩, *Method 1*.
Acceptance criteria
Ethylene oxide: NMT 1 µg/g
Dioxane: NMT 10 µg/g

SPECIFIC TESTS

- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩

Sample: 10.0 g
Acceptance criteria: NMT 1.0

- **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩: NMT 12.0
- **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩: See *Table 1*.
- **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩: See *Table 1*.
- **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩: See *Table 1*.

Table 1

	5–6 Ethylene Oxide Units	10 Ethylene Oxide Units
Hydroxyl value	50–70	65–90
Iodine value	50–60	27–34
Saponification value	105–120	68–85

- **FATS AND FIXED OILS,** *Fatty Acid Composition* ⟨401⟩: Polyoxyl Oleate exhibits the composition profile of fatty acids shown in *Table 2*.

Table 2

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
14	0	≤5.0
16	0	≤16.0
18	0	≤6.0
16	1	≤8.0
18	1	65.0–88.0
18	2	≤18.0
18	3	≤4.0
>18	—	≤4.0

- **ALKALINITY**

Sample solution: 100 mg/mL of Polyoxyl Oleate in alcohol
Analysis: To 2 mL of the *Sample solution* add 0.05 mL of phenol red TS.
Acceptance criteria: The solution is not red.

- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩

Sample: 1.0 g
Acceptance criteria: NMT 0.3%

- **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 2.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and store in a cool, dry place. Protect from moisture.
- **LABELING:** Label it to indicate the number of ethylene oxide units per molecule (nominal value), and the name and concentration of any added antioxidant.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Polyoxyl Oleate RS

Polyoxyl Stearate

$R\text{-}CO\text{-}(OCH_2CH_2)_n\text{-}OH$

$R\text{-}CO\text{-}(OCH_2CH_2)_n\text{-}OOC\text{-}R$

$R = CH_3(CH_2)_{16}$ or $CH_3(CH_2)_{14}$

n = 6–100
Polyethylene glycol stearate;
Polyethylene glycol monostearate;
Poly(oxy-1,2-ethanediyl), α-hydro-ω-hydroxyoctadecanoate [9004-99-3].

DEFINITION

Polyoxyl Stearate is a mixture of monoesters and diesters of mainly stearic (octadecanoic) acid and/or palmitic (hexadecanoic) acid and polyethylene glycols. The fatty acids may be of vegetable, animal, or synthetic origin. Polyoxyl Stearate Type I or Type II differs in its content of stearic acid. It may contain free polyethylene glycols. The average polymer length is equivalent to 6–100 ethylene oxide units per molecule (nominal value).

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197A**▲ (CN 1-May-2020)

Sample: Use an undried specimen.

- **B.** It meets the requirements of the test for *Content of Stearic Acid and Palmitic Acid*.

ASSAY

- **CONTENT OF STEARIC ACID AND PALMITIC ACID**

Polyoxyl Stearate exhibits the composition profiles of fatty acids shown in *Table 1* below, as determined in *Fats and Fixed Oils* ⟨401⟩, *Fatty Acid Composition*.

Table 1

	Content of Stearic Acid and Palmitic Acid
Polyoxyl Stearate Type I	Stearic Acid: 40.0%–60.0%; sum of Palmitic and Stearic acids: NLT 90.0%
Polyoxyl Stearate Type II	Stearic Acid: 90.0%–99.0%; sum of Palmitic and Stearic acids: NLT 96.0%

- **CONTENT OF FREE POLYETHYLENE GLYCOLS**

[NOTE—This test is for Polyoxyl 40 Stearate only.]

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Sample: 6 g of Polyoxyl 40 Stearate
Analysis: Transfer the *Sample* to a 500-mL separator containing 50 mL of ethyl acetate. Dissolve completely, then add 50 mL of sodium chloride solution (29 in 100), shake vigorously for 2 min, and allow to separate for 15 min. If separation is incomplete, carefully insert the separator into the well of a steam bath for short time intervals. Repeat this technique as many times as necessary to ensure the complete separation of the two phases. Cool, and drain the lower, aqueous phase into a second 500-mL separator. Extract the upper layer with a second 50-mL portion of sodium chloride solution (29 in 100). Repeat the separation as before, including the steam bath technique, to facilitate complete separation.
To the combined aqueous layers add 50 mL of ethyl acetate, shake vigorously for 2 min, and allow to separate as before. Drain the lower, aqueous phase into a third 500-mL separator, and extract it with two 50-mL portions of chloroform, shaking for 2 min each time. Repeat the steam bath technique to ensure complete separation.
Evaporate the combined chloroform extracts in a 150-mL beaker on a steam bath, with the aid of a stream of nitrogen, to apparent dryness.
Redissolve in about 15 mL of chloroform, and filter, collecting the filtrate in a weighed 150-mL beaker. Record the weight of the empty 150-mL beaker, W_1, in g. Rinse the funnel with several small portions of chloroform, and evaporate the combined filtrate and rinsings, as described above, to remove chloroform or ethyl acetate.
Dry in vacuum at 60° for 1 h. Cool in a desiccator, and weigh. Record the weight, W_2, in g.
Calculate the percentage of free polyethylene glycols in Polyoxyl 40 Stearate taken:

$$\text{Result} = [(W_2 - W_1)/W] \times 100$$

W = weight of Polyoxyl 40 Stearate (g)

Acceptance criteria: 17%–27% of free polyethylene glycols for Polyoxyl 40 Stearate only

IMPURITIES

- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 0.3%, determined on 1.0 g
- **LIMIT OF ETHYLENE OXIDE AND DIOXANE**
  Analysis: Proceed as directed in *Ethylene Oxide and Dioxane* ⟨228⟩, *Method II.*
  Acceptance criteria
  Ethylene oxide: 1 ppm
  Dioxane: 380 ppm

SPECIFIC TESTS

- **ALKALINITY**
  Phenol red solution: Dissolve 100 mg of phenolsulfonphthalein in a mixture of 2.82 mL of 0.1 M sodium hydroxide and 20 mL of alcohol, and dilute with water to 100 mL.
  Sample solution: 2.0 g of Polyoxyl Stearate
  Analysis: Dissolve the *Sample* in alcohol and dilute with alcohol to 20 mL. To 2 mL of this solution add 0.05 mL of *Phenol red solution.*
  Acceptance criteria: The solution does not turn red.
- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩**:** NMT 6.0
- **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩**:** Within the ranges specified in *Table 2*
- **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩**:** NMT 3.0
- **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩**:** NMT 10.0
- **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩**:** Within the ranges specified in *Table 2*
- **MELTING RANGE OR TEMPERATURE** ⟨741⟩
  Sample: 10 g
  Analysis: Melt the *Sample* at 80°–90°. Introduce a sufficient amount of the *Sample* into the tube by capillary action to form a column of the prescribed height in the tube. Allow to stand at 0° for 2 h.
  Acceptance criteria: Within the ranges specified in *Table 2*

Table 2

Ethylene Oxide Units/ Molecule (Nominal Value)	Melting Range or Temperature (°)	Hydroxyl Value	Saponification Value
6	26–37	80–110	90–115
8	26–35	80–105	88–100
32	46–50	20–40	30–45
40	Measure Congealing Temperature	25–40	25–35
75	53–59	15–35	8–25
100	48–60	15–30	5–20

- **CONGEALING TEMPERATURE** ⟨651⟩**:** 37°–47° for Polyoxyl 40 Stearate only
- **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 3.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and store at room temperature. Protect from light and moisture.
- **LABELING:** Label it to indicate the number of ethylene oxide units/molecule (nominal value), and the type of Polyoxyl Stearate. Label it to indicate whether the fatty acids are derived from vegetable, animal, or synthetic sources.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Polyoxyl 6 Stearate RS
  USP Polyoxyl 8 Stearate RS
  USP Polyoxyl 32 Stearate RS
  USP Polyoxyl 40 Stearate RS
  USP Polyoxyl 75 Stearate RS
  USP Polyoxyl 100 Stearate RS

Polyoxyl Stearyl Ether

Polyethylene glycol monostearyl ether

$CH_3(CH_2)_{17}(OCH_2CH_2)_nOH$, n = 2–20 [9005-00-9].

DEFINITION

Polyoxyl Stearyl Ether is a mixture of the monostearyl ethers of mixed polyethylene glycols, the average polymer length being equivalent to NLT 2 and NMT 20 oxyethylene units (nominal value). It may contain various amounts of free stearyl alcohol and some free polyethylene glycol.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F.▲** (CN 1-May-2020) Use a thin film of melted Polyoxyl Stearyl Ether.
- **B. PROCEDURE**
  Sample: 0.1 g
  Analysis: Dissolve or disperse the *Sample* in alcohol. Add 10 mL of diluted hydrochloric acid, 5 mL of barium chloride TS, and 10 mL of phosphomolybdic acid solution (1 in 10).

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Acceptance criteria: A precipitate is formed.

• **C.** It meets the requirements in the test for *Fats and Fixed Oils, Hydroxyl Value* ⟨401⟩.

IMPURITIES

ORGANIC IMPURITIES

• **Procedure: Limit of Free Ethylene Oxide and Dioxane**

Analysis: Proceed as directed in *Ethylene Oxide and Dioxane* ⟨228⟩, *Method I.*

Acceptance criteria

Ethylene oxide: NMT 1 µg/g (ppm)

Dioxane: NMT 10 µg/g (ppm)

SPECIFIC TESTS

• **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩**:** NMT 1.0, determined on 5.0 g

• **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩**:** Within the ranges specified in the table below

Oxyethylene Units/Molecule (Nominal Value)	Hydroxyl Value
2	150–180
10	75–90
20	40–60

• **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩**:** NMT 2.0

• **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩**:** NMT 3.0, determined on 10.0 g

• **ALKALINITY**

Sample: 2.0 g of Polyoxyl Stearyl Ether

Analysis: Dissolve the *Sample* to a hot mixture of 10 mL of alcohol and 10 mL of water. Add 0.05 mL of bromothymol blue TS. Titrate with 0.1 N hydrochloric acid to a yellow endpoint.

Acceptance criteria: NMT 0.5 mL of 0.1 N hydrochloric acid is required.

• **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 3.0%

• **APPEARANCE OF SOLUTION:** 5.0 g of Polyoxyl Stearyl Ether in 50.0 mL of alcohol. The solution is not more intensely colored than a solution prepared immediately before use by mixing 12.0 mL of ferric chloride CS, 5.0 mL of cobaltous chloride CS, and 2.0 mL of cupric sulfate CS with dilute hydrochloric acid (10 g/L) to make 50.0 mL, and diluting 12.5 mL of this solution with dilute hydrochloric acid (10 g/L) to make 100.0 mL. Make the comparison by viewing the substance and the solution downward in matched color-comparison tubes against a white surface (see *Color and Achromicity* ⟨631⟩).

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers, and store in a cool, dry place.

• **LABELING:** Label it to indicate the average nominal number of oxyethylene units.

• **USP REFERENCE STANDARDS** ⟨11⟩

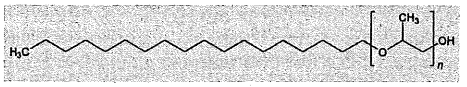
USP Polyoxyl 2 Stearyl Ether RS

USP Polyoxyl 10 Stearyl Ether RS

USP Polyoxyl 20 Stearyl Ether RS

Add the following:

▲Polypropylene Glycol 11 Stearyl Ether



$C_{18}H_{38}O(C_3H_6O)_n$, where the average value of *n* is 11.

Poly[oxy(methyl-1,2-ethanediyl)], α-octadecyl-ω-hydroxy-;

Polypropylene glycol monooctadecyl ether;

Polyoxypropylene 11 stearyl ether [25231-21-4].

DEFINITION

Mixture of ethers of polypropylene glycol with linear alcohols, mainly stearyl alcohol, obtained by the reaction of stearyl alcohol with propylene oxide. It may contain some free polypropylene glycol and various amounts of free stearyl alcohol. The number of moles of propylene oxide reacted per mole of stearyl alcohol is 11 (nominal value). A suitable antioxidant may be added.

IDENTIFICATION

• **A. SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy***:** 197A

• **B.** It meets the requirements in *Specific Tests* for *Fats and Fixed Oils* ⟨401⟩, *Procedures, Hydroxyl Value.*

• **C.** It meets the requirements in *Specific Tests* for *Fats and Fixed Oils* ⟨401⟩, *Procedures, Saponification Value.*

• **D. VISCOSITY**

Analysis: Follow the procedures in *Viscosity—Capillary Methods* ⟨911⟩, *Method I.* Immerse the viscometer in a water or oil bath stabilized at 20°. The measurement can also be carried out with a rotational rheometer or viscometer[1] following the procedures in *Viscosity—Rotational Methods* ⟨912⟩, *Method III.* The temperature under the test condition is at 20°. The samples are tested in the controlled stress mode with a shear stress increasing from 0–100 Pa for 120 s with 100 measured points. Each sample is equilibrated in the measuring gap of 0.052 mm for 60 s. The dynamic viscosity is determined from the slope of the flow curve.

Acceptance criteria: 83–95 mPa · s

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩

Sample: 1.0 g

Acceptance criteria: NMT 0.3%

• **LIMIT OF FREE PROPYLENE OXIDE**

Propylene oxide stock solution: Accurately weigh 45 g of polyethylene glycol 200 kept at 8°–15° and add 1.0 g of propylene oxide. Carefully mix the solution by swirling, ensuring a homogenous solution. Add further polyethylene glycol 200 until the total weight is 50.0 g and mix again. Allow the solution to reach room temperature. Dilute 0.5 g of this solution up to 100.0 mL in water. Note the exact mass. [NOTE—*Propylene oxide stock solution* is stable for 1 month, if stored at –20°.]

Propylene oxide standard solution: Dilute 10.0 mL of *Propylene oxide stock solution* with water to 100.0 mL.

Propionaldehyde stock solution: Accurately weigh 0.1 g of propionaldehyde into a volumetric flask and dilute with water to 100.0 mL.

Sample solution A: Accurately weigh 1.0 g of Polypropylene Glycol 11 Stearyl Ether into a 20-mL headspace vial. Add 0.5 mL of water kept at 8°–15° and seal

[1] Haake RheoStress 6000, Thermo Scientific, D-Karlsruhe, with a cone/plate geometry (diameter of 35 mm and cone angle of 1°) and other equivalent instruments can be used.

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the vial immediately with a polytetrafluoroethylene (PTFE)-coated silicon membrane and an aluminum cap. Mix carefully.

Sample solution B: Accurately weigh 1.0 g of Polypropylene Glycol 11 Stearyl Ether into a 20-mL headspace vial. Add 0.5 mL of *Propylene oxide standard solution* kept at 8°–15° and seal the vial immediately with a PTFE-coated silicon membrane and an aluminum cap. Mix carefully.

Reference solution: Add 50.0 mL of *Propylene oxide standard solution* kept at 8°–15° to a volumetric flask and add 0.5 mL of *Propionaldehyde stock solution* kept at 8°–15°. Dilute with water to 100.0 mL. Introduce 0.5 mL of this solution into a 20-mL headspace vial and seal the vial immediately with a PTFE-coated silicon membrane and an aluminum cap.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC, equipped with a headspace injector

Detector: Flame ionization

Column: 0.32-mm x 60-m fused-silica; coated with a 5-µm film of stationary phase G1

Temperatures

Injection port: 150°

Detector: 250°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
50	—	50	5
50	5	180	0
180	29.4	230	5

Carrier gas: Helium

Flow rate: 2.6 mL/min

Injection volume: A suitable volume (e.g., 1.0 mL) of the gaseous phase of *Sample solution A*, *Sample solution B*, and *Reference solution* (injected separately)

Injection type: Split ratio, 10:1

Static headspace conditions: The vials are maintained at 90° for 45 min before headspace injection. [NOTE—The static headspace conditions can be optimized/adjusted according to the instruments used, if needed.]

System suitability

Sample: *Reference solution*

[NOTE—The relative retention (with reference to the propylene oxide peak with a retention time at ~10.4 min) for the propionaldehyde peak is approximately 0.96.]

Suitability requirements

Resolution: NLT 1.5 between the propionaldehyde and propylene oxide peaks

Signal-to-noise ratio: NLT 10, determined from the propylene oxide peak

Analysis

Samples: *Sample solution A* and *Sample solution B*

Calculate the content of propylene oxide in parts per million (ppm) in the portion of Polypropylene Glycol 11 Stearyl Ether taken:

$$\text{Result} = (A_1 \times m \times 5)/[(A_2 \times M_1) - (A_1 \times M_2)]$$

A_1 = peak area of propylene oxide from *Sample solution A*

m = mass of propylene oxide used to prepare the *Propylene oxide stock solution* (g)

A_2 = peak area of propylene oxide from *Sample solution B*

M_1 = mass of Polypropylene Glycol 11 Stearyl Ether in *Sample solution A* (g)

M_2 = mass of Polypropylene Glycol 11 Stearyl Ether in *Sample solution B* (g)

Acceptance criteria: NMT 5 ppm

SPECIFIC TESTS

• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Acid Value*

Analysis: Accurately weigh 20 g of the sample into a wide-necked flask. To prepare the solvent, heat 50 mL of ethanol to the boiling point for 15–20 s in a second flask and add 0.5 mL of phenolphthalein TS. Neutralize the solvent with 0.1 N sodium hydroxide VS while keeping the temperature above 70°. Add the neutralized solvent to the sample flask and mix thoroughly. Bring the contents to a boil and titrate with 0.1 N sodium hydroxide VS until a single drop produces a slight but definite color change, from colorless to pink, persisting for at least 15 s. For additional information not mentioned here, follow the procedure as directed in the chapter.

Acceptance criteria: NMT 2.0

• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Hydroxyl Value*

Pyridine–acetic anhydride reagent: Just before use, mix 85 volumes of freshly opened or freshly distilled pyridine with 15 volumes of freshly opened or freshly distilled acetic anhydride.

Analysis: Accurately weigh about 1.5 g of the sample into a flat-bottomed flask and pipet 5.0 mL of *Pyridine–acetic anhydride reagent*. Attach an air condenser to the flask and place the solution onto the sand bath[2] for refluxing for 60 min. The sand bath temperature must be set such that the sample reflux temperature is between 85° and 100°. Add 10 mL of water through the top of the condenser, swirl the flask and contents, and allow the flask to remain on the sand bath for a further 5–10 min. Remove the flask from the sand bath and allow it to cool to room temperature. Wash down the condenser with 30 mL of ethanol. Add 0.5 mL of phenolphthalein TS to the flask. Titrate with 0.5 N sodium hydroxide VS until a single drop produces a slight but definite color change, from colorless to pink, persisting for at least 15 s. Perform the test in duplicate, along with a blank. For the blank, proceed exactly as above but omit the sample. Calculate the average hydroxyl value for the sample from the two determinations. For additional information not mentioned here, follow the procedure as directed in the chapter.

Acceptance criteria: 60–77

• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Iodine Value, Method I*

Analysis: Follow the procedure as directed in the chapter, except use 15 mL of chloroform to dissolve the sample instead of 10 mL and the volume of potassium iodide TS added in the process is 6 mL instead of 30 mL.

Acceptance criteria: NMT 3.0

• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Peroxide Value*

Analysis: Accurately weigh about 10 g of Polypropylene Glycol 11 Stearyl Ether. Follow the procedure as directed in

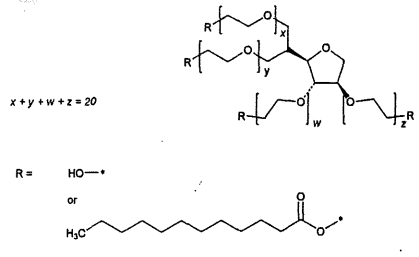
[2] Other types of heating devices may also be used if suitable for the intended use.

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the chapter, except use 0.1 N sodium thiosulfate VS in the titration instead of 0.01 N sodium thiosulfate solution.
Acceptance criteria: NMT 5.0
• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Saponification Value*
Analysis: Accurately weigh about 20 g of Polypropylene Glycol 11 Stearyl Ether. Follow the procedure as directed in the chapter, except that as the endpoint approaches in the titration reheat the solution to ensure that the endpoint (color change from pink to colorless) is achieved at the boiling point of the solution.
Acceptance criteria: NMT 3.0
• **WATER DETERMINATION** ⟨921⟩, *Method I, Method Ic:* NMT 0.7%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight containers.
• **LABELING:** Label to indicate the names and proportions of any added antioxidant.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Polypropylene Glycol 11 Stearyl Ether RS▲ (NF 1-May-2020)

Polysorbate 20



The ratio of the OH group to the ($C_{11}H_{23}COO$) group is mainly 3:1.
Polyethylene glycol 20 sorbitan ether monolaurate;
Polyoxyethylene 20 sorbitan monododecanoate;
Polyoxyethylene 20 sorbitan monolaurate [9005-64-5].

DEFINITION
Polysorbate 20 is a laurate ester of sorbitol and its anhydrides, copolymerized with approximately 20 moles of ethylene oxide for each mole of sorbitol and sorbitol anhydrides. The fatty acids may be of vegetable, animal, or synthetic origin.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F** ▲ (CN 1-May-2020)
• **B.** It meets the requirements in the *Assay* for *Composition of Fatty Acids*.

ASSAY
• **COMPOSITION OF FATTY ACIDS:** Polysorbate 20 exhibits the composition profiles of fatty acids shown in *Table 1*, as determined in *Fats and Fixed Oils* ⟨401⟩, *Fatty Acid Composition*.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
6	0	≤1.0
8	0	≤10.0
10	0	≤10.0
12	0	40.0–60.0
14	0	14.0–25.0
16	0	7.0–15.0
18	0	≤11.0
18	1	≤11.0
18	2	≤3.0

IMPURITIES
• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.25%
• **LIMIT OF ETHYLENE OXIDE AND DIOXANE,** *Method II* ⟨228⟩
Acceptance criteria
Ethylene oxide: NMT 1 ppm
Dioxane: NMT 10 ppm

SPECIFIC TESTS
• **BACTERIAL ENDOTOXINS TEST** ⟨85⟩
For Polysorbate 20 intended for use in the manufacture of injectable dosage forms: The level of bacterial endotoxins is such that the requirement in the relevant dosage form monograph(s) in which Polysorbate 20 is used can be met. Where the label states that Polysorbate 20 must be subjected to further processing during the preparation of injectable dosage forms, the level of bacterial endotoxins is such that the requirement in the relevant dosage form monograph(s) in which Polysorbate 20 is used can be met.
• **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩
Sample: 10.0 g
Analysis: Transfer the *Sample* to a wide-mouth, 250-mL conical flask, and add 50 mL of neutralized alcohol. Heat on a steam bath nearly to boiling, occasionally shaking thoroughly while heating. Invert a beaker over the mouth of the flask, cool under running water, and add 5 drops of phenolphthalein TS. Titrate with 0.1 N sodium hydroxide VS. Calculate the acid value as directed in the chapter.
Acceptance criteria: NMT 2.0
• **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩: 96–108
• **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩
Sample: 10.0 g
Saturated potassium iodide solution: Prepare a saturated solution of potassium iodide in carbon dioxide-free water. Make sure the solution remains saturated as indicated by the presence of undissolved crystals.
Analysis: Introduce the *Sample* into a 100-mL beaker, and dissolve with 20 mL of glacial acetic acid. Add 1 mL of *Saturated potassium iodide solution*, mix, and allow to stand for 1 min. Add 50 mL of carbon dioxide-free water and a magnetic stirring bar. Titrate with 0.01 M sodium thiosulfate VS, determining the endpoint potentiometrically (see *Titrimetry* ⟨541⟩). Perform a blank titration. Calculate the peroxide value as directed in the chapter.
Acceptance criteria: NMT 10.0
For Polysorbate 20 intended for use in the manufacture of injectable dosage forms: NMT 5.0
• **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩: 40–50
• **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 3.0%

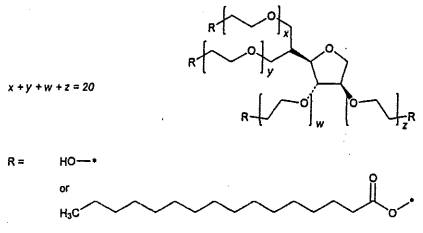
ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light and moisture. Store at room temperature.

NF Monographs

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- **LABELING:** Label it to indicate whether the fatty acids are derived from animal, vegetable, or synthetic sources. Where Polysorbate 20 is intended for use in the manufacture of injectable dosage forms, it is so labeled.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Polysorbate 20 RS

Polysorbate 40



The ratio of OH group to $C_{15}H_{31}COO$ group is mainly 3:1.
Polyethylene glycol 20 sorbitan ether monopalmitate;
Polyoxyethylene 20 sorbitan monohexadecanoate;
Polyoxyethylene 20 sorbitan monopalmitate [9005-66-7].

DEFINITION
Polysorbate 40 is a palmitate ester of sorbitol and its anhydrides copolymerized with about 20 moles of ethylene oxide for each mole of sorbitol and sorbitol anhydrides. The fatty acids may be of vegetable, animal, or synthetic origin.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F** ▲ (CN 1-May-2020)
- **B.** It meets the requirements in the *Assay* for *Composition of Fatty Acids*.

ASSAY
- **COMPOSITION OF FATTY ACIDS**
  Polysorbate 40 exhibits the composition profiles of fatty acids shown in *Table 1*, as determined in *Fats and Fixed Oils* ⟨401⟩, *Fatty Acid Composition*.

Table 1

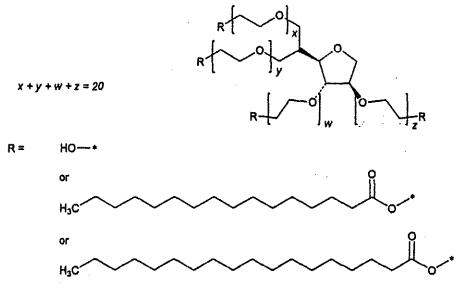
Carbon-Chain Length	Number of Double Bonds	Percentage (%)
16	0	≥92.0

IMPURITIES
- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.25%
- **ETHYLENE OXIDE AND DIOXANE,** *Method II* ⟨228⟩
  Acceptance criteria
  Ethylene oxide: NMT 1 ppm
  Dioxane: NMT 10 ppm

SPECIFIC TESTS
- **BACTERIAL ENDOTOXINS TEST** ⟨85⟩
  For Polysorbate 40 intended for use in the manufacture of injectable dosage forms: The level of bacterial endotoxins is such that the requirement in the relevant dosage form monograph(s) in which Polysorbate 40 is used can be met. Where the label states that Polysorbate 40 must be subjected to further processing during the preparation of injectable dosage forms, the level of bacterial endotoxins is such that the requirement in the relevant dosage form monograph(s) in which Polysorbate 40 is used can be met.
- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩
  Sample: 10.0 g
  Analysis: Transfer the *Sample* to a wide-mouth, 250-mL conical flask, and add 50 mL of neutralized alcohol. Heat on a steam bath nearly to boiling, shaking thoroughly occasionally while heating. Invert a beaker over the mouth of the flask, cool under running water, and add 5 drops of phenolphthalein TS. Titrate with 0.1 N sodium hydroxide VS. Calculate the acid value as directed in the chapter.
  Acceptance criteria: NMT 2.0
- **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩: 89–105
- **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩
  Sample: 10.0 g
  Saturated potassium iodide solution: Prepare a saturated solution of potassium iodide in carbon dioxide-free water. Make sure the solution remains saturated as indicated by the presence of undissolved crystals.
  Analysis: Introduce the *Sample* into a 100-mL beaker, and dissolve with 20 mL of glacial acetic acid. Add 1 mL of *Saturated potassium iodide solution*, mix, and allow to stand for 1 min. Add 50 mL of carbon dioxide-free water and a magnetic stirring bar. Titrate with 0.01 M sodium thiosulfate VS, determining the endpoint potentiometrically (see *Titrimetry* ⟨541⟩). Perform a blank titration.
  Calculate the peroxide value as directed in the chapter.
  Acceptance criteria: NMT 10.0
  For Polysorbate 40 intended for use in the manufacture of injectable dosage forms: NMT 5.0
- **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩: 41–52
- **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 3.0%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light and moisture. Store at room temperature.
- **LABELING:** Label it to indicate whether the fatty acids are derived from animal, vegetable, or synthetic sources. Where Polysorbate 40 is intended for use in the manufacture of injectable dosage forms, it is so labeled.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Polysorbate 40 RS

Polysorbate 60



The ratio of OH group to the sum of $C_{15}H_{31}COO$ and $C_{17}H_{35}COO$ groups is mainly 3:1.
Polyethylene glycol 20 sorbitan ether monostearate;
Polyoxyethylene 20 sorbitan monooctadecanoate;
Polyoxyethylene 20 sorbitan monostearate [9005-67-8].

DEFINITION
Polysorbate 60 is a mixture of stearate and palmitate esters of sorbitol and its anhydrides copolymerized with about 20 moles of ethylene oxide for each mole of sorbitol and sorbitol

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anhydrides. The fatty acids may be of vegetable, animal, or synthetic origin.

IDENTIFICATION

Change to read:

- **A.** **▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F** ▲ (CN 1-May-2020)
- **B.** It meets the requirements in the *Assay* for *Composition of Fatty Acids*.

ASSAY

- **COMPOSITION OF FATTY ACIDS**

Polysorbate 60 exhibits the composition profiles of fatty acids shown in *Table 1*, as determined in *Fats and Fixed Oils* ⟨401⟩, *Fatty Acid Composition*.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
18	0	40.0–60.0
Sum of stearic acid (C18:0) and palmitic acid (C16:0)		≥90.0

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.25%
- **ETHYLENE OXIDE AND DIOXANE,** *Method II* ⟨228⟩

Acceptance criteria

Ethylene oxide: NMT 1 ppm

Dioxane: NMT 10 ppm

SPECIFIC TESTS

- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩

Sample: 10.0 g of Polysorbate 60

Analysis: Transfer the *Sample* to a wide-mouth, 250-mL conical flask, and add 50 mL of neutralized alcohol. Heat on a steam bath nearly to boiling, shaking thoroughly occasionally while heating. Invert a beaker over the mouth of the flask, cool under running water, and add 5 drops of phenolphthalein TS. Titrate with 0.1 N sodium hydroxide VS. Calculate the acid value as directed in the chapter.

Acceptance criteria: NMT 2.0

- **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩: 81–96
- **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩

Sample: 10.0 g

Saturated potassium iodide solution: Prepare a saturated solution of potassium iodide in carbon dioxide-free water. Make sure the solution remains saturated as indicated by the presence of undissolved crystals.

Analysis: Introduce the *Sample* into a 100-mL beaker, and dissolve with 20 mL of glacial acetic acid. Add 1 mL of *Saturated potassium iodide solution*, mix, and allow to stand for 1 min. Add 50 mL of carbon dioxide-free water and a magnetic stirring bar. Titrate with 0.01 M sodium thiosulfate VS, determining the endpoint potentiometrically (see *Titrimetry* ⟨541⟩). Perform a blank titration.

Calculate the peroxide value as directed in the chapter.

Acceptance criteria: NMT 10.0

- **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩: 45–55
- **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 3.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light and moisture. Store at room temperature.
- **LABELING:** Label to indicate whether the fatty acids are derived from animal, vegetable, or synthetic sources.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Polysorbate 60 RS

Polysorbate 80

Portions of the monograph text that are national *USP* text, and are not part of the harmonized text, are marked with symbols (‣ ‣) to specify this fact.

Sorbitan, mono-9-octadecenoate, poly(oxy-1,2-ethanediyl) derivs., (*Z*)-;
Polyoxyethylene 20 sorbitan monooleate [9005-65-6].

DEFINITION

Polysorbate 80 is a mixture of partial esters of fatty acids, mainly oleic acid, with sorbitol and its anhydrides ethoxylated with approximately 20 moles of ethylene oxide for each mole of sorbitol and sorbitol anhydrides.

IDENTIFICATION

- **A.** It meets the requirements of the test for *Composition of Fatty Acids*.

Change to read:

- ‣**B.** **▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F**▲ (CN 1-May-2020) ‣

ASSAY

- **COMPOSITION OF FATTY ACIDS**

Diluent: 20 g/L of sodium hydroxide in methanol

Boron trifluoride–methanol solution: 140 g/L of boron trifluoride in methanol

Saturated sodium chloride solution: Sodium chloride and water (1:2). Before use, decant the solution from any undissolved substance and filter, if necessary.

Reference solution A: Prepare 0.50 g of the mixture of calibrating substances with the composition described in *Table 1*. Dissolve in heptane, and dilute with heptane to 50.0 mL.

Reference solution B: *Reference solution A* in heptane (1 in 10)

Reference solution C: Prepare 0.50 g of a mixture of fatty acid methyl esters, which corresponds to the composition of the substance to be examined. Dissolve in heptane, and dilute with heptane to 50.0 mL. [NOTE—Commercially available mixtures of fatty acid methyl esters may also be used.]

Sample solution: Dissolve 0.10 g of Polysorbate 80 in 2 mL of *Diluent* in a 25-mL conical flask, and boil under a reflux condenser for 30 min. Add 2.0 mL of *Boron trifluoride–methanol solution* through the condenser, and boil for 30 min. Add 4 mL of heptane through the condenser, and boil for 5 min. Cool, add 10.0 mL of *Saturated sodium chloride solution*, shake for about 15 s, and add a quantity of *Saturated sodium chloride solution* such that the upper phase is brought into the neck of the flask. Collect 2 mL of the upper phase, wash with three quantities, each of 2 mL, of water, and dry over anhydrous sodium sulfate.

Table 1

Mixture of the Following Substances	Composition (%)
Methyl myristate	5
Methyl palmitate	10
Methyl stearate	15

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Table 1 *(continued)*

Mixture of the Following Substances	Composition (%)
Methyl arachidate	20
Methyl oleate	20
Methyl eicosenoate	10
Methyl behenate	10
Methyl lignocerate	10

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability.*)
Mode: GC
Detector: Flame ionization
Column: 0.32-mm × 30-m G16 on fused silica; film thickness 0.5 µm
Temperatures
Injection port: 250°
Detector: 250°
Column: See *Table 2.*

Table 2

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
80	10	220	—
220	—	220	40

Carrier gas: Helium
Linear velocity: 50 cm/s
Injection volume: 1 µL
Injection type: Split ratio, 50:1
System suitability
Samples: *Reference solution A* and *Reference solution B*
Suitability requirements
Resolution: NLT 1.8 between the peaks due to methyl oleate and methyl stearate, *Reference solution A*
Theoretical plates: NLT 30,000 calculated for the peak of methyl stearate, *Reference solution A*
Signal-to-noise ratio: NLT 5 for the peak of methyl myristate, *Reference solution B*

Analysis
Sample: *Sample solution*
Identify the peaks from *Reference solution C.*
Calculate the percentage of each component in the *Sample solution:*

$$\text{Result} = (A_C/A_T) \times 100$$

A_C = peak area for the component of interest
A_T = total area of all peaks related to fatty acids

Acceptance criteria: See *Table 3.*

Table 3

Name	Acceptance Criteria, NMT (%)	Acceptance Criteria, NLT (%)
Myristic acid	5.0	—
Palmitic acid	16.0	—
Palmitoleic acid	8.0	—
Stearic acid	6.0	—
Oleic acid	—	58.0
Linoleic acid	18.0	—
Linolenic acid	4.0	—

IMPURITIES

• **Residue on Ignition**
Sample: 2.00 g
Analysis: Heat a silica or platinum crucible to redness for 30 min, allow to cool in a desiccator, and weigh. Evenly distribute the *Sample* in the crucible. Dry at 100°–105° for 1 h and ignite to constant mass in a muffle furnace at 600 ± 25°, allowing the crucible to cool in a desiccator after each ignition. Flames should not be produced at any time during the procedure. If after prolonged ignition the ash still contains black particles, take up with hot water, pass through an ashless filter paper, and ignite the residue and the filter paper. Combine the filtrate with the ash, carefully evaporate to dryness, and ignite to constant mass.
Acceptance criteria: NMT 0.25%

• **Ethylene Oxide and Dioxane**
Ethylene oxide standard solution: Dilute 0.5 mL of a commercially available solution of ethylene oxide in methylene chloride (50 mg/mL) with water to 50.0 mL. [Note—The solution is stable for 3 months, if stored in vials with a Teflon-coated, silicon membrane and crimped caps at –20°.] Allow to reach room temperature. Dilute 1.0 mL of this solution with water to 250.0 mL.
Dioxane standard solution: Dioxane in water (v/v) 1 in 20,000
Acetaldehyde standard solution: 0.01 mg/mL of acetaldehyde in water
Standard solution: Dilute 6.0 mL of *Ethylene oxide standard solution* and 2.5 mL of *Dioxane standard solution* with water to 25.0 mL.
Sample solution A: Transfer 1.0 g of Polysorbate 80 to a 10-mL headspace vial. Add 2.0 mL of water, and seal the vial immediately with a Teflon-coated, silicon membrane and an aluminum cap.
Sample solution B: Transfer 1.0 g of Polysorbate 80 to a 10-mL headspace vial. Add 2.0 mL of the *Standard solution,* and seal the vial immediately with a Teflon-coated, silicon membrane and an aluminum cap.
Reference solution: Transfer 2.0 mL of *Acetaldehyde standard solution* and 2.0 mL of *Ethylene oxide standard solution* to a 10-mL headspace vial, and seal the vial immediately with a Teflon-coated, silicon membrane and an aluminum cap.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability.*)
Mode: Headspace GC
Detector: Flame ionization
Column:[1] 0.53-mm × 50-m G27 on fused silica; film thickness 5 µm
Temperatures
Injection port: 85°
Detector: 250°
Column: See *Table 4.*

[1] CP-Sil 8 CB is suitable.

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Table 4

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	10	250	—
250	—	250	5

Split ratio: 3.5:1
Carrier gas: Helium
Flow rate: 4.0 mL/min
Injection volume: 1 mL
System suitability
Sample: *Reference solution*
[NOTE—The relative retention times for ethylene oxide, acetaldehyde, and dioxane are 1.0, 0.9, and 1.9, respectively. The retention time for ethylene oxide is about 6.5 min.]
Suitability requirements
Resolution: NLT 2.0 between the peaks due to acetaldehyde and ethylene oxide
Analysis
Samples: *Sample solution A* and *Sample solution B*
Calculate the content, in ppm, of ethylene oxide:

$$\text{Result} = (2 \times C_{EO} \times A_A)/(A_B - A_A)$$

C_{EO} = concentration of ethylene oxide in *Sample solution B* (µg/mL)
A_A = peak area of ethylene oxide from *Sample solution A*
A_B = peak area of ethylene oxide from *Sample solution B*

Calculate the content, in ppm, of dioxane:

$$\text{Result} = (2 \times D \times C_D \times A_{A'}) \times 1000/(A_{B'} - A_{A'})$$

D = density of dioxane, 1.03 g/mL
C_D = concentration of dioxane in *Sample solution B* (µL/mL)
$A_{A'}$ = peak area of dioxane from *Sample solution A*
$A_{B'}$ = peak area of dioxane from *Sample solution B*

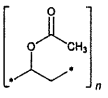
Acceptance criteria: NMT 1 ppm for ethylene oxide; NMT 10 ppm for dioxane

SPECIFIC TESTS
• ‣ **SPECIFIC GRAVITY** ⟨841⟩: 1.06–1.09 ‣
• ‣ **VISCOSITY—CAPILLARY METHODS** ⟨911⟩ or **VISCOSITY—ROTATIONAL METHODS** ⟨912⟩: 300–500 centistokes at 25° ‣
• **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩
Light petroleum: It has the following properties: a clear, colorless, flammable liquid without fluorescence; practically insoluble in water; miscible with alcohol; density at 20° about 0.720; distillation range 100°– 120°; water content NMT 0.03%.[2]
Sample solution: Dissolve 5.0 g in 50 mL of a mixture of equal volumes of alcohol and *Light petroleum* (previously neutralized with 0.1 N potassium hydroxide or 0.1 N sodium hydroxide), using 0.5 mL of phenolphthalein TS as the indicator. If necessary, heat to about 90° to dissolve the substance to be examined.
Analysis: Titrate the *Sample solution* with 0.1 N potassium hydroxide VS or 0.1 N sodium hydroxide VS until the pink color persists for at least 15 s. When heating has been applied to aid dissolution, maintain the temperature at about 90° during the titration.
Acceptance criteria: NMT 2.0
• **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩
Sample: 2.0 g
Analysis: Transfer the *Sample* into a 150-mL acetylation flask fitted with an air condenser. Add 5.0 mL of *Pyridine–Acetic Anhydride Reagent*, and attach the air condenser. Heat the flask in a water bath for 1 h keeping the level of the water about 2.5 cm above the level of the liquid in the flask. Withdraw the flask, and allow to cool. Add 5 mL of water through the upper end of the condenser. If a cloudiness appears, add sufficient pyridine to clear it, noting the volume added. Shake the flask, and replace in the water bath for 10 min. Withdraw the flask, and allow to cool. Rinse the condenser and the walls of the flask with 5 mL of alcohol, previously neutralized with phenolphthalein TS. Titrate with 0.5 N alcoholic potassium hydroxide VS using 0.2 mL of phenolphthalein TS as the indicator. Carry out a blank test under the same conditions.
Acceptance criteria: 65–80
• **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩
Sample: 10.0 g
Saturated potassium iodide solution: Prepare a saturated solution of potassium iodide in carbon dioxide-free water. Make sure the solution remains saturated as indicated by the presence of undissolved crystals.
Analysis: Transfer the *Sample* into a 100-mL beaker, and dissolve with 20 mL of glacial acetic acid. Add 1 mL of *Saturated potassium iodide solution*, and allow to stand for 1 min. Add 50 mL of carbon dioxide-free water and a magnetic stirring bar. Titrate with 0.01 M sodium thiosulfate VS, determining the endpoint potentiometrically. Carry out a blank titration.
Acceptance criteria: NMT 10
• **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩
Sample: 4.0 g
Analysis: Transfer the *Sample* into a 250-mL borosilicate glass flask fitted with a reflux condenser. Add 30.0 mL of 0.5 N alcoholic potassium hydroxide VS and a few glass beads. Attach the condenser, and heat under reflux for 60 min. Add 1 mL of phenolphthalein TS and 50 mL of dehydrated alcohol, and titrate immediately with 0.5 N hydrochloric acid VS. Carry out a blank test under the same conditions.
Acceptance criteria: 45–55
• **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 3.0%, determined on 1.0 g

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Store in an airtight container, protected from light.
• ‣ **USP REFERENCE STANDARDS** ⟨11⟩
USP Polysorbate 80 RS
‣

[2] Petroleum ether; boiling range 100°–140°; [CAS 64742-49-0] from Fisher Scientific; catalog number AC23302-0025 is suitable.

Polyvinyl Acetate



$(C_4H_6O_2)_n$
Vinyl acetate homopolymer
Vinyl acetate resin [9003-20-7].

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DEFINITION

Polyvinyl acetate is a thermoplastic polymer, represented by the formula:

$$(C_4H_6O_2)_n$$

in which the value of n lies between approximately 100 and 17,000.

IDENTIFICATION

• **A. PROCEDURE**

Sample: 100 mg of Polyvinyl Acetate

Analysis: Dissolve the *Sample* in 2.5 mL of acetone, place two drops on a potassium bromide plate, and dry to evaporate the solvent.

Acceptance criteria: The IR absorption spectrum of polyvinyl acetate exhibits maxima corresponding to the same wavelengths as that of a similar preparation of USP Polyvinyl Acetate RS, treated in the same manner.

• **B. PROCEDURE**

Sample: 0.5 g of Polyvinyl Acetate

Analysis: Saponify the *Sample* in a mixture of 25.0 mL of 0.5 N alcoholic potassium hydroxide and 25.0 mL of water.

Acceptance criteria: The solution so obtained meets the requirements of the tests for *Identification Tests—General* ⟨191⟩, *Acetate.*

IMPURITIES

INORGANIC IMPURITIES

• **Residue on Ignition** ⟨281⟩: NMT 0.1%

• **RESIDUAL PEROXIDES**

Sample: 0.85 g of Polyvinyl Acetate

Analysis: Place the *Sample* in a borosilicate glass flask with a ground-glass neck. Add 10.0 mL of ethyl acetate, and heat under a reflux condenser with constant agitation. Allow to cool. Replace the air in the container with oxygen-free nitrogen, and add a solution of 1.0 mL of glacial acetic acid and 0.5 g of sodium iodide in 40.0 mL of water. Shake thoroughly, and allow to stand protected from light for 20 min. Titrate with 0.005 N sodium thiosulfate VS until the yellow color is discharged. Perform a blank titration.

Acceptance criteria: The difference between the titration volumes is not greater than 1.0 mL; and NMT 100 ppm, calculated as hydrogen peroxide, is found.

ORGANIC IMPURITIES

• **Procedure: Limit of Vinyl Acetate**

Standard stock solution: 1.0 mg/mL of Vinyl Acetate in toluene

Standard solutions: 0.1, 0.3, 1, 3, and 10 µg/mL of vinyl acetate in toluene, prepared from *Standard stock solution*

Sample solution: 0.1 g/mL of Polyvinyl Acetate in toluene

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability.*)

Mode: GC

Detector: Hydrogen flame ionization

Column: 0.32-mm × 30-m fused-silica capillary column, 5-µm layer of phase G1

Temperature

Detector: 250°

Injector port: 150°

Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
100	—	100	8
100	20	250	5

Carrier gas: Helium

Flow rate: Adjusted so that the vinyl acetate peak appears after about 7 min

Injection size: 1.0 µL

Injection type: Split ratio is about 8:1.

System suitability

Sample: *Standard solution* containing 1 µg/mL of vinyl acetate in toluene

Suitability requirements

Relative standard deviation: NMT 15%

Analysis

Samples: *Standard solution* and *Sample solution*

Plot the peak responses of the vinyl acetate in the *Standard solutions* versus the concentration, in µg/mL, of vinyl acetate, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C, in µg/mL, of vinyl acetate in the *Sample solution.*

Calculate the quantity, in µg, of vinyl acetate in each g of Polyvinyl Acetate taken:

$$\text{Result} = (C/C_p)$$

C = as determined above

C_p = concentration of the Polyvinyl Acetate in the *Sample solution* (g/mL)

Acceptance criteria: NMT 5 µg/g (5 ppm) of vinyl acetate

SPECIFIC TESTS

• **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩

Sample: 10.0 g of Polyvinyl Acetate

Analysis: Transfer the *Sample* to a 250-mL glass-stoppered conical flask, dissolve in 75 mL of ethylene dichloride, add 60 mL of denatured alcoholic TS, and mix. Add 1 mL of phenolphthalein TS, and titrate with 0.02 N alcoholic potassium hydroxide VS until the solution remains faintly pink after shaking for 30 s. Perform a blank determination, and make any necessary correction.

Acceptance criteria: The acid value is NMT 0.5.

• **FATS AND FIXED OILS,** *Ester Value* ⟨401⟩

Sample: 0.5 g of Polyvinyl Acetate

Analysis: Saponify the *Sample* in a mixture of 25.0 mL of 0.5 N alcoholic potassium hydroxide VS and 25.0 mL of water. Proceed as directed under *Fats and Fixed Oils* ⟨401⟩, *Saponification Value,* beginning with "Heat the flask on a steam bath".

Acceptance criteria: The ester value, calculated from the *Saponification Value* and the *Acid Value,* is between 615 and 675.

• **LOSS ON DRYING** ⟨731⟩: Dry 1.5 g at 100° for 2 h in a vacuum: it loses NMT 1.0% of its weight.

• **AVERAGE MOLECULAR WEIGHT AND MOLECULAR WEIGHT DISTRIBUTION**

[**CAUTION**—Tetrahydrofuran (THF) is considered to be a carcinogen and embryo-fetal toxic substance. It is also a peroxide former and is flammable. A safe-handling practice must be in place in the laboratory. Carefully review appropriate Material Safety Data Sheets before use.]

Mobile phase: Tetrahydrofuran inhibited with 250 ppm butylated hydroxytoluene. Do not sparge or degas.

Standard solutions: Prepare two sets of mixtures, each set containing five narrow polystyrene standards of different known molecular weights, totaling 10 narrow polystyrene standards covering the molecular weight range from about 600 to 3,000,000 g/mol.[1] Prepare each set of five narrow

[1] Narrow polystyrene standards are available from polymer laboratories, such as EasiCal, or are available as various individual polystyrene standards.

NF Monographs

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polystyrene standards to have a known concentration at about 0.05% (w/v) for each standard in *Mobile phase*.

Sample solution: Transfer 0.025 g of polyvinyl acetate to a vial, and add 10 mL of *Mobile phase*. Cap and mix well, using an appropriate laboratory shaker, for 1 h. Pass the polyvinyl acetate solution through a polytetrafluoroethylene filter having a porosity of 0.45 µm, discard an appropriate volume of the initial filtrate, and use the rest of the filtered solution for analysis.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Refractive Index (RI)

Detector temperature: 35°

Columns: Two 10-mm × 50-cm analytical columns; 5-µm packing L73, and a 10-mm × 10-cm, 500-Å guard column; packing L73. [NOTE—The analytical column is suitable for molecular weight ranges from 100 to 10,000,000 g/mol.]

Flow rate: 1.1 mL/min

Injection size: 200 µL[2]

System suitability

Sample: *Standard solutions*

Suitability requirements

Resolution: NLT 1.7 between the polystyrene standards

Analysis

Samples: *Standard solutions* and *Sample solution*

Separately inject equal volumes of the *Standard solutions* and the *Sample solution* into the chromatograph, record the chromatograms, and determine the elution peak maxima and the corresponding retention volumes for the 10 polystyrene standards.

Universal calibration: Analyze each polystyrene standard, and use a data handling system or a suitable gel permeation chromatography or size exclusion chromatography (GPC/SEC) software to compute the data and calibration. Construct the *Universal calibration curve* as follows, and use it in the section *Data analysis for sample*.

Plot log ([η] × M_r) for each polystyrene standard in the *Standard solutions* versus its retention volume, V, in mL, at each standard peak maximum; and construct the best cubic line fitting the 10 points. In this expression, M_r is the molecular weight, in g/mol, of polystyrene standard; and [η] is the intrinsic viscosity of a polymer and is related to polymer molecular weight (M_r), especially viscosity-average molecular weight, M_v, by the following Mark-Houwink equation:

$$[\eta] = K \times M_v^{\,a}$$

K = constant for a given polymer/solvent system at a specified temperature

a = constant for a given polymer/solvent system at a specified temperature

For polystyrene in THF at 25°, K = 0.0128 mL/g, and a = 0.712

For polyvinyl acetate in THF at 25°, K = 0.025 mL/g, and a = 0.63

Based on the Mark-Houwink equation and the fact that M_v can represent the molecular weight (M_r), the following equation is given:

$$\log([\eta] \times M_r) = \log K + (a + 1)\log(M_r)$$

M_r can be obtained as M_v, a viscosity-average molecular weight of polystyrene standard.

Data analysis for sample: Analyze the polyvinyl acetate sample by identifying retention volumes V_a and V_b, corresponding to the beginning and end of the polyvinyl acetate chromatogram. The baseline between V_a and V_b is assumed to be linear. [NOTE—Draw a straight line between V_a and V_b.] Data analysis is based on a suitable GPC/SEC computer software or a real-time data acquisition system with either offline or online data processing that is able to provide a means of determining chromatographic peak heights or integrated area segments at prescribed intervals under the SEC chromatogram and a means of handling and reporting the data. The following describes the data processes, which can be computed either by the GPC/SEC software or by an equivalent data processing system. Upon acquisition, handle the data under the polyvinyl acetate elution peak in discrete segments A_i, integrated area slices, or as digitized chromatogram heights H_i, by recording the vertical displacements between the chromatogram trace and the baseline at retention volume, V_i, over designated intervals. A minimum of 40 area segments or heights is required. Obtain the corresponding molecular weight value M_i for Polyvinyl Acetate at its retention volume, V_i, from the *Universal calibration curve* obtained in the section *Universal calibration*, since the constants K and a for Polyvinyl Acetate are known and given above.

Calculate the number-, weight-, and viscosity-average molecular weights, M_n, M_w, and M_v, respectively, in g/mol, of polyvinyl acetate, using the following formula:

$$M_n = \frac{\sum_{i=1}^{N} A_i}{\sum_{i=1}^{N}\left(\frac{A_i}{M_i}\right)} \qquad M_w = \frac{\sum_{i=1}^{N}(A_i \bullet M_i)}{\sum_{i=1}^{N} A_i}$$

$$M_v = \left[\frac{\sum_{i=1}^{N}(A_i \bullet M_i^a)}{\sum_{i=1}^{N} A_i}\right]^{1/a}$$

If the retention volume internal ΔV_i (for instance, $V_2 - V_1 = V_3 - V_2$, etc.) is constant, parameters A_i and M_i are the chromatographic peak slice area and the Polyvinyl Acetate molecular weight associated with the retention volume, V_i; and N is the number of data points obtained from the chromatogram between V_a and V_b ($N \geq 40$). [NOTE—If N is sufficiently large, the use of area segments A_i or peak heights H_i will yield equivalent results.]

Calculate the molecular weight distribution or polydispersity for Polyvinyl Acetate:

$$\text{Result} = M_w/M_n$$

Acceptance criteria: The values of weight-average molecular weight and polydispersity are, respectively, NLT 85% and NMT 115% of their respective values as stated on the label.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirement specified.

[2] A sample loop of 400 µL and a syringe of 250 µL were used in the *Analysis*.

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- **LABELING:** Label it to indicate its weight-average molecular weight, M_w, and polydispersity (M_w/M_n).
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Polyvinyl Acetate RS

Polyvinyl Acetate Dispersion

DEFINITION
Dispersion of polyvinyl acetate in water. It contains 25.0% to 30.0% of polyvinyl acetate. It may contain povidone and sodium lauryl sulfate as stabilizers.

IDENTIFICATION
- **A. FILM FORMATION:** Place 1 drop of Dispersion on a glass plate and allow to dry. A clear and homogeneous film is formed.

Change to read:

- **B. INFRARED ABSORPTION**
  (See ▲*Spectroscopic Identification Tests* ⟨197⟩, *Infrared Spectroscopy*▲ (CN 1-May-2020).)
  Analysis: Place 1 drop of the Dispersion on a glass plate, and cover the test substance with a water-resistant crystal disk (silver chloride or KRS-5).[1] Gently press on, and then remove the crystal disk. Dry the crystal disk in a drying chamber until a homogeneous film is formed.
  Acceptance criteria: The IR absorption spectrum of the film so formed exhibits maxima corresponding to the same wavelengths as those of a similar preparation of USP Polyvinyl Acetate Dispersion RS treated in the same manner.

ASSAY
- **PROCEDURE**
  Sample 1: 10 g of Dispersion
  Solvent: 50 mL of a mixture of equal volumes of alcohol and petroleum ether with a 100°–120° boiling range, which is previously neutralized with 0.1 N potassium hydroxide or 0.1 N sodium hydroxide
  Analysis 1: Dissolve *Sample 1* in the *Solvent*. Add 0.5 mL of phenolphthalein TS, and titrate with 0.1 N potassium hydroxide or 0.1 N sodium hydroxide until the pink color persists for at least 15 s.
  Calculate the acid value, I_A:

$$\text{Result} = (M_{r1} \times V \times N)/W$$

M_{r1}	= molecular weight of potassium hydroxide, 56.11
V	= volume of 0.1 N potassium hydroxide or 0.1 N sodium hydroxide consumed in the actual test (mL)
N	= exact normality of the potassium hydroxide solution or sodium hydroxide solution
W	= weight of Dispersion taken for the test (g)

Sample 2: 1.5 g of Dispersion
Analysis 2: Transfer *Sample 2* to a 250-mL borosilicate glass flask fitted with a reflux condenser. Add 25.0 mL of 0.5 M alcoholic potassium hydroxide and a few glass beads. Attach the condenser and heat under reflux for 30 min. Add 1 mL of phenolphthalein TS, and titrate immediately (while still hot) with 0.5 N hydrochloric acid VS. Perform a blank determination under the same conditions (see *Titrimetry* ⟨541⟩, *Residual Titrations*.)
Calculate the saponification value, I_S:

$$\text{Result} = [M_{r1} \times (V_B - V_T) \times N]/W$$

M_{r1}	= molecular weight of potassium hydroxide, 56.11
V_B	= volume of 0.5 N hydrochloric acid consumed in the blank test (mL)
V_T	= volume of 0.5 N hydrochloric acid consumed in the actual test (mL)
N	= exact normality of the hydrochloric acid
W	= weight of Dispersion taken for the test (g)

Calculate the percentage content of polyvinyl acetate in the portion of Dispersion taken:

$$\text{Result} = F \times \{M_{r2} \times [(I_S - I_A)/M_{r1}]\} \times 100$$

F	= factor converting mg to g, 10^{-3} g/mg
M_{r2}	= molecular weight of vinyl acetate, 86.09
I_S	= saponification value
I_A	= acid value
M_{r1}	= molecular weight of potassium hydroxide, 56.11

Acceptance criteria: The content of polyvinyl acetate is 25.0%–30.0%.

OTHER COMPONENTS
STABILIZERS
- **Povidone**
  [NOTE—Perform this test only if the Dispersion contains povidone.]
  Sample: 0.25 g
  Analysis: Perform nitrogen determination by sulfuric acid digestion on the *Sample* as directed in *Nitrogen Determination* ⟨461⟩, *Method II*.
  Calculate the percentage content of povidone in the portion of Dispersion taken:

$$\text{Result} = N/N_V$$

N	= percentage content of nitrogen
N_V	= percentage content, expressed as a decimal number, of nitrogen in vinylpyrrolidone, 0.126

Acceptance criteria: The content of povidone is NMT 4.0%.

IMPURITIES
- **RESIDUE ON IGNITION** ⟨281⟩
  Sample: 1.0 g of Dispersion
  Analysis: Heat a silica crucible to redness for 30 min, allow to cool in a desiccator, and weigh. Evenly distribute the *Sample* in the crucible and weigh. Dry the crucible at 100°–105° for 1 h and ignite in a muffle furnace at 600 ± 25°, until the test substance is thoroughly charred. Continue the experiment as directed in *Residue on Ignition* ⟨281⟩ on the residue obtained, beginning with "Moisten the sample with a small amount (usually 1 mL) of sulfuric acid..."
  Acceptance criteria: NMT 0.5%
- **LIMIT OF VINYL ACETATE**
  Solution A: Acetonitrile, methanol, and water (5:5:90)
  Solution B: Acetonitrile, methanol, and water (45:5:50)
  Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0

[1] KRS-5 consists of 42% thallium(I) bromide and 58% thallium(I) iodine by molecular weight. Suitable disks of silver chloride and of KRS-5 are available from www.crystals.saint-gobain.com, www.almazoptics.com, and www.internationalcrystal.net.

Supplement 1

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Table 1 *(continued)*

Time (min)	Solution A (%)	Solution B (%)
2	100	0
40	85	15
42	0	100
48	0	100
51	100	0

Standard solution: Transfer 50 mg of vinyl acetate to a 100-mL volumetric flask, dissolve in and dilute with methanol to volume, and mix well. Dilute 5.0 mL of the solution with *Solution A* to 100 mL. Dilute 10.0 mL of this solution with *Solution A* to 100 mL. The *Standard solution* contains about 2.5 µg/mL of vinyl acetate. [NOTE—This solution should be analyzed within 1 h when stored at room temperature.]

System suitability solution: Transfer 50 mg of vinyl acetate and 50 mg of 1-vinylpyrrolidin-2-one to a 50-mL volumetric flask, add 10 mL of methanol, sonicate or gently shake the flask to dissolve the materials. Dilute with *Solution A* to volume. Dilute 10 mL of this solution with *Solution A* to 100 mL. Dilute 5 mL of this solution with *Solution A* to 100 mL. The *System suitability solution* contains about 5 µg/mL each of vinyl acetate and 1-vinylpyrrolidin-2-one.

Sample solution: Transfer 250 mg of Dispersion to a 10-mL volumetric flask, add about 4 mL of methanol, and sonicate. After cooling to ambient temperature, dilute with water to volume, and mix. Centrifuge at 4000 × g for 10 min, and pass through a 0.2-µm membrane filter. [NOTE—This solution should be analyzed within 1 h when stored at room temperature.]

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 205 nm
Columns
Precolumn: 4.0-mm × 3-cm; 5-µm packing L1 may be used if a matrix effect is observed. [NOTE—The matrix effect may result in poor reproducibility of the retention times and of the peak shapes.]
Analytical: 4.0-mm × 25-cm; 5-µm packing L1
Column temperature: 30°
Flow rate: 1 mL/min
Injection volume: 10 µL

System suitability
Sample: *System suitability solution*
[NOTE—The relative retention times for vinyl acetate and 1-vinylpyrrolidin-2-one are 1.0 and 1.2, respectively.]
Suitability requirements
Resolution: NLT 5.0 between vinyl acetate and 1-vinylpyrrolidin-2-one
Relative standard deviation: NMT 5.0% determined from the 1-vinylpyrrolidin-2-one peak

Analysis
Samples: *Standard solution* and *Sample solution*

Acceptance criteria: The response of the vinyl acetate peak from the *Sample solution* is NMT that of the vinyl acetate peak from the *Standard solution*, corresponding to NMT 100 ppm of vinyl acetate.

• **LIMIT OF ACETIC ACID/ACETATE**
Solution A: 5 mM sulfuric acid
Solution B: Acetonitrile and 5 mM sulfuric acid (1:1)
Mobile phase: See *Table 2*.

Table 2

Time (min)	Solution A (%)	Solution B (%)
0	100	0
10	100	0
10.5	0	100
20	0	100
20.5	100	0
30	100	0

System suitability solution: Transfer 30 mg of glacial acetic acid and 30 mg of malonic acid to a 25-mL volumetric flask, dilute with methanol to volume, and mix well. Transfer 1 mL of the solution to a 25-mL flask, dilute with water to volume, and mix well. The solution contains 0.048 mg/mL each of acetic acid and malonic acid.

Standard solution: 0.1 mg/mL of acetic acid in water

Sample solution: Transfer 330 mg of Dispersion to a 50-mL volumetric flask, add about 5 mL of methanol, and dilute with water to volume, which leads to a precipitation of sample. Pass the dispersion through a 0.2-µm regenerated cellulose membrane filter.[2] Use the filtrate.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 205 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 25°
Flow rate: 1 mL/min
Injection volume: 20 µL
Run time: 30 min

System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The relative retention times for malonic acid and acetic acid are 0.9 and 1.0, respectively.]
Suitability requirements
Resolution: NLT 2.0 between malonic acid and acetic acid, *System suitability solution*
Relative standard deviation: NMT 5.0% determined from the acetic acid peak, *Standard solution*

Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of acetic acid in the portion of Dispersion taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of acetic acid from the *Sample solution*
r_S = peak response of acetic acid from the *Standard solution*
C_S = concentration of acetic acid in the *Standard solution* (mg/mL)
C_U = concentration of Polyvinyl Acetate Dispersion in the *Sample solution* (mg/mL)

Acceptance criteria: NMT 1.5% of acetic acid

SPECIFIC TESTS

• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic microbial count is NMT 1000 cfu/g, and the total combined molds and yeasts count is NMT 100 cfu/g.

• **PH** ⟨791⟩**:** 3.0–5.5

[2] Whatman Spartan HPLC certified syringe filter, Whatman Cat # 10463060 or equivalent filter.

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• **LOSS ON DRYING** ⟨731⟩
Sample: 1.0 g of Dispersion
Analysis: Dry the *Sample* at 110° for 5 h.
Acceptance criteria: 68.5%–71.5%
• **COAGULUM CONTENT**
Sample: 100 g of Dispersion
Analysis: Accurately weigh a stainless steel sieve with 45-µm openings or a suitable single-woven wire cloth with a mesh width of 45 µm, and filter the *Sample* through it. [NOTE—Suitable single-woven wire cloth mesh meets the requirements set in ISO 9044.] Wash the sieve or the cloth with distilled water until a clear filtrate is obtained, and dry the sieve or the cloth to constant weight at 100°–105°.
Acceptance criteria: The weight of the residue is NMT 500 mg (0.5%).

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight containers at a temperature below 25°. Protect from freezing.
• **LABELING:** Label it to indicate the amounts of povidone and sodium lauryl sulfate.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Polyvinyl Acetate Dispersion RS

Polyvinyl Acetate Phthalate

DEFINITION
Polyvinyl Acetate Phthalate is a reaction product of phthalic anhydride and a partially hydrolyzed polyvinyl acetate. It contains NLT 55.0% and NMT 62.0% of phthalyl (*o*-carboxybenzoyl, $C_8H_5O_3$) groups, calculated on the anhydrous, acid-free basis.

IDENTIFICATION
• **A.** The *Sample solution* in the *Assay* exhibits a maximum at 277 ± 3 nm.
• **B.**
Sample: 10 mg
Analysis: Place the *Sample* in a small test tube, add 10 mg of resorcinol, and mix. Add 0.5 mL of sulfuric acid, and heat in a liquid bath at 160° for 3 min. Cool, and pour the solution into a mixture of 25 mL of 1 N sodium hydroxide and 200 mL of water.
Acceptance criteria: The solution shows a vivid green fluorescence.
• **C.**
Sample solution: 100 mg/mL of Polyvinyl Acetate Phthalate in methanol
Analysis: Pour 1 mL of the *Sample solution* onto a clear glass plate.
Acceptance criteria: A film is deposited as the methanol evaporates.

ASSAY
• **PHTHALYL CONTENT**
Standard solution: 0.05 mg/mL of phthalic anhydride in alcohol, prepared as follows. Transfer 50 mg of phthalic anhydride to a 1000-mL volumetric flask. Dissolve with heat in 100 mL of alcohol, dilute with alcohol to volume, and mix.
Sample solution: 0.1 mg/mL of Polyvinyl Acetate Phthalate in alcohol, prepared as follows. Transfer 100 mg of Polyvinyl Acetate Phthalate to a 1000-mL volumetric flask, dissolve in alcohol, and dilute with alcohol to volume.
Instrumental conditions
Mode: UV
Cell: 1 cm
Analytical wavelength: 275 nm
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate, on the acid-free basis, the percentage of phthalyl taken:

$$\text{Result} = (A_U/A_S) \times (C_S/C_U) \times 100$$

A_U = absorbance of the *Sample solution*
A_S = absorbance of the *Standard solution*
C_S = concentration of phthalic anhydride in the *Standard solution* (mg/mL)
C_U = concentration of Polyvinyl Acetate Phthalate in the *Sample solution* (mg/mL)

Acceptance criteria: 55.0%–62.0% of phthalyl (*o*-carboxybenzoyl, $C_8H_5O_3$) groups on the anhydrous, acid-free basis

IMPURITIES
• **RESIDUE ON IGNITION** ⟨281⟩: NMT 1.0%
• **FREE PHTHALIC ACID**
Standard solution: 0.05 mg/mL of phthalic anhydride
Sample solution: 6 mg/mL of polyvinyl acetate phthalate in water prepared as follows. Dissolve 1500 mg of Polyvinyl Acetate Phthalate in 50 mL of a mixture of methylene chloride and methanol (4:1). Transfer the solution to a separator with the aid of 75 mL of water, and swirl, taking care not to shake. Add 100 mL of hexanes, shake, and allow the mixture to stand until it separates into two layers. Transfer the water layer to a 250-mL volumetric flask. Add 100 mL of water to the separator, shake, and allow to stand until the layers separate. Transfer the water layer to the same volumetric flask, and dilute with water to volume. If the solution is cloudy, centrifuge a portion until clear.
Instrumental conditions
Mode: UV
Cell: 1 cm
Analytical wavelength: 277 nm
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of free phthalic acid in the portion taken:

$$\text{Result} = (A_U/A_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

A_U = absorbance of the *Sample solution*
A_S = absorbance of the *Standard solution*
C_S = concentration of phthalic anhydride in the *Standard solution* (mg/mL)
C_U = concentration of Polyvinyl Acetate Phthalate in the *Sample solution* (mg/mL)
M_{r1} = molecular weight of phthalic acid, 166.13
M_{r2} = molecular weight of phthalic anhydride, 148.12

Acceptance criteria: NMT 0.6% on the anhydrous basis
• **FREE ACID OTHER THAN PHTHALIC**
Sample solution: Proceed as directed for *Sample solution* in *Impurities, Free Phthalic Acid*, but instead of transferring the water extracts to a volumetric flask, transfer them to a 400-mL beaker.
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.1 N sodium hydroxide VS
Endpoint detection: Visual
Analysis: Titrate the *Sample solution* with *Titrant* to a phenolphthalein endpoint.
Calculate the volume, V_P, in mL, of 0.1 N sodium hydroxide consumed by the free phthalic acid in the sample taken:

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$$V_p = (1/M_r) \times (1/N) \times P \times W$$

M_r = equivalent weight of phthalic acid, 83.065 mg/mEq
N = actual normality of the *Titrant* (mEq/mL)
P = percentage of free phthalic acid, previously determined, in decimal form
W = sample weight of Polyvinyl Acetate Phthalate on the anhydrous basis (mg)

Calculate the percentage of free acid other than phthalic, as acetic acid, in the portion taken:

$$\text{Result} = [(V - V_p) \times M_{r3} \times N]/W \times 100$$

V = total volume of 0.1 N sodium hydroxide used (mL)
M_{r3} = equivalent weight of acetic acid, 60.05 mg/mEq
N = actual normality of the *Titrant*
W = sample weight on the anhydrous basis (mg)

Acceptance criteria: NMT 0.6% on the anhydrous basis

SPECIFIC TESTS

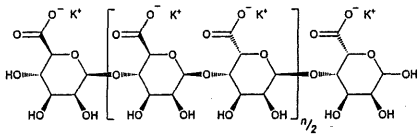
- **VISCOSITY—CAPILLARY METHODS** ⟨911⟩
  Sample solution: Dissolve a quantity, equivalent to 15 g on the anhydrous basis, in 85 g of methanol.
  Analysis: Determine the viscosity of the *Sample solution*, using a capillary viscometer at 25 ± 0.2°.
  Acceptance criteria: The apparent viscosity is 7–11 mPa · s (centipoises).
- **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 5.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.

Polyvinyl Alcohol—*see Polyvinyl Alcohol General Monographs*

Potassium Alginate



Alginic acid, potassium salt;
Potassium alginate [9005-36-1].

DEFINITION

Change to read:

Potassium Alginate is the purified carbohydrate product extracted from various species of brown seaweeds by the use of dilute alkali. It consists chiefly of the potassium salt of Alginic Acid, a linear, ▲unbranched copolymer of β-D-mannuronic acid (M) and α-L-guluronic acid (G) linked to each other by 1→4 glycosidic bonds. The M and G units in the alginates may be randomly or non-randomly arrayed as heterogeneous or homogeneous sequences.▲ (NF 1-May-2020) It yields NLT 16.5% and NMT 19.5% of carbon dioxide (CO_2), equivalent to NLT 89.2% and NMT 105.5% of potassium alginate, calculated on the dried basis. ▲Typical average molecular weight is in the range of 10,000–600,000 g/mol.▲ (NF 1-May-2020)

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: 197K or 197A ▲ (NF 1-May-2020)

Change to read:

- **B.**
  ▲Analysis 1: To 5 mL of a 1-in-100 solution in 0.1 N sodium hydroxide add 1 mL of calcium chloride TS.
  Acceptance criteria 1: A voluminous, gelatinous precipitate is formed.▲ (NF 1-May-2020)
  Analysis ▲2:▲ (NF 1-May-2020) To 10 mL of a 1-in-100 solution in 0.1 N sodium hydroxide add 1 mL of 2 N sulfuric acid
  Acceptance criteria ▲2:▲ (NF 1-May-2020) A heavy, gelatinous precipitate is formed.
- **C.**
  Analysis: To 5 mg in a test tube add 5 mL of water, 1 mL of a freshly prepared 1-in-100 solution of 1,3-naphthalenediol in alcohol, and 5 mL of hydrochloric acid. Heat the mixture to boiling, boil gently for 3 min, then cool to 15°. Transfer the contents of the test tube to a 30-mL separator with the aid of 5 mL of water, and extract with 15 mL of isopropyl ether.
  Acceptance criteria: The isopropyl ether extract exhibits a deeper purplish hue than that from a blank, similarly prepared.

Change to read:

- **D. IDENTIFICATION TESTS—GENERAL** ⟨191⟩, *Chemical Identification Tests, Potassium*
  ▲Sample solution: 5 mg/mL of Potassium Alginate▲ (NF 1-May-2020)
  Analysis ▲1: Transfer 1 mL of the *Sample solution* into a test tube, add 1 mL of sodium bitartrate TS, stir the inside of the test tube with a glass rod, and add 0.5 mL of alcohol.▲ (NF 1-May-2020)
  Acceptance criteria ▲1: A white crystalline precipitate is formed.
  Analysis 2: To the test tube from *Analysis 1* add 3 mL of 6 N ammonium hydroxide.
  Acceptance criteria 2: The white crystalline precipitate is dissolved.▲ (NF 1-May-2020)

ASSAY

- **ALGINATES ASSAY** ⟨311⟩**:** 16.5%–19.5% of carbon dioxide (CO_2), equivalent to 89.2%–105.5% of potassium alginate on the dried basis

IMPURITIES

- **ARSENIC** ⟨211⟩, *Procedures, Method II*: NMT 1.5 ppm
- **LEAD** ⟨251⟩
  Standard solution: 5 mL of *Diluted standard lead solution*
  Test preparation: Add 1.0 g to 20 mL of nitric acid in a 250-mL conical flask, mix, and heat carefully until the Potassium Alginate is dissolved. Continue the heating until the volume is reduced to 7 mL. Cool rapidly to room temperature, transfer to a 100-mL volumetric flask, and dilute with water to volume. Use a 50-mL sample of the *Test preparation*.
  Analysis: Proceed as directed in the chapter, using 15 mL of ammonium citrate solution, 3 mL of potassium cyanide solution, and 0.5 mL of hydroxylamine hydrochloride solution being used for the test. After the first dithizone

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extractions, wash the combined chloroform layers with 5 mL of water, discarding the water layer and continuing in the usual manner by extracting with 20 mL of 0.2 N nitric acid.
Acceptance criteria: A 50.0-mL portion of the *Test preparation* contains NMT 5 µg of lead (corresponding to NMT 10 ppm of lead).

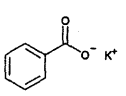
SPECIFIC TESTS
• **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Total Ash*
Analysis: Proceed as directed in the chapter, carefully igniting 3 g in a tared platinum dish until the residue is thoroughly carbonized (5 min), and then igniting in a muffle furnace at a temperature of 800 ± 25° until the carbon is completely burned off (approximately 75 min).
Acceptance criteria: 24.0%–32.0% of ash is found, calculated on the as-is basis.
• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.
• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry at 105° for 4 h.
Acceptance criteria: NMT 15%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.

Add the following:

▲• **USP REFERENCE STANDARDS** ⟨11⟩
USP Potassium Alginate RS▲ (NF 1-May-2020)

Potassium Benzoate



$C_7H_5KO_2$ 160.21
Benzoic acid, potassium salt;
Potassium benzoate [582-25-2].

DEFINITION
Potassium Benzoate contains NLT 99.0% and NMT 101.0% of potassium benzoate ($C_7H_5KO_2$), calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

• **A.** ▲**SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
Sample: Undried sample
Acceptance criteria: Meets the requirements
• **B.** Potassium Benzoate imparts a violet color to a nonluminous flame. Because the presence of small quantities of sodium masks the color, screen out the yellow color produced by sodium by viewing through a blue filter that blocks emission at 589 nm (sodium) but is transparent to emission at 404 nm (potassium). [NOTE—Traditionally, cobalt glass has been used, but other suitable filters are commercially available.]
• **C.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

ASSAY
• **PROCEDURE**
Solution A: Adjust a 20 mM solution of monobasic potassium phosphate with phosphoric acid to a pH of 2.5.
Mobile phase: *Solution A* and acetonitrile (70:30)
Diluent: Water and acetonitrile (50:50)
System suitability solution: 0.1 mg/mL of USP Salicylic Acid RS and 0.1 mg/mL of USP Benzoic Acid RS in *Diluent*
Standard solution: 0.1 mg/mL of USP Benzoic Acid RS in *Diluent*
Sample solution: 0.1 mg/mL of Potassium Benzoate in *Diluent*
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 230 nm
Column: 4.6-mm × 15-cm; 5-µm packing L1
Column temperature: 25°
Flow rate: 1.0 mL/min
Injection volume: 10 µL
System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The relative retention times for benzoic acid and salicylic acid are approximately 1 and 1.2, respectively.]
Suitability requirements
Resolution: NLT 3.0 between benzoic acid and salicylic acid, *System suitability solution*
Tailing factor: NMT 2.0 for benzoic acid, *Standard solution*
Relative standard deviation: NMT 0.5% for benzoic acid, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of potassium benzoate ($C_7H_5KO_2$) in the portion of Potassium Benzoate taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of benzoic acid from the *Sample solution*
r_S = peak area of benzoic acid from the *Standard solution*
C_S = concentration of USP Benzoic Acid RS in the *Standard solution*, corrected for purity (mg/mL)
C_U = concentration of Potassium Benzoate in the *Sample solution* (mg/mL)
M_{r1} = molecular weight of potassium benzoate, 160.21
M_{r2} = molecular weight of benzoic acid, 122.12

Acceptance criteria: 99.0%–101.0% on the anhydrous basis

SPECIFIC TESTS
• **ALKALINITY**
Sample: 2 g
Analysis: Dissolve the *Sample* in 20 mL of hot water, and add 2 drops of phenolphthalein TS.
Acceptance criteria: If a pink color is produced, it is discharged by the addition of 0.20 mL of 0.10 N sulfuric acid.
• **WATER DETERMINATION** ⟨921⟩, *Method I*: NMT 1.5%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

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• **USP REFERENCE STANDARDS** ⟨11⟩
USP Benzoic Acid RS
USP Potassium Benzoate RS
USP Salicylic Acid RS

Potassium Carbonate—*see Potassium Carbonate General Monographs*

Potassium Chloride—*see Potassium Chloride General Monographs*

Potassium Citrate—*see Potassium Citrate General Monographs*

Potassium Hydroxide

KOH 56.11
Potassium hydroxide [1310-58-3].

DEFINITION
Potassium Hydroxide contains NLT 85.0% of total alkali, calculated as potassium hydroxide (KOH), including NMT 3.5% of potassium carbonate (K_2CO_3). It also contains NLT 59.9% of potassium.
[**CAUTION**—Exercise great care in handling Potassium Hydroxide because it rapidly destroys tissues.]

IDENTIFICATION
• **A. IDENTIFICATION TESTS—GENERAL** ⟨191⟩, *Potassium*: A solution (1 in 25) meets the requirements.
• **B. PH** ⟨791⟩
Sample solution: 0.1 mg/mL of Potassium Hydroxide
Acceptance criteria: NLT 10.5

ASSAY
• **TOTAL ALKALI**
Sample: 1.5 g of Potassium Hydroxide
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 1 N sulfuric acid VS
Endpoint detection: Colorimetric
Analysis: Dissolve the *Sample* in 40 mL of carbon dioxide–free water. Cool the solution to 15° and add phenolphthalein TS. Titrate with 1 N sulfuric acid VS. At the discharge of the pink color of the indicator, record the volume of acid solution required, then add methyl orange TS and continue the titration to a persistent pink color. Each milliliter of 1 N sulfuric acid is equivalent to 56.11 mg of total alkali, calculated as potassium hydroxide (KOH), and each milliliter of acid consumed in the titration with methyl orange is equivalent to 138.2 mg of potassium carbonate (K_2CO_3).
Acceptance criteria: NLT 85.0% of total alkali, calculated as potassium hydroxide (KOH), including NMT 3.5% of potassium carbonate (K_2CO_3)
• **CONTENT OF POTASSIUM**
Diluent: 1% hydrochloric acid solution
Sodium chloride solution: 0.2 g/mL of sodium chloride
Blank solution: Transfer 2.0 mL of the *Sodium chloride solution* to a 100-mL volumetric flask and dilute with *Diluent* to volume.
Standard stock solution: 57.21 µg/mL of potassium chloride, previously dried at 105° for 2 h, in water. This solution contains 30 µg/mL of potassium.
Standard solutions: Transfer 2.0-, 4.0-, and 6.0-mL portions of the *Standard stock solution* to separate 100-mL volumetric flasks. To each flask, add 2.0 mL of the *Sodium chloride solution*. Dilute the content of each flask with *Diluent* to volume and mix to obtain solutions with known concentrations of 0.6, 1.2, and 1.8 µg/mL of potassium.
Sample stock solution: 0.5 mg/mL of Potassium Hydroxide
Sample solution: Transfer 1.0 mL of the *Sample stock solution* to a 250-mL volumetric flask. Add 5.0 mL of the *Sodium chloride solution* and dilute with *Diluent* to volume.
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Analytical wavelength: 766.5 nm (potassium emission line)
Lamp: Potassium hollow-cathode
Flame: Air–acetylene
Blank: *Blank solution*
Standard curve
Samples: *Standard solutions*
Plot: Absorbance values versus their corresponding concentrations (µg/mL) of potassium. The correlation coefficient is NLT 0.999.
Analysis
Sample: *Sample solution*
From the *Standard curve*, determine the concentration of potassium in the *Sample solution*.
Calculate the percentage of potassium in the portion of Potassium Hydroxide taken:

$$\text{Result} = (C_S/C_U) \times 100$$

C_S = concentration of potassium in the *Sample solution* from the *Standard curve* (µg/mL)
C_U = concentration of Potassium Hydroxide in the *Sample solution* (µg/mL)

Acceptance criteria: NLT 59.9%

IMPURITIES
• **LIMIT OF SODIUM**
Diluent: 1% hydrochloric acid solution
Standard stock solution: 12.71 µg/mL of sodium chloride, previously dried at 105° for 2 h, in water. This solution contains 5 µg/mL of sodium.
Standard solutions: Transfer 1.0-, 10.0-, and 15.0-mL portions of the *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the content of each flask with *Diluent* to volume and mix to obtain solutions with known concentrations of 0.05, 0.5, and 0.75 µg/mL of sodium.
Sample stock solution: 0.5 mg/mL of Potassium Hydroxide
Sample solution: 50 µg/mL of Potassium Hydroxide in *Diluent*, prepared from the *Sample stock solution*
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Analytical wavelength: 589.0 nm
Lamp: Sodium hollow-cathode
Flame: Air–acetylene
Blank: *Diluent*
Standard curve
Samples: *Standard solutions*

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Plot: Absorbance values versus their corresponding concentrations (µg/mL) of sodium. The correlation coefficient is NLT 0.995.

Analysis

Sample: *Sample solution*

From the *Standard curve*, determine the concentration of sodium in the *Sample solution*.

Calculate the percentage of sodium in the portion of Potassium Hydroxide taken:

$$\text{Result} = (C_S/C_U) \times 100$$

C_S = concentration of sodium in the *Sample solution* from the *Standard curve* (µg/mL)

C_U = concentration of Potassium Hydroxide in the *Sample solution* (µg/mL)

Acceptance criteria: NMT 1.0%

SPECIFIC TESTS

- **INSOLUBLE SUBSTANCES**

Sample solution: 1 g of Potassium Hydroxide in 20 mL of water

Acceptance criteria: The *Sample solution* is complete, clear, and colorless.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.

Potassium Metabisulfite

$K_2S_2O_5$ 222.32

Disulfurous acid, dipotassium salt;
Dipotassium pyrosulfite [16731-55-8].

DEFINITION

Potassium Metabisulfite contains an amount of $K_2S_2O_5$ equivalent to NLT 51.8% and NMT 57.6% of SO_2.

IDENTIFICATION

- **A. IDENTIFICATION TESTS—GENERAL,** *Potassium* ⟨191⟩ and *Sulfite* ⟨191⟩**:** A solution (1 in 20) meets the requirements.

ASSAY

- **PROCEDURE**

Sample: 250 mg of Potassium Metabisulfite

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Residual titration

Titrant: 0.1 N iodine VS

Back titrant: 0.1 N sodium thiosulfate VS

Endpoint detection: Visual

Analysis: Transfer the *Sample* to a glass-stoppered conical flask containing 50.0 mL of 0.1 N iodine VS, and swirl to dissolve. Allow to stand for 5 min, protected from light, and then add 1 mL of hydrochloric acid. Titrate the excess iodine with 0.1 N sodium thiosulfate VS, adding 3 mL of starch TS as the endpoint is approached.

Calculate the percentage of SO_2 in the portion of *Sample* taken:

$$\text{Result} = \{[(V_B - V_S) \times N \times F]/W\} \times 100$$

V_B = *Back titrant* volume consumed by the *Blank* (mL)

V_S = *Back titrant* volume consumed by the *Sample* (mL)

N = *Back titrant* actual normality (mEq/mL)

F = equivalency factor, 32.03 mg/mEq

W = weight of the *Sample* (mg)

Acceptance criteria: 51.8%–57.6% of SO_2

IMPURITIES

- **IRON** ⟨241⟩

Test preparation: Dissolve 1.00 g in 14 mL of dilute hydrochloric acid (2 in 7), and evaporate on a steam bath to dryness. Dissolve the residue in 7 mL of dilute hydrochloric acid (2 in 7), and again evaporate to dryness. Dissolve the resulting residue in a mixture of 2 mL of hydrochloric acid and 20 mL of water. Add 3 drops of bromine TS, and boil to expel the bromine. Cool, and dilute with water to 47 mL.

Acceptance criteria: NMT 10 ppm

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-fitted, tight containers, and avoid exposure to excessive heat.

Potassium Metaphosphate

$(KPO_3)_n$

Metaphosphoric acid (HPO_3), potassium salt;
Potassium metaphosphate;
Potassium polymetaphosphate [7790-53-6].

DEFINITION

Potassium Metaphosphate is a straight-chain polyphosphate, having a high degree of polymerization. It contains the equivalent of NLT 59.0% and NMT 61.0% of phosphorus pentoxide (P_2O_5).

IDENTIFICATION

- **A.**

Sample: 1 g, finely powdered

Analysis: Add the *Sample*, slowly and with vigorous stirring, to 100 mL of sodium chloride solution (20 mg/mL).

Acceptance criteria: A gelatinous mass is formed.

- **B. IDENTIFICATION TESTS—GENERAL,** *Potassium* ⟨191⟩

Sample: 0.5 g

Analysis: Boil a mixture of the *Sample*, 10 mL of nitric acid, and 50 mL of water for 30 min, and cool.

Acceptance criteria: Meets the requirements

- **C. IDENTIFICATION TESTS—GENERAL,** *Phosphate* ⟨191⟩

Sample: 0.5 g

Analysis: Boil a mixture of the *Sample*, 10 mL of nitric acid, and 50 mL of water for 30 min, and cool.

Acceptance criteria: Meets the requirements

ASSAY

- **PROCEDURE**

Sample: 200 mg

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Residual titration

Titrant: 1 N sodium hydroxide VS

Back-titrant: 1 N sulfuric acid VS

Endpoint detection: Visual

Analysis: Mix the *Sample* with 15 mL of nitric acid and 30 mL of water. Boil for 30 min, cool, and dilute with water to 100 mL. Heat to 60°, add an excess of ammonium molybdate TS, and heat at 50° for 30 min. Filter, and wash the precipitate, first with 0.5 N nitric acid, and then with 10 mg/mL of potassium nitrate until the filtrate is no longer acid to litmus. Add 25 mL of water to the precipitate, dissolve it in 50.0 mL of *Titrant*, and add phenolphthalein TS. Titrate the excess sodium hydroxide with *Back-titrant*. Each mL of *Titrant* is equivalent to 3.086 mg of phosphorus pentoxide (P_2O_5).

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Acceptance criteria: 59.0%–61.0%

IMPURITIES

• **LEAD** ⟨251⟩
Sample solution: 1 g of Potassium Metaphosphate in 10 mL of 3 N hydrochloric acid
Acceptance criteria: The *Sample solution* contains NMT 5 µg of lead (corresponding to NMT 5 ppm of lead).

• **LIMIT OF FLUORIDE**
Control: Water
Sample solution: Place 5.0 g of Potassium Metaphosphate, 25 mL of water, 50 mL of perchloric acid, 5 drops of 500 mg/mL silver nitrate, and a few glass beads in a 250-mL distilling flask connected with a condenser and carrying a thermometer and a capillary tube, both of which extend into the liquid. Connect a small dropping funnel, filled with water, or a steam generator to the capillary tube. Support the flask on a distillation shield with a hole that exposes one-third of the bottom of the flask to the flame. Distill into a 250-mL volumetric flask until the temperature reaches 135°. Add water from the funnel or introduce steam through the capillary to maintain the temperature between 135° and 140°. Continue the distillation until 225–240 mL has been collected, then dilute with water to volume.
Analysis: Transfer 50.0 mL of the *Sample solution* to a 100-mL color-comparison tube, and transfer 50.0 mL of the *Control* to a similar tube. Add to each tube 0.1 mL of a filtered solution of sodium alizarinsulfonate TS and 1 mL of freshly prepared 0.25 mg/mL hydroxylamine hydrochloride. Add, dropwise and with stirring, 0.05 N sodium hydroxide to the tube containing the distillate until its color just matches that of the *Control*, which is faintly pink. Then add to each tube 1.0 mL of 0.1 N hydrochloric acid. From a buret, graduated in 0.05-mL increments, add slowly to the tube containing the distillate, enough 0.25-mg/mL thorium nitrate solution so that, after mixing, the color of the liquid just changes to a faint pink. Note the volume of the solution added, add the same volume to the *Control*, and mix. Then add sodium fluoride TS (10 µg/mL of fluorine) to the *Control* from a buret to make the colors of the two tubes match after dilution to the same volume. Mix, and allow all air bubbles to escape before making the final color comparison. Check the endpoint by adding 1 or 2 drops of sodium fluoride TS to the *Control*. A distinct change in color appears.
Acceptance criteria: 10 µg/g; the volume of sodium fluoride TS required for the *Control* is NMT 1.0 mL.

SPECIFIC TESTS

• **VISCOSITY—CAPILLARY METHODS** ⟨911⟩
Sample solution: Mix 300 mg with 200 mL of 3.5 mg/mL sodium pyrophosphate, using a magnetic stirrer.
Analysis: Using an Ostwald-Fenske viscometer maintained at 25°, determine the viscosity of the clear solution obtained, or of the liquid phase of the mixture obtained after 30 min of continuous stirring.
Acceptance criteria: 6.5–15 mPa · s

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Monobasic Potassium Phosphate

KH_2PO_4 136.09
Phosphoric acid, monopotassium salt;
Monopotassium phosphate [7778-77-0].

DEFINITION

Monobasic Potassium Phosphate, dried at 105° for 4 h, contains NLT 98.0% and NMT 100.5% of monobasic potassium phosphate (KH_2PO_4).

IDENTIFICATION

• **A. IDENTIFICATION TESTS—GENERAL,** *Potassium* ⟨191⟩
Sample solution: 50 mg/mL
Acceptance criteria: Meets the requirements

• **B. IDENTIFICATION TESTS—GENERAL,** *Phosphate* ⟨191⟩
Sample solution: 50 mg/mL
Acceptance criteria: Meets the requirements

ASSAY

• **PROCEDURE**
Sample solution: Transfer 5 g of Monobasic Potassium Phosphate, previously dried, to a 250-mL beaker. Add 100 mL of water and 5.0 mL of 1 N hydrochloric acid VS, and stir until the assay specimen is completely dissolved.
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 1 N sodium hydroxide VS
Endpoint detection: Potentiometrically
Analysis: Slowly titrate the excess acid in the *Sample solution*, stirring constantly, with *Titrant* to the inflection point occurring at about pH 4 (V_{S1}). Continue the titration with *Titrant* until the inflection point occurring at about pH 8.8 is reached (V_{S2}).
Calculate the percentage of monobasic potassium phosphate (KH_2PO_4) in the sample taken:

$$\text{Result} = \{[(V_{S2} - V_{S1}) \times N \times F]/W\} \times 100$$

V_{S2} = *Titrant* volume consumed by the *Sample solution* to the second inflection point (mL)
V_{S1} = *Titrant* volume consumed by the *Sample solution* to the first inflection point (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 0.1361 g/mEq
W = weight of monobasic potassium phosphate taken to prepare the *Sample solution* (g)

Acceptance criteria: 98.0%–100.5% on the previously dried basis

IMPURITIES

• **ARSENIC,** *Method I* ⟨211⟩**:** NMT 3 µg/g

• **LEAD** ⟨251⟩
Test preparation: 1 g in 20 mL of water
Acceptance criteria: NMT 5 µg/g

• **LIMIT OF FLUORIDE**
[NOTE—Prepare and store all solutions in plastic containers.]
Buffer solution: 294 mg/mL of sodium citrate dihydrate
Standard stock solution: 1.1052 mg/mL of USP Sodium Fluoride RS
Standard solution: Dilute 20.0 mL of *Standard stock solution* and 50.0 mL of *Buffer solution* with water to 100 mL. Each mL of this solution contains 100 µg of fluoride ion.
Sample solution: Transfer 2.0 g of Monobasic Potassium Phosphate to a beaker containing a plastic-coated stirring bar. Add 20 mL of water and 2.0 mL of hydrochloric acid, and stir until dissolved. Add 50.0 mL of *Buffer solution* and sufficient water to make 100 mL.
Electrode system: Use a fluoride-specific ion-indicating electrode and a silver–silver chloride reference electrode connected to a pH meter capable of measuring potentials with a minimum reproducibility of ±0.2 mV (see *pH* ⟨791⟩).

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Analysis

Standard response line: Transfer 50.0 mL of *Buffer solution* and 2.0 mL of hydrochloric acid to a beaker, and add water to make 100 mL. Add a plastic-coated stirring bar, insert the electrodes into the solution, stir for 15 min, and read the potential, in mV. Continue stirring, and at 5-min intervals add 100, 100, 300, and 500 µL of *Standard solution*, reading the potential 5 min after each addition. Plot the logarithms of the cumulative fluoride ion concentrations (0.1, 0.2, 0.5, and 1.0 µg/mL) versus potential, in mV.

Rinse and dry the electrodes, insert them into the *Sample solution*, stir for 5 min, and read the potential in mV. From the measured potential and the *Standard response line* determine the concentration, C (in µg/mL), of fluoride ion in the *Sample solution*.

Calculate the content of fluoride in the portion of Monobasic Potassium Phosphate taken:

$$\text{Result} = (V \times C)/W$$

V = volume of the *Sample solution* (mL)
C = concentration of fluoride ion, determined from the *Standard response line*, in the *Sample solution* (µg/mL)
W = weight of Monobasic Potassium Phosphate taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 10 µg/g

SPECIFIC TESTS

• **INSOLUBLE SUBSTANCES**

Sample solution: 10 g in 100 mL of hot water

Analysis: Filter the *Sample solution* through a tared filtering crucible, wash the insoluble residue with hot water, and dry at 105° for 2 h.

Acceptance criteria: NMT 20 mg (0.2%)

• **LOSS ON DRYING** ⟨731⟩

Analysis: Dry a sample at 105° for 4 h.

Acceptance criteria: NMT 1.0%

ADDITIONAL REQUIREMENTS

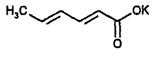
• **PACKAGING AND STORAGE:** Preserve in tight containers.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Sodium Fluoride RS

Potassium Sorbate

Change to read:



$C_6H_7KO_2$ 150.22

2,4-Hexadienoic acid, (*E*,*E*)-, potassium salt;

▲Potassium (2*E*,4*E*)-hexa-2,4-dienoate;▲ (NF 1-May-2020)

Potassium (*E*,*E*)-sorbate ▲▲ (NF 1-May-2020) [590-00-1; 24634-61-5].

DEFINITION

Change to read:

Potassium Sorbate contains NLT 98.0% and NMT ▲102.0%▲ (NF 1-May-2020) of potassium sorbate ($C_6H_7KO_2$), calculated on the dried basis.

IDENTIFICATION

• **A. IDENTIFICATION TESTS—GENERAL** ⟨191⟩, *Chemical Identification Tests, Potassium*

Sample: Dissolve 1 g of Potassium Sorbate in 10 mL of water.

Acceptance criteria: Meets the requirements

Change to read:

• **B. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: 197A or 197K▲ (NF 1-May-2020)

ASSAY

Delete the following:

▲• **PROCEDURE**

Sample: 300 mg of Potassium Sorbate

Blank: 40 mL of glacial acetic acid

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Direct titration

Titrant: 0.1 N perchloric acid VS

Endpoint detection: Visual

Analysis: Dissolve the *Sample* in 40 mL of glacial acetic acid, warming, if necessary, to dissolve the solution. Cool to room temperature, and add 1 drop of crystal violet TS. Titrate with *Titrant* to a blue-green endpoint. Perform a blank determination.

Calculate the percentage of potassium sorbate ($C_6H_7KO_2$) in the *Sample* taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

V_S = *Titrant* consumed by the *Sample* (mL)
V_B = *Titrant* consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 150.2 mg/mEq
W = *Sample* weight (mg)

Acceptance criteria: 98.0%–101.0% on the dried basis▲ (NF 1-May-2020)

Add the following:

▲• **PROCEDURE 1: CONTENT OF POTASSIUM SORBATE**

Solution A: 0.1% (v/v) trifluoroacetic acid in water

Solution B: 0.1% (v/v) trifluoroacetic acid in methanol

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	75	25
17.0	75	25
18.0	5	95
23.0	5	95
23.1	75	25
30.0	75	25

Diluent: Water and methanol (1:1, v/v)

System suitability solution: Prepare 0.1 mg/mL of USP Potassium Sorbate RS in *Diluent* first and then treat the

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solution with UV irradiation to generate ~1% degradation of potassium sorbate (based on area%).[1]

Standard solution: 0.1 mg/mL of USP Potassium Sorbate RS in *Diluent*

Sample solution: 0.1 mg/mL of Potassium Sorbate in *Diluent*

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 264 nm

Column: 4.6-mm × 15-cm; 3.5-µm packing L1

Column temperature: 40°

Flow rate: 1.0 mL/min

Injection volume: 10 µL

Run time: 30 min

System suitability

Samples: *System suitability solution* and *Standard solution*

[NOTE—The approximate relative retention times of related substances are listed in *Table 2*.]

Suitability requirements

Resolution: NLT 1.5 between potassium sorbate and potassium sorbate *trans, cis* isomer, *System suitability solution*

Tailing factor: NMT 2.0, determined from potassium sorbate, *Standard solution*

Relative standard deviation: NMT 2.0%, determined from potassium sorbate, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of potassium sorbate in the sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of potassium sorbate from the *Sample solution*

r_S = peak area of potassium sorbate from the *Standard solution*

C_S = concentration of USP Potassium Sorbate RS in the *Standard solution* (mg/mL)

C_U = concentration of Potassium Sorbate in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–102.0% on the dried basis

Table 2

Name	Relative Retention Time
Potassium sorbate *cis, cis* isomer[a]	0.83
Potassium sorbate *cis, trans* isomer[b]	0.89
Potassium sorbate *trans, cis* isomer[c]	0.92
Potassium sorbate	1.0

[a] Potassium (*Z,Z*)-sorbate.
[b] Potassium (*Z,E*)-sorbate.
[c] Potassium (*E,Z*)-sorbate.

[NOTE—Potassium sorbate *cis, trans* isomer and potassium sorbate *trans, cis* isomer might co-elute in some columns, which has no effects on assay analysis. All the four isomers are in their corresponding sorbic acid forms in the HPLC column because of the acidic mobile phase condition.▲ (NF 1-May-2020)]

Add the following:

▲• PROCEDURE 2: CONTENT OF POTASSIUM

Diluent: 1% hydrochloric acid solution

Sodium chloride solution: 0.2 g/mL of sodium chloride in *Diluent*

Blank solution: 4 mg/mL of sodium chloride from *Sodium chloride solution* in *Diluent*

Standard stock solution: 57.21 µg/mL of potassium chloride, previously dried at 105° for 2 h, in water. This solution contains 30 µg/mL of potassium.

Standard solutions: Transfer 2.0-, 4.0-, and 6.0-mL portions of the *Standard stock solution* to separate 100-mL volumetric flasks. To each flask add 2.0 mL of the *Sodium chloride solution*. Dilute the content of each flask with *Diluent* to volume and mix to obtain solutions with known concentrations of 0.6, 1.2, and 1.8 µg/mL of potassium.

Sample stock solution: 0.46 mg/mL of Potassium Sorbate in water

Sample solution: Transfer 1.0 mL of the *Sample stock solution* to a 100-mL volumetric flask. Add 2.0 mL of the *Sodium chloride solution* and dilute with *Diluent* to volume. The concentration of this solution is 4.6 µg/mL of Potassium Sorbate.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: 766.5 nm (potassium emission line)

Lamp: Potassium hollow-cathode

Flame: Air–acetylene

Standard curve

Samples: *Standard solutions*

Plot: Absorbance values versus their corresponding concentrations (µg/mL) of potassium. The correlation coefficient is NLT 0.995.

Analysis

Sample: *Sample solution*

From the *Standard curve*, determine the concentration of potassium in the *Sample solution*.

Calculate the percentage of potassium in the portion of Potassium Sorbate taken:

$$\text{Result} = (C_S/C_U) \times 100$$

C_S = concentration of potassium in the *Sample solution* from the *Standard curve* (µg/mL)

C_U = concentration of Potassium Sorbate in the *Sample solution* (µg/mL)

Acceptance criteria: 24.5%–27.6%▲ (NF 1-May-2020)

IMPURITIES

Add the following:

▲• LIMIT OF ALDEHYDE

Decolorized fuchsin solution: Dissolve 0.1 g of basic fuchsin in 60 mL of water. Add a solution containing 1 g of anhydrous sodium sulfite in 10 mL of water. Slowly and with continuous shaking add 2 mL of hydrochloric acid. Dilute with water to 100 mL. Allow to stand protected from light for at least 12 h, decolorize with activated charcoal, and filter. If the solution becomes cloudy, filter before use.

[1] UV irradiation conditions of wavelengths of 254, 300, and 365 nm at 18 Watts for 2 h have been used for the solution. Other equivalent UV conditions are also suitable as long as ~1% degradation of potassium sorbate (based on area%) can be achieved after the irradiation. Quartz glassware is preferred.

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If on standing the solution becomes violet, decolorize again by adding activated charcoal.

Test for sensitivity: To 1.0 mL of *Decolorized fuchsin solution* add 1.0 mL of water and 0.1 mL of alcohol, aldehyde-free. Add 0.2 mL of a solution containing 0.1 g/L of formaldehyde (CH_2O). A pale-pink color develops within 5 min.

Storage: Protected from light

Acetaldehyde standard solution: 100 µg/mL of acetaldehyde (C_2H_4O). Dissolve 1.0 g of acetaldehyde in 2-propanol and dilute with the same solvent to 100.0 mL. Dilute 5.0 mL of the solution with 2-propanol to 500.0 mL. Prepare immediately before use.

Standard solution: Add 1 mL of *Decolorized fuchsin solution* to a mixture of 1.5 mL of *Acetaldehyde standard solution*, 4 mL of 2-propanol, and 4.5 mL of water.

Sample solution: Dissolve 1.0 g of the sample in a mixture of 30 mL of water and 50 mL of 2-propanol, adjust with 1 N hydrochloric acid to a pH of 4, and dilute with water to 100 mL.

Analysis: To 10 mL of the *Sample solution* add 1 mL of *Decolorized fuchsin solution* and allow to stand for 30 min. Any color in the solution is not more intense than that in the *Standard solution* prepared at the same time.

Acceptance criteria: NMT 0.15%, as acetaldehyde (C_2H_4O)▲ (NF 1-May-2020)

SPECIFIC TESTS

• **ACIDITY OR ALKALINITY**

Sample solution: 1.1 g of Potassium Sorbate in 20 mL of water

Analysis: Add phenolphthalein TS to the *Sample solution*.

Acceptance criteria: If the solution is colorless, titrate with 0.10 N sodium hydroxide to a pink color that persists for 15 s: NMT 1.1 mL of 0.10 N sodium hydroxide is required. If the solution is pink in color, titrate with 0.10 N hydrochloric acid to discharge the pink color: NMT 0.80 mL of 0.10 N hydrochloric acid is required.

• **LOSS ON DRYING** ⟨731⟩

Analysis: Dry at 105° for 3 h.

Acceptance criteria: NMT 1.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light, and avoid exposure to excessive heat.

Add the following:

▲• **USP REFERENCE STANDARDS** ⟨11⟩

USP Potassium Sorbate RS▲ (NF 1-May-2020)

Povidone—*see Povidone General Monographs*

Propane

C_3H_8 44.10

[74-98-6].

DEFINITION

Propane contains NLT 98.0% of propane (C_3H_8).

[**CAUTION**—Propane is highly flammable and explosive.]

IDENTIFICATION

• **A. INFRARED ABSORPTION:** Exhibits maxima, among others, at the following wavelengths, in µm: 3.4 (vs), 6.8 (s), and 7.2 (m).

• **B.**

Sample: Use an empty stainless steel cylinder equipped with a stainless steel valve, having a capacity of NLT 200 mL, and a pressure rating of 240 psi or more. Dry the cylinder with the valve open at 110° for 2 h, and evacuate the hot cylinder to less than 1 mm of mercury. Close the valve, cool, and weigh. Connect one end of a charging line tightly to the propane container and the other end loosely to the empty cylinder. Carefully open the propane container, and allow the propane to flush out the charging line through the loose connection. Avoid excessive flushing, which causes moisture to freeze in the charging line and connections. Tighten the fitting on the empty cylinder, and open the empty cylinder valve, allowing the propane to flow into the evacuated cylinder. Continue sampling until the desired amount of propane is obtained, then close the propane container valve, and finally close the sample cylinder valve. [**CAUTION**—Do not overload the sample cylinder; hydraulic expansion due to temperature change can cause overloaded cylinders to explode.] Weigh the charged sample cylinder, and determine the weight.

Analysis: Determine the vapor pressure of the *Sample* at 21° by means of a suitable pressure gauge.

Acceptance criteria: 820–875 kPa absolute (119–127 psia)

ASSAY

• **PROCEDURE**

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Thermal conductivity

Column: 6-m × 3-mm aluminum; packed with 10 weight percent of liquid phase G30 on support S1D

Column temperature: 33°

Carrier gas: Helium

Flow rate: 50 mL/min

Injection volume: 2 µL

System suitability

Sample: Propane

Suitability requirements: The peak responses for Propane from duplicate determinations agree within 1%.

Analysis: Connect one Propane cylinder to the chromatograph through a suitable sampling valve and a flow control valve downstream from the sampling valve. Flush the liquid specimen through the sampling valve, taking care to avoid entrapment of gas or air in the sampling valve. Calculate the percentage purity by dividing 100 times the propane response by the sum of all of the responses.

Acceptance criteria: NLT 98.0%

SPECIFIC TESTS

• **HIGH-BOILING RESIDUES**

Sample: Use the *Sample* from *Identification* test *B*.

Analysis: Prepare a cooling coil from copper tubing (about 6-mm outside diameter × about 6.1-m long) to fit into a vacuum-jacketed flask. Immerse the cooling coil in a mixture of dry ice and acetone in a vacuum-jacketed flask, and connect one end of the tubing to the *Sample*. Carefully open the sample cylinder valve, flush the cooling coil with about 50 mL of the *Sample*, and discard this portion of liquefied sample. Continue delivering liquefied sample from the cooling coil, and collect it in a previously chilled 1000-mL sedimentation cone until the cone is filled to the 1000-mL mark. Allow the sample to evaporate, using a

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warm water bath maintained at about 40° to reduce evaporating time. When all of the liquid has evaporated, rinse the sedimentation cone with two 50-mL portions of pentane, and combine the rinsings in a tared 150-mL evaporating dish. Transfer 100 mL of the pentane solvent to a second tared 150-mL evaporating dish, place both evaporating dishes on a water bath, evaporate to dryness, and heat the dishes in an oven at 100° for 60 min. Cool the dishes in a desiccator, and weigh. Repeat the heating for 15-min periods until successive weighings are within 0.1 mg, and calculate the weight of the residue obtained from the *Sample* as the difference between the weights of the residues in the two evaporating dishes.

Acceptance criteria: NMT 5 µg/mL

• **ACIDITY OF RESIDUE**

Sample solution: Add 10 mL of water to the residue obtained in the test for *High-Boiling Residues*, mix by swirling for 30 s, add 2 drops of methyl orange TS, insert the stopper in the tube, and shake vigorously.

Acceptance criteria: No pink or red color appears in the aqueous layer.

• **LIMIT OF SULFUR COMPOUNDS**

Analysis: Carefully open the container valve to produce a moderate flow of gas. Do not direct the gas stream toward the face, but deflect a portion of the stream toward the nose.

Acceptance criteria: The odor is free from the characteristic odor of sulfur compounds.

• **WATER DETERMINATION** ⟨921⟩

Sample: 100 g of the *Sample* from *Identification* test *B*

Analysis: Proceed as directed in the chapter with the following modifications. (a) Provide the closed-system titrating vessel with an opening through which passes a coarse-porosity gas dispersion tube connected to a sampling cylinder. (b) Dilute the *Reagent* with anhydrous methanol to give a water equivalence factor of 0.2–1.0 mg/mL; age this diluted solution for NLT 16 h before standardization. (c) Introduce the *Sample* into the titration vessel through the gas dispersion tube at a rate of about 100 mL/min; if necessary, heat the sample cylinder gently to maintain this flow rate.

Acceptance criteria: NMT 0.001%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight cylinders, and prevent exposure to excessive heat.

Propanediol



$C_3H_8O_2$ 76.09

1,3-Propanediol;
1,3-Dihydroxypropane;
Propane, 1-3-diol;
Trimethylene glycol [504-63-2].

DEFINITION

Propanediol contains NLT 99.7% of 1,3-propanediol ($C_3H_8O_2$). It may be of vegetable, other natural source, or synthetic origin.

IDENTIFICATION

Change to read:

• **A.** ▲**SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F** ▲ (CN 1-May-2020)

• **B.** The retention time of the major peak of the *Sample solution* corresponds to the 1,3-propanediol peak of the *System suitability solution*, as obtained in the *Assay*.

ASSAY

• **PROCEDURE**

System suitability solution: Mix quantities of USP Propylene Glycol RS and USP 1,3-Propanediol RS to obtain a solution containing about 5% propylene glycol and 95% propanediol.

Sample solution: Propanediol (neat)

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.25-mm × 30-m capillary column; bonded with a 0.25-µm layer of phase G16

Temperatures

Detector: 250°

Injection port: 250°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
50	15	200	—
200	40	250	17

Carrier gas: Helium

Flow rate: 1.1 mL/min

Injection volume: 0.2 µL

Split type: Split ratio of 18:1

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for propylene glycol and propanediol are 0.7 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 2.0 between the peaks due to propylene glycol and propanediol

Analysis

Sample: *Sample solution*

Calculate the percentage of propanediol in the portion of sample taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response for propanediol in the *Sample solution*

r_T = sum of all peak responses in the *Sample solution*

Acceptance criteria: NLT 99.7%

IMPURITIES

• **LIMIT OF RELATED GLYCOL SUBSTANCES**

System suitability solution, Sample solution, Chromatographic system, and System suitability: Proceed as directed in the *Assay*.

Analysis

Sample: *Sample solution*

Calculate the percentage of each individual impurity in the portion of Propanediol taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of each individual impurity in the *Sample solution*

r_T = sum of all peak responses in the *Sample solution*

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Acceptance criteria
Each individual impurity: NMT 0.1%
Total impurities: NMT 0.3%

• **LIMIT OF ALDEHYDES**

Formaldehyde methanol solution:[1] A solution containing 37% (w/w) of formaldehyde and 10%–15% (w/w) of methanol in water

Phenolphthalein solution: Dissolve 0.1 g of phenolphthalein in 80 mL of alcohol, and dilute with water to 100 mL.

Quantification of Formaldehyde methanol solution: To 2.0 g of *Formaldehyde methanol solution*, add 100 mL of a freshly prepared 100 mg/mL solution of sodium sulfite in carbon-dioxide free water. Add 0.1 mL of *Phenolphthalein solution*, and titrate with 0.5 N sulfuric acid until the color changes from pink to colorless. Carry out a blank titration. Calculate the percentage content of formaldehyde in *Formaldehyde methanol solution* using the following expression:

$$\text{Result } (P_{HCHO}) = \{[(V_S - V_B) \times N \times M_W \times F]/W\} \times 100$$

V_S = volume of 0.5 N sulfuric acid used in the assay (mL)
V_B = volume of 0.5 N sulfuric acid used in the blank (mL)
N = normality of the titrant (mEq/mL)
M_W = milliequivalent weight of formaldehyde, 30.03 mg/mEq
F = unit conversion factor, 10^{-3} g/mg
W = weight of sample (g)

Standard stock solution: 1.2 µg/mL of *Formaldehyde methanol solution* in carbon-dioxide free water, prepared from appropriately diluting *Formaldehyde methanol solution* in carbon-dioxide free water

Standard solutions: Introduce into 50-mL volumetric flasks 1.0-, 3.0-, 5.0-, 10.0-, 15.0-, and 25.0-mL of *Standard stock solution*, respectively. Calculate the content of formaldehyde, in µg, in the *Standard solutions* using the following expression. Proceed as directed in the *Analysis* below.

$$\text{Result (content of formaldehyde)} = V \times C \times P_{HCHO} \times 0.01$$

V = volume of the *Standard stock solution* added into the *Standard solution* (mL)
C = concentration of *Formaldehyde methanol solution* in the *Standard stock solution* (µg/mL)
P_{HCHO} = percentage content of formaldehyde in *Formaldehyde methanol solution*, as determined above

Sample solution: Introduce 5.0 mL of 0.2 g/mL of propanediol in carbon-dioxide free water into a 50-mL volumetric flask. Proceed as directed in the *Analysis* below.

Blank solution: Prepare in the same manner as for the *Standard solutions* but omitting the *Standard stock solution*.

Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: Vis spectrophotometry
Analytical wavelength: 655 nm

Analysis
Samples: *Blank solution*, *Standard solutions*, and *Sample solution*

To each flask of the *Blank solution*, *Standard solutions*, and *Sample solution* add 2 mL of a freshly prepared 5 mg/mL solution of methylbenzothiazolone hydrazone hydrochloride adjusted with 0.02 N sodium hydroxide to a pH of 4.0. Allow the solutions to stand for 30 min. Add 5 mL of a freshly prepared 7 mg/mL solution of ferric chloride. Cap and swirl the flasks. Allow to stand for 5 min. Add methanol to each flask, and dilute with methanol to 50.0 mL. Mix thoroughly, then allow to stand for 1 min.

Measure the absorbance of the solutions using the treated *Blank solution* as compensation liquid.

Plot the absorbance of the treated *Standard solution* versus the content of formaldehyde, in µg, in the *Standard solution*. Obtain the content of formaldehyde W_{HCHO}, in µg, in the treated *Sample solution* based on the calibration curve.

Calculate the content of aldehydes expressed as formaldehyde (HCHO) in the portion of Propanediol taken:

$$\text{Result} = W_{HCHO}/(C \times V)$$

W_{HCHO} = content of formaldehyde in the treated *Sample solution*, determined from the calibration curve (µg)
C = concentration of Propanediol in the *Sample solution* (g/mL)
V = volume of the *Sample solution* in the analysis (mL)

Acceptance criteria: NMT 20 µg/g, expressed as HCHO.

SPECIFIC TESTS

• **ACIDITY**

Sample: 50 mL of Propanediol

Phenolphthalein solution: Dissolve 0.1 g of phenolphthalein in 80 mL of alcohol, and dilute with water to 100 mL.

Titrimetric system
(See *Titrimetry* ⟨541⟩)
Mode: Direct titration
Titrant: 0.01 N sodium hydroxide VS
Endpoint detection: Visual

Analysis: To 50 mL of water, add 1 mL of *Phenolphthalein solution*, then add *Titrant* until the solution remains pink for 30 s. Add the *Sample*, and titrate with *Titrant* until the color turns back to pink and remains for more than 30 s. Calculate the acidity, as acetic acid (CH_3COOH):

$$\text{Result} = (V_T \times N \times W_{Meq})/V_S$$

V_T = *Titrant* volume (mL)
N = *Titrant* normality (mEq/mL)
W_{MEq} = milliequivalent weight of acetic acid, 60.05 mg/mEq
V_S = volume of Propanediol in the *Sample* (mL)

Acceptance criteria: NMT 0.1 mg/mL, calculated as acetic acid (CH_3COOH)

• **WATER DETERMINATION,** *Method Ic* ⟨921⟩**:** NMT 0.1%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers. Do not store above 50°. Protect from moisture.

• **LABELING:** Label it to indicate whether Propanediol is derived from vegetable, other natural source, or synthetic origin.

• **USP REFERENCE STANDARDS** ⟨11⟩
USP 1,3-Propanediol RS
USP Propylene Glycol RS

[1] Formaldehyde TS can be used for *Formaldehyde methanol solution*.

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Propionic Acid

DEFINITION
Propionic Acid contains NLT 99.5% and NMT 100.5%, by weight, of propionic acid ($C_3H_6O_2$).

ASSAY
• **PROCEDURE**
Sample: 1.5 g of Propionic Acid
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.5 N sodium hydroxide VS
Endpoint detection: Visual
Analysis: Mix the *Sample* with 100 mL of recently boiled and cooled water in a 250-mL conical flask. Add phenolphthalein TS to the *Sample solution*, and titrate with *Titrant* to the first appearance of a faint pink endpoint that persists for NLT 30 s.
Calculate the percentage of propionic acid ($C_3H_6O_2$) in the sample taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

V_S = volume of *Titrant* consumed by the sample (mL)
V_B = volume of *Titrant* consumed by the blank (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 74.08 mg/mEq
W = weight of the sample (mg)

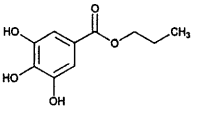
Acceptance criteria: 99.5%–100.5% by weight

IMPURITIES
• **LIMIT OF NONVOLATILE RESIDUE:** Evaporate 20 g of Propionic Acid in a tared dish, and dry at 105° for 1 h: the weight of the residue does not exceed 2.0 mg.
• **LIMIT OF ALDEHYDES**
Sample solution: Transfer 10.0 mL of Propionic Acid to a glass-stoppered 250-mL conical flask containing 50 mL of water and 10.0 mL of sodium bisulfite solution (1 in 80), insert the stopper, and shake vigorously. Allow the mixture to stand for 30 min.
Blank: Add 50 mL of water and 10.0 mL of sodium bisulfite solution (1 in 80) to a glass-stoppered 250-mL conical flask, insert the stopper, and shake vigorously. Allow the mixture to stand for 30 min.
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Residual titration
Titrant: 0.1 N iodine VS
Endpoint detection: Visual
Analysis: Titrate the *Sample solution* and the *Blank* with *Titrant* to the same brownish-yellow endpoint.
Acceptance criteria: The difference between the volume of 0.1 N iodine required for the *Blank* and that required for the *Sample solution* is NMT 1.75 mL.

SPECIFIC TESTS
• **DISTILLING RANGE,** *Method I* ⟨721⟩**:** 138.5°–142.5°
• **SPECIFIC GRAVITY** ⟨841⟩**:** 0.988–0.993
• **READILY OXIDIZABLE SUBSTANCES**
Sample solution: Dissolve 15 g of sodium hydroxide in 50 mL of water. Cool, add 6 mL of bromine, stirring to effect complete solution, and dilute with water to 2000 mL. Transfer 25.0 mL of this solution to a glass-stoppered, 250-mL conical flask containing 100 mL of water, and add 10 mL of sodium acetate solution (1 in 5) and 10.0 mL of Propionic Acid. Allow to stand for 15 min, and add 5 mL of potassium iodide solution (1 in 4) and 10 mL of hydrochloric acid.
Blank: Dissolve 15 g of sodium hydroxide in 50 mL of water. Cool, add 6 mL of bromine, stirring to effect complete solution, and dilute with water to 2000 mL. Transfer 25.0 mL of this solution to a glass-stoppered, 250-mL conical flask containing 100 mL of water, and add 10 mL of sodium acetate solution (1 in 5). Allow to stand for 15 min, and add 5 mL of potassium iodide solution (1 in 4) and 10 mL of hydrochloric acid.
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Residual titration
Titrant: 0.1 N sodium thiosulfate VS
Endpoint detection: Visual
Analysis: Titrate the *Sample solution* and the *Blank* with *Titrant* just to the disappearance of the brown color.
Acceptance criteria: The difference between the volume of 0.1 N sodium thiosulfate required for the *Blank* and that required for the *Sample solution* is NMT 2.2 mL.

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight containers.

Propyl Gallate



$C_{10}H_{12}O_5$ 212.20
Benzoic acid, 3,4,5-trihydroxy-, propyl ester;
Propyl gallate [121-79-9].

DEFINITION
Propyl Gallate contains NLT 98.0% and NMT 102.0% of propyl gallate ($C_{10}H_{12}O_5$), calculated on the dried basis.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197M**▲ (CN 1-May-2020)

Change to read:

• **B. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Ultraviolet-Visible Spectroscopy*: **197U**▲ (CN 1-May-2020)
Sample solution: 10 µg/mL in methanol
Acceptance criteria: Meets the requirements

ASSAY
• **PROCEDURE**
Standard solution: 10 µg/mL of USP Propyl Gallate RS in methanol
Sample solution: 10 µg/mL of Propyl Gallate in methanol
Blank: Methanol
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: UV
Analytical wavelength: Maximum at about 273 nm
Cell: 1 cm
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of propyl gallate ($C_{10}H_{12}O_5$) in the portion of the sample taken:

$$\text{Result} = (A_U/A_S) \times (C_S/C_U) \times 100$$

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A_U = absorbance of the *Sample solution*
A_S = absorbance of the *Standard solution*
C_S = concentration of USP Propyl Gallate RS in the *Standard solution* (µg/mL)
C_U = concentration of the *Sample solution* (µg/mL)

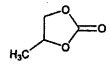
Acceptance criteria: 98.0%–102.0% on the dried basis

IMPURITIES
• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%

SPECIFIC TESTS
• **MELTING RANGE OR TEMPERATURE** ⟨741⟩: 146°–150°
• **LOSS ON DRYING** ⟨731⟩: Dry a sample at 105° for 4 h: it loses NMT 0.5% of its weight.

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light, and avoid contact with metals.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Propyl Gallate RS

Propylene Carbonate



$C_4H_6O_3$ 102.09
4-Methyl-1,3-dioxolan-2-one;
Cyclic propylene carbonate [108-32-7].

DEFINITION
Propylene Carbonate contains NLT 99.0% and NMT 100.5% of propylene carbonate ($C_4H_6O_3$).

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F▲** (CN 1-May-2020)

ASSAY
• **PROCEDURE**
Barium hydroxide solution: 75 mg/mL of barium hydroxide (octahydrate). Filter the solution before use.
Sample: 600 mg
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.5 N hydrochloric acid VS
Endpoint detection: Visual
Analysis: Flush a 250-mL iodine flask with nitrogen to expel the air, and insert the stopper in the flask to exclude carbon dioxide. Transfer 50.0 mL of *Barium hydroxide solution* and the *Sample* to the flask, and loosely reinsert the stopper. Moisten the stopper with 3 drops of water, and heat the flask on a steam bath for 10 min. Remove the flask from the steam bath, add 6 drops of phenolphthalein TS, and titrate while hot with *Titrant* until only a trace of pink color remains. Perform a blank determination, using the same *Barium hydroxide solution*. Each mL of 0.5 N hydrochloric acid consumed is equivalent to 25.52 mg of propylene carbonate ($C_4H_6O_3$).
Acceptance criteria: 99.0%–100.5%

IMPURITIES
• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.01%

SPECIFIC TESTS
• **SPECIFIC GRAVITY** ⟨841⟩: 1.203–1.210 at 20°
• **pH** ⟨791⟩
Sample solution: Mix 10 mL of propylene carbonate with 0.3 mL of saturated potassium chloride solution in a 100-mL borosilicate volumetric flask. Dilute with carbon dioxide-free water having a pH of 7.0 ± 0.5 to volume.
Analysis: Completely purge the solution by vigorous nitrogen bubbling, and continue the bubbling during the pH measurement. Determine the pH potentiometrically when the reading stabilizes.
Acceptance criteria: 6.0–7.5

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight containers.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Propylene Carbonate RS

Propylene Glycol—*see Propylene Glycol General Monographs*

Propylene Glycol Alginate

DEFINITION
Propylene Glycol Alginate is a propylene glycol ester of alginic acid. Each gram yields NLT 0.16 and NMT 0.20 g of carbon dioxide, calculated on the dried basis.

IDENTIFICATION
• **A.**
Sample solution: Place 20 mL of the saponified solution obtained in the determination of *Esterified Carboxyl Groups* in a 250-mL conical flask. Add 50 mL of a solution of periodic acid (1 in 50), swirl, and allow to stand for 30 min. Add 2 g of potassium iodide, titrate with sodium thiosulfate TS to a faint yellow color, dilute the mixture with water to 200 mL, and mix to obtain the *Sample solution* for *Identification* test *A* and *Identification* test *B*.
Modified Schiff's reagent: Dissolve 200 mg of rosaniline hydrochloride ($C_{20}H_{20}ClN_3$) in 120 mL of hot water. Cool, add 2 g of sodium bisulfite ($NaHSO_3$), followed by 2 mL of hydrochloric acid, and dilute with water to 200 mL. [NOTE—Store this solution in a brown bottle at 15° or lower.]
Analysis: To 10 mL of the *Sample solution* add 5 mL of hydrochloric acid and 10 mL of *Modified Schiff's reagent*.
Acceptance criteria: A blue to blue-violet color, due to formaldehyde, develops in about 20 min.
• **B.**
Analysis: To 10 mL of the *Sample solution* prepared in *Identification* test *A* add 1 mL of a saturated solution of piperazine and 0.5 mL of sodium nitroferricyanide TS.
Acceptance criteria: A green color, due to acetaldehyde, develops.

ASSAY
• **CONTENT OF ALGINATE**
Analysis: Proceed as directed for *Procedure* in *Alginates Assay* ⟨311⟩, without preliminary drying of the Propylene Glycol Alginate.
Acceptance criteria: 0.16–0.20 g of carbon dioxide/g of Propylene Glycol Alginate, calculated on the dried basis

OTHER COMPONENTS
• **FREE CARBOXYL GROUPS**
Sample: 1 g

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Titrimetric system
Mode: Direct titration
Titrant: 0.1 N sodium hydroxide VS
Endpoint detection: Potentiometric
Analysis: Transfer the *Sample* to a 600-mL beaker. Dissolve in 200 mL of water, stirring by mechanical means for NLT 30 min. Titrate with 0.1 N sodium hydroxide VS to a pH of 7.0.
Calculate the weight, in g, of free carboxyl groups in the *Sample* taken:

$$\text{Result} = [(V \times M_r \times N)/W] \times F$$

V = *Titrant* volume consumed (mL)
M_r = mEq of CO_2, 44 mg/mEq
N = actual normality of the *Titrant* (mEq/mL)
W = *Sample* weight (g)
F = conversion factor, 10^{-3} g/mg

Acceptance criteria: The weight of free carboxyl groups found, calculated on the dried basis, is NMT 35% of the weight of carbon dioxide yielded by an equal weight of specimen in the *Assay*.

• **ESTERIFIED CARBOXYL GROUPS**
Sample solution: The solution obtained in the test for *Free Carboxyl Groups*
Analysis: Transfer the *Sample solution* with the aid of water to a 1000-mL conical flask. Add phenolphthalein TS and 50.0 mL of 0.1 N sodium hydroxide VS, insert a stopper in the flask, mix, and allow to stand for 30 min at ambient temperature. Titrate the excess sodium hydroxide with 0.1 N hydrochloric acid VS to a faint pink endpoint. Transfer the solution with the aid of water to a 600-mL beaker, and complete the titration to a pH of 7.0, determining the endpoint potentiometrically.
Calculate the weight, in g, of esterified carboxyl groups in the weight, W, in g, of the specimen taken:

$$\text{Result} = [(V \times M_r \times N)/W] \times F$$

V = volume of 0.1 N sodium hydroxide consumed (mL)
M_r = mEq of CO_2, 44 mg/mEq
N = actual normality of 0.1 N sodium hydroxide (mEq/mL)
W = specimen weight (g)
F = conversion factor, 10^{-3} g/mg

Acceptance criteria: The weight of esterified carboxyl groups found, calculated on the dried basis, is 40%–85% of the weight of carbon dioxide yielded by an equal weight of specimen in the *Assay*.

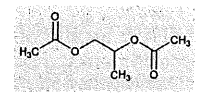
IMPURITIES
• **ARSENIC,** *Method II* ⟨211⟩ **:** 3 ppm
• **LEAD** ⟨251⟩
Standard solution: 5 mL of *Diluted Standard Lead Solution*
Test preparation: Add 1.0 g to 20 mL of nitric acid in a 250-mL conical flask, mix, and heat carefully until the specimen is dissolved. Continue the heating until the volume is reduced to 7 mL. Cool rapidly to room temperature, transfer to a 100-mL volumetric flask, dilute with water to volume, and mix. Use a 50-mL portion.
Analysis: Proceed as directed in the chapter, using 15 mL of ammonium citrate solution, 3 mL of potassium cyanide solution, and 0.5 mL of hydroxylamine hydrochloride solution for the test. After the first dithizone extractions, wash the combined chloroform layers with 5 mL of water, discarding the water layer and continuing in the usual manner by extracting with 20 mL of 0.2 N nitric acid.
Acceptance criteria: A 50.0-mL portion of this solution contains NMT 5 µg of lead (corresponding to NMT 10 ppm of Pb).

SPECIFIC TESTS
• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total bacterial count does not exceed 200 cfu/g, and the tests for *Salmonella* species and *Escherichia coli* are negative.
• **LOSS ON DRYING** ⟨731⟩**:** Dry a sample at 105° for 4 h: it loses NMT 20.0% of its weight.
• **ASH**
Sample: 3 g
Analysis: Weigh the *Sample* in a tared crucible, and incinerate at 650 ± 25° until free from carbon. Cool in a desiccator, weigh, and determine the weight of the ash.
Acceptance criteria: NMT 10.0% on the dried basis

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Add the following:

▲Propylene Glycol Diacetate



$C_7H_{12}O_4$ 160.17
1,2-Propanediol, 1,2-diacetate;
Propane-1,2-diyl diacetate [623-84-7].

DEFINITION
Propylene Glycol Diacetate contains NLT 98.0% and NMT 102.0% of propylene glycol diacetate ($C_7H_{12}O_4$).

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩**,** *Infrared Spectroscopy*: **197F** ▲ (CN 1-May-2020)
• **B. CHROMATOGRAPHIC IDENTITY**
Analysis: Proceed as directed in the *Assay*.
Acceptance criteria: The retention time of the major peak of the *Sample solution*, excluding the solvent and internal standard peaks, corresponds to the propylene glycol diacetate peak of the *Standard solution*.

ASSAY
• **PROCEDURE**
Internal standard solution: 20 mg/mL of triacetin in methanol
System suitability solution: 20 mg/mL each of USP Propylene Glycol Diacetate RS and USP Benzyl Alcohol RS in *Internal standard solution*
Standard solution: 20.0 mg/mL of USP Propylene Glycol Diacetate RS in *Internal standard solution*
Sample solution: 20.0 mg/mL of Propylene Glycol Diacetate in *Internal standard solution*
Chromatographic system
(See *Chromatography* ⟨621⟩*, System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.53-mm × 30-m fused silica; coated with a 1.0-µm film of phase G46

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Temperatures
Injection port: 280°
Detector: 280°
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
80	—	80	2
80	25	250	2

Carrier gas: Hydrogen
Flow rate: 5.0 mL/min
Injection volume: 1 µL
Injection type: Split ratio, 60:1
Run time: 10.8 min
System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The relative retention times for propylene glycol diacetate, benzyl alcohol, and triacetin are 1.0, 1.1, and 1.4, respectively.]
Suitability requirements
Resolution: NLT 10 between the propylene glycol diacetate and benzyl alcohol peaks, *System suitability solution*
Tailing factor: NMT 1.5 for the propylene glycol diacetate and triacetin peaks, *Standard solution*
Relative standard deviation: NMT 1.0% for the peak area ratio of propylene glycol diacetate to the internal standard, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of propylene glycol diacetate in the portion of Propylene Glycol Diacetate taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times P \times 100$$

R_U = peak area ratio of propylene glycol diacetate to the internal standard from the *Sample solution*
R_S = peak area ratio of propylene glycol diacetate to the internal standard from the *Standard solution*
C_S = concentration of USP Propylene Glycol Diacetate RS in the *Standard solution* (mg/mL)
C_U = concentration of Propylene Glycol Diacetate in the *Sample solution* (mg/mL)
P = labeled purity of USP Propylene Glycol Diacetate RS

Acceptance criteria: 98.0%–102.0%

IMPURITIES

• **ORGANIC IMPURITIES**
System suitability solution and **Chromatographic system:** Proceed as directed in the *Assay*.
Sensitivity solution: 0.2 mg/mL of USP Propylene Glycol Diacetate RS in methanol
Sample solution: 20.0 mg/mL of Propylene Glycol Diacetate in methanol
System suitability
Samples: *System suitability solution* and *Sensitivity solution*
[NOTE—The relative retention times for propylene glycol diacetate, benzyl alcohol, and triacetin are 1.0, 1.1, and 1.4, respectively.]
Suitability requirements
Resolution: NLT 10 between the propylene glycol diacetate and benzyl alcohol peaks, *System suitability solution*
Signal-to-noise ratio: NLT 20 for the propylene glycol diacetate peak, *Sensitivity solution*
Analysis
Sample: *Sample solution*
Identify the impurities in the *Sample solution* by comparing with those listed in *Table 2*.
Calculate the percentage of any individual impurity in the portion of Propylene Glycol Diacetate taken:

$$\text{Result} = (r_U/r_S) \times 100$$

r_U = peak area of any individual impurity from the *Sample solution*
r_S = sum of all the peak areas, excluding the solvent peaks from the *Standard solution*

Table 2

Name	Relative Retention Time
Propylene glycol	0.67
Propylene glycol monoacetate, isomer 1	0.85
Propylene glycol monoacetate, isomer 2	0.88
Propylene glycol diacetate	1.0
Propylene glycol monoacetate monopropionate	1.11

Acceptance criteria
Individual impurity: NMT 0.1%
Total impurities: NMT 0.5%
• **ACETIC ACID**
Sample: 10 g
Analysis: Dissolve the *Sample* in 30 mL of a mixture of alcohol and water (1:1), previously neutralized to phenolphthalein. Titrate with 0.1 N sodium hydroxide VS to a phenolphthalein endpoint. Each milliliter of 0.1 N sodium hydroxide is equivalent to 6.005 mg of acetic acid ($C_2H_4O_2$).
Acceptance criteria: NMT 0.2%

SPECIFIC TESTS

• **PH** ⟨791⟩
Sample: 1 in 20 solution
Acceptance criteria: 4.0–6.0
• **SPECIFIC GRAVITY** ⟨841⟩**:** 1.040–1.060
• **REFRACTIVE INDEX** ⟨831⟩**:** 1.4130–1.4150 at 20°
• **WATER DETERMINATION** ⟨921⟩, *Method I*, *Method Ic*: NMT 0.1%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Benzyl Alcohol RS
USP Propylene Glycol Diacetate RS▲ (NF 1-May-2019)

Propylene Glycol Dicaprylate/Dicaprate

Decanoic acid, mixed diesters with octanoic acid and propylene glycol [68583-51-7].

NF Monographs

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DEFINITION

Propylene Glycol Dicaprylate/Dicaprate is a mixture of the propylene glycol mono- and diesters of caprylic acid ($C_8H_{16}O_2$) and capric acid ($C_{10}H_{20}O_2$), the diesters fraction being predominant.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F▲** (CN 1-May-2020)
- **B. THIN-LAYER CHROMATOGRAPHY IDENTIFICATION TEST** ⟨201⟩

Standard solution: 50 mg/mL of USP Propylene Glycol Dicaprylate/Dicaprate RS in methylene chloride
Sample solution: 50 mg/mL of Propylene Glycol Dicaprylate/Dicaprate in methylene chloride
Application volume: 10 µL, as streaks
Developing solvent system: Ether and hexane (7:3)
Spray reagent: 0.1 mg/mL of rhodamine 6G in alcohol
Analysis
Samples: *Standard solution* and *Sample solution*
Develop the chromatogram over a path of 15 cm, and dry the plate in a current of air. Spray the plate with *Spray reagent*, and locate the spots on the plate by examination under UV light at a wavelength of 365 nm.
Acceptance criteria: The R_F values of the principal spots from the *Sample solution* correspond to those from the *Standard solution*.

- **C.** It meets the requirements of the test for *Fats and Fixed Oils* ⟨401⟩, *Fatty Acid Composition*.

IMPURITIES

INORGANIC IMPURITIES

- **Articles of Botanical Origin,** *Total Ash* ⟨561⟩: NMT 0.1%
- **ALKALINE IMPURITIES**

Sample: 2.0 g of Propylene Glycol Dicaprylate/Dicaprate
Analysis: Dissolve the *Sample* in a mixture of 1.5 mL of alcohol and 3.0 mL of ether. Add 0.05 mL of bromophenol blue TS.
Acceptance criteria: NMT 0.15 mL of 0.01 N hydrochloric acid is required in order to change the color of the indicator to yellow.

SPECIFIC TESTS

- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩: NMT 0.2
- **FATS AND FIXED OILS,** *Fatty Acid Composition* ⟨401⟩: Propylene Glycol Dicaprylate/Dicaprate exhibits the composition profile of fatty acids shown in the following table:

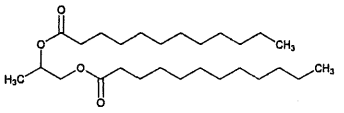
Carbon-Chain Length	Number of Double Bonds	Percentage (%)
6	0	≤2.0
8	0	50.0–80.0
10	0	20.0–50.0
12	0	≤3.0
14	0	≤1.0

- **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩: NMT 10
- **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩: NMT 1.0
- **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩: NMT 1.0
- **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩: 320–340
- **FATS AND FIXED OILS,** *Unsaponifiable Matter* ⟨401⟩: NMT 0.5%, determined on 5.0 g
- **WATER DETERMINATION,** *Method Ia* ⟨921⟩: NMT 0.1%, using a mixture of methanol and methylene chloride (1:1) in place of methanol in the titration vessel

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, and protect from moisture. No storage requirements specified.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Propylene Glycol Dicaprylate/Dicaprate RS

Propylene Glycol Dilaurate



$C_{27}H_{52}O_4$
Lauric acid, diester with propane-1,2-diol;
Propane-1,2-diyl didodecanoate 440.70
[22788-19-8].

DEFINITION

Propylene Glycol Dilaurate is a mixture of the propylene glycol mono- and diesters of lauric acid. It contains NLT 70.0% of diesters and NMT 30.0% of monoesters.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩

Standard solution: 50 mg/mL of USP Propylene Glycol Dilaurate RS in methylene chloride
Sample solution: 50 mg/mL of Propylene Glycol Dilaurate in methylene chloride
Developing solvent system: Hexane and ether (3:7)
Spray reagent: 0.1 mg/mL of rhodamine 6G in alcohol
Analysis: Apply 200 µg of the *Standard solution* and *Sample solution*. Develop the chromatogram over a path of 15 cm, and dry the plate in a current of air. Spray the plate with *Spray reagent*, and locate the spots on the plate by examination under UV light at a wavelength of 365 nm.
Acceptance criteria: The R_F values of the principal spots of the *Sample solution* correspond to those of the *Standard solution*.

- **B. FATS AND FIXED OILS,** *Fatty Acid Composition* ⟨401⟩: Meets the requirements

ASSAY

- **PROCEDURE**

Mobile phase: Tetrahydrofuran
Sample solution: 200 mg of Propylene Glycol Dilaurate in 5 mL of tetrahydrofuran
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Refractive index
Column: 7-mm × 60-cm; 5-µm packing L21 (100Å)
[NOTE—Two 7-mm × 30-cm L21 columns may be used in place of one 60-cm column, provided system suitability requirements are met.]
Temperature
Column: 40°
Detector: 40°
Flow rate: 1 mL/min
Injection size: 40 µL
System suitability
Sample: *Sample solution*

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[NOTE—Elution order: diesters, monoesters, propylene glycol.]

Suitability requirements

Relative standard deviation: NMT 1.0% is determined from the monoester peak.

Analysis

Sample: *Sample solution*

Calculate the percentage of monoesters or diesters in the portion of Propylene Glycol Dilaurate taken:

$$\text{Result} = (r_U/r_T) \times (100 - D)$$

r_U = peak response for monoesters or diesters

r_T = sum of the responses of the monoester and diester peaks

D = sum of the percentage content of propylene glycol and the percentage content of free fatty acids

Calculate the percentage content of free fatty acids:

$$\text{Result} = (A/561.1) \times 200$$

A = acid value

Acceptance criteria: NLT 70.0% of diesters and NMT 30.0% of monoesters

IMPURITIES

INORGANIC IMPURITIES

• **Articles of Botanical Origin,** *Total Ash* ⟨561⟩: NMT 0.1%

ORGANIC IMPURITIES

• **Procedure: Limit of Propylene Glycol**

Mobile phase: Proceed as directed in the *Assay*.

Standard stock solution: 4 mg/mL of USP Propylene Glycol RS in tetrahydrofuran

Standard solutions: Into four 15-mL flasks, introduce respectively 0.25, 0.5, 1.0, and 2.5 mL of *Standard stock solution*, and dilute with tetrahydrofuran to 5.0 mL. In a fifth 15-mL flask, introduce 5.0 mL of *Standard stock solution*.

Sample solution: Use the *Sample solution* from the *Assay*.

Chromatographic system: Proceed as directed in the *Assay*.

Analysis

Samples: *Standard solutions* and *Sample solution*

Prepare a standard curve of peak area versus concentration, in mg/mL, of propylene glycol in the *Standard solutions*. Obtain the concentration, C, in mg/mL, of propylene glycol in the *Sample solution* from the standard curve.

Acceptance criteria: NMT 2.0% of propylene glycol is found.

SPECIFIC TESTS

• **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩: NMT 4

• **FATS AND FIXED OILS,** *Fatty Acid Composition* ⟨401⟩: Propylene Glycol Dilaurate exhibits the following composition profile of fatty acids, determined as directed in the chapter.

Fatty Acids	Carbon-Chain Length	Percentage (%)
Caprylic acid	C8	NMT 0.5
Capric acid	C10	NMT 2.0
Lauric acid	C12	NLT 95.0
Myristic acid	C14	NMT 3.0
Palmitic acid	C16	NMT 1.0

• **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩: NMT 1

• **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩: 230–250

• **WATER DETERMINATION,** *Method Ia* ⟨921⟩: NMT 1.0%, using a mixture of methanol and methylene chloride (1:1) in place of methanol in the titration vessel

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, and protect from moisture. No storage requirements are specified.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Propylene Glycol RS

USP Propylene Glycol Dilaurate RS

Propylene Glycol Monocaprylate

Propylene glycol monooctanoate;
Octanoic acid, monoester with 1,2-propane diol;
Caprylic acid, monoester with propane-1,2-diol [31565-12-5].

DEFINITION

Propylene Glycol Monocaprylate is a mixture of the propylene glycol monoesters and diesters of fatty acids composed predominately of caprylic acid. The requirements for monoester and diester content differ for the two types of Propylene Glycol Monocaprylate, as shown in the table below.

	Content of Monoesters (%)		Content of Diesters (%)	
	Min.	Max.	Min.	Max.
Type I	55.0	80.0	20.0	45.0
Type II	90.0	—	—	10.0

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197A▲** (CN 1-May-2020)

• **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩

Standard solution: 50 mg/mL of USP Propylene Glycol Monocaprylate Type I RS or USP Propylene Glycol Monocaprylate Type II RS in methylene chloride

Sample solution: 50 mg/mL in methylene chloride

Chromatographic system

Application volume: 10 µL

Developing solvent system: Ether and hexane (70:30)

Spray reagent: 0.1 mg/mL of rhodamine 6G in alcohol

Analysis: Develop the chromatogram over a path of 15 cm, and dry the plate in a current of air. Spray the plate with *Spray reagent*, and locate the spots on the plate by examination under UV light at a wavelength of 365 nm.

Acceptance criteria: The R_F values of the principal spots from the *Sample solution* correspond to those from the *Standard solution*.

• **C.** It meets the requirements in *Specific Tests* for *Fats and Fixed Oils, Fatty Acid Composition* ⟨401⟩.

ASSAY

• **PROCEDURE**

Mobile phase: Tetrahydrofuran

Sample solution: 40 mg/mL of Propylene Glycol Monocaprylate in tetrahydrofuran

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

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Mode: LC
Detector: Refractive index
Column: 7-mm × 60-cm; 5-µm packing L21 (100 Å).
[NOTE—Two 7-mm × 30-cm L21 columns may be used in place of one 60-cm column, provided *System suitability* requirements are met.]
Temperatures
Column: 40°
Detector: 40°
Flow rate: 1 mL/min
Injection volume: 40 µL
System suitability
Sample: *Sample solution*
[NOTE—The relative retention times with reference to propylene glycol for diesters and monoesters are about 0.85 and 0.90, respectively.]
Suitability requirements
Relative standard deviation: NMT 2.0% for the monoester peak
Analysis
Sample: *Sample solution*
Calculate the percentage of monoesters or diesters in the portion of Propylene Glycol Monocaprylate taken:

$$\text{Result} = (r_U/r_T) \times (100 - D)$$

r_U = peak response for monoesters or diesters
r_T = sum of the peak responses of the monoesters and diesters
D = sum of the percentage content of propylene glycol and the percentage content of free fatty acids

Calculate the percentage content of free fatty acids:

$$\text{Result} = (A/561.1) \times 144$$

A = acid value

Acceptance criteria
Type I
Monoesters: 55.0%–80.0%
Diesters: 20.0%–45.0%
Type II
Monoesters: NLT 90.0%
Diesters: NMT 10.0%

IMPURITIES

• **LIMIT OF PROPYLENE GLYCOL**
Mobile phase, Sample solution, and **Chromatographic system:** Proceed as directed in the *Assay*.
Standard stock solution: 4 mg/mL of USP Propylene Glycol RS in tetrahydrofuran
Standard solutions: Into four 5-mL volumetric flasks, introduce respectively 0.25, 0.5, 1.0, and 2.5 mL of *Standard stock solution*, and dilute with tetrahydrofuran to volume. In a fifth 5-mL volumetric flask, introduce 5.0 mL of *Standard stock solution*.
Analysis
Samples: *Standard solutions* and *Sample solution*
Prepare a standard curve of peak area versus concentration, in mg/mL, of propylene glycol in the *Standard solutions*. Obtain the concentration of propylene glycol in the *Sample solution* from the standard curve.
Calculate the percentage of free propylene glycol in the portion of Propylene Glycol Monocaprylate taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of propylene glycol in the *Sample solution* from the standard curve
C_U = concentration of the *Sample solution* (mg/mL)

Acceptance criteria
Propylene glycol monocaprylate (Type I): NMT 1.5%
Propylene glycol monocaprylate (Type II): NMT 1.5%

SPECIFIC TESTS

• **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩**:** NMT 1.5
• **FATS AND FIXED OILS,** *Fatty Acid Composition* ⟨401⟩**:** Propylene Glycol Monocaprylate exhibits the composition profile of fatty acids shown in *Table 1*.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
8	0	≥90.0
10	0	≤3.0
12	0	≤3.0
14	0	≤3.0
16	0	≤1.0

• **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩**:** NMT 1.0
• **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩
Propylene glycol monocaprylate (Type I): 285–315
Propylene glycol monocaprylate (Type II): 270–295
• **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩**:** NMT 6.0
• **WATER DETERMINATION,** *Method Ia* ⟨921⟩
Analysis: Use a mixture of methanol and methylene chloride (1:1) in place of methanol in the titration vessel.
Acceptance criteria: NMT 1.0%
• **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 0.1%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, and protect from moisture. No storage requirements specified.
• **LABELING:** Label it to indicate the type (Type I or Type II).
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Propylene Glycol RS
USP Propylene Glycol Monocaprylate Type I RS
USP Propylene Glycol Monocaprylate Type II RS

Propylene Glycol Monolaurate

Dodecanoic acid, monoester with 1,2-propanediol; Lauric acid, monoester with propane-1,2-diol.

DEFINITION

Propylene Glycol Monolaurate is a mixture of the propylene glycol mono- and diesters of lauric acid. The requirements for monoester and diester content differ for the two types of Propylene Glycol Monolaurate, as set forth in the table below.

	Content of Monoesters (%)		Content of Diesters (%)	
	Min.	Max.	Min.	Max.
Type I	45.0	70.0	30.0	55.0
Type II	90.0	—	—	10.0

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IDENTIFICATION

• **A. Thin-Layer Chromatographic Identification Test** ⟨201⟩

Standard solution: 50 mg/mL of USP Propylene Glycol Monolaurate Type I RS (or USP Propylene Glycol Monolaurate Type II RS) in methylene chloride

Sample solution: 50 mg/mL in methylene chloride

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Developing solvent system: Ether and hexane (70:30)

Spray reagent: 0.1 mg/mL of rhodamine 6G in alcohol

Analysis: Develop the chromatogram over a path of 15 cm, and dry the plate in a current of air. Spray the plate with *Spray reagent*, and locate the spots on the plate by examination under UV light at a wavelength of 365 nm.

Acceptance criteria: The R_F values of the principal spots from the *Sample solution* correspond to those from the *Standard solution*.

• **B.** It meets the requirements in *Specific Tests* for *Fats and Fixed Oils, Fatty Acid Composition* ⟨401⟩.

ASSAY

• **Procedure**

Mobile phase: Tetrahydrofuran

Sample solution: 40 mg/mL of Propylene Glycol Monolaurate in tetrahydrofuran

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 7-mm × 60-cm; 5-µm packing L21 (100 Å)

[Note—Two 7-mm × 30-cm L21 columns may be used in place of one 60-cm column, provided *System suitability* requirements are met.]

Temperatures

Column: 40°

Detector: 40°

Flow rate: 1 mL/min

Injection volume: 40 µL

System suitability

Sample: *Sample solution*

Suitability requirements

[Note—The order of elution is diesters, monoesters, and propylene glycol.]

Relative standard deviation: NMT 1.0% for the monoester peak

Analysis

Sample: *Sample solution*

Calculate the percentage of monoesters or diesters in the portion of Propylene Glycol Monolaurate taken:

$$\text{Result} = (r_U/r_T) \times (100 - D)$$

r_U = peak response for monoesters or diesters

r_T = sum of the peak responses of the monoesters and diesters

D = sum of the percentage content of propylene glycol and the percentage content of free fatty acids

Calculate the percentage content of free fatty acids in the portion taken:

$$\text{Result} = (A/561.1) \times 200$$

A = acid value

Acceptance criteria

Type I

Monoesters: 45.0%–70.0%

Diesters: 30.0%–55.0%

Type II

Monoesters: NLT 90.0%

Diesters: NMT 10.0%

IMPURITIES

• **Limit of Propylene Glycol**

Mobile phase, Sample solution, and **Chromatographic system:** Proceed as directed in the *Assay*.

Standard stock solution: 4 mg/mL of USP Propylene Glycol RS in tetrahydrofuran

Standard solutions: Into four 15-mL flasks introduce, respectively, 0.25, 0.5, 1.0, and 2.5 mL of *Standard stock solution*, and dilute with tetrahydrofuran to 5.0 mL. In a fifth 15-mL flask, introduce 5.0 mL of *Standard stock solution*.

Analysis

Samples: *Standard solutions* and *Sample solution*

Prepare a standard curve of peak area versus concentration, in mg/mL, of propylene glycol in the *Standard solutions*. Obtain the concentration of propylene glycol in the *Sample solution* from the standard curve.

Calculate the percentage of free propylene glycol in the portion of Propylene Glycol Monolaurate taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of propylene glycol in the *Sample solution* from the standard curve

C_U = concentration of the *Sample solution* (mg/mL)

Acceptance criteria

Propylene glycol monolaurate (Type I): NMT 5.0%

Propylene glycol monolaurate (Type II): NMT 1.0%

SPECIFIC TESTS

• **Fats and Fixed Oils,** *Acid Value* ⟨401⟩**:** NMT 4

• **Fats and Fixed Oils,** *Fatty Acid Composition* ⟨401⟩**:** Propylene Glycol Monolaurate exhibits the composition profile of fatty acids shown in *Table 1*.

Table 1

Fatty Acids	Carbon-Chain Length	Percentage (%)
Caprylic acid	C8	NMT 0.5
Capric acid	C10	NMT 2.0
Lauric acid	C12	NLT 95.0
Myristic acid	C14	NMT 3.0
Palmitic acid	C16	NMT 1.0

• **Fats and Fixed Oils,** *Iodine Value* ⟨401⟩**:** NMT 1

• **Fats and Fixed Oils,** *Saponification Value* ⟨401⟩

Propylene glycol monolaurate (Type I): 210–245

Propylene glycol monolaurate (Type II): 200–230

• **Water Determination,** *Method Ia* ⟨921⟩

Analysis: Use a mixture of methanol and methylene chloride (1:1) in place of methanol in the titration vessel.

Acceptance criteria: NMT 1.0%

• **Articles of Botanical Origin,** *Total Ash* ⟨561⟩**:** NMT 0.1%

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Preserve in well-closed containers, and protect from moisture. No storage requirements specified.

• **Labeling:** Label it to indicate the type (Type I or Type II).

• **USP Reference Standards** ⟨11⟩

USP Propylene Glycol RS

NF Monographs

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USP Propylene Glycol Monolaurate Type I RS
USP Propylene Glycol Monolaurate Type II RS

Propylene Glycol Monostearate

Octadecanoic acid, monoester with 1,2-propanediol; 1,2-Propanediol monostearate [1323-39-3].

DEFINITION

Propylene Glycol Monostearate is a mixture of the propylene glycol mono- and di-esters of stearic and palmitic acids. It contains NLT 90.0% of monoesters of saturated fatty acids, chiefly propylene glycol monostearate ($C_{21}H_{42}O_3$) and propylene glycol monopalmitate ($C_{19}H_{38}O_3$).

ASSAY

• **PROPYLENE GLYCOL MONOESTERS**

Sample: 25 g

Analysis: Place the *Sample* in a 500-mL, round-bottom flask, and add 250 mL of alcohol and 7.5 g of potassium hydroxide. Connect the flask to a suitable condenser, reflux the mixture for 2 h, cool, and transfer to an 800-mL beaker, rinsing the flask with 100 mL of water and combining the rinsing with the mixture in the beaker. Heat on a steam bath to evaporate the alcohol, adding water occasionally to replace the alcohol, and continue the evaporation until the odor of alcohol can no longer be detected. Adjust the volume, with hot water, to 250 mL, neutralize with a mixture of equal volumes of sulfuric acid and water, noting the volume used, and add a 10% excess of the dilute acid. Heat with stirring until the fatty acid layer separates, and transfer the fatty acids to a 500-mL separator. Wash the fatty acids with four 200-mL portions of hot water, and discard the washings. Dry the fatty acids at 105° for 1 h, cool, and determine the acid value on a 1-g portion, as directed in *Fats and Fixed Oils* ⟨401⟩, *Acid Value (Free Fatty Acids)*.

Calculate the average molecular weight of the monoesters, in the portion of Propylene Glycol Monostearate taken:

$$M_{rAvg} = (M_{r1}/A) + M_{r2} - M_{r3}$$

M_{r1} = 1000 times the molecular weight of potassium hydroxide, 56,110
A = acid value
M_{r2} = molecular weight of propylene glycol, 76.10
M_{r3} = molecular weight of water, 18.02

Calculate F in the portion taken:

$$F = (M_{r4} \times G)/M_{r5}$$

M_{r4} = 10 times the molecular weight of potassium hydroxide, 561.1
G = content, in percentage, of glycerin and propylene glycol in propylene glycol monostearate
M_{r5} = one-half of the molecular weight of propylene glycol, 38.05

Calculate the percentage of propylene glycol monoesters:

$$\text{Result} = [(H - F) \times M_{rAvg}]/M_{r4}$$

H = hydroxyl value of propylene glycol monostearate

Acceptance criteria: NLT 90.0% of monoesters of saturated fatty acids, chiefly propylene glycol monostearate and propylene glycol monopalmitate

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.5%

• **FREE GLYCERIN AND PROPYLENE GLYCOL**

Periodic acid solution: Dissolve 5.4 g of periodic acid in 100 mL of water, and add 1900 mL of glacial acetic acid. Store in a glass-stoppered bottle, protected from light.

Chloroform: Use chloroform that meets the following additional requirement. To each of three glass-stoppered, 500-mL conical flasks add 50.0 mL of *Periodic acid solution*, then add 50 mL of chloroform and 10 mL of water to two of the flasks and 50 mL of water to the third flask. To each flask add 20 mL of potassium iodide TS, mix gently, and proceed as directed for *Analysis*, beginning with "allow to stand for 1–5 min". The difference between the volumes of 0.1 N sodium thiosulfate required in the titrations with and without the chloroform does not exceed 100 µL.

Sample solution: Melt the Propylene Glycol Monostearate at a temperature NMT 55°. Transfer a 3-g portion to a 100-mL beaker, and dissolve in 25 mL of *Chloroform*.

Analysis: Transfer the *Sample solution*, with the aid of another 25-mL portion of *Chloroform*, to a separator, wash the beaker with 25 mL of water, and add the washing to the separator. Insert the stopper, shake vigorously for 30–60 s, and allow the layers to separate, adding 1–2 mL of glacial acetic acid, if necessary, to break any emulsion. Transfer the aqueous layer to a glass-stoppered, 500-mL conical flask, wash the chloroform layer with two 25-mL portions of water, combining the washings with the aqueous layer, and discard the chloroform layer. Add, with swirling, 50.0 mL of *Periodic acid solution* to the solution and to another glass-stoppered, 500-mL conical flask containing 75 mL of water to provide the blank. Allow to stand for 30–90 min. To each flask add 20 mL of potassium iodide TS, mix gently, and allow to stand for 1–5 min before titrating. Add 100 mL of water, and titrate with 0.1 N sodium thiosulfate VS until the brown iodine color fades to pale yellow, add 3 mL of starch TS, and continue the titration to the disappearance of the blue color.

Calculate the percentage of free glycerin and propylene glycol, calculated as propylene glycol, in the portion of Propylene Glycol Monostearate taken:

$$\text{Result} = [(V_B - V_S) \times M_r \times N]/W$$

V_B = volume of sodium thiosulfate consumed by the blank solution (mL)
V_S = volume of sodium thiosulfate consumed by the *Sample solution* (mL)
M_r = molecular weight of propylene glycol divided by 20, 3.805
N = actual normality of the sodium thiosulfate solution
W = weight of Propylene Glycol Monostearate taken (g)

Acceptance criteria: NMT 1.0% free glycerin and propylene glycol, calculated as propylene glycol

SPECIFIC TESTS

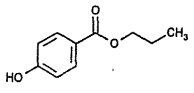
• **CONGEALING TEMPERATURE** ⟨651⟩: NLT 45°
• **FATS AND FIXED OILS,** *Acid Value (Free Fatty Acids)* ⟨401⟩: NMT 4
• **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩: 160–175
• **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩: NMT 3
• **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩: 155–165

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

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Propylparaben



$C_{10}H_{12}O_3$ 180.20
Benzoic acid, 4-hydroxy-, propyl ester;
Propyl *p*-hydroxybenzoate [94-13-3].

DEFINITION
Propylparaben contains NLT 98.0% and NMT 102.0% of $C_{10}H_{12}O_3$.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197M**▲ (CN 1-May-2020)
- **B. MELTING RANGE OR TEMPERATURE** ⟨741⟩: 96°–99°

ASSAY
- **PROCEDURE**

Mobile phase, Sample solution, Standard solution B, and **Chromatographic system:** Proceed as described in the procedure for *Related Substances.*

System suitability
Sample: *Standard solution B*
Suitability requirements
Relative standard deviation: NMT 0.85% for 6 injections

Analysis
Samples: *Sample solution* and *Standard solution B*
Calculate the percentage of Propylparaben in the *Sample solution*:

$$\text{Result} = P \times (r_U \times C_S)/(r_S \times C_U)$$

P	= labeled purity of USP Propylparaben RS expressed as a percentage
r_U	= peak area of propylparaben from the *Sample solution*
C_S	= concentration of propylparaben in *Standard solution B*
r_S	= peak area of propylparaben from *Standard solution B*
C_U	= concentration of Propylparaben in the *Sample solution*

Acceptance criteria: 98.0%–102.0%

IMPURITIES
INORGANIC IMPURITIES
- **Residue on Ignition** ⟨281⟩: NMT 0.1%, determined on 1.0 g

ORGANIC IMPURITIES
- **Procedure: Related Substances**

Mobile phase: Methanol and a 6.8 g/L solution of potassium dihydrogen phosphate (65:35 v/v)
Sample solution: Dissolve 50.0 mg of Propylparaben in 2.5 mL of methanol, and dilute with *Mobile phase* to 50.0 mL. Dilute 10.0 mL of this solution with *Mobile phase* to 100.0 mL.
Standard solution A: 5.0 µg/mL each of *p*-hydroxy benzoic acid, USP Ethylparaben RS, and USP Propylparaben RS in *Mobile phase*
Standard solution B: Dissolve 50.0 mg of USP Propylparaben RS in 2.5 mL of methanol, and dilute with *Mobile phase* to 50.0 mL. Dilute 10.0 mL of this solution with *Mobile phase* to 100.0 mL.
Standard solution C: Dilute 1.0 mL of the *Sample solution* with *Mobile phase* to 20.0 mL. Dilute 1.0 mL of this solution with *Mobile phase* to 10.0 mL.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability.*)
Mode: LC
Detector: UV 272 nm
Column: 4.6-mm × 15-cm; 5-µm packing L1
Flow rate: 1.3 mL/min
Injection size: 10 µL
Run time: About 2.5 times the retention time of propylparaben
System suitability
Sample: *Standard solution A*
[NOTE—The retention time of propylparaben is about 4.5 min; the relative retention times for *p*-hydroxy benzoic acid and ethylparaben are about 0.3 and 0.7, respectively.]
Suitability requirements
Resolution: NLT 3.0 between the ethylparaben and propylparaben peaks
Analysis
Samples: *Sample solution* and *Standard solution C*
[NOTE—Disregard any limit that is 0.2 times the area of the principal peak in the chromatogram obtained with *Standard solution C* (0.1%).]
Acceptance criteria
***p*-Hydroxybenzoic acid:** The peak area in the *Sample solution,* multiplied by 1.4 to correct for the calculation of content, is NMT the area of the principal peak in *Standard solution C* (0.5%).
Unspecified impurities: The peak area of each impurity in the *Sample solution* is NMT the area of the principal peak in *Standard solution C* (0.5%).
Total impurities: The total peak area for all impurities in the *Sample solution* is NMT twice the area of the principal peak in *Standard solution C* (1.0%).

SPECIFIC TESTS
- **COLOR OF SOLUTION**

Sample solution: 100 mg/mL in alcohol
Comparison solution: Mix 2.4 mL of ferric chloride CS, 1.0 mL of cobaltous chloride CS, and 0.4 mL of cupric sulfate CS with 0.3 N hydrochloric acid to make 10 mL. Dilute 5 mL of this solution with 0.3 N hydrochloric acid to make 100 mL. [NOTE—Prepare and use this solution immediately.]
Analysis
Samples: Alcohol, *Sample solution,* and *Comparison solution*
Make the comparison by viewing the solutions downward in matched color-comparison tubes against a white surface (see *Color and Achromicity* ⟨631⟩).
Acceptance criteria: The *Sample solution* is clear and not more intensely colored than alcohol or the *Comparison solution.*

- **ACIDITY**

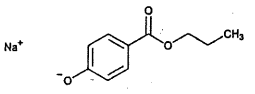
Sample solution: To 2 mL of *Sample solution* prepared in the test for *Color of Solution,* add 3 mL of alcohol, 5 mL of carbon dioxide-free water, and 0.1 mL of bromocresol green TS.
Analysis: Titrate with 0.10 N sodium hydroxide.
Acceptance criteria: NMT 0.1 mL is required to produce a blue color.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers.

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• **USP REFERENCE STANDARDS** ⟨11⟩
USP Ethylparaben RS
USP Propylparaben RS

Propylparaben Sodium



$C_{10}H_{11}NaO_3$ 202.20
Benzoic acid, 4-hydroxy-, propyl ester, sodium salt;
Propyl *p*-hydroxybenzoate, sodium salt;
Sodium 4-propoxycarbonylphenolate [35285-69-9].

DEFINITION
Propylparaben Sodium contains NLT 94.0% and NMT 102.0% of propylparaben sodium ($C_{10}H_{11}NaO_3$), calculated on the anhydrous basis.

IDENTIFICATION
• **A.**
Standard: 0.5 g of USP Propylparaben RS
Sample: 0.5 g
Analysis: Dissolve the *Sample* in 5 mL of water. Acidify with hydrochloric acid, and filter the resulting precipitate. Wash the precipitate with water, and dry over silica gel for 5 h. Repeat with the *Standard*.
Acceptance criteria: The IR absorption spectrum of a mineral oil dispersion of the *Sample* exhibits maxima only at the same wavelengths as those of a mineral oil dispersion of the *Standard*.
• **B.**
Sample solution: Ignite 0.3 g of Propylparaben Sodium, cool, and dissolve the residue in 3 mL of 3 N hydrochloric acid.
Acceptance criteria: A platinum wire dipped in the *Sample solution* imparts an intense, persistent yellow color to a nonluminous flame.

ASSAY
• **PROCEDURE**
Mobile phase: Methanol and a 6.8-g/L solution of potassium dihydrogen phosphate (65:35, v/v)
System suitability solution: 5.0 µg/mL each of *p*-hydroxybenzoic acid, USP Ethylparaben RS, and USP Propylparaben RS in *Mobile phase*
Standard solution: Dissolve 50.0 mg of USP Propylparaben RS in 2.5 mL of methanol, and dilute with *Mobile phase* to 50.0 mL. Dilute 10.0 mL of this solution with *Mobile phase* to 100.0 mL.
Sample solution: Dissolve 50.0 mg of Propylparaben Sodium in 2.5 mL of methanol, and dilute with *Mobile phase* to 50.0 mL. Dilute 10.0 mL of this solution with *Mobile phase* to 100.0 mL.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 272 nm
Column: 4.6-mm × 15-cm; 5-µm packing L1
Flow rate: 1.3 mL/min
Injection volume: 10 µL
Run time: About 2.5 times the retention time of the propylparaben peak
System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The retention time of propylparaben is about 4.0 min; the relative retention times for *p*-hydroxybenzoic acid, ethylparaben, and propylparaben are about 0.4, 0.7, and 1.0, respectively.]
Suitability requirements
Resolution: NLT 3.0 between the ethylparaben and propylparaben peaks, *System suitability solution*
Relative standard deviation: NMT 0.85% for six injections, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of propylparaben sodium ($C_{10}H_{11}NaO_3$) in the portion of Propylparaben Sodium taken:

$$\text{Result} = P \times (r_U \times C_S)/(r_S \times C_U) \times (M_{r1}/M_{r2})$$

P = labeled purity of USP Propylparaben RS expressed as a percentage
r_U = peak area of propylparaben from the *Sample solution*
C_S = concentration of propylparaben in the *Standard solution*
r_S = peak area of propylparaben from the *Standard solution*
C_U = concentration of Propylparaben Sodium in the *Sample solution*
M_{r1} = molecular weight of propylparaben sodium, 202.20
M_{r2} = molecular weight of propylparaben, 180.20

Acceptance criteria: 94.0%–102.0% on the anhydrous basis

IMPURITIES
• **RELATED COMPOUNDS**
Mobile phase, System suitability solution, Sample solution, and Chromatographic system: Proceed as directed in the *Assay*.
Standard solution: Dilute 1.0 mL of the *Sample solution* with *Mobile phase* to 20.0 mL. Dilute 1.0 mL of this solution with *Mobile phase* to 10.0 mL.
System suitability
Sample: *System suitability solution*
[NOTE—The retention time of propylparaben is about 4.0 min; the relative retention times for *p*-hydroxybenzoic acid, ethylparaben, and propylparaben are about 0.4, 0.7, and 1.0, respectively.]
Suitability requirements
Resolution: NLT 3.0 between the ethylparaben and propylparaben peaks
Analysis
Samples: *Standard solution* and *Sample solution*
Acceptance criteria
***p*-Hydroxybenzoic acid:** NMT 4.0%; the peak area in the *Sample solution*, multiplied by 1.4 to correct for the calculation of content, is NMT 8 times the area of the principal peak in the *Standard solution*.
Unspecified impurities: NMT 0.5%; the peak area of each impurity in the *Sample solution* is NMT the area of the principal peak in the *Standard solution*.
Total impurities: NMT 1.0%; the total peak area for all unspecified impurities in the *Sample solution* is NMT twice the area of the principal peak in the *Standard solution*.
• **CHLORIDE AND SULFATE,** *Chloride* ⟨221⟩
Standard solution: 0.10 mL of 0.020 N hydrochloric acid
Sample: 0.2 g
Analysis: Proceed as directed in the chapter.
Acceptance criteria: 0.035%; the *Sample* shows no more chloride than the *Standard solution*.

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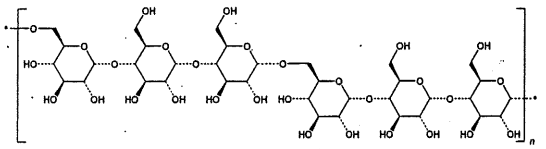
• **CHLORIDE AND SULFATE,** *Sulfate* ⟨221⟩
Standard solution: 0.30 mL of 0.020 N sulfuric acid
Sample: 0.25 g
Analysis: Proceed as directed in the chapter.
Acceptance criteria: 0.12%; the *Sample* shows no more sulfate than the *Standard solution*.

SPECIFIC TESTS
• **pH** ⟨791⟩
Sample solution: 1 mg/mL
Acceptance criteria: 9.5–10.5
• **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 5.0%
• **COMPLETENESS OF SOLUTION** ⟨641⟩
Sample solution: Dissolve 1 g in water.
Acceptance criteria: Meets the requirements

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight containers.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Ethylparaben RS
USP Propylparaben RS

Protamine Sulfate—*see Protamine Sulfate General Monographs*

Pullulan



$(C_{36}H_{60}O_{30})_n$
Poly[6)-α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→] [9057-02-7].

DEFINITION
Pullulan is a neutral, simple polysaccharide produced by the growth of *Aureobasidium pullulans*. It has a chain structure of repeated α-1,6-bonds of maltotriose composed of three glucoses in α-1,4-bonds. It may contain some maltotetraosyl units. It contains NLT 90% of glucan, calculated on the dried basis.

IDENTIFICATION
• **A.**
Sample: 10 g
Analysis: Dissolve the *Sample* in 100 mL of water by adding in small portions with stirring.
Acceptance criteria: A viscous solution is produced.
• **B.**
Pullulanase sample solution: 10 units/mL of pullulanase
Sample solution: The viscous solution obtained in *Identification* test *A*
Analysis: Mix 10 mL of the *Sample solution* with 0.1 mL of *Pullulanase sample solution*, and allow to incubate at 25° for 20 min.
Acceptance criteria: A substantial loss of viscosity is observed.
• **C.**
Sample solution: 20 mg/mL
Analysis: To 10 mL of the *Sample solution* add 2 mL of polyethylene glycol 600.
Acceptance criteria: A white precipitate is formed immediately.

ASSAY
• **CONTENT OF MONOSACCHARIDE, DISACCHARIDE, AND OLIGOSACCHARIDES**
Sample stock solution: 8 mg/mL, on previously dried material
Sample solution: To 1.0 mL of the *Sample stock solution* add 0.1 mL of saturated potassium chloride solution, and shake vigorously with 3 mL of methyl alcohol. Centrifuge, and use the supernatant.
Standard solution: Dilute 1.0 mL of the *Sample stock solution* with water to 50 mL.
Blank: Water
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: Vis
Analytical wavelength: 620 nm
Analysis
Samples: *Sample solution*, *Standard solution*, and *Blank*
Transfer 0.2 mL each of the *Standard solution*, *Sample solution*, and *Blank* to a test tube containing 5 mL of a 1-in-500 solution of anthrone in 75% (v/v) sulfuric acid, with the test tube placed in ice water. Mix each tube immediately, and then heat the test tube at 90° for 10 min. Remove the tube, and allow it to cool in cold running water.
Determine the absorbances of the resulting solutions at the specified wavelength.
Determine the percentage of monosaccharide, disaccharide, and oligosaccharides in the portion of the sample taken:

$$\text{Result} = (D_U/D_S) \times (A_U - A_B)/(A_S - A_B) \times 100$$

D_U = dilution factor for the *Sample solution*, 4.1
D_S = dilution factor for the *Standard solution*, 50
A_U = absorbance of the *Sample solution*
A_S = absorbance of the *Standard solution*
A_B = absorbance of the *Blank*

Acceptance criteria: The total content of monosaccharide, disaccharide, and oligosaccharides is NMT 10.0%, corresponding to NLT 90% of glucan on the dried basis.

IMPURITIES
• **RESIDUE ON IGNITION** ⟨281⟩
Sample: 2.0 g
Acceptance criteria: NMT 0.3%
• **NITROGEN DETERMINATION,** *Method II* ⟨461⟩
Sample: 3 g, previously dried
Analysis: Proceed as directed in the chapter, replacing the 7 mL of sulfuric acid with 12 mL of sulfuric acid for the decomposition and replacing the 30 mL of sodium hydroxide solution (2 in 5) with 40 mL of a solution of sodium hydroxide (2 in 5).
Acceptance criteria: NMT 0.05%

SPECIFIC TESTS
• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic microbial count does not exceed 10^2 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.
• **pH** ⟨791⟩
Sample: 1.0 g
Analysis: Dissolve the *Sample* in 10 mL of freshly boiled and cooled water.
Acceptance criteria: 4.5–6.5

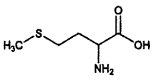
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• **Loss on Drying** ⟨731⟩
Analysis: Dry at 90° under vacuum for 6 h.
Acceptance criteria: NMT 6.0%
• **Viscosity—Capillary Methods** ⟨911⟩
Sample: Exactly 10.0 g, previously dried
Analysis: Dissolve the *Sample* in water to make exactly 100 g, and perform the test at 30 ± 0.1° using a Ubbelohde-type viscometer.
Acceptance criteria: The kinematic viscosity is 100–180 $mm^2 \cdot s^{-1}$.

ADDITIONAL REQUIREMENTS
• **Packaging and Storage:** Preserve in well-closed containers. No storage requirements specified.
• **Labeling:** Label it to indicate the viscosity, giving the type of viscosity parameter, concentration of the solution, and the type of equipment used.

Pumice—*see Pumice General Monographs*

Racemethionine



$C_5H_{11}NO_2S$ 149.21
Methionine, DL-;
DL-2-Amino-4-(methylthio)-butyric acid [59-51-8].

DEFINITION
Racemethionine contains NLT 99.0% and NMT 101.0% of $C_5H_{11}NO_2S$, as DL-methionine, calculated on the dried basis.

IDENTIFICATION

Change to read:

• **A. ▲Spectroscopic Identification Tests** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
Sample: Dry the substances at 105°.
Acceptance criteria: Meets the requirements
• **B.** The principal spot from *Sample solution B* is similar in size, color, and position to the principal spot from *Standard solution A*, as obtained in the test for *Organic Impurities, Related Substances.*
• **C. Optical Rotation,** *Angular Rotation* ⟨781A⟩
Sample: 50 mg/mL in 1 M hydrochloric acid
Acceptance criteria: −0.05° to +0.05°
• **D. Procedure**
Analysis: Dissolve 0.1 g of Racemethionine and 0.1 g of glycine in 4.5 mL of dilute sodium hydroxide solution (85 mg/mL). Add 1 mL of sodium nitroferricyanide solution (25 mg/mL). Heat to 40° for 10 min. Allow to cool, and add 2 mL of a mixture of hydrochloric acid and phosphoric acid (90:10).
Acceptance criteria: A deep red color develops.

ASSAY
• **Procedure**
Sample: 140 mg of Racemethionine
Analysis: Dissolve the *Sample* in a mixture of 3 mL of formic acid and 50 mL of glacial acetic acid. Titrate with 0.1 N perchloric acid VS, determining the endpoint potentiometrically. Perform a blank determination, and make any necessary corrections (see *Titrimetry* ⟨541⟩). Each mL of 0.1 N perchloric acid is equivalent to 14.92 mg of $C_5H_{11}NO_2S$.
Acceptance criteria: 99.0%–101.0% on the dried basis

IMPURITIES
Inorganic Impurities
• **Residue on Ignition** ⟨281⟩: NMT 0.1%, determined on 1.0 g
• **Chloride and Sulfate,** *Chloride* ⟨221⟩: [Note—Prepare the *Sample solution* and the *Standard solution* at the same time.]
Chloride standard solution (5 ppm Cl): 0.824 mg/mL of NaCl. Just before use, dilute 1 mL of this solution with water to 100 mL.
Standard solution: To 10 mL of *Chloride standard solution* add 10 mL of 0.1 N silver nitrate and 25 mL of water, and mix.
Sample solution: Dissolve 0.25 g in 35 mL of water. Add 5 mL of dilute nitric acid and 10 mL of 0.1 N silver nitrate. Allow to stand protected from light for 5 min.
Analysis: Examine the *Sample solution* and *Standard solution* laterally against a black background.
Acceptance criteria: Any opalescence in the *Sample solution* is not more intense than that in the *Standard solution* (200 ppm).
• **Chloride and Sulfate,** *Sulfate* ⟨221⟩: [Note—Prepare the *Sample solution* and the *Control solution* at the same time.]
Barium chloride solution: 250 mg/mL
Sulfate standard solution (10 ppm SO_4): 1.81 mg/mL of potassium sulfate in 30% alcohol (v/v). Just before use, dilute 1 mL of this solution with 30% alcohol (v/v) to 100 mL.
Standard solution: Mix 3 mL of the *Barium chloride solution* and 4.5 mL of the *Sulfate standard solution*, and allow to stand for 1 min.
Sample stock solution: 50.0 mg/mL, heated to 60°. Cool to 10°, and filter.
Sample solution: To 2.5 mL of the *Standard solution* add 15 mL of the *Sample stock solution* and 0.5 mL of 5 N acetic acid.
Control solution: To 2.5 mL of the *Standard solution* add 15 mL of the *Sulfate standard solution* and 0.5 mL of 5 N acetic acid.
Analysis
Samples: *Sample solution* and *Control solution*
Acceptance criteria: After 5 min, any opalescence in the *Sample solution* is not more intense than that in the *Control solution* (200 ppm).
• **Limit of Iron**
Standard stock solution (125 ppm): Dissolve 1.727 g of ferric ammonium sulfate [$FeNH_4(SO_4)_2 \cdot 12H_2O$] in water. Add 50 mL of 10% hydrochloric acid, dilute with water to 1000 mL, and mix. Dilute 1 mL of this solution with water to 40 mL. Pipet 5 mL of this solution into a 200-mL volumetric flask, dilute with water to volume, and mix.
Standard solution: Transfer 2 mL of the *Standard stock solution* to a 25-mL volumetric flask. Add 5 mL of 16% hydrochloric acid, 50 mg of ammonium persulfate, and 3 mL of 30% ammonium thiocyanate, and dilute with water to volume.
Sample solution: Transfer 1 g of Racemethionine to a 25-mL volumetric flask. Add 5 mL of 16% hydrochloric acid, and dissolve. Add 50 mg of ammonium persulfate and 3 mL of 30% ammonium thiocyanate, and dilute with water to volume.
Blank: Transfer 5 mL of 16% hydrochloric acid to a 25-mL volumetric flask. Add 50 mg of ammonium persulfate and 3 mL of 30% ammonium thiocyanate, and dilute with water to volume.

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Spectrometric conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: UV-Vis
Analytical wavelength: 475 nm
Cell: 1 cm
Analysis
Samples: *Standard solution*, *Sample solution*, and *Blank*
Without delay, concomitantly determine the absorbances of each sample, correcting for the *Blank*.
Acceptance criteria: The absorbance of the *Sample solution* is NMT that of the *Standard solution* (NMT 10 ppm).

• **LIMIT OF AMMONIUM**
Standard solution A: 0.297 mg/mL of USP Ammonium Chloride RS. This solution contains 0.1 mg/mL or 100 ppm of NH_4^+.
Standard solution B: 0.297 µg/mL of USP Ammonium Chloride RS. This solution contains 0.1 µg/mL or 0.1 ppm of NH_4^+.
Standard solution C: 2.97 µg/mL of USP Ammonium Chloride RS. This solution contains 1.0 µg/mL or 1 ppm of NH_4^+.
Standard solution D: 29.7 µg/mL of USP Ammonium Chloride RS. This solution contains 10 µg/mL or 10 ppm of NH_4^+.
Sample solution: 10 mg/mL of Racemethionine
Electrode system: Use an ammonia-specific,[1] ion-indicating electrode connected to a pH meter capable of measuring potentials (see *pH* ⟨791⟩).
Analysis
Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, *Standard solution D*, and *Sample solution*
Add 100 mL of water to a 150-mL beaker, place the electrode in the beaker, stir, and measure the potential. Add 1 mL of 10 N sodium hydroxide. Stir, and measure the potential after stabilization. [NOTE—It may take about 5 min.] The potential difference must be less than 20 mV.
Add 100.0 mL each of *Standard solutions A*, *B*, *C*, and *D* to four different 150-mL beakers. To each beaker, add 1 mL of 10 N sodium hydroxide. Place the ammonia electrode in the beaker, stir, and concomitantly measure the potential after stabilization. [NOTE—It may take about 5 min.] Draw a calibration curve of the potential, in mV, versus, the quantity of ammonium (NH_4^+), in mg.
Add 100.0 mL of the *Sample solution* to a 150-mL beaker. Add 1 mL of 10 N sodium hydroxide. Adjust the pH, if necessary, with 10 N sodium hydroxide to a pH of NLT 11. Place the ammonia electrode in the beaker, stir, and measure the potential after stabilization. [NOTE—It may take about 5 min.] Obtain the quantity of NH_4^+, in mg, in the 100 mL of the *Sample solution* based on the calibration curve.
Calculate the percentage of ammonium (NH_4^+), in the portion of Racemethionine taken:

$$\text{Result} = (C/W) \times F$$

C = quantity of ammonium in the *Sample solution* from the standard curve (mg)
W = weight of Racemethionine taken to prepare the *Sample solution* (mg)
F = conversion factor to µg/g (ppm), 1×10^6

Acceptance criteria: NMT 200 ppm

ORGANIC IMPURITIES
• **Procedure: Related Substances**
Standard solution A: 0.40 mg/mL of USP Racemethionine RS
Standard solution B: 40 µg/mL of USP Racemethionine RS
Sample solution A: 20 mg/mL of Racemethionine
Sample solution B: 0.40 mg/mL of Racemethionine
Chromatographic system
(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
Mode: TLC
Adsorbent: 0.25-mm layer of chromatographic silica gel mixture
Application volume: 5 µL
Developing solvent system: Butyl alcohol, glacial acetic acid, and water (3:1:1)
Spray reagent: 2 mg/mL of ninhydrin in a mixture of butyl alcohol and 2 N acetic acid (95:5)
Analysis
Samples: *Standard solution A*, *Standard solution B*, *Sample solution A*, and *Sample solution B*
Develop over a path of 10 cm using the *Developing solvent system*. After air-drying the plate, spray with *Spray reagent*, and heat between 100° and 105° for 15 min. Examine the plate under white light.
Acceptance criteria: Any spot obtained from *Sample solution A*, apart from the principal spot, is not more intense than the spot obtained from *Standard solution B* (NMT 0.2%).

SPECIFIC TESTS
• **pH** ⟨791⟩**:** 5.4–6.1, in a 20 mg/mL solution
• **LOSS ON DRYING** ⟨731⟩**:** Dry a sample at 105° for 3 h: it loses NMT 0.5% of its weight, determined on 1.000 g.
• **TRANSMITTANCE**
Sample solution: 10% of Racemethionine in 2 N hydrochloric acid, prepared by sonication
Analysis: Determine the transmittance in a 1-cm cell at 430 nm with a suitable spectrophotometer.
Acceptance criteria: Transmittance of NLT 0.98, corresponding to an absorbance of NMT about 0.009

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Ammonium Chloride RS
USP Racemethionine RS

[1] Orion 95-12 is suitable.

Fully Hydrogenated Rapeseed Oil

Fully hydrogenated rapeseed oil [84681-71-0].

DEFINITION
Fully Hydrogenated Rapeseed Oil is the product obtained by refining and hydrogenating oil obtained from the seeds of *Brassica napus* and *Brassica campestris* (Fam. Cruciferae). The product is a mixture of triglycerides in which the fatty acid composition is a mixture of saturated fatty acids.

IDENTIFICATION
• **A.** It meets the requirements of the test for *Fats and Fixed Oils, Fatty Acid Composition* ⟨401⟩.

IMPURITIES
• **ALKALINE IMPURITIES**
Sample solution: Prepare a mixture of 2.0 g of Fully Hydrogenated Rapeseed Oil, 1.5 mL of alcohol, and 3.0 mL of toluene. Dissolve by gentle heating.
Analysis: To the *Sample solution* add 0.05 mL of bromophenol blue TS, and titrate with 0.01 N hydrochloric acid to a yellow endpoint.
Acceptance criteria: NMT 0.4 mL of 0.01 N hydrochloric acid is required.

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• **Residue on Ignition** ⟨281⟩: NMT 0.5%, when a 5-g sample of Fully Hydrogenated Rapeseed Oil is ignited at an ignition temperature of 800 ± 25°

• **Limit of Nickel**

Sample solution: Weigh 5.0 g of Fully Hydrogenated Rapeseed Oil into a previously tared platinum or silica crucible. Cautiously heat the substance, and introduce into it a wick formed from twisted ashless filter paper. Ignite the wick. When the substance ignites, stop heating. After combustion, ignite in a muffle furnace at about 600°. Continue the incineration until white ash is obtained. After cooling, transfer the residue, with the aid of two 2-mL portions of diluted hydrochloric acid, to a 25-mL volumetric flask. Add 0.3 mL of nitric acid, and dilute with water to volume.

Standard stock solution: 0.2 µg/mL of nickel prepared from nickel standard solution TS and water. Prepare immediately before use.

Standard solutions: Into three identical 10-mL volumetric flasks introduce 1.0, 2.0, and 4.0 mL of *Standard stock solution*, respectively. To each flask add a 2.0-mL portion of the *Sample solution*, and dilute with water to volume.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometer equipped with a graphite furnace

Absorbance: 232.0 nm

Lamp: Nickel hollow-cathode

Analysis

Samples: *Sample solution* and *Standard solutions*

Concomitantly determine the absorbances of the *Samples* at least three times each. Record the average of the steady readings for each of the *Standard solutions* and the *Sample solution*. Plot the absorbances of the *Standard solutions* and the *Sample solution* versus the added quantity of nickel. [Note—The *Sample solution* should be plotted as if it had a content of added nickel equivalent to 0 µg.] Extrapolate the line joining the points on the graph until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of nickel, *C*, in µg/mL, in the *Sample solution*.

Calculate the content of nickel in the portion of Fully Hydrogenated Rapeseed Oil taken:

$$\text{Result} = (C/W) \times V$$

C = concentration of nickel in the *Sample solution* (µg/mL)
W = weight of Fully Hydrogenated Rapeseed Oil (g)
V = volume of the *Sample solution*, 25 mL

Acceptance criteria: NMT 1 ppm

• **Limit of Erucic Acid:** NMT 1.0%, as determined under *Specific Tests*, in the test *Fats and Fixed Oils, Fatty Acid Composition* ⟨401⟩

SPECIFIC TESTS

• **Fats and Fixed Oils,** *Fatty Acid Composition* ⟨401⟩: Fully Hydrogenated Rapeseed Oil exhibits the fatty acid composition profile shown in *Table 1*.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
14	0	<1.0
16	0	3–5
18	0	38–42
20	0	8–10
22	0	42–50
24	0	1.0–2.0
18	1	≤1.0
18	2	<1.0
20	1	<1.0
22[a]	1	≤1.0

[a] Erucic acid.

• **Fats and Fixed Oils,** *Acid Value* ⟨401⟩: NMT 6.0
• **Fats and Fixed Oils,** *Iodine Value* ⟨401⟩: NMT 4
• **Fats and Fixed Oils,** *Peroxide Value* ⟨401⟩: NMT 2.0
• **Fats and Fixed Oils,** *Unsaponifiable Matter* ⟨401⟩: NMT 1.5%

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Preserve in tight, light-resistant containers. No storage requirements specified.

Superglycerinated Fully Hydrogenated Rapeseed Oil

Superglycerinated fully hydrogenated rapeseed oil.

DEFINITION

Superglycerinated Fully Hydrogenated Rapeseed Oil is the product obtained by refining, hydrogenating, and glycerinating oil obtained from the seeds of *Brassica napus* and *Brassica campestris* (Fam. Cruciferae). The product is a mixture of mono-, di-, and triglycerides, with triglycerides as a minor component.

[Note—Use compendial grade glycerin as a starting material.]

IDENTIFICATION

• **A.** It meets the requirements of the test for *Fats and Fixed Oils, Fatty Acid Composition* ⟨401⟩.

COMPOSITION

• **Content of 1-Monoglycerides**

Periodic acid solution: Dissolve 5.4 g of periodic acid in 100 mL of water, add 1900 mL of glacial acetic acid, and mix. Preserve in a light-resistant, glass-stoppered bottle.

Chloroform: Use chloroform that meets the following test. Add 50.0 mL of *Periodic acid solution* to each of three 500-mL flasks. Add 50 mL of chloroform and 10 mL of water to two of the flasks, and add 50 mL of water to the third flask. Add 20 mL of potassium iodide TS to each flask, mix gently, and continue as directed in the *Analysis*, beginning with "and allow to stand at least 1 min, but no longer than 5 min, before titrating". The difference between the volume of 0.1 N sodium thiosulfate VS required in the titrations with and without the chloroform is not greater than 0.5 mL.

Sample solution: Melt Superglycerinated Fully Hydrogenated Rapeseed Oil at a temperature not higher than 10° above its melting point, and mix thoroughly. Transfer an accurately weighed quantity of it, equivalent to about 150 mg of 1-monoglycerides, to a 100-mL beaker, dissolve in 25 mL of *Chloroform*, and mix.

Analysis: Transfer the *Sample solution*, with the aid of an additional 25 mL of *Chloroform*, to a separator. Wash the

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beaker with 25 mL of water, and add the washing to the separator. Close the separator tightly with a stopper, shake vigorously for 30–60 s, and allow the layers to separate. [NOTE—Add 1–2 mL of glacial acetic acid to break emulsions due to the presence of soap.]

Collect the aqueous layer in a 500-mL glass-stoppered Erlenmeyer flask, and again extract the chloroform solution in the separator with two 25-mL portions of water. Retain the combined aqueous extracts, which will be used in the test for *Limit of Free Glycerin*. Transfer the chloroform layer to a 500-mL glass-stoppered Erlenmeyer flask, and add 50.0 mL of *Periodic acid solution* to this flask and to each of two blank flasks containing a mixture of 50 mL of *Chloroform* and 10 mL of water. Swirl the flasks during the addition of *Periodic acid solution*, and allow to stand for at least 30 min, but no longer than 90 min. To each flask, add 20 mL of potassium iodide TS, and allow to stand at least 1 min, but no longer than 5 min, before titrating. Add 100 mL of water, and titrate with 0.1 N sodium thiosulfate VS, using a magnetic stirrer to keep the solution thoroughly mixed, to the disappearance of the brown iodine color. Add 2 mL of starch TS, and continue the titration to the disappearance of the blue color.

Calculate the percentage of 1-monoglycerides in the portion of Superglycerinated Fully Hydrogenated Rapeseed Oil taken:

$$\text{Result} = \{[M_1 \times (V_B - V_S) \times N]/(W \times A)\} \times 100$$

M_1 = molecular weight of glyceryl monostearate, 358
V_B = volume of sodium thiosulfate VS consumed in the blank determination (mL)
V_S = volume of sodium thiosulfate VS required in the titration of the Superglycerinated Fully Hydrogenated Rapeseed Oil (mL)
N = normality of the sodium thiosulfate VS
W = weight of the Superglycerinated Fully Hydrogenated Rapeseed Oil taken to prepare the *Sample solution* (mg)
A = factor number, 2

Acceptance criteria: 90.0%–110.0% of that indicated on the label

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 0.5%, when a 5-g sample of Superglycerinated Fully Hydrogenated Rapeseed Oil is ignited at an ignition temperature of 800 ± 25°

• **LIMIT OF NICKEL**

Sample solution: Weigh 5.0 g of Superglycerinated Fully Hydrogenated Rapeseed Oil into a previously tared platinum or silica crucible. Cautiously heat the substance, and introduce into it a wick formed from twisted ashless filter paper. Ignite the wick. When the substance ignites, stop heating. After combustion, ignite in a muffle furnace at about 600°. Continue the incineration until white ash is obtained. After cooling, transfer the residue, with the aid of two 2-mL portions of diluted hydrochloric acid, to a 25-mL volumetric flask. Add 0.3 mL of nitric acid, and dilute with water to volume.

Nickel standard solution: Immediately before use, dilute 10 mL of nickel standard solution TS with water to 500 mL. This solution contains the equivalent of 0.2 µg/mL of nickel.

Standard solutions: Into three identical 10-mL volumetric flasks, introduce respectively 1.0, 2.0, and 4.0 mL of *Nickel standard solution*. To each flask, add a 2.0-mL portion of the *Sample solution*, and dilute with water to volume.

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption spectrophotometer equipped with a graphite furnace

Analytical wavelength: 232.0 nm

Lamp: Nickel hollow-cathode

Analysis

Samples: *Sample solution* and *Standard solutions*

Concomitantly determine the absorbances at least three times each, at the wavelength of maximum absorbance. Record the average of the steady readings for each of the *Standard solutions* and the *Sample solution*. Plot the absorbances of the *Sample solution* and the *Standard solutions* versus the added quantity of nickel. [NOTE—The *Sample solution* should be plotted as if it had a content of added nickel equivalent to 0 µg.]

Extrapolate the line joining the points on the graph until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of nickel, C, in µg/mL, in the *Sample solution*.

Calculate the content of nickel in the portion of Superglycerinated Fully Hydrogenated Rapeseed Oil taken:

$$\text{Result} = (V \times C)/W$$

V = volume of the *Sample solution*, 25 mL
C = concentration of nickel in the *Sample solution* (µg/mL)
W = weight of Superglycerinated Fully Hydrogenated Rapeseed Oil taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 1 µg/g (ppm)

• **LIMIT OF ERUCIC ACID:** NMT 1.0%, as determined in the test for *Fats and Fixed Oils* ⟨401⟩, *Fatty Acid Composition*

• **LIMIT OF FREE GLYCERIN**

Periodic acid solution and **Chloroform:** Prepare as directed in the test for *Content of 1-Monoglycerides*.

Sample solution: Use the combined aqueous extracts obtained as directed in the test for *Content of 1-Monoglycerides*.

Analysis: Transfer 50.0 mL of *Periodic acid solution* to each of two flasks: a 500-mL glass-stoppered Erlenmeyer flask containing the *Sample solution* and a 500-mL glass-stoppered Erlenmeyer blank flask containing 75 mL of water. Continue as directed for *Analysis* in the test for *Content of 1-Monoglycerides*, beginning with "Swirl the flasks during the addition of *Periodic acid solution*, and allow to stand for at least 30 min, but no longer than 90 min".

Calculate the percentage of free glycerin in the portion of Superglycerinated Fully Hydrogenated Rapeseed Oil taken:

$$\text{Result} = \{[M_1 \times (V_B - V_S) \times N]/(W \times A)\} \times 100$$

M_1 = molecular weight of glycerin, 92
V_B = volume of sodium thiosulfate VS consumed in the blank determination (mL)
V_S = volume of sodium thiosulfate VS required in the titration of the Superglycerinated Fully Hydrogenated Rapeseed Oil (mL)
N = normality of the sodium thiosulfate VS
W = weight of the Superglycerinated Fully Hydrogenated Rapeseed Oil taken to prepare the *Sample solution* as directed in the test for *Content of 1-Monoglycerides* (mg)
A = factor number, 4

Acceptance criteria: NMT 1%

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SPECIFIC TESTS
- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩: NMT 6.0
- **FATS AND FIXED OILS,** *Fatty Acid Composition* ⟨401⟩: Superglycerinated Fully Hydrogenated Rapeseed Oil exhibits the fatty acid composition profile shown in *Table 1*.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
14	0	<1.0
16	0	3–5
18	0	38–42
20	0	8–10
22	0	42–50
24	0	1.0–2.0
18	1	≤1.0
18	2	<1.0
20	1	<1.0
22[a]	1	≤1.0

[a] Erucic acid.

- **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩: NLT 90.0% and NMT 110.0% of that indicated on the label
- **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩: NMT 4
- **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩: NMT 2.0
- **FATS AND FIXED OILS,** *Unsaponifiable Matter* ⟨401⟩: NMT 1.5%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers. No storage requirements specified.
- **LABELING:** Label it to indicate the hydroxyl value and the content of 1-monoglycerides.

Rose Oil

DEFINITION
Rose Oil is volatile oil distilled with steam from the fresh flowers of *Rosa gallica L, Rosa damascena* Miller, *Rosa alba* L., *Rosa centifolia* L., and varieties of these species (Fam. Rosaceae).

SPECIFIC TESTS
- **SPECIFIC GRAVITY** ⟨841⟩
  Analysis: Measure at 30° compared with water at 15°.
  Acceptance criteria: 0.848–0.863
- **OPTICAL ROTATION,** *Angular Rotation* ⟨781A⟩: −1° to −4°
- **REFRACTIVE INDEX** ⟨831⟩: 1.457–1.463 at 30°
- **SOLUBILITY TEST**
  Analysis: 1 mL is miscible with 1 mL of chloroform without turbidity. Add 20 mL of 90% alcohol to this mixture.
  Acceptance criteria: The resulting liquid is neutral or acid to moistened litmus paper and, upon standing at 20°, deposits crystals within 5 min.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-filled, tight containers.

Rose Water Ointment—*see Rose Water Ointment General Monographs*

Stronger Rose Water

DEFINITION
Stronger Rose Water is a saturated solution of the odoriferous principals of the flowers of *Rosa centifolia* L. (Fam. Rosaceae) prepared by distilling the fresh flowers with water and separating the excess volatile oil from the clear, water portion of the distillate. [NOTE—Stronger Rose Water, diluted with an equal volume of purified water, may be supplied when "Rose Water" is required.]

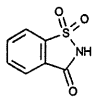
SPECIFIC TESTS
- **REACTION:** Neutral or acidic to litmus
- **RESIDUE ON EVAPORATION**
  Sample: 100 mL
  Analysis: Evaporate the sample on a steam bath, and dry the residue at 105° for 1 h.
  Acceptance criteria: NMT 15 mg (0.015%)

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** The odor of Stronger Rose Water is best preserved by allowing a limited access of fresh air to the container.

Saccharin

Change to read:

▲▲ (NF 1-Dec-2019)



$C_7H_5NO_3S$ 183.18
1,2-Benzisothiazol-3(2*H*)-one, 1,1-dioxide;
1,2-Benzisothiazolin-3-one 1,1-dioxide [81-07-2].

DEFINITION

Change to read:

Saccharin contains ▲NLT 98.0% and NMT 102.0%▲ (NF 1-Dec-2019) of saccharin ($C_7H_5NO_3S$), calculated on the dried basis.

IDENTIFICATION

Change to read:

- **A.** ▲**SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

ASSAY

Change to read:

- **PROCEDURE**
  ▲**Solution A:** 50 mM dibasic potassium phosphate (K_2HPO_4) buffer in 0.1% (v/v) phosphoric acid solution
  Solution B: Methanol

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Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	90	10
7.0	90	10
8.0	5	95
10.0	5	95
10.1	90	10
15.0	90	10

Diluent: Methanol and water (50:50 v/v)
System suitability solution: 0.1 mg/mL of phthalic anhydride and 0.1 mg/mL of USP Saccharin RS in *Diluent*
Standard solution: 0.1 mg/mL of USP Saccharin RS in *Diluent*
Sample solution: 0.1 mg/mL of Saccharin in *Diluent*
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 230 nm
Column: 4.6-mm × 15-cm; 3.5-µm packing L1
Column temperature: 20 ± 5°
Flow rate: 1.0 mL/min
Injection volume: 10 µL
Run time: 15 min
System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The retention times for phthalic anhydride and saccharin are about 6.3 and 7.3 min, respectively. Phthalic anhydride is a potential impurity.]
Suitability requirements
Resolution: NLT 1.5 between the phthalic anhydride and saccharin peaks, *System suitability solution*
Tailing factor: NMT 1.5, *Standard solution*
Relative standard deviation: NMT 0.73% for five replicate injections, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of saccharin in the portion of Saccharin taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of saccharin from the *Sample solution*
r_S = peak area of saccharin from the *Standard solution*
C_S = concentration of USP Saccharin RS in the *Standard solution* (mg/mL)
C_U = concentration of Saccharin in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–102.0% on the dried basis ▲(NF 1-Dec-2019)

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.2%, using an ignition temperature of 600 ± 50°

Change to read:

• ▲▲(NF 1-Dec-2019)**LIMIT OF TOLUENESULFONAMIDES**
Internal standard solution: 0.25 mg/mL of caffeine in methylene chloride
Standard stock solution: 20.0 µg/mL of USP *o*-Toluenesulfonamide RS and 20.0 µg/mL of USP *p*-Toluenesulfonamide RS in methylene chloride
Standard solution: Evaporate 5.0 mL of the *Standard stock solution* to dryness in a stream of nitrogen. Dissolve the residue in 1 mL of the *Internal standard solution*.
Sample solution: Suspend 10 g of Saccharin in 20 mL of water, and dissolve using 5–6 mL of 10 N sodium hydroxide. If necessary, adjust the solution with 1 N sodium hydroxide or 1 N hydrochloric acid to a pH of 7–8, and dilute with water to 50 mL. Shake the solution with four quantities each of 50 mL of methylene chloride. Combine the lower layers, dry over anhydrous sodium sulfate, and filter. Wash the filter and the sodium sulfate with 10 mL of methylene chloride. Combine the solution and the washings, and evaporate almost to dryness in a water bath at a temperature not exceeding 40°. Using a small quantity of methylene chloride, quantitatively transfer the residue into a suitable 10-mL tube, evaporate to dryness in a stream of nitrogen, and dissolve the residue in 1.0 mL of the *Internal standard solution*.
Blank solution: Evaporate 200 mL of methylene chloride to dryness in a water bath at a temperature not exceeding 40°. Dissolve the residue in 1 mL of methylene chloride.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.53-mm × 10-m fused silica; coated with a 2-µm film of phase G3
Temperatures
Injector: 250°
Column: 180°
Detector: 250°
Carrier gas: Nitrogen
Flow rate: 10 mL/min
Injection volume: 1 µL
Injection type: Split ratio, 2:1
System suitability
Samples: *Standard solution* and *Blank solution*
[NOTE—The substances are eluted in the following order: *o*-toluenesulfonamide, *p*-toluenesulfonamide, and caffeine.]
Suitability requirements: No peaks at the retention times for the internal standard, *o*-toluenesulfonamide, or *p*-toluenesulfonamide; *Blank solution*
Resolution: NLT 1.5 between *o*-toluenesulfonamide and *p*-toluenesulfonamide, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Acceptance criteria: See *Table 2*. If any peaks due to *o*-toluenesulfonamide and *p*-toluenesulfonamide appear in the chromatogram of the *Sample solution*, the ratio of their areas to that of the *Internal standard solution* is NMT the corresponding ratio in the chromatogram of the *Standard solution*.

Table 2

Name	Acceptance Criteria, ▲NMT▲ (NF 1-Dec-2019) (ppm)
o-Toluenesulfonamide	10
p-Toluenesulfonamide	10

▲▲(NF 1-Dec-2019)
• **LIMIT OF BENZOATE AND SALICYLATE**
Sample solution: 10 mL of a hot, saturated solution of saccharin

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Analysis: Add ferric chloride TS dropwise to the *Sample solution*.
Acceptance criteria: No precipitate or violet color appears in the liquid.

SPECIFIC TESTS

Change to read:

- ▲▲ (NF 1-Dec-2019)**MELTING RANGE OR TEMPERATURE** ⟨741⟩: 226°–230°▲▲ (NF 1-Dec-2019)
- **LOSS ON DRYING** ⟨731⟩
  Analysis: Dry at 105° for 2 h.
  Acceptance criteria: NMT 1.0%

Change to read:

- **READILY CARBONIZABLE SUBSTANCES TEST** ⟨271⟩
  ▲Matching fluid A: Cobaltous chloride CS, ferric chloride TS, cupric sulfate CS, and water (0.1: 0.4: 0.1: 4.4)▲ (NF 1-Dec-2019)
  Sample solution: 40 mg/mL in sulfuric acid ▲▲ (NF 1-Dec-2019) maintained at 48°–50° for 10 min
  Acceptance criteria: The *Sample solution* has no more color than *Matching fluid A*, when viewed against a white background.
- **CLARITY OF SOLUTION**
  [NOTE—The *Sample solution* is to be compared to *Reference suspension A* in diffused daylight 5 min after preparation of *Reference suspension A*.]
  Diluent: 200-g/L solution of sodium acetate
  Hydrazine solution: 10.0 mg/mL of hydrazine sulfate. [NOTE—Allow to stand for 4–6 h.]
  Methenamine solution: Transfer 2.5 g of methenamine to a 100-mL glass-stoppered flask, add 25.0 mL of water, insert the glass stopper, and mix to dissolve.
  Primary opalescent suspension: Transfer 25.0 mL of *Hydrazine solution* to the *Methenamine solution* in the 100-mL glass-stoppered flask. Mix, and allow to stand for 24 h. [NOTE—This suspension is stable for 2 months, provided it is stored in a glass container free from surface defects. The suspension must not adhere to the glass and must be well mixed before use.]
  Opalescence standard: Dilute 15.0 mL of the *Primary opalescent suspension* with water to 1000 mL. [NOTE—This suspension should not be used beyond 24 h after preparation.]
  Reference suspension A: *Opalescence standard* and water (1 in 20)
  Reference suspension B: *Opalescence standard* and water (1 in 10)
  Sample solution: 200 mg/mL in *Diluent*
  Analysis
  Samples: *Diluent*, *Reference suspension A*, *Reference suspension B*, *Sample solution*, and water
  Transfer a sufficient portion of the *Sample solution* to a test tube of colorless, transparent, neutral glass with a flat base and an internal diameter of 15–25 mm to obtain a depth of 40 mm. Similarly transfer portions of *Reference suspension A*, *Reference suspension B*, water, and *Diluent* to separate matching test tubes. Compare the solutions in diffused daylight, viewing vertically against a black background (see *Visual Comparison* ⟨630⟩). [NOTE—The diffusion of light must be such that *Reference suspension A* can readily be distinguished from water, and that *Reference suspension B* can readily be distinguished from *Reference suspension A*.]
  Acceptance criteria: The *Sample solution* shows the same clarity as that of water or *Diluent*, or its opalescence is NMT that of *Reference suspension A*.
- **COLOR OF SOLUTION**
  Diluent A: 200-g/L solution of sodium acetate
  Diluent B: 10-g/L solution of hydrochloric acid
  Standard stock solution: Ferric chloride CS, cobaltous chloride CS, cupric sulfate CS, and *Diluent B* (3.0: 3.0: 2.4: 1.6)
  Standard solution: *Standard stock solution* and *Diluent B* (1 in 100). [NOTE—Prepare the *Standard stock solution* and *Standard solution* immediately before use.]
  Sample solution: Use the *Sample solution* from the test for *Clarity of Solution*.
  Analysis
  Samples: *Diluent A*, *Standard solution*, *Sample solution*, and water
  Transfer a sufficient portion of the *Sample solution* to a test tube of colorless, transparent, neutral glass with a flat base and an internal diameter of 15–25 mm to obtain a depth of 40 mm. Similarly transfer portions of the *Standard solution*, *Diluent A*, and water to separate, matching test tubes. Compare the solutions in diffused daylight, viewing vertically against a white background (see *Visual Comparison* ⟨630⟩).
  Acceptance criteria: The *Sample solution* has the appearance of water or *Diluent A*, or is not more intensely colored than the *Standard solution*.

ADDITIONAL REQUIREMENTS

Change to read:

- ▲▲ (NF 1-Dec-2019)**PACKAGING AND STORAGE:** Preserve in well-closed containers. Store at room temperature.▲▲ (NF 1-Dec-2019)
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Saccharin RS
  USP *o*-Toluenesulfonamide RS
  USP *p*-Toluenesulfonamide RS

Saccharin Calcium—*see Saccharin Calcium General Monographs*

Saccharin Sodium—*see Saccharin Sodium General Monographs*

Safflower Oil—*see Safflower Oil General Monographs*

Sesame Oil

DEFINITION
Sesame Oil is the refined fixed oil obtained from the seed of one or more cultivated varieties of *Sesamum indicum* L. (Fam. Pedaliaceae). It may contain suitable antioxidants.

IDENTIFICATION
- **A. IDENTITY BY TRIGLYCERIDE PROFILE**
  Analysis: Proceed as directed in the test for *Triglyceride Composition*.
  Acceptance criteria: The peak responses of the eight major triglycerides—LLL, OLL, PLL, OOL, POL, OOO, SOL, and

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POO—elute between 0 and about 40 min, in the order specified, and at relative retention times of about 0.55, 0.65, 0.69, 0.77, 0.82, 0.93, 0.97, and 1.0, respectively, as obtained in the *Sample solution* in the test for *Triglyceride Composition*.

ASSAY

• **TRIGLYCERIDE COMPOSITION**

[NOTE—The fatty acid radicals are designated as linoleic (L), oleic (O), palmitic (P), and stearic (S), and the common abbreviations for triglycerides used are as follows: trilinolein (LLL), 1,2-dilinoleoyl-3-oleoyl-rac-glycerol (OLL), 1,2-dilinoleoyl-3-palmitoyl-rac-glycerol (PLL), 1,2-dioleoyl-3-linoleoyl-rac-glycerol (OOL), 1-palmitoyl-2-oleoyl-3-linoleoyl-rac-glycerol (POL), triolein (OOO), 1-linoleoyl-2-oleoyl-3-stearoyl-rac-glycerol (SOL), and 1,2-dioleoyl-3-palmitoyl-rac-glycerol (POO).]

Mobile phase: Acetonitrile and methylene chloride (60:40)

System suitability solution: 3.0 mg/mL each of USP Sesame Oil Related Compound A RS and USP Sesame Oil Related Compound B RS in *Mobile phase*. [NOTE—USP Sesame Oil Related Compound A RS is OLL, and USP Sesame Oil Related Compound B RS is PLL.]

Sample solution: 20 mg/mL of Sesame Oil in *Mobile phase*

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Refractive index

Columns: Two 4.6-mm × 25-cm in series; packings L1

Column temperature: 30°

Flow rate: 1.0 mL/min

Injection volume: 20 µL

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for OLL and PLL are about 0.93 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 1.8 between OLL and PLL

Relative standard deviation: NMT 1.5% determined from peak areas; NMT 2.2% determined from the peak area ratio of OLL to PLL

Analysis

[NOTE—The relative retention times for the eight major triglyceride peaks are listed in *Table 1*.]

Sample: *Sample solution*

Calculate the percentage of each of these triglycerides in the portion of the *Sample* taken:

$$\text{Result} = (A/B) \times 100$$

A = peak area of each individual triglyceride

B = sum of the areas of all the peaks, excluding the solvent peak

Table 1

Triglyceride	Relative Retention Time	Composition (%)
LLL	0.55	7.0–19.0
OLL	0.65	13.0–30.0
PLL	0.69	5.0–9.0
OOL	0.77	14.0–25.0
POL	0.82	8.0–16.0
OOO	0.93	5.0–14.0
SOL	0.97	2.0–8.0
POO	1.0	2.0–8.0

Table 1 *(continued)*

IMPURITIES

• **ALKALINE IMPURITIES**

Sample: 10 mL of Sesame Oil

Analysis: Mix 10 mL of freshly opened acetone and 0.3 mL of water, and add 0.05 mL of bromophenol blue TS. Add the *Sample*, shake, and allow to stand. Titrate with 0.01 N hydrochloric acid VS to change the color of the upper layer to yellow.

Acceptance criteria: NMT 0.1 mL of 0.01 N hydrochloric acid is required.

SPECIFIC TESTS

• **SPECIFIC GRAVITY** ⟨841⟩: 0.912–0.921

• **FATS AND FIXED OILS** ⟨401⟩, *Acid Value (Free Fatty Acids)*

Sample: 10 g

Acceptance criteria: NMT 2.0 mL of 0.020 N sodium hydroxide is required for neutralization.

• **FATS AND FIXED OILS** ⟨401⟩, *Iodine Value*: 103–116

• **FATS AND FIXED OILS** ⟨401⟩, *Saponification Value*: 188–195

• **FATS AND FIXED OILS** ⟨401⟩, *Solidification Temperature of Fatty Acids*: 20°–25°

• **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩: NMT 10.0

• **FATS AND FIXED OILS** ⟨401⟩, *Unsaponifiable Matter*: NMT 1.5%

• **COTTONSEED OIL**

Sample: 5 mL

Analysis: Mix the *Sample* in a test tube with 5 mL of a mixture of equal volumes of amyl alcohol and a 10-mg/mL solution of sulfur in carbon disulfide. Warm the mixture carefully until the carbon disulfide is expelled, and immerse the tube to one-third of its depth in a boiling saturated solution of sodium chloride.

Acceptance criteria: No reddish color develops within 15 min.

• **WATER DETERMINATION,** *Method Ic* ⟨921⟩: NMT 0.1%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and prevent exposure to excessive heat.

• **LABELING:** Label it to indicate the name and quantity of any added antioxidant. Where Sesame Oil is intended for use in the manufacture of injectable dosage forms, it is so labeled.

• **OTHER REQUIREMENTS:** For Sesame Oil intended for use in injectable dosage forms, which is specified in the labeling, the requirements must be met for *Unsaponifiable Matter*, *Acid Value*, *Peroxide Value*, and *Water, Method Ic*, under *Specific Tests* in *Injections and Implanted Drug Products* ⟨1⟩, *Vehicles and added substances, Nonaqueous vehicles*.

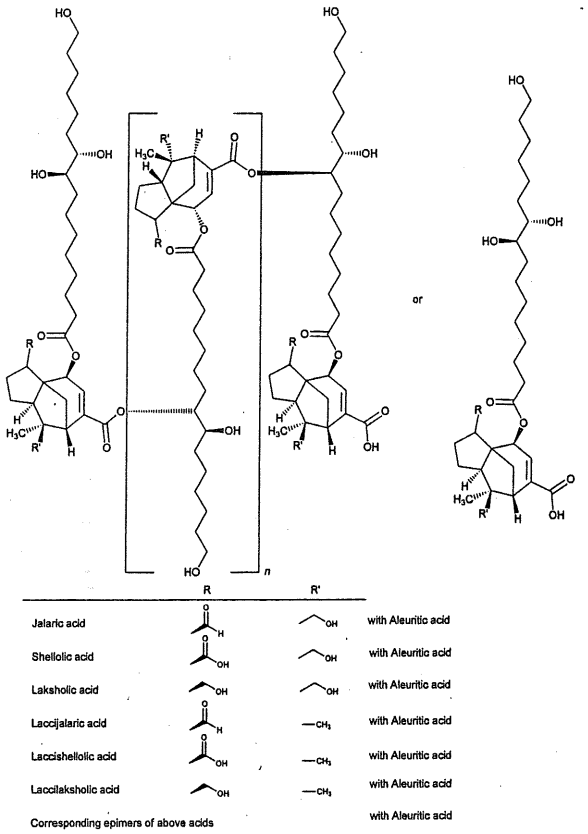
• **USP REFERENCE STANDARDS** ⟨11⟩

USP Sesame Oil Related Compound A RS

USP Sesame Oil Related Compound B RS

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Shellac



[9000-59-3].

DEFINITION

Shellac is obtained by the purification of lac, the resinuous secretion of the insect *Kerria lacca* (Kerr) Lindinger (*Laccifer lacca* Kerr) (Fam. Coccideae). Shellac is a polyester resin consisting of inter- and intra-esters of polyhydroxyl carboxylic acids formed from certain hydroxyl acids and sesquiterpenic acids, and also contains variable amounts of wax. There are four types of Shellac depending on the nature of the treatment of crude secretion (seedlac).

1. Orange Shellac is produced by a process of filtration in the molten state and/or by a process of solvent extraction. Orange Shellac retains most of its wax.
2. Refined (Dewaxed) Orange Shellac is produced by filtration of the wax in a solvent process. It may also be decolorized by activated carbon.
3. Regular Bleached (white) Shellac is prepared by dissolving the lac in an alkaline solution and bleaching the solution with sodium hypochlorite. It is precipitated by dilute acid and dried.
4. Refined Bleached Shellac is prepared by dissolving the lac in an alkaline solution and bleaching the solution with sodium hypochlorite. During the process, wax is removed by filtration. It is precipitated by dilute acid and dried.

Shellac conforms to the specifications in *Table 1*.

Table 1

	Acid Value (on dried basis)	Loss on Drying (%)	Wax (%)
Orange Shellac	68–76	NMT 2.0	NMT 5.5
Refined Orange Shellac	68–79	NMT 2.0	NMT 0.2
Regular Bleached Shellac	73–89	NMT 6.0	NMT 5.5
Refined Bleached Shellac	75–91	NMT 6.0	NMT 0.2

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197A** or **197K.▲** (CN 1-May-2020) Due to the degree of polymerization, the intensity of some absorption bands may vary.

Use USP Regular Bleached Shellac RS for the following two types:

- Orange Shellac
- Regular Bleached Shellac

Use USP Refined Bleached Shellac RS for the following two types:

- Refined Bleached Shellac
- Refined Orange Shellac

• **B. IDENTIFICATION OF ALEURITIC ACID AND SHELLOLIC ACID BY THIN-LAYER CHROMATOGRAPHY**

Standard solution: 6 mg/mL of USP Aleuritic Acid RS in methanol, heating slightly if necessary.

Sample solution: Weigh and finely powder 500 mg of Shellac. Transfer 500 mg of the finely powdered Shellac to a test tube and heat with 4 mL of 85-mg/mL sodium hydroxide solution in a vigorously boiling water bath for 5 min. Cool, add 10 mL of ethyl acetate, and transfer the content to a separatory funnel. With stirring, add slowly 4 mL of a 120-mg/mL solution of glacial acetic acid to the funnel. Shake the solution thoroughly and withdraw the lower layer. Transfer the upper layer to a small flask, add anhydrous sodium sulfate, and pass through a membrane disk syringe of 0.45-µm pore size. Collect the filtrate and use it as the sample.

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Mode: TLC

Plate: 10-cm × 20-cm or 20-cm × 20-cm, silica gel 60 F_{254}

Application volume: 10 µL, as 8-mm bands. [NOTE—An automated apparatus may be used.]

Developing solvent system: Ethyl acetate, methylene chloride, methyl alcohol, and acetic acid (60:32:8:1)

Spray reagent: Prepare the anisaldehyde solution by mixing in the following order. In 0.5 mL of anisaldehyde, add 10 mL of glacial acetic acid, 85 mL of methyl alcohol, and 5 mL of sulfuric acid.

Analysis

Samples: *Standard solution* and *Sample solution*

Development: Apply the *Samples* in different bands to the previously marked starting point on a TLC plate, and develop the plate two times over a path of 15 cm. Dry the plate in air.

Detection: Spray with the *Spray reagent*. Heat the plate at 100°–105° for 10 min, and examine in daylight (or white light).

[NOTE—The principal band of aleuritic acid shows strong intensity and purple color. The retardation factor (R_F) for the principal band of aleuritic acid is 0.41. A

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blue-gray band with medium intensity at R_F 0.22 could be assigned to shellolic acid.]

Acceptance criteria: The chromatogram from the *Sample solution* shows several colored bands. One of the colored bands is similar in position and color to the band in the chromatogram from the *Standard solution*, and it is assigned to aleuritic acid. Below the aleuritic acid band, a blue-gray band is assigned to shellolic acid.

IMPURITIES

• **Limit of Chloride**

Dilute nitric acid: Dilute 10.5 mL of nitric acid with water to 100 mL (10%).

Silver nitrate solution: Dissolve 1.75 g of silver nitrate in water to 100 mL (0.1 mol/L).

Sample: 0.4 g

Sample solution: Shake and dissolve the *Sample* in 5 mL of alcohol while warming. Add 40 mL of water, and cool. Add 12 mL of *Dilute nitric acid* and water to make 100 mL, and filter. Perform the analysis using 50-mL of the filtrate as the *Sample solution*.

Control solution: 0.8 mL of 0.01 M hydrochloric acid VS, 2.5 mL of alcohol, 6 mL of *Dilute nitric acid*, and water to make 50 mL

Analysis: Add 1 mL of *Silver nitrate solution* to the *Sample solution* and *Control solution*, mix well, and protect from light for 5 min. Compare the opalescence developed in both solutions against a black background by viewing downward or transversely.

Acceptance criteria: The opalescence of the *Sample solution* is NMT that of the *Control solution*, corresponding to NMT 0.14%.

• **Total Ash**

Sample: 1 g

Analysis: Before sampling, ignite a crucible of platinum, quartz, or porcelain at 500°–550° for 1 h. Cool and weigh the crucible. Transfer the *Sample* to this crucible. Take off the lid or open slightly if necessary. Heat the crucible at a low temperature at first, then gradually heat to 500°–550°. Ignite to incinerate the residue for more than 4 h until no carbonized substance remains in the ash. Cool and weigh the ash. Incinerate repeatedly to constant weight, cool, weigh accurately, and determine the percentage of total ash. If a carbonized substance remains and a constant weight cannot be obtained, extract the charred mass with hot water, collect the insoluble residue on filter paper for assay, and incinerate the residue and filter paper until no carbonized substance remains in the ash. Then add the filtrate, evaporate to dryness, and incinerate. Cool, weigh accurately, and determine the percentage of the total ash. If a carbon-free ash still cannot be obtained, moisten the ash with a small amount of alcohol (ethanol). Break up the ash with a glass rod, and wash the rod with a small amount of alcohol. Evaporate carefully, and determine the mass of the total ash as described above. A desiccator (silica gel) is used for cooling.

Acceptance criteria: NMT 1.0%

• **Ethanol-Insoluble Substances**

Sample: 5 g

Analysis: Dissolve the *Sample* in 50 mL of alcohol (ethanol) in a water bath while shaking. Transfer the ethanol solution to a tared extraction thimble that was previously dried at 105° for 2 h in a Soxhlet extractor, and extract with alcohol for 3 h. Dry the extraction thimble at 105° for 3 h. Weigh the residue in the thimble.

Acceptance criteria: The mass of the residue is NMT 2.0%.

SPECIFIC TESTS

• **Loss on Drying** ⟨731⟩

Analysis: Dry at 41 ± 2° in a well-ventilated oven for 24 h.

Acceptance criteria: See *Table 1*.

• **Acid Value**

Sample solution: Dissolve 2 g of finely ground Shellac in 50 mL of alcohol that has been neutralized to phenolphthalein with 0.1 N sodium hydroxide.

Analysis: Add additional phenolphthalein TS if necessary and titrate with 0.1 N sodium hydroxide VS to a pink endpoint, or determine the endpoint potentiometrically. Express the acid value in terms of the number of mg of potassium hydroxide required per g of dried Shellac, and calculate the acid value as directed in *Fats and Fixed Oils* ⟨401⟩, *Acid Value*.

[NOTE—For orange Shellac titrate slowly, stirring vigorously, until a glass rod dipped into the titrated solution produces a color change when touched to a drop of thymol blue TS on a spot plate.]

Acceptance criteria: See *Table 1*.

• **Wax**

Sample: 10 g of finely ground Shellac

Analysis: Transfer the *Sample* and 2.50 g of sodium carbonate to a 200-mL, tall-form beaker. Add 150 mL of hot water, immerse the beaker in a boiling water bath, and stir until dissolved. Cover the beaker with a watch glass, and maintain the heat for more than 3 h without agitation. Remove the beaker to a cold water bath. When the wax has floated to the surface, pass the solution through medium-speed quantitative ashless filter paper, transferring the wax to the paper, and wash the filter with water. Pour 5–10 mL of alcohol onto the filter to facilitate drying. Wrap the paper loosely in a larger piece of filter paper, bind with a piece of fine wire, and dry with the aid of gentle heat. Extract with chloroform in a suitable continuous extraction apparatus for 2 h, using a weighed flask to receive the extracted wax and solvent. Evaporate the solvent, and dry the wax at 105° to constant weight.

Acceptance criteria: See *Table 1*.

• **Rosin**

Sample solution: 200 mg/mL in dehydrated alcohol

Analysis: To 10 mL of *Sample solution* add slowly, with shaking, 50 mL of solvent hexane, wash with two successive 50-mL portions of water, filter the washed alcohol–solvent hexane solution, and evaporate to dryness. To the residue add 2 mL of a mixture of liquefied phenol, dehydrated alcohol, and solvent hexane (1: 0.5: 2). Stir, and transfer a portion of the solution to the cavity of a color-reaction plate. Fill an adjacent cavity with a mixture of bromine and solvent hexane (1:4), and cover both cavities with an inverted watch glass.

Acceptance criteria: No purple or deep indigo-blue color is produced in or above the liquid containing the residue.

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Preserve in well-closed containers. Store in a dry place below 15°. Protect from light.

• **Labeling:** Label it to indicate the type of Shellac.

• **USP Reference Standards** ⟨11⟩

USP Aleuritic Acid RS

USP Refined Bleached Shellac RS

USP Regular Bleached Shellac RS

Dental-Type Silica

DEFINITION

Dental-Type Silica is obtained from sodium silicate solution by destabilizing with acid in such a way as to yield very fine

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particles. The sum of the *Assay* values for Silicon Dioxide and Sodium Sulfate is NLT 98.0%.

ASSAY

- **SILICON DIOXIDE**

Sample: 1 g

Analysis: Ignite the *Sample* at 1000° for 1 h, cool in a desiccator, and weigh. Carefully wet with water, and add 10 mL of hydrofluoric acid, in small increments. Evaporate on a steam bath to dryness, and cool. Add about 10 mL of hydrofluoric acid and about 0.5 mL of sulfuric acid, and evaporate to dryness. Slowly increase the temperature until all of the acids have been volatilized, and ignite at 1000°. Cool in a desiccator, and weigh. The difference between the final weight and the weight of the initially ignited portion represents the weight of SiO_2.

- **SODIUM SULFATE**

Sample: 1 g

Analysis 1: In a platinum dish, wet the *Sample* with a few drops of water, add 15 mL of perchloric acid, and place the dish on a hot plate. Add 10 mL of hydrofluoric acid. Heat until copious fumes are evolved. Add 5 mL of hydrofluoric acid, and again heat to copious fumes. Add 5 mL of boric acid solution (1 in 25), and heat to fumes. Cool, and transfer the residue to a 400-mL beaker with the aid of 10 mL of hydrochloric acid. Adjust the volume with water to about 300 mL, and bring to boiling on a hot plate. Add 20 mL of hot barium chloride TS. Keep the beaker on the hot plate for 2 h, maintaining the volume above 200 mL. After cooling, transfer the precipitate and solution to a dried, tared crucible with a filter of 0.8-µm pore size. Wash the filter and precipitate 8 times with hot water, dry the crucible at 105° for 1 h, and weigh. The weight, multiplied by 0.6085, is the sodium sulfate content of the amount of specimen taken.

Acceptance criteria 1: NMT 4.0%

Analysis 2: Calculate the sum of the *Assay* values for the silicon dioxide and the sodium sulfate, and calculate the percentage in the Dental-Type Silica taken.

Acceptance criteria 2: NLT 98.0%

SPECIFIC TESTS

- **pH** ⟨791⟩

Sample solution: 50 mg/mL of slurry

Acceptance criteria: 4.0–8.5

- **LOSS ON DRYING** ⟨731⟩

Analysis: Dry a sample at 105° for 2 h.

Acceptance criteria: It loses NMT the maximum percentage of its weight as indicated in the labeling.

- **LOSS ON IGNITION** ⟨733⟩

Sample: 1 g, previously dried

Analysis: Ignite the *Sample* at 1000° for NLT 1 h.

Acceptance criteria: NMT 8.5%

- **CHLORIDE AND SULFATE,** *Chloride* ⟨221⟩

Sample solution: Boil 5 g in 50 mL of water under a reflux condenser for 2 h, cool, and filter.

Control: 1.0 mL of 0.020 N hydrochloric acid

Analysis: Use a 7-mL portion of *Sample solution*.

Acceptance criteria: 0.1%; the *Sample solution* shows no more chloride than the *Control*.

- **ARSENIC,** *Method I* ⟨211⟩

Sample solution: Transfer 4.0 g of Dental-Type Silica to a platinum dish, add 5 mL of nitric acid and 35 mL of hydrofluoric acid, and evaporate on a steam bath. Cool, add 5 mL of perchloric acid, 10 mL of hydrofluoric acid, and 10 mL of sulfuric acid, and evaporate on a hot plate to the production of heavy fumes. Cool, cautiously transfer to a 100-mL beaker with the aid of a few mL of hydrochloric acid, and evaporate to dryness. Cool, add 5 mL of hydrochloric acid, dilute with water to about 40 mL, and heat to dissolve any residue. Cool, transfer to a 100-mL volumetric flask, and dilute with water to volume.

Analysis: Use a 25.0-mL portion of *Sample solution*.

Acceptance criteria: NMT 3 ppm

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **LABELING:** Label it to indicate the maximum percentage of loss on drying.

Hydrophobic Colloidal Silica

[68611-44-9].

DEFINITION

Hydrophobic Colloidal Silica is prepared by partial alkylation for hydrophobation. It contains NLT 99.0% and NMT 101.0% of silicon dioxide (SiO_2), calculated on the ignited basis.

IDENTIFICATION

- **A.**

Sample: 25 mg

Analysis: Add the *Sample* to a platinum crucible, and ignite at 900° for 2 h. Using a copper wire, mix the ignited substance with 10 mg of sodium fluoride and a few drops of sulfuric acid to give a thin slurry. Cover the crucible with a thin, transparent plate of plastic under which a drop of water is suspended, and warm gently.

Acceptance criteria: Within a short time, a white ring is rapidly formed around the drop of water.

- **B.** It meets the requirements for *Water-Dispersible Substances* in *Specific Tests*.

ASSAY

- **PROCEDURE**

Sample: The residue obtained in the test for *Loss on Ignition*

Analysis: To the *Sample* add sufficient alcohol to moisten the residue completely, and then add 0.2 mL of sulfuric acid. Add 6 mL of hydrofluoric acid, and evaporate to dryness on a hot plate at about 100°, taking care to avoid loss from sputtering. Wash down the sides of the platinum crucible with 6 mL of hydrofluoric acid, and evaporate to dryness. Ignite at 900° for 2 h, cool in a desiccator, and weigh. The difference between the weight of the residue obtained in the test for *Loss on Ignition* and the weight of the final residue gives the amount of silicon dioxide (SiO_2) in the quantity of the substance to be examined.

Acceptance criteria: 99.0%–101.0% on the ignited basis

IMPURITIES

- **LOSS ON IGNITION** ⟨733⟩

Sample: 0.2 g

Analysis: Ignite the *Sample* in a platinum crucible at 900° for 2 h. Cool in a desiccator before weighing. [NOTE—It is advisable to place the crucible in a cold oven and then heat up the oven.]

Acceptance criteria: NMT 6.0%

- **LIMIT OF LEAD**

Select reagents having as low a lead content as practicable, and store all solutions in containers of borosilicate glass. Rinse all glassware thoroughly with warm, dilute nitric acid (1 in 2) followed by water.

Ammonium acetate buffer solution, pH 3.5: Weigh 25.0 g of ammonium acetate, and dissolve in 25 mL of water. Add 38.0 mL of dilute hydrochloric acid. Adjust the pH, if necessary, with dilute hydrochloric acid or weak ammonia

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solution (containing 460 mL/L of strong ammonia solution). Dilute with water to 100.0 mL.

Thioacetamide solution: Prepare immediately before use. To 0.2 mL of thioacetamide TS add 1 mL of a mixture of 5 mL of water, 15 mL of 1 N sodium hydroxide, and 20 mL of 85% glycerol. Heat in a water bath for 20 s.

Sample solution: Suspend 2.5 g of Hydrophobic Colloidal Silica in 30 mL of methanol, stir, and add 30 mL of weak ammonia solution (containing 460 mL/L of strong ammonia solution). With frequent stirring, evaporate on a water bath, and dry the residue in an oven at 140°. When the dried substance is white, break up the mass with a glass rod. Reduce the residue to a powder, and add 15 mL of methanol and 25 mL of 1 N hydrochloric acid. Boil gently for 5 min, stirring frequently with the glass rod. Centrifuge for 20 min, and pass the supernatant through a membrane filter. To the residue in the centrifuge tube add 3 mL of dilute hydrochloric acid and 9 mL of water, and bring to a boil. Centrifuge for 20 min, and pass the supernatant through the same membrane filter. Wash the residue with small quantities of water, combine the filtrates and washings, and dilute with water to 50 mL. To 20 mL of this solution add 50 mg of ascorbic acid and 1 mL of strong ammonia solution. Neutralize with diluted weak ammonia solution (containing 160 mL/L of strong ammonia solution). Dilute with water to 25 mL.

Reference solution: Pipet 10 mL of standard lead solution TS, and mix with 2 mL of the *Sample solution*.

Blank solution: A mixture of 10 mL of water and 2 mL of the *Sample solution*

Analysis: To the *Sample solution*, *Reference solution*, and *Blank solution* add 2 mL of *Ammonium acetate buffer solution, pH 3.5*. Mix, and add 1.2 mL of *Thioacetamide solution*. Mix immediately. Examine the solutions after 2 min. The test is invalid if the *Reference solution* does not show a slight brown color compared to the *Blank solution*.

Acceptance criteria: The brown color in the *Sample solution* is not more intense than that in the *Reference solution* (25 µg/g). [NOTE—If the result is difficult to judge, pass the solutions through a membrane filter of 3-µm pore size, and carry out the filtration slowly and uniformly. Compare the spots on the filters obtained with the different solutions.]

• **LIMIT OF CHLORIDE**

Standard solution: Add 10 mL of 0.15 mM sodium chloride and 5 mL of water. Add 1 mL of dilute nitric acid, and pour into a test tube containing 1 mL of silver nitrate TS.

Sample solution: To 1 g of Hydrophobic Colloidal Silica add 30 mL of methanol and 20 mL of dilute nitric acid. Heat on a water bath for 15 min with frequent stirring. Cool, dilute with water to 50 mL, and filter. Dilute 10 mL of the filtrate with water to 15 mL. Add 1 mL of dilute nitric acid, and pour into a test tube containing 1 mL of silver nitrate TS.

Analysis: Examine the tubes laterally against a black background.

Acceptance criteria: After standing for 5 min protected from light, any opalescence in the *Sample solution* is not more intense than that in the *Standard solution* (0.025%).

SPECIFIC TESTS

• **WATER-DISPERSIBLE SUBSTANCES**

Sample: 0.4 g

Analysis: Place the *Sample* in a 500-mL separating funnel, add 100 mL of water, and shake for 1 min. Allow to stand for 1 h. Allow 90 mL of the aqueous phase to run out dropwise without filtration into a suitable dish dried at 140°, and cool in a desiccator. Evaporate to dryness at 140°, starting at a low temperature to avoid splashing. Cool in a desiccator.

Acceptance criteria: NMT 3.0%; the weight of the residue does not exceed 12 mg.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.

Purified Siliceous Earth

DEFINITION

Purified Siliceous Earth is a form of silica (SiO_2) consisting of the frustules and fragments of diatoms, purified by calcining.

IMPURITIES

• **LOSS ON IGNITION** ⟨733⟩

Sample: 1 g, previously dried

Analysis: Ignite the *Sample* at 980 ± 25° for 1 h in a tared platinum or porcelain crucible.

Acceptance criteria: NMT 2.0%

• **LEACHABLE ARSENIC**

Sample solution: To 10.0 g in a 250-mL beaker add 50 mL of 0.5 N hydrochloric acid, cover with a watch glass, and heat at 70° for 15 min. Cool, and decant through a Whatman No. 3 filter paper into a 100-mL volumetric flask. Wash the slurry with three 10-mL portions of water, preheated to 70°, and dilute with water to volume.

Analysis: A 3.0-mL portion of the *Sample solution* meets the requirements in *Arsenic, Method 1* ⟨211⟩.

Acceptance criteria: NMT 10 µg/g

• **LEACHABLE LEAD**

Sample: A 10.0-mL portion of the *Sample solution* prepared in the test for *Leachable Arsenic*

Control: 10 mL of *Diluted Standard Lead Solution* in *Lead* ⟨251⟩

Analysis: The *Sample* meets the requirements in *Lead* ⟨251⟩.

Acceptance criteria: NMT 10 µg/g

• **LIMIT OF NONSILICEOUS SUBSTANCES**

Sample: 200 mg

Analysis: Transfer the *Sample* to a tared platinum crucible, add 5 mL of hydrofluoric acid and 2 drops of dilute sulfuric acid (1 in 2), and evaporate gently to dryness. Cool, add 5 mL of hydrofluoric acid, evaporate again to dryness, and ignite to constant weight.

Acceptance criteria: The weight of the residue is NMT 50 mg.

SPECIFIC TESTS

• **LOSS ON DRYING** ⟨731⟩

Analysis: Dry a sample at 105° for 2 h.

Acceptance criteria: NMT 0.5%

• **ACID-SOLUBLE SUBSTANCES**

Sample: 10.0 g

Analysis: Digest the *Sample* with 50 mL of 0.5 N hydrochloric acid at 70° for 15 min, and filter. Wash the residue, adding the washings to the filtrate to obtain a total volume of 100 mL. Evaporate at 110° in a tared porcelain dish to dryness.

Acceptance criteria: NMT 2.0% (weight of the dried residue is NMT 200 mg)

• **WATER-SOLUBLE SUBSTANCES**

Sample: 12.5 g

Analysis: Place the *Sample* in a 500-mL conical flask, add 250 mL of water, and shake for 2 h at room temperature. Filter with the aid of vacuum, and again filter if necessary to obtain a clear filtrate. Evaporate in a tared platinum or porcelain dish, and dry at 110°.

Acceptance criteria: NMT 0.2% (weight of the residue is NMT 25 mg)

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ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Silicon Dioxide

$SiO_2 \cdot xH_2O$
Anhydrous 60.08

DEFINITION
Silicon Dioxide is obtained by insolubilizing the dissolved silica in sodium silicate solution. Where obtained by the addition of sodium silicate to a mineral acid, the product is termed silica gel. Where obtained by the destabilization of a solution of sodium silicate in such manner as to yield very fine particles, the product is termed precipitated silica. After ignition at 1000° for NLT 1 h, it contains NLT 99.0% of SiO_2.

IDENTIFICATION
- **PROCEDURE**

Sample: 5 mg
Analysis: Transfer the *Sample* to a platinum crucible, mix with 200 mg of anhydrous potassium carbonate, ignite at a red heat over a burner for 10 min, and cool. Dissolve the melt in 2 mL of recently distilled water, warming if necessary, and slowly add 2 mL of ammonium molybdate TS.
Acceptance criteria: A deep yellow color is produced.

ASSAY
- **PROCEDURE**

Sample: 1 g
Analysis: Ignite the *Sample* in a tared platinum dish at 1000° for 1 h, cool in a desiccator, and weigh. Carefully wet with water, and add 10 mL of hydrofluoric acid in small increments. Evaporate on a steam bath to dryness, and cool. Add 10 mL of hydrofluoric acid and 0.5 mL of sulfuric acid, and evaporate to dryness. Slowly increase the temperature until all of the acids have been volatilized, and ignite at 1000°. Cool in a desiccator, and weigh. The difference between the final weight and the weight of the initially ignited portion represents the weight of SiO_2.
Acceptance criteria: NLT 99.0% on the previously ignited basis

IMPURITIES
INORGANIC IMPURITIES
- **Loss on Ignition** ⟨733⟩

Sample: 1 g
Analysis: Ignite the *Sample*, previously dried and weighed, at 1000° for NLT 1 h.
Acceptance criteria: It loses NMT 8.5% of its weight.
- **Chloride and Sulfate,** *Chloride* ⟨221⟩: Boil 5 g in 50 mL of water under a reflux condenser for 2 h, cool, and filter. A 7-mL portion of the filtrate shows no more chloride than corresponds to 1.0 mL of 0.020 N hydrochloric acid (0.1%).
- **Chloride and Sulfate,** *Sulfate* ⟨221⟩: A 10-mL portion of the filtrate from the test for *Chloride* shows no more sulfate than corresponds to 5.0 mL of 0.020 N sulfuric acid (0.5%).
- **Arsenic,** *Method I* ⟨211⟩

Sample solution: Transfer 4.0 g to a platinum dish. Add 5 mL of nitric acid and 35 mL of hydrofluoric acid, and evaporate on a steam bath. Cool. Add 5 mL of perchloric acid, 10 mL of hydrofluoric acid, and 10 mL of sulfuric acid, and evaporate on a hot plate to the production of heavy fumes. Cool. Cautiously transfer to a 100-mL beaker with the aid of a few mL of hydrochloric acid, and evaporate to dryness. Cool. Add 5 mL of hydrochloric acid, dilute with water to 40 mL, and heat to dissolve any residue. Cool. Transfer to a 100-mL volumetric flask, and dilute with water to volume.
Analysis: Use a 25.0-mL portion of the *Sample solution*.
Acceptance criteria: Meets the requirements of the test (NMT 3 ppm)

SPECIFIC TESTS
- **pH** ⟨791⟩: 4–8 in a slurry (1 in 20)
- **LOSS ON DRYING** ⟨731⟩: Dry a sample at 145° for 4 h; it loses NMT 5.0% of its weight.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from moisture.
- **LABELING:** Label it to state whether it is silica gel or precipitated silica.

Colloidal Silicon Dioxide

SiO_2 60.08
Silica [7631-86-9].

DEFINITION
Colloidal Silicon Dioxide is a submicroscopic fumed silica prepared by the vapor-phase hydrolysis of a silicon compound. When ignited at 1000° for 2 h, it contains NLT 99.0% and NMT 100.5% of SiO_2.

IDENTIFICATION
- **A. PROCEDURE**

Analysis: Transfer 5 mg to a platinum crucible, and mix with 200 mg of anhydrous potassium carbonate. Heat the crucible to a red color with the aid of a Bunsen burner for 10 min, and cool. Dissolve the melt in 2 mL of freshly distilled water, warming if necessary, and slowly add 2 mL of ammonium molybdate TS to the solution.
Acceptance criteria: A deep yellow color is produced.
- **B. PROCEDURE**

[NOTE—Avoid contact with *o*-tolidine when performing this test, and conduct the test in a well-ventilated hood.]
Analysis: Place 1 drop of the yellow silicomolybdate solution from *Identification* test *A* on a filter paper, and evaporate the solvent. Add 1 drop of a saturated solution of *o*-tolidine in glacial acetic acid to reduce the silicomolybdate to molybdenum blue, and place the paper over ammonium hydroxide.
Acceptance criteria: A greenish blue spot is produced.

ASSAY
- **PROCEDURE**

Sample: 500 mg
Analysis: Ignite the *Sample* in a tared platinum crucible at 1000 ± 25° for 2 h, cool in a desiccator, and weigh. Add 3 drops of sulfuric acid, and add enough alcohol to just moisten the sample completely. Add 15 mL of hydrofluoric acid, and in a well-ventilated hood evaporate on a hot plate to dryness, using medium heat (95°–105°) and taking care that the sample does not spatter as dryness is approached. Heat the crucible to a red color with the aid of a Bunsen burner. Ignite the residue at 1000 ± 25° for 30 min, cool in a desiccator, and weigh. If a residue remains, repeat the *Analysis*, beginning with "Add 15 mL of hydrofluoric acid". The weight lost by the assay specimen, previously ignited at 1000 ± 25°, represents the weight of SiO_2 in the portion taken.
Acceptance criteria: 99.0%–100.5% on the previously ignited basis

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IMPURITIES

INORGANIC IMPURITIES

- **Loss on Ignition** ⟨733⟩: Ignite the portion of Colloidal Silicon Dioxide, retained from the test for *Loss on Drying*, at 1000 ± 25° to constant weight: the previously dried Colloidal Silicon Dioxide loses NMT 2.0% of its weight.
- **Arsenic,** *Method 1* ⟨211⟩

Sample solution: To 2.5 g add 50 mL of 3 N hydrochloric acid, and reflux for 30 min using a water condenser. Cool, filter with the aid of suction, and transfer the filtrate to a 100-mL volumetric flask. Wash the filter and flask with several portions of hot water, and add the washings to the flask. Cool, and dilute with water to volume.

Analysis: A 15.0-mL portion of *Sample solution*, to which 3 mL of hydrochloric acid has been added, meets the requirements of the test, the addition of the 7 N sulfuric acid being omitted.

Acceptance criteria: NMT 8 ppm

SPECIFIC TESTS

- **PH** ⟨791⟩: 3.5–5.5, in a (1 in 25) dispersion
- **LOSS ON DRYING** ⟨731⟩: Dry in a tared platinum crucible at 105° for 2 h: it loses NMT 2.5% of its weight. Retain the dried specimen in the crucible for the test for *Loss on Ignition*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Simethicone—*see Simethicone General Monographs*

Simethicone Emulsion—*see Simethicone Emulsion General Monographs*

Soda Lime

DEFINITION

Soda Lime is a mixture of Calcium Hydroxide and Sodium or Potassium Hydroxide or both. It may contain an indicator that is inert toward anesthetic gases such as Ether, Cyclopropane, and Nitrous Oxide and that changes color when the Soda Lime can no longer absorb Carbon Dioxide.

IDENTIFICATION

- **A.**

Analysis: Place a granule on a piece of moistened red litmus paper.

Acceptance criteria: The paper turns blue immediately.

- **B. IDENTIFICATION TESTS—GENERAL,** *Calcium* ⟨191⟩

Sample solution: A solution in 6 N acetic acid

Acceptance criteria: Meets the requirements. It also imparts a yellow color to a nonluminous flame that, when viewed through cobalt glass, may show a violet color.

SPECIFIC TESTS

- **LOSS ON DRYING** ⟨731⟩

Sample: 10 g

Analysis: Dry at 105° for 2 h.

Acceptance criteria: 12.0%–19.0%

- **CARBON DIOXIDE ABSORBENCY**

Analysis: Fill the lower transverse section of a U-shaped drying tube of 15-mm internal diameter and 15-cm height with loosely packed glass wool. In one arm of the tube, place 5 g of anhydrous calcium chloride, and weigh the tube and the contents. In the other arm, place 9.5–10.5 g of Soda Lime, and again weigh. Insert stoppers in the open arms of the tube, and connect the side tube of the arm filled with Soda Lime to a calcium chloride drying tube, which in turn is connected to a suitable source of supply of carbon dioxide. Pass the carbon dioxide through the tube at 75 mL/min for 20 min, accurately timed. Disconnect the tube, cool to room temperature, remove the stoppers, and weigh.

Acceptance criteria: NLT 19.0% increase in weight of the Soda Lime used for the test

- **HARDNESS**

Sample: 200 g

Analysis: Screen the *Sample* on a mechanical sieve shaker (see *Particle Size Distribution Estimation by Analytical Sieving* ⟨786⟩) having a frequency of oscillation of 285 ± 3 cycles/min, for 3 min, to remove granules both coarser and finer than the labeled particle size. Weigh 50 g of the granules retained on the screen, and place them in a hardness pan that has a diameter of 200 mm and a concave brass bottom 7.9 mm thick at the circumference and 3.2 mm thick at the center, with an inside spherical radius of curvature of 109 cm. Add 15 steel balls of 7.9-mm diameter, and shake on a mechanical sieve shaker for 30 min. Remove the steel balls, brush the contents of the hardness pan onto a sieve of the fine-mesh size designated on the label, shake for 3 min on the mechanical sieve shaker, and weigh.

Acceptance criteria: NLT 75.0% of Soda Lime is retained on the screen.

- **MOISTURE ABSORPTION**

Sample: 10 g

Analysis: Place the *Sample* in a tared 50-mL weighing bottle having a diameter of 50 mm and a height of 30 mm, and weigh. Then place the bottle, with cover removed, for 24 h in a closed container in which the atmosphere is maintained at 85% relative humidity by being in equilibrium with sulfuric acid having a specific gravity of 1.16. Weigh again.

Acceptance criteria: The weight increase is NMT 7.5%.

- **PARTICLE SIZE DISTRIBUTION ESTIMATION BY ANALYTICAL SIEVING,** *Method I* ⟨786⟩

Sample: 100 g

Analysis: Screen the *Sample* for 5 min as directed, using a mechanical shaker.

Acceptance criteria: It passes completely through a No. 2 standard-mesh sieve, and NMT 2.0% passes through a No. 40 standard-mesh sieve. NMT 7.0% is retained on the coarse-mesh sieve, and NMT 15.0% passes through the fine-mesh sieve designated on the label.

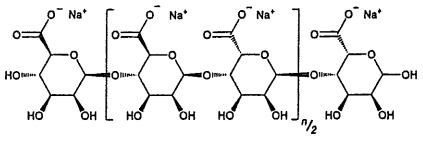
ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **LABELING:** If an indicator has been added, the name and color change of such indicator are stated on the container label. The container label also indicates the mesh size in terms of standard-mesh sieve sizes (see *Powder Fineness* ⟨811⟩).

Sodium Acetate—*see Sodium Acetate General Monographs*

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Sodium Alginate



$(C_6H_7NaO_6)_n$
Alginic acid, sodium salt;
Sodium alginate [9005-38-3].

DEFINITION

Change to read:

Sodium Alginate is the purified carbohydrate product extracted from brown seaweeds by the use of dilute alkali. It consists chiefly of the sodium salt of Alginic Acid, a ▲linear, unbranched, amorphous copolymer of β-D-mannuronic acid (M) and α-L-guluronic acid (G) linked to each other by 1→4 glycosidic bonds. The M and G units in the alginates may be randomly or non-randomly arrayed as heterogeneous or homogeneous sequences. It contains NLT 90.8% and NMT 106.0% of sodium alginate, calculated on the dried basis. The typical average molecular weight for Sodium Alginate is in the range of 10,000–600,000 g/mol.▲ (NF 1-May-2020)

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: 197K or 197A▲ (NF 1-May-2020)

Change to read:

• **B.**
▲Sample solution 1: A solution (1 in 100)
Analysis 1: To 5 mL of *Sample solution 1* add 1 mL of calcium chloride TS.
Acceptance criteria 1: A voluminous, gelatinous precipitate is formed immediately.▲ (NF 1-May-2020)
Sample solution ▲2:▲ (NF 1-May-2020) A solution (1 in 100)
Analysis ▲2:▲ (NF 1-May-2020) To 10 mL of *Sample solution ▲2▲* (NF 1-May-2020) add 1 mL of 4 N sulfuric acid.
Acceptance criteria ▲2:▲ (NF 1-May-2020) A heavy, gelatinous precipitate is formed.

Add the following:

▲• C. IDENTIFICATION TESTS—GENERAL ⟨191⟩, *Chemical Identification Tests, Sodium*
Sample solution: Ignite 0.2 g of Sodium Alginate at 700° for 2 h, and dissolve the residue in 2 mL of water.
15% Potassium carbonate solution: Dissolve 7.5 g of potassium carbonate in 50 mL of water and mix well.
Analysis 1: Transfer 2 mL of the *Sample solution* to a test tube, add 2 mL of *15% Potassium carbonate solution*, and heat to boiling.
Acceptance criteria 1: No precipitate is formed.
Analysis 2: To the test tube from *Analysis 1* add 4 mL of potassium pyroantimonate TS, heat to boiling, cool in ice water, and rub the inside of the test tube with a glass rod.
Acceptance criteria 2: A dense precipitate is formed.▲ (NF 1-May-2020)

ASSAY
• **ALGINATES**
(See *Alginates Assay* ⟨311⟩.)
Sample: 250 mg
Analysis: Each milliliter of 0.2500 N sodium hydroxide consumed is equivalent to 27.75 mg of sodium alginate.
Acceptance criteria: 90.8%–106.0% on the dried basis

IMPURITIES
• **ARSENIC** ⟨211⟩, *Procedures, Method II*: NMT 1.5 ppm
• **LEAD** ⟨251⟩
Standard solution: 5 mL of *Diluted standard lead solution*
Test preparation: Add 1.0 g to 20 mL of nitric acid in a 250-mL conical flask, mix, and heat carefully until the Sodium Alginate is dissolved. Continue heating until the volume is reduced to 7 mL. Cool rapidly to room temperature, transfer to a 100-mL volumetric flask, and dilute with water to volume.
Analysis: Proceed as directed in the chapter, using 15 mL of ammonium citrate solution, 3 mL of potassium cyanide solution, and 0.5 mL of hydroxylamine hydrochloride solution. After the first dithizone extraction, wash the combined chloroform layers with 5 mL of water, discarding the water layer and continuing in the usual manner by extracting with 20 mL of 0.2 N nitric acid.
Acceptance criteria: A 50-mL portion of the *Test preparation* contains NMT 5 µg of lead (corresponding to NMT 10 ppm).

SPECIFIC TESTS
• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩: The total bacterial count does not exceed 200 cfu/g. The tests for *Salmonella* species and *Escherichia coli* are negative.
• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry at 105° for 4 h.
Acceptance criteria: NMT 15.0%
• **ARTICLES OF BOTANICAL ORIGIN,** ⟨561⟩, *Methods of Analysis, Total Ash*
Analysis: Proceed as directed in the chapter, carefully igniting 3 g in a tared platinum dish until the residue is thoroughly carbonized (5 min), and then igniting in a muffle furnace at a temperature of 800 ± 25° until the carbon is completely burned off (approximately 75 min).
Acceptance criteria: 18.0%–27.0% of ash on the dried basis

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight containers.

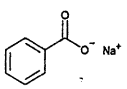
Add the following:

▲• USP REFERENCE STANDARDS ⟨11⟩
USP Sodium Alginate RS▲ (NF 1-May-2020)

Sodium Ascorbate

—see Sodium Ascorbate General Monographs

Sodium Benzoate



$C_7H_5NaO_2$ 144.10

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Benzoic acid, sodium salt;
Sodium benzoate [532-32-1].

DEFINITION
Sodium Benzoate contains NLT 99.0% and NMT 101.0% of sodium benzoate ($C_7H_5NaO_2$), calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
  Sample: Undried sample
  Acceptance criteria: Meets the requirements
- **B. IDENTIFICATION TESTS—GENERAL,** *Sodium* ⟨191⟩**:** Meets the requirements
- **C.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

ASSAY
- **PROCEDURE**
  Solution A: Adjust a 20-mM solution of monobasic potassium phosphate with phosphoric acid to a pH of 2.5.
  Mobile phase: *Solution A* and acetonitrile (70:30)
  Diluent: Water and acetonitrile (50:50)
  System suitability solution: 0.1 mg/mL of USP Salicylic Acid RS and 0.1 mg/mL of USP Benzoic Acid RS in *Diluent*
  Standard solution: 0.1 mg/mL of USP Benzoic Acid RS in *Diluent*
  Sample solution: 0.1 mg/mL of Sodium Benzoate in *Diluent*
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: LC
  Detector: UV 230 nm
  Column: 4.6-mm × 15-cm; 5-µm packing L1
  Column temperature: 25°
  Flow rate: 1.0 mL/min
  Injection volume: 10 µL
  System suitability
  Samples: *System suitability solution* and *Standard solution*
  [NOTE—The relative retention times for benzoic acid and salicylic acid are approximately 1.0 and 1.2, respectively.]
  Suitability requirements
  Resolution: NLT 3.0 between benzoic acid and salicylic acid, *System suitability solution*
  Relative standard deviation: NMT 0.5% for benzoic acid, *Standard solution*
  Tailing factor: NMT 2.0 for benzoic acid, *Standard solution*
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Calculate the percentage of sodium benzoate ($C_7H_5NaO_2$) in the portion of Sodium Benzoate taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of benzoic acid from the *Sample solution*
r_S = peak area of benzoic acid from the *Standard solution*
C_S = concentration of benzoic acid in the *Standard solution*, corrected for purity (mg/mL)
C_U = concentration of Sodium Benzoate in the *Sample solution* (mg/mL)
M_{r1} = molecular weight of sodium benzoate, 144.10
M_{r2} = molecular weight of benzoic acid, 122.12

Acceptance criteria: 99.0%–101.0% on the anhydrous basis

SPECIFIC TESTS
- **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 1.5%
- **ALKALINITY**
  Sample solution: 2 g in 20 mL of hot water
  Analysis: To the *Sample solution* add 2 drops of phenolphthalein TS.
  Acceptance criteria: The pink color produced, if any, is discharged by the addition of 0.20 mL of 0.10 N sulfuric acid.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Benzoic Acid RS
  USP Salicylic Acid RS
  USP Sodium Benzoate RS

Sodium Bicarbonate—*see Sodium Bicarbonate General Monographs*

Sodium Borate

$Na_2B_4O_7 \cdot 10H_2O$ 381.37
$Na_2B_4O_7$ 201.22
Borax [1303-96-4].
Anhydrous [1330-43-4].

DEFINITION
Sodium Borate contains an amount of $Na_2B_4O_7$ equivalent to NLT 99.0% and NMT 105.0% of $Na_2B_4O_7 \cdot 10H_2O$.

IDENTIFICATION
- **A. IDENTIFICATION TESTS—GENERAL,** *Sodium* ⟨191⟩
  Sample solution: 1 in 20
  Acceptance criteria: Meets the requirements
- **B. IDENTIFICATION TESTS—GENERAL,** *Borate* ⟨191⟩
  Sample solution: 1 in 20
  Acceptance criteria: Meets the requirements

ASSAY
- **PROCEDURE**
  Sample: 3 g of Sodium Borate
  Titrimetric system
  (See *Titrimetry* ⟨541⟩.)
  Mode: Direct titration
  Titrant: 0.5 N hydrochloric acid VS
  Blank: 50 mL of water
  Endpoint detection: Visual
  Analysis: Dissolve the *Sample* in 50 mL of water, add methyl red TS, and titrate with 0.5 N hydrochloric acid VS.
  [NOTE—Heating on a steam bath may be required initially to effect solution.]
  Calculate the percentage of sodium borate ($Na_2B_4O_7 \cdot 10H_2O$) in the *Sample* taken:

$$\text{Result} = [(V - B) \times N \times F] \times 100/W$$

V = volume of *Titrant* consumed by the *Sample* (mL)
B = volume of *Titrant* consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 190.7 mg/mEq

NF Monographs

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W = weight of the *Sample* (mg)

Acceptance criteria: 99.0%–105.0%

IMPURITIES

• **CARBONATE AND BICARBONATE**

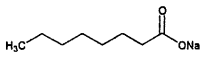
Sample solution: To 5 mL of a solution (1 in 20), contained in a test tube, add 1 mL of 3 N hydrochloric acid.

Acceptance criteria: No effervescence is observed.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers.

Sodium Caprylate



$C_8H_{15}NaO_2$ 166.19

Sodium octanoate [1984-06-1].

DEFINITION

Sodium Caprylate contains NLT 99.0% and NMT 101.0% of sodium caprylate ($C_8H_{15}NaO_2$), calculated on the anhydrous basis.

IDENTIFICATION

• **A.** The retention time of the major peak of *Sample solution A* corresponds to that of the *Standard solution*, as obtained in the test for *Chromatographic Purity* in *Impurities*.

• **B.**

Methoxyphenylacetic reagent: Dissolve 2.7 g of methoxyphenylacetic acid in 6 mL of 10% tetramethylammonium hydroxide solution in methanol, and add 20 mL of alcohol. Store in a polyethylene container.

Sample solution: 20 mg

Analysis: Dissolve the *Sample* in 0.5 mL of water, add 1.5 mL of *Methoxyphenylacetic reagent*, and cool in ice water for 30 min. A voluminous, white, crystalline precipitate is formed. Place in water at 20°, and stir for 5 min. The precipitate does not disappear. Add 1 mL of ammonia TS. The precipitate dissolves completely. Add 1 mL of ammonium carbonate solution (160 mg/mL).

Acceptance criteria: No precipitate is formed.

ASSAY

• **PROCEDURE**

Sample: 150 mg

Blank: Glacial acetic acid

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Direct titration

Titrant: 0.1 N perchloric acid VS

Endpoint detection: Potentiometric

Analysis: Transfer the *Sample* to a 125-mL volumetric flask, and dissolve in 50 mL of glacial acetic acid. Titrate with *Titrant*. Perform a blank determination, and make any necessary correction. Each mL of 0.1 N perchloric acid is equivalent to 16.62 mg of sodium caprylate ($C_8H_{15}NaO_2$).

Acceptance criteria: 99.0%–101.0% on the anhydrous basis

IMPURITIES

• **CHROMATOGRAPHIC PURITY**

Standard solution: 1.0 mg/mL of USP Caprylic Acid RS in ethyl acetate

Sample solution A: Dissolve 116 mg of Sodium Caprylate in 5 mL of water, add 1 mL of dilute sulfuric acid (1 in 35), and extract with 10 mL of ethyl acetate. Separate the organic layer, and dry it over anhydrous sodium sulfate.

Sample solution B: Dilute 1.0 mL of *Sample solution A* with ethyl acetate to 100 mL, transfer 5.0 mL of the solution obtained, and dilute with ethyl acetate to 50 mL.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.25-mm × 30-m fused silica; coated with a 0.25-µm layer of phase G25

Temperatures

Injection port: 250°

Detector: 250°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
100	—	100	1
100	5	220	10

Flow rate: 1.5 mL/min

Carrier gas: Helium

Injection volume: 1 µL

Injection type: Split ratio, 100:1

System suitability

Sample: *Sample solution B*

Suitability requirements

Signal-to-noise ratio: NLT 5

Analysis

Samples: *Standard solution*, *Sample solution A*, and *Sample solution B*

Disregard any peaks with an area less than half of the area of the principal peak from *Sample solution B* and any peak due to the solvent.

Calculate the percentage of each impurity in the portion of Sodium Caprylate taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of the individual impurity

r_T = sum of all the peak responses

Acceptance criteria

Individual impurities: NMT 0.3%

Total impurities: NMT 0.5%

SPECIFIC TESTS

• **APPEARANCE OF SOLUTION**

Standard stock solution: Combine 30.0 mL of ferric chloride CS, 30.0 mL of cobaltous chloride CS, and 24.0 mL of cupric sulfate CS, and dilute with 1% (w/v) hydrochloric acid to 100.0 mL.

Standard solution: Dilute 1.0 mL of *Standard stock solution* with 1% (w/v) hydrochloric acid to 100.0 mL.

Sample solution: Dissolve 2.5 g of Sodium Caprylate in 25.0 mL of freshly boiled and cooled water.

Acceptance criteria: The *Sample solution* is clear and colorless, or not more intensely colored than the *Standard solution*.

• **pH** ⟨791⟩

Sample solution: Use the *Sample solution* in the test for *Appearance of Solution*.

Acceptance criteria: 8.0–10.5

• **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 3.0%

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ADDITIONAL REQUIREMENTS
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Caprylic Acid RS

Sodium Carbonate

Na_2CO_3 (anhydrous) 105.99

$Na_2CO_3 \cdot H_2O$ 124.00

Carbonic acid, disodium salt;
Disodium carbonate [497-19-8].
Monohydrate [5968-11-6].

DEFINITION
Sodium Carbonate is anhydrous or contains one molecule of water of hydration. It contains NLT 99.5% and NMT 100.5% of Na_2CO_3, calculated on the anhydrous basis.

IDENTIFICATION
- **A. IDENTIFICATION TESTS—GENERAL,** *Sodium* ⟨191⟩**:** Meets the requirements
- **B. IDENTIFICATION TESTS—GENERAL,** *Carbonate* ⟨191⟩**:** Meets the requirements

ASSAY
- **PROCEDURE**

Sample: 2 g of Sodium Carbonate, previously dried, from the test for *Water Determination*
Blank: 50 mL of water
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 1 N sulfuric acid VS
Endpoint detection: Visual
Analysis: Transfer the *Sample* to a flask with the aid of 50 mL of water. Add methyl red TS, and titrate with 1 N sulfuric acid VS. Add the acid slowly, with constant stirring, until the solution becomes faintly pink. Heat the solution to boiling, cool, and continue the titration. Heat again to boiling, and titrate further as necessary until the faint pink color is no longer affected by continued boiling.
Calculate the percentage of sodium carbonate (Na_2CO_3) in the *Sample* taken.

$$\text{Result} = [(V - B) \times N \times F \times 100]/W$$

V = *Titrant* volume consumed by the *Sample* (mL)
B = *Titrant* volume consumed by the *Blank* (mL)
N = *Titrant* actual normality (mEq/mL)
F = equivalency factor, 52.99 mg/mEq
W = weight of the *Sample* (mg)

Acceptance criteria: 99.5%–100.5% on the anhydrous basis

SPECIFIC TESTS
- **WATER DETERMINATION,** *Method III* ⟨921⟩

Sample: 2 g
Analysis: Dry the *Sample* at 105° for 4 h.
Acceptance criteria: The anhydrous form loses NMT 0.5% of its weight, and the hydrous form loses 12.0%–15.0% of its weight.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **LABELING:** Label it to indicate whether it is anhydrous or hydrous.

Sodium Cetostearyl Sulfate

DEFINITION
Sodium Cetostearyl Sulfate is a mixture of sodium cetyl sulfate and sodium stearyl sulfate. It contains NLT 40.0% of sodium cetyl sulfate ($C_{16}H_{33}NaSO_4$), and the sum of the sodium cetyl sulfate content and sodium stearyl sulfate ($C_{18}H_{37}NaSO_4$) content is NLT 90.0% (both contents calculated on the anhydrous basis). It may contain a suitable buffer.

IDENTIFICATION
- **A.** The retention times of the two major peaks of *Sample solution C* correspond to those of the *System suitability solution*, as obtained in the *Assay*.
- **B.** Sodium Cetostearyl Sulfate imparts an intense yellow color to a nonluminous flame.
- **C.**

Sample solution: 1.0 mg/mL in alcohol
Analysis: Heat 10 mL of the *Sample solution* to boiling on a water bath, shaking frequently. Filter immediately, and evaporate to dryness. Dissolve the residue in 7 mL of water, add 3 mL of diluted hydrochloric acid, and evaporate the solution to half its volume. Allow to cool, and filter. To the filtrate add 1 mL of barium chloride solution (60 mg/mL).
Acceptance criteria: A white crystalline precipitate is formed.

ASSAY
- **PROCEDURE**

System suitability solution: 5 mg/mL each of USP Cetyl Alcohol RS and USP Stearyl Alcohol RS in alcohol
Internal standard solution: 4 mg/mL of 1-heptadecanol in alcohol
Sample solution A: Dissolve 300 mg of Sodium Cetostearyl Sulfate in 50 mL of alcohol, and add 2 mL of the *Internal standard solution* and 48 mL of water. Extract the solution with four 25-mL portions of pentane, adding 10–15 mL of saturated sodium chloride solution, if necessary, to facilitate the separation of the layers. Combine the organic layers, and reserve the hydroalcoholic layers for the preparation of *Sample solution C* and *Sample solution D*. Wash the organic layer with two 30-mL portions of water, dry over anhydrous sodium sulfate, and filter.
Sample solution B: Dissolve 300 mg of Sodium Cetostearyl Sulfate in 50 mL of alcohol, and add 50 mL of water. Extract the solution with four 25-mL portions of pentane, adding 10–15 mL of saturated sodium chloride solution, if necessary, to facilitate the separation of the layers. Combine the organic layers, wash with two 30-mL portions of water, dry over anhydrous sodium sulfate, and filter.
Sample solution C: Transfer 25 mL of the hydroalcoholic solution obtained in the preparation of *Sample solution A* to a 200-mL flask that can be fitted with a reflux condenser. Add 20 mL of hydrochloric acid and 10 mL of the *Internal standard solution*, and boil under reflux for 2 h. Allow to cool. Extract with four 20-mL portions of pentane. Wash the combined organic layer with two 20-mL portions of water, dry over anhydrous sodium sulfate, and filter.
Sample solution D: Transfer 25 mL of the hydroalcoholic solution obtained in the preparation of *Sample solution A* to a 200-mL flask that can be fitted with a reflux condenser. Add 20 mL of hydrochloric acid and 10 mL of alcohol, and boil under reflux for 2 h. Allow to cool. Extract with four 20-mL portions of pentane. Wash the combined organic layer with two 20-mL portions of water, dry over anhydrous sodium sulfate, and filter.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC

NF Monographs

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Detector: Flame ionization
Column: 0.25-mm × 25-m fused silica capillary; phase G2
Temperatures
Injection port: 250°
Detector: 250°
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
150	5	250	Duration of analysis

Carrier gas: Nitrogen
Flow rate: 1 mL/min
Injection volume: 1 µL
Injection type: Split ratio 100:1
System suitability
Sample: *System suitability solution*
Suitability requirements
Resolution: NLT 4.0 between cetyl alcohol and stearyl alcohol
Relative standard deviation: NMT 1.5%
Analysis
Correction for interference: Inject *Sample solution A* and *Sample solution B* into the chromatograph, record the chromatograms, and measure the areas for the major peaks.
If *Sample solution B* shows a peak at the same retention time as the internal standard peak of *Sample solution A*, calculate the ratio, *R*:

$$R = S_{CB}/S_I$$

S_{CB} = peak response of cetyl alcohol from *Sample solution B*
S_I = peak response with the same retention time as the internal standard of *Sample solution B*

If *R* is less than 300, calculate the corrected area, $S_{A(corr)}$, of the peak corresponding to the internal standard of *Sample solution A*:

$$S_{A(corr)} = S_{HA} - (S_I \times S_{CA}/S_{CB})$$

S_{HA} = peak response of the internal standard from *Sample solution A*
S_I = peak response with the same retention time as the internal standard of *Sample solution B*
S_{CA} = peak response of cetyl alcohol from *Sample solution A*
S_{CB} = peak response of cetyl alcohol from *Sample solution B*

Inject *Sample solution C* and *Sample solution D* into the chromatograph, record the chromatograms, and measure the areas for the major peaks. Carry out the *Correction for interference* in the same manner as for *Sample solution A*, and calculate the corrected area of the peak corresponding to the internal standard of *Sample solution C*, $S_{C(corr)}$.
Samples: *System suitability solution*, *Sample solution C*, and *Sample solution D*
[NOTE—The substances are eluted in the following order: cetyl alcohol, 1-heptadecanol (internal standard), and stearyl alcohol. Identify the cetyl alcohol and stearyl alcohol peaks in the chromatograms of the *Sample solutions* by comparison with the *System suitability solution*.]
Calculate the percentage of sodium cetyl sulfate ($C_{16}H_{33}NaSO_4$) in the portion of Sodium Cetostearyl Sulfate taken:

$$\text{Result} = (r_C \times W_{CH})/(S_{C(corr)} \times W_C) \times F \times 100$$

r_C = peak response of cetyl alcohol from *Sample solution C*
W_{CH} = weight of the internal standard added in the preparation of *Sample solution C* (mg)
$S_{C(corr)}$ = corrected area of the peak corresponding to the internal standard of *Sample solution C*
W_C = weight of Sodium Cetostearyl Sulfate taken to prepare *Sample solution C*, calculated on the anhydrous basis (mg)
F = correction factor, 1.421

Calculate the percentage of sodium stearyl sulfate ($C_{18}H_{37}NaSO_4$) in the portion of Sodium Cetostearyl Sulfate taken:

$$\text{Result} = (B_C \times W_{CH})/(S_{C(corr)} \times W_C) \times F \times 100$$

B_C = peak response of stearyl alcohol from *Sample solution C*
W_{CH} = weight of the internal standard added in the preparation of *Sample solution C* (mg)
$S_{C(corr)}$ = corrected area of the peak corresponding to the internal standard of *Sample solution C*
W_C = weight of Sodium Cetostearyl Sulfate taken to prepare *Sample solution C*, calculated on the anhydrous basis (mg)
F = correction factor, 1.377

Acceptance criteria
Sodium cetyl sulfate: NLT 40.0% on the anhydrous basis
Sum of sodium cetyl sulfate and sodium stearyl sulfate: NLT 90.0% on the anhydrous basis

IMPURITIES

• LIMIT OF SODIUM CHLORIDE AND SODIUM SULFATE
Sodium chloride
Sample: 5 g
Titrimetric system
Mode: Direct titration
Titrant: 0.1 N silver nitrate VS
Endpoint detection: Potentiometric
Analysis: Dissolve the *Sample* in 50 mL of water, and add diluted nitric acid dropwise until the solution is neutral to blue litmus paper. To the resulting solution add 1 mL of potassium chromate TS and titrate with *Titrant*.
Calculate the percentage of sodium chloride (NaCl) in the portion of Sodium Cetostearyl Sulfate taken:

$$\text{Result} = (V \times N)/W \times F$$

V = volume of the *Titrant* (mL)
N = actual normality of the *Titrant*
W = weight of Sodium Cetostearyl Sulfate (g)
F = equivalence factor for sodium chloride, 5.844

Sodium sulfate
Dichloroacetic acid solution: Dilute 67 mL of dichloroacetic acid with water to 300 mL, and neutralize to blue litmus paper using ammonia TS. Cool, add 33 mL of dichloroacetic acid, and dilute with water to 600 mL.
Sample: 0.5 g

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Titrimetric system
Mode: Direct titration
Titrant: 0.01 M lead nitrate VS
Endpoint detection: Visual
Analysis: Dissolve the *Sample* in 20 mL of water, warming gently if necessary, and add 1 mL of a solution containing 0.5 g/L of dithizone in acetone. If the solution is red, add 1 N nitric acid dropwise until a bluish-green color is obtained. To the resulting solution add 2.0 mL of *Dichloroacetic acid solution* and 80 mL of acetone, and titrate with *Titrant* until a persistent orange-red color is obtained.
Calculate the percentage of sodium sulfate (Na_2SO_4) in the portion of Sodium Cetostearyl Sulfate taken:

$$\text{Result} = (V \times M)/W \times F$$

V = volume of *Titrant* (mL)
M = actual molarity of *Titrant*
W = weight of Sodium Cetostearyl Sulfate (g)
F = equivalence factor for sodium sulfate, 14.20

Acceptance criteria: The sum of the percentages of sodium chloride and sodium sulfate is NMT 8.0%.

• **LIMIT OF FREE CETOSTEARYL ALCOHOL**
Analysis: Examine the chromatogram of *Sample solution A*, obtained as directed in the *Assay*.
Calculate the percentage of free cetostearyl alcohol in the portion of Sodium Cetostearyl Sulfate taken:

$$\text{Result} = 100(r_A + r_B) \times W_{IS}/(S_{A(corr)} \times W)$$

r_A = peak response of the cetyl alcohol peak from *Sample solution A*
r_B = peak response of stearyl alcohol from *Sample solution A*
W_{IS} = weight of the internal standard added in the preparation of *Sample solution A* (mg)
$S_{A(corr)}$ = corrected peak area corresponding to the internal standard of *Sample solution A* (see *Assay*)
W = weight of Sodium Cetostearyl Sulfate taken to prepare *Sample solution A* (mg)

Acceptance criteria: NMT 4.0%

SPECIFIC TESTS

• **ACIDITY OR ALKALINITY**
Sample: 500 mg
Analysis: Dissolve the *Sample* by heating in a mixture of 10 mL of water and 15 mL of 90% alcohol. Add 0.1 mL of phenolphthalein TS.
Acceptance criteria: The resulting solution is colorless. Add 0.1 mL of 0.1 N sodium hydroxide, and the resulting solution becomes red.

• **WATER DETERMINATION** ⟨921⟩, *Method I*: NMT 1.5%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.
• **LABELING:** Label it to indicate the name and concentration of any added buffer.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Cetyl Alcohol RS
USP Stearyl Alcohol RS

Sodium Chloride—*see Sodium Chloride General Monographs*

Sodium Chloride Injection—*see Sodium Chloride Injection General Monographs*

Sodium Chloride Injection, Bacteriostatic
—*see Bacteriostatic Sodium Chloride Injection General Monographs*

Sodium Citrate—*see Sodium Citrate General Monographs*

Sodium Dehydroacetate

$C_8H_7NaO_4$ 190.13
2*H*-Pyran-2,4(3*H*)-dione, 3-acetyl-6-methyl-, monosodium salt [4418-26-2].

DEFINITION
Sodium Dehydroacetate contains NLT 98.0% and NMT 100.5% of sodium dehydroacetate ($C_8H_7NaO_4$), calculated on the anhydrous basis.

IDENTIFICATION

• **A. MELTING RANGE OR TEMPERATURE** ⟨741⟩
Sample solution: 150 mg/mL
Analysis: To 10 mL of the *Sample solution* add 5 mL of 3 N hydrochloric acid, collect the crystals by filtration with suction, wash with 10 mL of water, and dry at 80° for 4 h. Determine the melting point as directed in the chapter.
Acceptance criteria: 109°–111°

• **B. IDENTIFICATION TESTS—GENERAL,** *Sodium* ⟨191⟩
Sample solution: 1 in 20
Acceptance criteria: Meets the requirements

ASSAY

• **PROCEDURE**
Sample: 500 mg
Blank: 25 mL of glacial acetic acid containing *p*-naphtholbenzein TS, which has been previously neutralized to a blue color
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.1 N perchloric acid VS
Endpoint detection: Visual
Analysis: Transfer the *Sample* to a 125-mL conical flask, and dissolve it in 25 mL of glacial acetic acid containing *p*-naphtholbenzein TS, which has been previously neutralized to a blue color. Titrate with 0.1 N perchloric acid VS to the original blue color. Perform a blank determination.
Calculate the percentage of dehydroacetate ($C_8H_7NaO_4$) in the *Sample* taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

V_S = volume of the *Titrant* consumed by the *Sample* (mL)
V_B = volume of the *Titrant* consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 190.1 mg/mEq

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W = weight of the *Sample* (mg)

Acceptance criteria: 98.0%–100.5% on the anhydrous basis

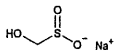
SPECIFIC TESTS

• **WATER DETERMINATION,** *Method I* ⟨921⟩**:** 8.5%–10.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Sodium Formaldehyde Sulfoxylate



CH_3NaO_3S 118.09

$CH_3NaO_3S \cdot 2H_2O$ 154.11

Methanesulfinic acid, hydroxy-, monosodium salt; Monosodium hydroxymethanesulfinate [149-44-0]. Dihydrate [6035-47-8].

DEFINITION

Sodium Formaldehyde Sulfoxylate contains an amount of sodium formaldehyde sulfoxylate (CH_3NaO_3S) equivalent to NLT 45.5% and NMT 54.5% of SO_2, calculated on the dried basis. It may contain a suitable stabilizer, such as sodium carbonate.

IDENTIFICATION

• **A.**

Sample solution: Dissolve 4 g in 10 mL of water in a test tube.

Analysis: To the *Sample solution* add 1 mL of silver–ammonia–nitrate TS.

Acceptance criteria: Metallic silver is produced, either as a finely divided, gray precipitate or as a bright metallic mirror on the inner surface of the tube.

• **B.**

Sample solution: Dissolve 40 mg of salicylic acid in 5 mL of sulfuric acid, and add 50 mg of Sodium Formaldehyde Sulfoxylate.

Analysis: Warm very gently.

Acceptance criteria: A permanent, deep red color appears.

ASSAY

• **PROCEDURE**

Sample: 1 g

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Direct titration

Titrant: 0.1 N iodine VS. [NOTE—Prepare an adequate amount for both the *Assay* and the test for *Sodium Sulfite*.]

Endpoint detection: Visual

Analysis: Transfer the *Sample* to a 50-mL volumetric flask, dissolve in 25 mL of water, and dilute with water to volume. Reserve a portion of this solution for the test for *Sodium Sulfite*. Transfer 4.0 mL of the remaining solution to a conical flask containing 100 mL of water. Titrate with *Titrant*, adding 3 mL of starch TS as the endpoint is approached. Each mL of 0.1 N iodine is equivalent to 1.602 mg of SO_2.

Acceptance criteria: 45.5%–54.5% of SO_2 on the dried basis

IMPURITIES

• **SULFIDE**

Analysis: Dissolve 6 g in 14 mL of water in a test tube, and wet a strip of lead acetate test paper with the clear solution.

Acceptance criteria: No discoloration is evident within 5 min.

• **IRON**

Standard solution: Dissolve 43.2 mg of ferric ammonium sulfate in 10 mL of 2 N sulfuric acid, and add water to make 1000 mL, each mL representing 5 µg of Fe.

Sample solution: Transfer 1.0 g of Sodium Formaldehyde Sulfoxylate to a suitable crucible, and carefully ignite, initially at a low temperature until thoroughly charred, and finally, preferably in a muffle furnace, at 500°–600° until the carbon is all burned off. Cool, dissolve the residue in 2 mL of hydrochloric acid, and dilute with water to 50 mL.

Analysis: To 5.0 mL of the *Standard solution* and 50 mL of the *Sample solution* add 50 mg of ammonium persulfate and 5 mL of ammonium thiocyanate TS, and transfer each to a separate color comparison tube.

Acceptance criteria: 0.0025%; the color of the *Sample* is not deeper than that of the *Standard solution*.

• **SODIUM SULFITE**

Sample solution: 4.0 mL of the solution prepared for the *Assay* in a conical flask containing 100 mL of water

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Direct titration

Titrant: 0.1 N iodine VS, prepared in the *Assay*

Endpoint detection: Visual

Analysis: Add 2 mL of formaldehyde TS to the *Sample solution*, and titrate with the *Titrant*, adding 3 mL of starch TS as the endpoint is approached.

Calculate the percentage of sodium sulfite (Na_2SO_3) in the Sodium Formaldehyde Sulfoxylate taken:

$$\text{Result} = (V_2 - V_1) \times (N/W) \times (F \times 1.25)$$

V_2 = volume of 0.1 N iodine VS consumed in the titration performed in the *Assay* (mL)

V_1 = volume of 0.1 N iodine VS consumed in this titration (mL)

N = actual normality of the *Titrant* (mEq/mL)

W = weight of the *Sample* in the *Assay* (g)

F = equivalency weight of sodium sulfite, 63.02 mg/mEq

Acceptance criteria: NMT 5.0% on the dried basis

SPECIFIC TESTS

• **PH** ⟨791⟩

Sample solution: 20 mg/mL

Acceptance criteria: 9.5–10.5

• **LOSS ON DRYING** ⟨731⟩

Analysis: Dry at 105° for 3 h.

Acceptance criteria: NMT 27.0%

• **ALKALINITY**

Sample solution: 1.0 g of Sodium Formaldehyde Sulfoxylate in 50 mL of water

Analysis: To the *Sample solution* add phenolphthalein TS, and titrate with 0.10 N sulfuric acid.

Acceptance criteria: NMT 3.5 mL is required for neutralization.

• **CLARITY AND COLOR OF SOLUTION**

Sample solution: 1 g of Sodium Formaldehyde Sulfoxylate in 20 mL of water

Analysis: Transfer 10 mL of the *Sample solution* to a 20- × 150-mm test tube. Compare with water in a similar test tube.

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Acceptance criteria: The *Sample solution* and the water are equally clear and, when viewed transversely by transmitted light, exhibit no apparent difference in color.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed, light-resistant containers, and store at controlled room temperature.

Sodium Hydroxide

NaOH 40.00
Sodium hydroxide [1310-73-2].

DEFINITION

Sodium Hydroxide contains NLT 95.0% and NMT 100.5% of total alkali, calculated as sodium hydroxide (NaOH), including NMT 3.0% of sodium carbonate (Na_2CO_3). It also contains NLT 54.0% and NMT 59.8% of sodium.
[**CAUTION**—Exercise great care in handling sodium hydroxide, because it rapidly destroys tissues.]

IDENTIFICATION

- **A. IDENTIFICATION TESTS—GENERAL** ⟨191⟩, *Chemical Identification Tests, Sodium*: A solution (1 in 25) meets the requirements.
- **B. pH** ⟨791⟩
  Sample solution: 0.1 mg/mL of Sodium Hydroxide
  Acceptance criteria: NLT 11.0

ASSAY

- **TOTAL ALKALI**
  Sample: 1.5 g
  Blank: 40.0 mL of carbon dioxide-free water
  Titrimetric system
  (See *Titrimetry* ⟨541⟩.)
  Mode: Direct titration
  Titrant: 1 N sulfuric acid VS
  Endpoint detection: Visual
  Analysis: Dissolve the *Sample* in 40 mL of carbon dioxide-free water. Cool the solution to room temperature, and add phenolphthalein TS. Titrate with 1 N sulfuric acid VS. At the discharge of the pink color of the indicator, record the volume of *Titrant* (V_{S1}). Add methyl orange TS, and continue the titration until a persistent pink color is produced. Record the volume of *Titrant* (V_{S2}). Perform a blank determination, and make any necessary corrections. Calculate the percentage of total alkali, calculated as sodium hydroxide (NaOH), in the *Sample* taken:

$$\text{Result} = \{[(V_{S2} - V_B) \times N \times F_1]/W\} \times 100$$

V_{S2} = volume of *Titrant* consumed by the *Sample* to the second endpoint (mL)
V_B = volume of *Titrant* consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F_1 = equivalency factor, 40.00 mg/mEq
W = weight of the *Sample* (mg)

Calculate the percentage of sodium carbonate (Na_2CO_3) in the *Sample* taken:

$$\text{Result} = \{[(V_{S2} - V_{S1}) \times N \times F_2]/W\} \times 100$$

V_{S2} = volume of *Titrant* consumed by the *Sample* to the second endpoint (mL)
V_{S1} = volume of *Titrant* consumed by the *Sample* to the first endpoint (mL)
N = actual normality of the *Titrant* (mEq/mL)
F_2 = equivalency factor, 106.0 mg/mEq
W = weight of the *Sample* (mg)

Acceptance criteria: 95.0%–100.5% of total alkali; NMT 3.0% of sodium carbonate (Na_2CO_3)

- **CONTENT OF SODIUM**
  Diluent: 1% hydrochloric acid solution
  Standard stock solution: 25.41 µg/mL of sodium chloride in *Diluent*. This solution contains 10 µg/mL of sodium.
  Standard solutions: Transfer 6.0-, 7.5-, and 9.0-mL portions of *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the content of each flask with *Diluent* to volume, and mix to obtain solutions having known concentrations of 0.6, 0.75, and 0.9 µg/mL of sodium, respectively.
  Sample stock solution: 1.303 mg/mL of Sodium Hydroxide in *Diluent*
  Sample solution: Transfer 0.1 mL of *Sample stock solution* to a 100-mL volumetric flask and dilute with *Diluent* to volume.
  Instrumental conditions
  (See *Atomic Absorption Spectroscopy* ⟨852⟩.)
  Mode: Atomic absorption spectrophotometry
  Analytical wavelength: 589.0 nm (sodium emission line)
  Lamp: Sodium hollow-cathode
  Flame: Air–acetylene
  Blank: *Diluent*
  Standard curve
  Samples: *Standard solutions*
  Plot: Absorbance values versus their corresponding concentration (µg/mL) of sodium. The correlation coefficient is NLT 0.995.
  Analysis
  Sample: *Sample solution*
  From the *Standard curve*, determine the concentration of sodium in the *Sample solution*.
  Calculate the percentage of sodium in the portion of Sodium Hydroxide taken:

$$\text{Result} = (C_S/C_U) \times 100$$

C_S = concentration of sodium in the *Sample solution* from the *Standard curve* (µg/mL)
C_U = concentration of Sodium Hydroxide in the *Sample solution* (µg/mL)

Acceptance criteria: 54.0%–59.8%

IMPURITIES

- **POTASSIUM**
  Diluent: 1% hydrochloric acid solution
  Standard stock solution: 1.907 mg/mL of potassium chloride, previously dried at 105° for 2 h, in water. Transfer 5.0 mL of this solution to a 1.0-L volumetric flask and dilute with *Diluent* to volume. This solution contains 5 µg/mL of potassium.
  Standard solutions: Transfer 2.0-, 5.0-, and 10.0-mL portions of the *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the content of each flask with *Diluent* to volume, and mix to obtain solutions having known concentrations of 0.10, 0.25, and 0.50 µg/mL of potassium, respectively.
  Sample stock solution: 0.5 mg/mL of Sodium Hydroxide
  Sample solution: 50 µg/mL of Sodium Hydroxide in *Diluent*, prepared from the *Sample stock solution*
  Instrumental conditions
  (See *Atomic Absorption Spectroscopy* ⟨852⟩.)
  Mode: Atomic absorption spectrophotometry

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Analytical wavelength: 766.5 nm (potassium emission line)
Lamp: Potassium hollow-cathode
Flame: Air–acetylene
Blank: *Diluent*
Standard curve
Samples: *Standard solutions*
Plot: Absorbance values versus their corresponding concentration (µg/mL) of potassium. The correlation coefficient is NLT 0.99.
Analysis
Sample: *Sample solution*
From the *Standard curve*, determine the concentration of potassium in the *Sample solution*.
Calculate the percentage of potassium in the portion of Sodium Hydroxide taken:

$$\text{Result} = (C_S/C_U) \times 100$$

C_S = concentration of potassium in the *Sample solution* from the *Standard curve* (µg/mL)
C_U = concentration of Sodium Hydroxide in the *Sample solution* (µg/mL)

Acceptance criteria: NMT 0.5%

SPECIFIC TESTS
• **INSOLUBLE SUBSTANCES AND ORGANIC MATTER:** A solution (1 in 20) is complete, clear, and colorless to slightly colored.

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight containers.

Sodium Lauryl Sulfate

Portions of this monograph that are national *USP* text, and are not part of the harmonized text, are marked with symbols (• •) to specify this.

Sulfuric acid monododecyl ester sodium salt;
Sodium monododecyl sulfate [151-21-3].

DEFINITION
Sodium Lauryl Sulfate is a mixture of sodium alkyl sulfates consisting chiefly of sodium lauryl sulfate ($C_{12}H_{25}NaO_4S$). It contains NLT 85.0% of sodium alkyl sulfates calculated as sodium lauryl sulfate ($C_{12}H_{25}NaO_4S$).

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197A** or **197K**▲ (CN 1-May-2020)•
• **B. SODIUM**
Potassium pyroantimonate solution: To 2 g of potassium pyroantimonate add 100 mL of water. Boil the solution for about 5 min, cool quickly, and add 10 mL of a solution of potassium hydroxide (3 in 20). Allow to stand for 24 h, and filter.
Sample: 2.5 g
Analysis: Place the *Sample* in a silica or platinum crucible, and add 2 mL of 10 N sulfuric acid. Heat on a water bath, then cautiously raise the temperature progressively over an open flame. Ignite, preferably in a muffle furnace, at 600 ± 25°. Continue heating until all black particles have disappeared. Cool, add a few drops of 2 N sulfuric acid, and heat and ignite as above. Add a few drops of ammonium carbonate TS, evaporate to dryness, and ignite as above. Cool, dissolve the residue in 50 mL of water, and mix. To a 2-mL portion of this solution, add 4 mL of *Potassium pyroantimonate solution*. If necessary, rub the inside of the test tube with a glass rod.
Acceptance criteria: A white, crystalline precipitate is formed.
• **C. SULFATE**
Sample: Solution (1 in 10)
Analysis: After acidification with hydrochloric acid and boiling for 20 min, no precipitate is formed. Add barium chloride TS; a white precipitate is produced.
Acceptance criteria: Meets the requirements
• **D.**
Sample: 0.1 g
Analysis: Dissolve the *Sample* in 10 mL of water and shake.
Acceptance criteria: A copious foam is formed.
• **E.**
Sample: Solution prepared for *Identification D*
Analysis: To 0.1 mL of the *Sample*, add 0.1 mL of a 1 g/L solution of methylene blue and 2 mL of sulfuric acid, diluted. Add 2 mL of methylene chloride and shake.
Acceptance criteria: An intense blue color develops in the methylene chloride layer.

ASSAY
• **CONTENT OF SODIUM ALKYL SULFATES**
Methylene blue solution: Dissolve 0.003 g of methylene blue, 5.0 g of anhydrous sodium sulfate, and 1.2 g of sulfuric acid in 100 mL of water.
[NOTE—Anhydrous sodium sulfate is the emulsion breaker.]
Sample: 1.5 g of Sodium Lauryl Sulfate
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.004 M benzethonium chloride VS
Endpoint detection: Visual
Analysis
Dissolve the *Sample* in water, warming if necessary, and dilute with water to exactly 1000.0 mL. To 10.0 mL of the solution, add 25 mL of *Methylene blue solution*, 15 mL of methylene chloride, and 20 mL of water. Titrate with *Titrant*, shaking vigorously and allowing the layers to separate before each addition, until the two layers are almost the same blue color. One mL of *Titrant* is equivalent to 1.154 mg of sodium alkyl sulfates, calculated as sodium lauryl sulfate ($C_{12}H_{25}NaO_4S$).
Acceptance criteria: NLT 85.0%, calculated as sodium lauryl sulfate ($C_{12}H_{25}NaO_4S$)

IMPURITIES
• **SODIUM CHLORIDE**
Fluorescein sodium solution: Dissolve 0.2 g of fluorescein sodium in water to 100 mL.
Dilute nitric acid: Dilute 105 mL of nitric acid with water to 1000 mL.
Sample solution: 100 mg/mL of Sodium Lauryl Sulfate in water
Analysis: Neutralize 50 mL of *Sample solution* with *Dilute nitric acid*, using litmus paper as the indicator, if necessary. Add exactly 5.0 mL of 0.1 N sodium chloride and titrate with 0.1 N silver nitrate VS (indicator: 2 drops of *Fluorescein sodium solution*) to the first appearance of turbidity with solution color change from yellow-green to orange through yellow. Perform a blank determination, and make any necessary correction. Each milliliter of 0.1 N silver nitrate is equivalent to 5.844 mg of sodium chloride.
Acceptance criteria: The combined content of sodium chloride and sodium sulfate is NMT 8.0%.

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• **SODIUM SULFATE**
Sample solution: 100 mg/mL of Sodium Lauryl Sulfate in water
Analysis: To 10 mL of *Sample solution*, add 100 mL of alcohol and heat at a temperature just below the boiling point for 2 h. Pass through a glass filter (pore size equivalent to 5–10 µm) while hot, and wash with 100 mL of boiling alcohol. Dissolve the precipitate by washing with 150 mL of water, collecting the washings in a beaker. Add 10 mL of hydrochloric acid, diluted, heat to boiling, add 25 mL of barium chloride TS, and allow to stand overnight. Collect the precipitate and wash with water until the last washing shows no opalescence with 0.1 N silver nitrate. Dry the precipitate, ignite to constant mass between 500° and 600° by raising the temperature gradually, and weigh as barium sulfate ($BaSO_4$; 233.39).

Amount (mg) of sodium sulfate (Na_2SO_4) = amount (mg) of barium sulfate ($BaSO_4$) × 0.6086

Acceptance criteria: The combined content of sodium chloride and sodium sulfate is NMT 8.0%.

SPECIFIC TESTS

• **ALKALINITY**
Sample solution: Dissolve 1.0 g in 100 mL of water, add 0.1 mL of phenol red TS, and titrate with 0.10 N hydrochloric acid.
Acceptance criteria: NMT 0.5 mL for neutralization

• **▸TOTAL ALCOHOLS:** Transfer 5 g to an 800-mL Kjeldahl flask, and add 150 mL of water, 50 mL of hydrochloric acid, and a few boiling chips. Attach a reflux condenser to the Kjeldahl flask, heat carefully to avoid excessive frothing, and boil for 4 h. Cool the flask, rinse the condenser with ether, collecting the ether in the flask, and transfer the contents to a 500-mL separator, rinsing the flask twice with ether and adding the washings to the separator. Extract the solution with two 75-mL portions of ether, evaporate the combined ether extracts in a tared beaker on a steam bath, dry the residue at 105° for 30 min, cool, and weigh.
Acceptance criteria: The residue represents the total alcohols and is NLT 59.0% of the weight of Sodium Lauryl Sulfate taken.◂

• **UNSULFATED ALCOHOLS**
Sample solution: Dissolve 10 g in 100 mL of water, and add 100 mL of alcohol.
Analysis: Transfer the solution to a separator, and extract with three 50-mL portions of petroleum ether. If an emulsion forms, sodium chloride may be added to promote separation of the two layers. Wash the combined petroleum ether extracts with three 50-mL portions of water, and dry with anhydrous sodium sulfate. Filter the petroleum ether extract into a tared beaker. Evaporate on a water bath until the odor of petroleum ether no longer is perceptible, dry the residue at 105° for 30 min, cool, and weigh.
Acceptance criteria: The weight of the residue is NMT 4.0% of the weight of Sodium Lauryl Sulfate taken.

ADDITIONAL REQUIREMENTS

• **▸PACKAGING AND STORAGE:** Preserve in well-closed containers.◂
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Sodium Lauryl Sulfate RS

Sodium Metabisulfite

$Na_2S_5O_5$ 190.11

Disulfurous acid, disodium salt;
Disodium pyrosulfite [7681-57-4].

DEFINITION

Sodium Metabisulfite contains an amount of sodium metabisulfite ($Na_2S_2O_5$) equivalent to NLT 65.0% and NMT 67.4% of SO_2.

IDENTIFICATION

• **A. IDENTIFICATION TESTS—GENERAL,** *Sodium* ⟨191⟩ and *Sulfite* ⟨191⟩**:** A solution (1 in 20) meets the requirements.

ASSAY

Change to read:

• **PROCEDURE**
Sample: 200 mg of Sodium Metabisulfite
Blank: 50.0 mL of 0.1 N iodine VS, accurately measured
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Residual titration
Titrant: 0.1 N iodine VS
Back-titrant: 0.1 N sodium thiosulfate VS
Endpoint detection: Visual
Analysis: Add the *Sample* to 50.0 mL of 0.1 N iodine VS in a glass-stoppered conical flask, and swirl to dissolve. Allow to stand for 5 min, protected from light. Add 1 mL of hydrochloric acid, and titrate the excess iodine with *Back-titrant*, adding 3 mL of starch TS as the endpoint is approached. Perform a blank determination.
Calculate the percentage of ▸sulfur dioxide (SO_2)◂ (ERR 1-Jun-2018) in the portion of Sodium Metabisulfite taken:

$$\text{Result} = \{[(V_B - V_S) \times N \times F]/W\} \times 100$$

V_B = *Back-titrant* volume consumed by the *Blank* (mL)
V_S = *Back-titrant* volume consumed by the *Sample* (mL)
N = *Back-titrant* normality (mEq/mL)
F = equivalency factor, 32.03 mg/mEq
W = *Sample* weight (mg)

Acceptance criteria: 65.0%–67.4% of SO_2

IMPURITIES

• **LIMIT OF CHLORIDE**
Standard solution: 0.71 mL of 0.020 N hydrochloric acid in 100 mL of water
Sample solution: 1.0 g in 10 mL of water. [NOTE—Pass through a small chloride-free filter, if necessary.] Add 6 mL of 30% hydrogen peroxide. Add 1 N sodium hydroxide until the solution is slightly alkaline to phenolphthalein, and dilute with water to 100 mL.
Analysis
Samples: *Standard solution* and *Sample solution*
Dilute 2.0 mL of the *Samples* with water to 20 mL. Add 1 mL of nitric acid and 1 mL of silver nitrate TS. Allow to stand for 5 min protected from direct sunlight, and compare the turbidity from the *Samples* (see *Nephelometry, Turbidimetry, and Visual Comparison* ⟨855⟩).
Acceptance criteria: Any turbidity produced by the *Sample solution* does not exceed that of the *Standard solution* (0.05%).

• **LIMIT OF THIOSULFATE**
Standard solution: Mix 0.10 mL of 0.10 N sodium thiosulfate with 10 mL of 1 N hydrochloric acid.
Sample solution: Mix 2.2 g with 10 mL of 1 N hydrochloric acid.

NF Monographs

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Analysis
Samples: *Standard solution* and *Sample solution*
Gently boil the *Samples* for 5 min. Cool, then transfer each solution to a small test tube.
Acceptance criteria: Any turbidity produced by the *Sample solution* does not exceed that of the *Standard solution* (0.05%).

• **IRON** ⟨241⟩
Test preparation: Dissolve 500 mg of Sodium Metabisulfite in 14 mL of dilute hydrochloric acid (2 in 7), and evaporate on a steam bath to dryness. Dissolve the residue in 7 mL of dilute hydrochloric acid (2 in 7), and again evaporate to dryness. Dissolve the resulting residue in a mixture of 2 mL of hydrochloric acid and 20 mL of water. Add 3 drops of bromine TS, and boil to expel the bromine. Cool, then dilute with water to 47 mL.
Analysis: Proceed as directed in the chapter.
Acceptance criteria: NMT 20 ppm

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-filled, tight containers, and avoid exposure to excessive heat.

Sodium Phosphate, Dibasic
—see Dibasic Sodium Phosphate General Monographs

Sodium Phosphate, Monobasic
—see Monobasic Sodium Phosphate General Monographs

Tribasic Sodium Phosphate

Na_3PO_4 (anhydrous)	163.94
Trisodium phosphate, monohydrate	181.96
Phosphoric acid, trisodium salt, dodecahydrate; Trisodium phosphate, dodecahydrate	380.13

[10101-89-0].
Anhydrous [7601-54-9].

DEFINITION
Tribasic Sodium Phosphate is anhydrous or contains one to twelve molecules of water of hydration. Na_3PO_4 (anhydrous) and $Na_3PO_4 \cdot H_2O$ (monohydrate) contain NLT 97.0% of Na_3PO_4, calculated on the ignited basis. $Na_3PO_4 \cdot 12H_2O$ (dodecahydrate) contains NLT 92.0% of Na_3PO_4, calculated on the ignited basis.

IDENTIFICATION
• **A. IDENTIFICATION TESTS—GENERAL,** *Sodium* ⟨191⟩ and *Phosphate* ⟨191⟩**:** A solution (1 in 20) meets the requirements.

ASSAY
• **PROCEDURE**
Sample: 5.5 g of Tribasic Sodium Phosphate, on the anhydrous basis
Blank: 100.0 mL of 1 N hydrochloric acid, accurately measured
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Residual titration
Titrant: 1 N sodium hydroxide VS
Endpoint detection: Potentiometric
Analysis: Transfer the *Blank* to a 400-mL beaker, and titrate with the *Titrant* to the endpoint at a pH of 7.0. Record as the volume consumed, and designate as *A*. Transfer the *Sample* to a 400-mL beaker, add 100.0 mL of 1 N hydrochloric acid, and stir until dissolved. Pass a stream of carbon dioxide-free air, in fine bubbles, through the solution for 30 min to expel carbon dioxide, covering the beaker loosely to prevent any loss by spraying. Wash the cover and sides of the beaker with a few mL of water. Titrate the excess acid potentiometrically with the *Titrant* to the inflection point at a pH of 4. Record the buret reading, and designate as *B*. Protect the solution from carbon dioxide absorbed from the air, and continue the titration with 1 N sodium hydroxide VS to the inflection point at a pH of 8.8. Record the buret reading, and designate as *C*.
Calculate the amount of *Titrant* consumed by the *Sample* to the first inflection point, correcting for the *Blank* ($V_1 = A - B$) and the amount of *Titrant* consumed by the *Sample* between the two inflection points ($V_2 = C - B$). If V_1 is equal to or greater than $2V_2$, calculate the amount of Na_3PO_4 in the portion of *Sample* taken:

$$D = V_2 \times N \times F$$

V_2 = volume of *Titrant* consumed between the two inflection points (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 163.9 mg/mEq

If V_1 is less than $2V_2$, calculate the amount of Na_3PO_4 in the portion of *Sample* taken:

$$D = (V_1 - V_2) \times N \times F$$

V_1 = volume of the *Titrant* consumed to the first inflection point, correcting for the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 163.9 mg/mEq

Calculate the percentage of Na_3PO_4 on the ignited basis in the portion of Tribasic Sodium Phosphate taken:

$$\text{Result} = [10/(100 - L)] \times (D/W)$$

L = percentage calculated in the test for *Loss on Ignition* ⟨733⟩
D = amount of Na_3PO_4 found (mg)
W = weight of the *Sample* (g)

Acceptance criteria: NLT 97.0% of Na_3PO_4 on the ignited basis. $Na_3PO_4 \cdot 12H_2O$ (dodecahydrate) contains NLT 92.0% of Na_3PO_4 on the ignited basis.

IMPURITIES
• **LOSS ON IGNITION** ⟨733⟩
Sample: 2 g
Analysis: Dry the *Sample* at 110° for 5 h, and then ignite at 800° for 30 min.
Acceptance criteria: The anhydrous form loses NMT 2.0% of its weight, the monohydrate loses 8.0%–11.0% of its weight, and the dodecahydrate loses 45.0%–57.0% of its weight.
• **ARSENIC,** *Method I* ⟨211⟩
Test preparation: Dissolve a portion equivalent to 1.0 g of anhydrous tribasic sodium phosphate in 35 mL of water.
Analysis: Proceed as directed in the chapter.
Acceptance criteria: NMT 3 ppm

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SPECIFIC TESTS

• **INSOLUBLE SUBSTANCES**

Sample solution: Dissolve a portion equivalent to 10.0 g of anhydrous tribasic sodium phosphate in 100 mL of hot water.

Analysis: Filter the *Sample solution* through a tared filtering crucible. [NOTE—Do not use glass.] Wash the insoluble residue with hot water, and dry at 105° for 2 h.

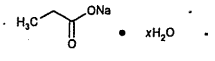
Acceptance criteria: The weight of the residue so obtained does not exceed 20 mg (0.2%).

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.

• **LABELING:** Label it to indicate whether it is anhydrous, the monohydrate, or the dodecahydrate.

Sodium Propionate



$C_3H_5NaO_2 \cdot xH_2O$

$C_3H_5NaO_2$ 96.06

Propanoic acid, sodium salt, hydrate;
Sodium propionate hydrate [6700-17-0].
Anhydrous [137-40-6].

DEFINITION

Sodium Propionate, dried at 105° for 2 h, contains NLT 99.0% and NMT 100.5% of sodium propionate ($C_3H_5NaO_2$).

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

Analysis: Perform test on an undried sample.

Acceptance criteria: Meets the requirements

• **B. IDENTIFICATION TESTS—GENERAL,** *Sodium* ⟨191⟩

Sample solution: 1 in 20

Acceptance criteria: Meets the requirements

ASSAY

• **PROCEDURE**

Sample: 200 mg of Sodium Propionate, previously dried at 105° for 2 h

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Direct titration

Titrant: 0.1 N perchloric acid VS

Blank: 50 mL of glacial acetic acid

Endpoint detection: Visual

Analysis: Dissolve the *Sample* in 50 mL of glacial acetic acid, and add 1 drop of crystal violet TS. Titrate with 0.1 N perchloric acid VS to a green endpoint. Perform a blank determination, and make any necessary correction. Calculate the percentage of sodium propionate ($C_3H_5NaO_2$) in the *Sample* taken:

$$\text{Result} = [(V_S - V_B) \times N \times F \times 100]/W$$

V_S = volume of the *Titrant* consumed by the *Sample* (mL)

V_B = volume of the *Titrant* consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 96.06 mg/mEq

W = weight of the *Sample* (mg)

Acceptance criteria: 99.0%–100.5% on the dried basis

SPECIFIC TESTS

• **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 1.0%

• **ALKALINITY**

Sample solution: 2.0 g of Sodium Propionate in 20 mL of water

Analysis: Add phenolphthalein TS to the *Sample solution*.

Acceptance criteria: If a pink color is produced, it is discharged by 0.60 mL of 0.10 N sulfuric acid.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Sodium Propionate RS

Sodium Starch Glycolate

Portions of this monograph that are national *USP–NF* text, and are not part of the harmonized text, are marked with symbols (‣ ◂) to specify this fact.

Starch carboxymethyl ether, sodium salt.

DEFINITION

Sodium Starch Glycolate is the sodium salt of a carboxymethyl ether of starch or of a cross-linked carboxymethyl ether of starch. It may contain NMT 7.0% of Sodium Chloride. The pH and assay requirements for Type A and Type B are set forth in the accompanying table.

	pH		% Sodium, Combined as Sodium Starch Glycolate	
Type	Min.	Max.	Min.	Max.
A	5.5	7.5	2.8	4.2
B	3.0	5.0	2.0	3.4

IDENTIFICATION

Change to read:

• **‣A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

[NOTE—Disregard any peaks at about 845, 1285, and 1305 cm^{-1}, which are attributed to the presence of citrate.]

• **B.** An acidified solution of it is colored blue to violet by the addition of iodine and potassium iodide TS 1.

• **C.**

Potassium pyroantimonate solution: Dissolve 2 g of potassium pyroantimonate in 85 mL of hot water. Cool quickly, and add 10 mL of a solution of potassium hydroxide (3 in 20). Allow to stand for 24 h, filter, and dilute with water to 100 mL.

Analysis: To a 2-mL portion of the *Sample solution* prepared for the test for *Limit of Iron*, add 2 mL of 15% potassium carbonate, and heat to boiling. No precipitate is formed. Add 4 mL of *Potassium pyroantimonate solution*, and heat to boiling. Allow to cool in ice water and, if necessary, rub the inside of the test tube with a glass rod.

Acceptance criteria: A dense precipitate is formed.

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• **D.** Sodium Starch Glycolate imparts an intense yellow color to a nonluminous flame.

ASSAY

• **PROCEDURE**

Sample: 1 g

Analysis: Transfer the *Sample* to a conical flask, add 20 mL of 80% alcohol, stir for 10 min, and filter. Repeat the extraction until the chloride has been completely extracted, as shown by a test with silver nitrate. Dry the insoluble portion at 105° to constant weight, and transfer an accurately weighed portion (700 mg) of the dried 80% alcohol–insoluble portion to a suitable flask. Add 80 mL of glacial acetic acid, and heat the mixture under reflux on a boiling water bath for 2 h. Cool to room temperature, and titrate with 0.1 N perchloric acid VS, determining the endpoint potentiometrically.

Calculate the percentage of sodium combined in the form of sodium starch glycolate:

$$\text{Result} = 100 \times 22.99 \times V \times (N/W)$$

V = volume of perchloric acid consumed (mL)
N = normality of the perchloric acid
W = weight of the dried alcohol–insoluble residue taken for the *Assay* (mg)

Acceptance criteria: 2.8%–4.2% for Type A; 2.0%–3.4% for Type B

OTHER COMPONENTS

• **LIMIT OF SODIUM CHLORIDE**

Sample: 500 mg of Sodium Starch Glycolate

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Direct titration

Titrant: 0.1 N silver nitrate VS

Endpoint detection: Potentiometric

Electrodes

Indicator: Suitable silver-based

Reference: Double junction electrode containing a 10% potassium nitrate filling solution in the outer jacket, and a standard filling solution in the inner jacket

Analysis: Transfer the *Sample* to a beaker, and suspend in 100 mL of water. Add 1 mL of nitric acid. Titrate with the *Titrant*. Each mL of 0.1 N silver nitrate is equivalent to 5.844 mg of sodium chloride.

Acceptance criteria: NMT 7.0%

• **LIMIT OF SODIUM GLYCOLATE**

[NOTE—Conduct this test without exposure to daylight. Use low-actinic glassware.]

Solution A: 0.1 mg/mL of 2,7-dihydroxynaphthalene in sulfuric acid; allow to stand until decolorized, and use within 2 days.

Standard solution: Transfer 310 mg of glycolic acid, previously dried over phosphorus pentoxide in a desiccator at room temperature overnight, to a 500-mL volumetric flask. Dissolve in and dilute with water to volume. Transfer 5.0 mL of this solution to a 100-mL beaker, add 4 mL of 6 N acetic acid, and allow to stand for about 30 min. Add 50 mL of acetone and 1 g of sodium chloride, mix, and pass through fast filter paper moistened with acetone into a 100-mL volumetric flask. Rinse the beaker and the filter paper with acetone. Combine the filtrate and washings, dilute with acetone to volume, and mix. Allow to stand for 24 h without shaking. Use the clear supernatant as the *Standard solution*.

Sample solution: Transfer 200 mg to a 100-mL beaker. Add 4 mL of 6 N acetic acid and 5 mL of water. Stir until dissolution is complete (about 10 min). Add 50 mL of acetone and 1 g of sodium chloride, mix, and pass through fast filter paper moistened with acetone into a 100-mL volumetric flask. Rinse the beaker and filter paper with acetone. Combine the filtrate and washings, dilute with acetone to volume, and mix. Allow to stand for 24 h without shaking. Use the clear supernatant as the *Sample solution*.

Analysis: Treat the *Sample solution* and the *Standard solution* as follows. Heat 2.0 mL of the solution on a water bath for 20 min to remove the acetone. Cool to room temperature. Add 20.0 mL of *Solution A* to the solution under test, mix, and heat on a water bath for 20 min. Cool under running water, and quantitatively transfer to a 25-mL volumetric flask. Maintain the flask under running water, and dilute with sulfuric acid to volume. Within 10 min, determine the absorbance of the solution at 540 nm with a suitable spectrophotometer, using water as the blank.

Acceptance criteria: The absorbance of the *Sample solution* is NMT that of the *Standard solution* (2.0%).

IMPURITIES

• **LIMIT OF IRON**

Standard solution: Dissolve 863.4 mg of ferric ammonium sulfate [$FeNH_4(SO_4)_2 \cdot 12H_2O$] in water, add 25 mL of 2 N sulfuric acid, dilute with water to 500.0 mL, and mix. Pipet 10 mL of this solution into a 100-mL volumetric flask, dilute with water to volume, and mix. Pipet 5 mL of this solution into a 100-mL volumetric flask, dilute with water to volume, and mix. This solution contains the equivalent of 1.0 µg/mL of iron.

Sample solution: Place 2.5 g in a silica or platinum crucible, and add 2 mL of 10 N sulfuric acid. Heat on a water bath, then cautiously raise the temperature progressively over an open flame. Ignite, preferably in a muffle furnace, at 600 ± 25°. Continue heating until all black particles have disappeared. Cool, add a few drops of 2 N sulfuric acid, and heat and ignite as above. Add a few drops of 2 M ammonium carbonate, evaporate to dryness, and ignite as above. Cool, dissolve the residue in 50 mL of water, and mix.

[NOTE—Reserve a portion of this solution for *Identification* test C.]

Analysis: Treat the *Sample solution* and the *Standard solution* as follows. Transfer 10 mL of the solution to a suitable beaker, add 2 mL of citric acid solution (1 in 5) and 0.1 mL of thioglycolic acid, and mix. Render the solution alkaline, using litmus paper as an external indicator, by the addition of ammonium hydroxide. Dilute with water to 20 mL, and mix. Allow the solutions to stand for 5 min.

Acceptance criteria: The color of the solution from the *Sample solution* is a shade of pink no deeper than that of the solution from the *Standard solution* (0.002%).

SPECIFIC TESTS

• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩: It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

• **pH** ⟨791⟩: Disperse 1 g in 30 mL of water. The pH of the resulting suspension is either 5.5–7.5 for Type A or 3.0–5.0 for Type B.

• **LOSS ON DRYING** ⟨731⟩

Analysis: Dry at 130° for 90 min.

Acceptance criteria: NMT 10.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, preferably protected from wide variations in temperature and humidity, which may cause caking.

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• ***LABELING:** Label it to indicate the botanical source of the starch from which it was derived, the cross-linking agent (if used), the pH range, and whether it is Type A or Type B.

• **USP REFERENCE STANDARDS** ⟨11⟩
USP Sodium Starch Glycolate Type A RS
USP Sodium Starch Glycolate Type B RS

Sodium Stearate

Octadecanoic acid, sodium salt;
Sodium stearate [822-16-2].

DEFINITION

Sodium Stearate is a compound of sodium with a mixture of solid organic acids obtained from sources of vegetable or animal origin and consists mainly of variable proportions of sodium stearate ($C_{18}H_{35}NaO_2$) and sodium palmitate ($C_{16}H_{31}NaO_2$). The content of stearic acid in the fatty acid fraction is NLT 40.0% of the total content. The sum of stearic acid and palmitic acid in the fatty acid fraction is NLT 90.0% of the total content. Sodium stearate contains small amounts of the sodium salts of other fatty acids.

IDENTIFICATION

• **A.**
Analysis: Heat a small quantity of Sodium Stearate in a crucible over a flame until it fuses. Continue heating the sample until it decomposes with emission of flammable vapors that should burn when ignited. Moisten the residue with water, and test with red litmus paper. The paper must turn blue. Add a small amount of acid to the crucible, and observe the solution effervesce. The solution must impart an intense yellow color to a nonluminous flame.
Acceptance criteria: Meets the requirements

• **B.** The retention times of the major peaks of the *Sample solution* correspond to those of the *Standard solution*, as obtained in the *Assay*.

ASSAY

• **PROCEDURE**
Boron trifluoride–methanol solution: 140 g/L of boron trifluoride in methanol
Sample solution: Dissolve 100 mg of Sodium Stearate in a small conical flask, fitted with a suitable reflux attachment, with 5 mL of *Boron trifluoride–methanol solution*. Boil under reflux for 10 min. Add 4.0 mL of *n*-heptane through the condenser, and boil again under reflux for 10 min. Allow to cool. Add 20 mL of a saturated solution of sodium chloride. Shake, and allow the layers to separate. Remove about 2 mL of the organic layer, and dry it over 0.2 g of anhydrous sodium sulfate. Dilute 1.0 mL of this solution with *n*-heptane to 10.0 mL.
Standard solution: Prepare as directed in the *Sample solution* using 50 mg of USP Stearic Acid RS and 50 mg of USP Palmitic Acid RS.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 30-m × 0.32-mm fused silica; 0.5-µm layer of phase G16
Temperatures
Injector: 220°
Detector: 260°
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	—	70	2
70	5	240	5

Carrier gas: Helium, passed through a bed of molecular sieve for drying, if necessary
Flow rate: 2.4 mL/min
Injection type: Splitless
Injection volume: 1 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Resolution: NLT 5.0 between the methyl palmitate and methyl stearate peaks. [NOTE—The relative retention times for methyl palmitate and methyl stearate are about 0.9 and 1.0, respectively.]
Relative standard deviation: NMT 3.0% for the methyl stearate and methyl palmitate peaks; NMT 1.0% for the ratio of the peak areas of methyl palmitate to the peak areas of methyl stearate, from 6 replicate injections
Analysis: Calculate the percentage of stearic acid ($C_{18}H_{36}O_2$) in the fatty acid fraction of the sample taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak area due to methyl stearate
r_T = sum of all the peak areas, excluding the solvent peak

Calculate the percentage of palmitic acid ($C_{16}H_{32}O_2$) in the fatty acid fraction of the sample taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak area due to methyl palmitate
r_T = sum of all the peak areas, excluding the solvent peak

Acceptance criteria
Stearic acid: NLT 40.0%
Sum of stearic acid and palmitic acid: NLT 90.0%

SPECIFIC TESTS

• **ACIDITY**
Sample solution: Heat 50 mL of alcohol to the same temperature, ±5°, as that attained when the pink endpoint is reached in the titration of the sample specimen. Add 3 drops of phenolphthalein TS and sufficient 0.020 N sodium hydroxide to produce a faint pink color. Add 2.00 g of Sodium Stearate, and dissolve with the aid of a small amount of heat. No pink color is produced.
Analysis: Titrate the solution with 0.020 N sodium hydroxide until a pink color is produced.
Acceptance criteria: 1.00–4.25 mL of 0.020 N sodium hydroxide is required (0.28%–1.2% as stearic acid).

• **LOSS ON DRYING** ⟨731⟩
Sample: Tare a beaker containing 1 g of washed sand, previously dried at 105°, add 500 mg of Sodium Stearate, and again weigh.
Analysis: To the *Sample* add 10 mL of alcohol, and evaporate at 80° to dryness. Dry at 105° for 4 h.
Acceptance criteria: NMT 5.0%

• **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩
Sample: 1 g of the fatty acids obtained as follows. Dissolve 25 g of Sodium Stearate in 300 mL of hot water, add 60 mL

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of 2 N sulfuric acid, and heat the solution, with frequent stirring, until the separated fatty acid layer is clear. Wash the fatty acids with boiling water until they are free from sulfate, collect in a small beaker, and warm on a steam bath until the water has settled and the fatty acids are clear. Allow the acids to cool, pour off the water layer, then melt the acids, filter into a dry beaker while hot, and dry at 105° for 20 min.

Acceptance criteria: 196–211

- **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩

Sample: The fatty acids obtained in *Fats and Fixed Oils, Acid Value* ⟨401⟩

Acceptance criteria: NMT 4.0

- **ALCOHOL-INSOLUBLE SUBSTANCES**

Sample: 1.0 g of Sodium Stearate

Analysis: Reflux the *Sample* with 25 mL of alcohol until it dissolves completely.

Acceptance criteria: The resulting solution is clear or NMT slightly opalescent.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed, light-resistant containers.
- **LABELING:** Label it to indicate the content of stearic acid in the fatty acid fraction and to indicate the fatty acids used to produce sodium stearate are from sources of vegetable or animal origin.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Palmitic Acid RS
USP Stearic Acid RS

Sodium Stearyl Fumarate

DEFINITION

Sodium Stearyl Fumarate contains NLT 99.0% and NMT 101.5% of sodium stearyl fumarate ($C_{22}H_{39}NaO_4$), calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

- **▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

Analysis: Perform test on an undried specimen (1 in 300).

Acceptance criteria: Meets the requirements

ASSAY

- **PROCEDURE**

Sample: 250 mg

Blank: 10 mL of chloroform

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Direct titration

Titrant: 0.1 N perchloric acid VS

Endpoint detection: Visual

Analysis: Transfer the *Sample* to a 50-mL conical flask, mix with 10 mL of chloroform, and add 20 mL of glacial acetic acid to dissolve. Add quinaldine red TS, and titrate with 0.1 N perchloric acid VS.

Calculate the percentage of sodium stearyl fumarate ($C_{22}H_{39}NaO_4$) in the *Sample* taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

V_S = volume of *Titrant* consumed by the *Sample* (mL)
V_B = volume of *Titrant* consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 390.5 mg/mEq
W = weight of the *Sample* (mg)

Acceptance criteria: 99.0%–101.5% on the anhydrous basis

OTHER COMPONENTS

- **LIMIT OF SODIUM STEARYL MALEATE AND STEARYL ALCOHOL**

Solvent: Chloroform and glacial acetic acid (4:1)

Standard monostearyl maleate stock solution: 1 mg/mL of USP Monostearyl Maleate RS in *Solvent*

Standard monostearyl maleate solution: Pipet 5.0 mL of *Standard monostearyl maleate stock solution*, and dilute with chloroform to 50 mL.

Standard stearyl alcohol stock solution: 1 mg/mL of USP Stearyl Alcohol RS in *Solvent*

Standard stearyl alcohol solution: Pipet 5.0 mL of *Standard stearyl alcohol stock solution*, and dilute with chloroform to 50 mL.

Sample solution: Transfer 200 mg of Sodium Stearyl Fumarate to a small, glass-stoppered conical flask. Add 10.0 mL of *Solvent*. Dissolve by placing the flask in an ultrasonic bath for 10 min.

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Mode: TLC

Adsorbent: 0.25-mm layer of chromatographic silica gel (TLC plates)

Spray reagent: Sulfuric acid and alcohol (1:9). [NOTE—Add cautiously and with stirring.]

Developing solvent system: Hexanes, toluene, and glacial acetic acid (5:5:1)

Analysis

Samples: *Standard monostearyl maleate solution*, *Standard stearyl alcohol solution*, and *Sample solution*

Apply 5 µL of *Standard monostearyl maleate solution* and 10 µL each of *Standard stearyl alcohol solution* and *Sample solution* to the plate. Immerse the plate in a tank containing a layer of 10 mm of chloroform on the bottom. Allow the solvent front to reach the upper edge of the spots. Withdraw the plate, and dry in a current of cold air. Repeat the immersion, development, and drying. This results in spots having a linear shape. Develop the chromatograph in a saturated chamber containing the *Developing solvent system* until the solvent front has moved 15 cm, and remove the plate from the chamber. Allow to dry for 10 min, and heat in an oven at 90° for 2 min. Allow to cool to room temperature. Replace the plate in the chamber for another 15-cm development, remove the plate, and allow to dry at room temperature for 15 min. Spray the plate with *Spray reagent*. Dry the plate in an oven at 150° for 15 min. Dark spots appear on a light background. Allow to cool. Faint spots at an R_F value of 0.9 may result from traces of distearyl maleate and distearyl fumarate.

Acceptance criteria: The intensity of any spot from the *Sample solution* is not greater than that from the corresponding spot from the *Standard monostearyl maleate solution* and *Standard stearyl alcohol solution* (NMT 0.25% sodium stearyl maleate, NMT 0.5% stearyl alcohol).

IMPURITIES

- **LEAD** ⟨251⟩: NMT 10 ppm

SPECIFIC TESTS

- **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 5.0%
- **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩

Sample: 450 mg

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Ethanolic potassium hydroxide: Dissolve 5.5 g of potassium hydroxide in absolute alcohol, heating if necessary to dissolve, and dilute with absolute alcohol to about 1000 mL. Prepare fresh daily, and filter if necessary to remove carbonate.

Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Residual titration
Titrant: 0.1 N hydrochloric acid VS
Blank: 50.0 mL of *Ethanolic potassium hydroxide*
Endpoint detection: Visual

Analysis: Transfer the *Sample* to a 300-mL conical flask, and add 50.0 mL of *Ethanolic potassium hydroxide*, rinsing down the inside of the flask during the addition. Gently reflux the mixture on a steam bath for NLT 2 h, occasionally swirl gently, but avoid splashing the mixture up into the condenser. Rinse the condenser with 10 mL of 70% alcohol, followed by three 10-mL portions of water, collecting the rinsings in the flask. Cool, rinse the sides of the flask with two 10-mL portions of 70% alcohol, add phenolphthalein TS, and titrate with 0.1 N hydrochloric acid VS to the disappearance of any pink color. Perform a blank determination.

Calculate the *Saponification Value* for Sodium Stearyl Fumarate in the *Sample* taken:

$$\text{Result} = [(V_B - V_S) \times N \times F]/W$$

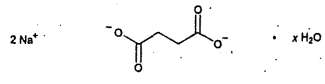
V_B = volume of the *Titrant* consumed by the *Blank* (mL)
V_S = volume of the *Titrant* consumed by the *Sample* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = molecular weight of potassium hydroxide, 56.11
W = weight of the *Sample* (g)

Acceptance criteria: 142.2–146.0, calculated on the anhydrous basis

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Monostearyl Maleate RS
  USP Sodium Stearyl Fumarate RS
  USP Stearyl Alcohol RS

Sodium Succinate



x = 0 or 6

$NaOOC\text{-}CH_2CH_2\text{-}COONa$ ($C_4H_4Na_2O_4$) 162.05
Anhydrous disodium 1,4-butanedioate;
Anhydrous butanedioic acid disodium salt [150-90-3].

$NaOOC\text{-}CH_2CH_2\text{-}COONa \cdot 6H_2O$ ($C_4H_4Na_2O_4 \cdot 6H_2O$) 270.14
Disodium 1,4-butanedioate hexahydrate;
Butanedioic acid disodium salt hexahydrate [6106-21-4].

DEFINITION

Sodium Succinate, when dried at 120° for 2 h, contains NLT 98.0% and NMT 102.0% of disodium succinate ($C_4H_4Na_2O_4$).

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197A OR 197K.▲** (CN 1-May-2020) Dry the Anhydrous Sodium Succinate or Sodium Succinate Hexahydrate sample at 120° for 2 h before use.
- **B. CHROMATOGRAPHIC IDENTITY**
  Analysis: Proceed as directed in the *Assay*.
  Acceptance criteria: The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*.
- **C. SODIUM**
  Analysis: Proceed as directed in *Identification Tests—General* ⟨191⟩, *Sodium*.
  Acceptance criteria: Meets the requirements

ASSAY

- **PROCEDURE**

Solution A: Dissolve 6.8 g of monobasic potassium phosphate in 2 L of water. Adjust with phosphoric acid to a pH of 2.3. Pass under vacuum through an HNWP (nylon hydrophilic) membrane filter of 0.45-µm pore size. This is a 25 mM potassium phosphate buffer with a pH of 2.3.

Mobile phase: Add 100 mL of methanol to 1900 mL of *Solution A* and mix well. Sonicate for 30 min and cool to room temperature.

Diluent: Add 10 mL of phosphoric acid to 1 L of water and mix well. This is a 1% phosphoric acid solution.

System suitability solution: 3.0 mg/mL of USP Anhydrous Sodium Succinate RS and 2.2 µg/mL of USP Fumaric Acid RS in *Diluent*

Standard solution: 3.0 mg/mL of USP Anhydrous Sodium Succinate RS in *Diluent*

Sample solution: 3.0 mg/mL of Anhydrous Sodium Succinate or Sodium Succinate Hexahydrate in *Diluent*. Dry Anhydrous Sodium Succinate or Sodium Succinate Hexahydrate at 120° for 2 h before use.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 204 nm
Column: 4.6-mm × 15-cm; 3-µm packing L1
Column temperature: 30°
Flow rate: 1.0 mL/min
Injection volume: 10 µL
Run time: 10 min

System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The relative retention times for succinic acid and fumaric acid are 1.0 and 1.2, respectively.]
Suitability requirements
Resolution: NLT 2.0 between succinic acid and fumaric acid, *System suitability solution*
Tailing factor: 0.8–2.0, *Standard solution*
Relative standard deviation: NMT 0.5%, *Standard solution*

Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of sodium succinate ($C_4H_4Na_2O_4$) in the portion of sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP Anhydrous Sodium Succinate RS in the *Standard solution* (mg/mL)

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C_U = concentration of Anhydrous Sodium Succinate or Sodium Succinate Hexahydrate in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–102.0%

IMPURITIES

• **LIMIT OF SODIUM ACETATE, SODIUM MALEATE, AND SODIUM FUMARATE**

Solution A, Mobile phase, Diluent, and **Chromatographic system:** Proceed as directed in the *Assay*.

Acetic acid stock solution: Transfer 37.5 mg of USP Glacial Acetic Acid RS to a 25-mL volumetric flask that contains 10 mL of *Diluent*. Dissolve and dilute with *Diluent* to volume. Transfer 1.0 mL of this solution to a 10-mL volumetric flask and dilute with *Diluent* to volume. This solution is equivalent to 200 µg/mL of sodium acetate in *Diluent*.

Maleic acid stock solution: Transfer 36.5 mg of USP Maleic Acid RS to a 50-mL volumetric flask. Dissolve and dilute with *Diluent* to volume. Transfer 1.0 mL of this solution to a 10-mL volumetric flask and dilute with *Diluent* to volume. This solution is equivalent to 100 µg/mL of sodium maleate in *Diluent*.

Fumaric acid stock solution: Transfer 36.5 mg of USP Fumaric Acid RS to a 50-mL volumetric flask. Dissolve and dilute with *Diluent* to volume. Transfer 1.0 mL of this solution to a 10-mL volumetric flask and dilute with *Diluent* to volume. This solution is equivalent to 100 µg/mL of sodium fumarate in *Diluent*.

System suitability solution: 10 mg/mL of USP Anhydrous Sodium Succinate RS, 15 µg/mL of USP Glacial Acetic Acid RS, and 7.3 µg/mL each of USP Maleic Acid RS and USP Fumaric Acid RS in *Diluent*

Standard solution: Transfer 1 mL each of *Acetic acid stock solution*, *Maleic acid stock solution*, and *Fumaric acid stock solution* to a 10-mL volumetric flask and dilute with *Diluent* to volume.

Sample solution: 10 mg/mL of Anhydrous Sodium Succinate or Sodium Succinate Hexahydrate in *Diluent*

System suitability

Samples: *System suitability solution* and *Standard solution*

[NOTE—The relative retention times for acetic acid, maleic acid, succinic acid, and fumaric acid are 0.7, 0.8, 1.0, and 1.2, respectively.]

Suitability requirements

Resolution: NLT 1.5 between acetic acid and maleic acid; NLT 2.0 between succinic acid and fumaric acid, *System suitability solution*

Relative standard deviation: NMT 5.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Based on the *Standard solution*, identify the peaks of acetic acid, maleic acid, and fumaric acid. Compare peak areas of acetic acid, maleic acid, and fumaric acid in the *Standard solution* and the *Sample solution*.

Acceptance criteria

Sodium acetate: The peak area of acetic acid in the *Sample solution* is NMT the peak area of acetic acid in the *Standard solution*, corresponding to NMT 0.2% of sodium acetate in Sodium Succinate.

Sodium maleate: The peak area of maleic acid in the *Sample solution* is NMT the peak area of maleic acid in the *Standard solution*, corresponding to NMT 0.1% of sodium maleate in Sodium Succinate.

Sodium fumarate: The peak area of fumaric acid in the *Sample solution* is NMT the peak area of fumaric acid in the *Standard solution*, corresponding to NMT 0.1% of sodium fumarate in Sodium Succinate.

• **LIMIT OF SULFATE**

Standard solution: 0.4 mL of 0.005 mol/L sulfuric acid

Sample solution: Dissolve 1.0 g of Anhydrous Sodium Succinate or Sodium Succinate Hexahydrate in 30 mL of water and neutralize with a diluted hydrochloric acid (1 in 40).

Analysis: Proceed as directed in *Chloride and Sulfate* ⟨221⟩, *Sulfate*.

Acceptance criteria: NMT 0.019% as SO_4

SPECIFIC TESTS

• **ACIDITY AND ALKALINITY**

Sample solution: Dissolve 1.0 g of Anhydrous Sodium Succinate or Sodium Succinate Hexahydrate in carbon dioxide-free water and dilute with water to 20 mL.

Analysis: Proceed as directed in *pH* ⟨791⟩.

Acceptance criteria: 7.0–9.0

• **LOSS ON DRYING** ⟨731⟩

Analysis: Proceed as directed in *Loss on Drying* ⟨731⟩. Dry at 120° for 2 h.

Acceptance criteria

Anhydrous Sodium Succinate: NMT 2.0%

Sodium Succinate Hexahydrate: 37.0%–41.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers. Store at room temperature.

• **LABELING:** Label it to state, as part of the official title, anhydrous or hexahydrate for sodium succinate.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Anhydrous Sodium Succinate RS
USP Fumaric Acid RS
USP Glacial Acetic Acid RS
USP Maleic Acid RS

Sodium Sulfite

Na_2SO_3 126.04
[7757-83-7].

DEFINITION

Sodium Sulfite contains NLT 95.0% and NMT 100.5% of sodium sulfite (Na_2SO_3).

IDENTIFICATION

• **A.**

Sample solution: 50 mg/mL of Sodium Sulfite.

[NOTE—Reserve portions of the solution so obtained for use in *Identification* test *B* and in the test for *Color and Clarity of Solution*.]

Analysis: Add a drop of phenolphthalein TS.

Acceptance criteria: A pink color is produced.

• **B. IDENTIFICATION TESTS—GENERAL** ⟨191⟩, *Sulfate*

Analysis: To 5 mL of the solution from *Identification* test *A* add 0.5 mL of iodine TS.

Acceptance criteria: The solution is colorless and meets the requirements of test *A*

• **C. IDENTIFICATION TESTS—GENERAL** ⟨191⟩, *Sodium*: Meets the requirements of test *A*

ASSAY

• **PROCEDURE**

Sample: 250 mg

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Residual titration

Titrant: 0.1 N iodine VS

Back titrant: 0.1 N sodium thiosulfate VS

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Blank: 50.0 mL of 0.1 N iodine VS, accurately measured
Endpoint detection: Colorimetric
Analysis: Add the *Sample* to a 500-mL beaker, add 50.0 mL of 0.1 N iodine VS, accurately measured, and shake to dissolve. Add 1 mL of starch TS, and titrate with 0.1 N sodium thiosulfate VS to a clear endpoint. Perform a blank determination, and make any necessary correction. Calculate the percentage of sodium sulfite (Na_2SO_3) in the *Sample* taken:

$$\text{Result} = [(B - V) \times N \times F \times 100]/W$$

B = 0.1 N sodium thiosulfate VS volume consumed by the *Blank*
V = 0.1 N sodium thiosulfate VS volume consumed by the *Sample*
N = actual normality of the *Back titrant* (mEq/mL)
F = equivalency factor, 63.0 mg/mEq
W = weight of *Sample* (mg)

Acceptance criteria: 95.0%–100.5%

IMPURITIES

• **LIMIT OF IRON**
Standard solution: Immediately before use, dilute 1 volume of *Standard Iron Solution*, prepared as directed under *Iron* ⟨241⟩, to 10 mL with water. [NOTE—This solution contains the equivalent of 1 µg/mL of iron.]
Sample solution: 10.0 g of Sodium Sulfite in 25 mL of water. Shake until mostly dissolved, and add 15 mL of hydrochloric acid. Heat to boiling. Cool, and dilute with water to 100.0 mL. Use a 10-mL portion.
Analysis: To the *Standard solution* and the *Sample solution*, separately add 2 mL of a citric acid solution (200 g/L), and then add 0.1 mL of thioglycolic acid. Make alkaline with stronger ammonia water, and dilute with water to 20 mL. Allow to stand for 5 min.
Acceptance criteria: Any pink color in the *Sample solution* is not more intense than that in the *Standard solution* (NMT 10 ppm).

• **LIMIT OF SELENIUM**
[**CAUTION**—Selenium is toxic; handle with care.]
Selenium standard solution: 100 µg/mL of selenium is prepared as follows. Dissolve 0.1 g of metallic selenium in 2 mL of nitric acid. Evaporate to dryness, add 2 mL of water, and evaporate to dryness. Repeat the addition of water and the evaporation to dryness three more times. Dissolve the residue so obtained in 50 mL of diluted hydrochloric acid. Transfer to a 1000-mL volumetric flask, and dilute with diluted hydrochloric acid to volume.
Standard solution: To 1.0 g of Sodium Sulfite add 0.2 mL of *Selenium standard solution* and 10 mL of formaldehyde TS, and slowly add 2 mL of hydrochloric acid. Heat in a water bath for 20 min.
Sample solution: To 3.0 g of Sodium Sulfite add 10 mL of formaldehyde TS, and slowly add 2 mL of hydrochloric acid.
Analysis: Heat the *Standard solution* and the *Sample solution* in a water bath for 20 min.
Acceptance criteria: Any pink color in the *Sample solution* is not more intense than that in the *Standard solution* (NMT 10 ppm).

• **LIMIT OF THIOSULFATES**
Sample solution: 20 mg/mL of Sodium Sulfite
Analysis: To 100 mL of the *Sample solution*, add 10 mL of formaldehyde TS and 10 mL of acetic acid. Allow to stand for 5 min. Add 0.5 mL of starch TS, and titrate with 0.1 N iodine VS. Perform a blank determination (see *Titrimetry* ⟨541⟩, *Residual Titrations*), and note the difference in volumes required.
Acceptance criteria: The difference in volumes is NMT 0.15 mL (NMT 0.1%).

• **LIMIT OF ZINC**
Zinc standard stock solution: A solution of 1 mL of acetic acid and the amount of zinc sulfate equivalent to 0.440 g of $ZnSO_4 \cdot 7H_2O$ in 100.0 mL of water. [NOTE—This solution contains the equivalent of 1000 µg/mL of Zn.]
Zinc standard solution: 25 µg/mL of zinc from *Zinc standard stock solution*
Standard solutions: Transfer 1.0-, 2.0-, and 4.0-mL portions of *Zinc standard solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with water to volume, and mix to obtain solutions having known concentrations of 0.25, 0.5, and 1.0 µg/mL of zinc.
Sample stock solution: 100 mg/mL of Sodium Sulfite is prepared as follows. To 10.0 g of Sodium Sulfite add 25 mL of water. Shake until mostly dissolved, and slowly add 15 mL of hydrochloric acid. Heat to boiling. Cool, and dilute with water to 100.0 mL.
Sample solution: 20.0 mg/mL of Sodium Sulfite from the *Sample stock solution*
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Analytical wavelength: Zinc emission line at 213.9 nm
Lamp: Zinc hollow-cathode
Flame: Air–acetylene
Analysis
Samples: *Standard solutions* and the *Sample solution*
Plot the absorbances of the *Standard solutions* versus concentration of zinc, in µg/mL, and draw the straight line best fitting the plotted points. From the graph so obtained, determine the concentration of zinc, in µg/mL, in the *Sample solution*.
Acceptance criteria: NMT 25 ppm

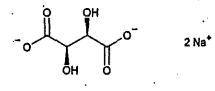
SPECIFIC TESTS

• **COLOR AND CLARITY OF SOLUTION**
Analysis: Examine the solution prepared for *Identification* test *A*.
Acceptance criteria: The solution is clear and colorless.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers. Store at room temperature.

Sodium Tartrate



$C_4H_4Na_2O_6 \cdot 2H_2O$ 230.08
Disodium L-tartrate;
Disodium (+)-2,3-dihydroxybutanedioic acid [868-18-8].

DEFINITION

Sodium Tartrate contains NLT 99.0% and NMT 100.5% of sodium tartrate ($C_4H_4Na_2O_6$), calculated on the dried basis.

IDENTIFICATION

• **A. IDENTIFICATION TESTS—GENERAL,** *Sodium* ⟨191⟩: Meets the requirements
• **B. IDENTIFICATION TESTS—GENERAL,** *Tartrate* ⟨191⟩: Meets the requirements

ASSAY

• **PROCEDURE**
Sample: 250 mg, previously dried at 150° for 3 h

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Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.1 N Perchloric acid VS (in glacial acetic acid)
Endpoint detection: Potentiometric
Analysis: Dissolve the *Sample* in 150 mL of glacial acetic acid by stirring and heating to near the boiling point. Cool to room temperature. Titrate with 0.1 N perchloric acid VS (in glacial acetic acid) to a potentiometric endpoint. Perform a blank determination, and make any necessary adjustments. Each mL of 0.1 N perchloric acid is equivalent to 9.703 mg of sodium tartrate ($C_4H_4Na_2O_6$).
Acceptance criteria: 99.0%–100.5% on the dried basis

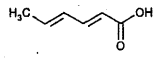
SPECIFIC TESTS
- **pH** ⟨791⟩: 7–9 (1 in 10 solution)
- **Loss on Drying** ⟨731⟩: Dry a sample at 150° for 3 h: it loses 14.0%–17.0% of its weight.

ADDITIONAL REQUIREMENTS
- **Packaging and Storage:** Preserve in a tight container. No storage requirements specified.

Sodium Thiosulfate—*see Sodium Thiosulfate General Monographs*

Sorbic Acid

Change to read:



$C_6H_8O_2$ 112.13
2,4-Hexadienoic acid, (*E*,*E*)-; ▲(2*E*,4*E*)-Hexa-2,4-dienoic acid;▲ (NF 1-Dec-2019)
(*E*,*E*)-Sorbic acid ▲▲ (NF 1-Dec-2019) [110-44-1].

DEFINITION

Change to read:

Sorbic Acid contains NLT ▲98.0%▲ (NF 1-Dec-2019) and NMT ▲102.0%▲ (NF 1-Dec-2019) of sorbic acid ($C_6H_8O_2$), calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

- **A. ▲Spectroscopic Identification Tests** ⟨197⟩, *Infrared Spectroscopy*: 197A or 197K▲ (CN 1-May-2020)
- **B.** A 1-in-400,000 solution in isopropyl alcohol exhibits an absorbance maximum at 254 ± 2 nm.

ASSAY

Change to read:

- **Procedure**
▲Solution A: 0.1% (v/v) trifluoroacetic acid in water
Solution B: 0.1% (v/v) trifluoroacetic acid in methanol
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	75	25
17.0	75	25
18.0	5	95
23.0	5	95
23.1	75	25
30.0	75	25

Diluent: Water and methanol (1:1, v/v)
System suitability solution: Prepare 0.1 mg/mL of USP Sorbic Acid RS in *Diluent* first and then treat the solution with UV irradiation to generate ~1% degradation of sorbic acid (based on area%).[1]
Standard solution: 0.1 mg/mL of USP Sorbic Acid RS in *Diluent*
Sample solution: 0.1 mg/mL of Sorbic Acid in *Diluent*
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: UV 264 nm
Column: 4.6-mm × 15-cm; 3.5-µm packing L1
Column temperature: 40°
Flow rate: 1.0 mL/min
Injection volume: 10 µL
Run time: 30 min
System suitability
Samples: *System suitability solution* and *Standard solution*
[Note—The approximate relative retention times of related substances are listed in *Table 2*.]
Suitability requirements
Resolution: NLT 1.5 between sorbic acid and sorbic acid *trans*, *cis* isomer, *System suitability solution*
Tailing factor: NMT 2.0, determined from sorbic acid, *Standard solution*
Relative standard deviation: NMT 2.0%, determined from sorbic acid, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of sorbic acid in the sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of sorbic acid from the *Sample solution*
r_S = peak area of sorbic acid from the *Standard solution*
C_S = concentration of USP Sorbic Acid RS in the *Standard solution* (mg/mL)
C_U = concentration of Sorbic Acid in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–102.0% on the anhydrous basis

[1] UV irradiation conditions of wavelengths of 254, 300, and 365 nm at 18 Watts for 2 h have been used for the solution. Other equivalent UV conditions are also suitable as long as ~1% degradation of sorbic acid (based on area%) can be achieved after the irradiation. Quartz glassware is preferred.

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Table 2

Name	Relative Retention Time
Sorbic acid *cis, cis* isomer[a]	0.83
Sorbic acid *cis, trans* isomer[b]	0.89
Sorbic acid *trans, cis* isomer[c]	0.92
Sorbic acid	1.0

[a] (*Z,Z*)-Sorbic acid.
[b] (*Z,E*)-Sorbic acid.
[c] (*E,Z*)-Sorbic acid.

[NOTE—Sorbic acid *cis, trans* isomer and sorbic acid *trans, cis* isomer might co-elute in some columns, which has no effect on assay analysis.]▲ (NF 1-Dec-2019)

IMPURITIES

Add the following:

▲• **LIMIT OF ALDEHYDE**

Decolorized fuchsin solution: Dissolve 0.1 g of basic fuchsin in 60 mL of water. Add a solution containing 1 g of anhydrous sodium sulfite in 10 mL of water. Slowly and with continuous shaking add 2 mL of hydrochloric acid. Dilute with water to 100 mL. Allow to stand protected from light for at least 12 h, decolorize with activated charcoal and filter. If the solution becomes cloudy, filter before use. If on standing the solution becomes violet, decolorize again by adding activated charcoal.

Test for sensitivity: To 1.0 mL of the *Decolorized fuchsin solution* add 1.0 mL of water and 1.0 mL of alcohol, aldehyde-free. Add 0.2 mL of a solution containing 0.1 g/L of formaldehyde (CH_2O). A pale-pink color develops within 5 min.

Storage of *Decolorized fuchsin solution*: Protected from light

Acetaldehyde standard solution: 100 µg/mL of acetaldehyde (C_2H_4O). Dissolve 1.0 g of acetaldehyde in 2-propanol and dilute with the same solvent to 100.0 mL. Dilute 5.0 mL of the solution with 2-propanol to 500.0 mL. Prepare immediately before use.

Standard solution: Add 1 mL of *Decolorized fuchsin solution* to a mixture of 1.5 mL of *Acetaldehyde standard solution*, 4 mL of 2-propanol, and 4.5 mL of water.

Sample solution: Dissolve 1.0 g of the sample in a mixture of 30 mL of water and 50 mL of 2-propanol, adjust with 0.1 N hydrochloric acid or 0.1 N sodium hydroxide to a pH of 4 and dilute with water to 100 mL.

Analysis: To 10 mL of the *Sample solution* add 1 mL of *Decolorized fuchsin solution* and allow to stand for 30 min. Any color in the solution is not more intense than that in the *Standard solution* prepared at the same time.

Acceptance criteria: NMT 0.15%, as acetaldehyde (C_2H_4O)▲ (NF 1-Dec-2019)

• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.2%

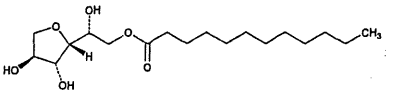
SPECIFIC TESTS

• **MELTING RANGE OR TEMPERATURE** ⟨741⟩: 132°–135°
• **WATER DETERMINATION** ⟨921⟩, *Method I*: NMT 0.5%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light, and avoid exposure to excessive heat.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Sorbic Acid RS

Sorbitan Monolaurate



Sorbitan, esters, monododecanoate;
Sorbitan monolaurate [1338-39-2].

DEFINITION

Sorbitan Monolaurate is a partial ester of lauric acid with Sorbitol and its mono- and dianhydrides. It yields, upon saponification, NLT 55.0% and NMT 63.0% of fatty acids, and NLT 39.0% and NMT 45.0% of polyols (w/w).

IDENTIFICATION

• **A.**
Sample: Residue obtained in the *Assay for Fatty Acids*
Acceptance criteria
Fats and Fixed Oils, Acid Value ⟨401⟩: 260–280 on 1-g sample
Fats and Fixed Oils, Iodine Value ⟨401⟩: NMT 5

• **B.**
Standard solution: 33 mg/mL of USP Isosorbide RS, 25 mg/mL of USP 1,4-Sorbitan RS, and 25 mg/mL of sorbitol
Sample solution: 250 mg/mL of the polyols from the *Assay for Polyols*
Chromatographic system
(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
Mode: TLC
Adsorbent: 0.25-mm layer of chromatographic silica gel
Application volume: 2 µL
Developing solvent system: Acetone and glacial acetic acid (50:1)
Spray reagent: Sulfuric acid and water (50:50)
Analysis
Samples: *Standard solution* and *Sample solution*
Apply the *Standard solution* and *Sample solution*, and allow the spots to dry. Develop the chromatogram until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the developing chamber, mark the solvent front, and allow the solvent to evaporate. Spray evenly with *Spray reagent* until the surface is uniformly wet (do not overspray), and immediately place the sprayed plate on a 200° hot plate in a well-ventilated hood. Char until white fumes of sulfur trioxide cease, and cool.
Acceptance criteria: The R_F values of the spots of the *Sample solution* correspond to those of the spots of the *Standard solution*.

ASSAY

• **FATTY ACIDS**
Sample: 10 g
Analysis: Transfer the *Sample* to a 500-mL conical flask. Cautiously add 100 mL of alcohol and 3.0 g of potassium hydroxide, and a few glass beads. Connect a suitable condenser to the flask, reflux the mixture on a hot plate for 2 h, add 100 mL of water, and heat on a steam bath to evaporate the alcohol, adding water occasionally to replace the alcohol. Continue the evaporation until the odor of alcohol can no longer be detected, and transfer the saponification mixture, with the aid of 100 mL of hot water, to a 500-mL separator. Using extreme caution, neutralize to litmus paper with a mixture of equal volumes of sulfuric acid and water, noting the volume used, and add a 10% excess of the dilute acid. Allow the solution to cool. If salts appear, add sufficient water to produce a clear solution. Cautiously add 100 mL of solvent hexane, shake

NF Monographs

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thoroughly, and withdraw the lower layer into a second 500-mL separator. Similarly extract with two more 100-mL portions of solvent hexane. Extract the combined hexane layers with 50-mL portions of water until neutral to litmus paper. Combine the extracts with the original aqueous phase to use for the *Assay* for *Polyols*. Evaporate the solvent hexane in a tared beaker on a steam bath nearly to dryness, dry under vacuum at 60° for 1 h, cool in a desiccator, and weigh the fatty acids.

Acceptance criteria: 55.0%–63.0%

• **POLYOLS**

Sample solution: Aqueous solution retained from the *Assay* for *Fatty Acids*

Analysis: Neutralize the *Sample solution* with potassium hydroxide solution (1 in 10) to a pH of 7, using a pH meter. Evaporate on a steam bath to a moist residue, extract the polyols from the salts with three 150-mL portions of dehydrated alcohol, boiling the salt residue for 3 min, and crushing it, as necessary, with the flattened end of a stirring rod during each extraction; filtering each extract while hot through a medium-porosity sintered-glass funnel provided with a sheet of retentive filter paper on which a layer of purified siliceous earth has been superimposed; and receiving the filtrates in a 1-L suction flask. Transfer the clear alcoholic polyols solution to a tared beaker, evaporate the alcohol on a steam bath, dry under vacuum at 60° for 1 h, cool in a desiccator, and weigh the polyols.

Acceptance criteria: 39.0%–45.0% (w/w)

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.5%

SPECIFIC TESTS

• **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 1.5%

• **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩: NMT 8

• **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩: 330–358

• **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩: 158–170

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers.

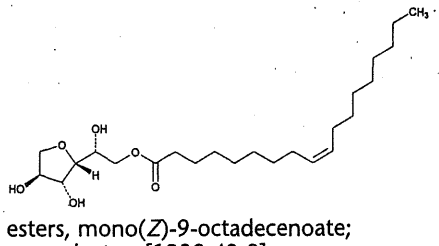
• **USP REFERENCE STANDARDS** ⟨11⟩

USP Isosorbide RS

USP 1,4-Sorbitan RS

$C_6H_{12}O_5$ 164.16

Sorbitan Monooleate



Sorbitan, esters, mono(*Z*)-9-octadecenoate;
Sorbitan monooleate [1338-43-8].

DEFINITION

Sorbitan Monooleate is a partial oleate ester of Sorbitol and its mono- and dianhydrides. It yields, upon saponification, NLT 72.0% and NMT 78.0% of fatty acids, and NLT 25.0% and NMT 31.0% of polyols (w/w).

IDENTIFICATION

• **A.**

Sample: Residue obtained in the *Assay for Fatty Acids*

Acceptance criteria

Fats and Fixed Oils, Acid Value ⟨401⟩: 192–204 on 1-g sample

Fats and Fixed Oils, Iodine Value ⟨401⟩: 75–95

• **B.**

Standard solution: 33 mg/mL of USP Isosorbide RS, 25 mg/mL of USP 1,4-Sorbitan RS, and 25 mg/mL of sorbitol

Sample solution: 250 mg/mL of the polyols, obtained in the *Assay* for *Polyols*

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Mode: TLC

Adsorbent: 0.25-mm layer of chromatographic silica gel

Application volume: 2 µL

Developing solvent system: Acetone and glacial acetic acid (50:1)

Spray reagent: Sulfuric acid and water (50:50)

Analysis

Samples: *Standard solution* and *Sample solution*

Apply the *Standard solution* and *Sample solution*, and allow the spots to dry. Develop the chromatogram until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the developing chamber, mark the solvent front, and allow the solvent to evaporate. Spray evenly with *Spray reagent* until the surface is uniformly wet (do not overspray), and immediately place the sprayed plate on a 200° hot plate in a well-ventilated hood. Char until white fumes of sulfur trioxide cease, and cool.

Acceptance criteria: The R_F values of the spots from the *Sample solution* correspond to those of the spots from the *Standard solution*.

ASSAY

• **FATTY ACIDS**

Sample: 10 g

Analysis: Transfer the *Sample* to a 500-mL conical flask. Cautiously add 100 mL of alcohol and 3.5 g of potassium hydroxide, and a few glass beads. Connect a suitable condenser to the flask, reflux the mixture on a hot plate for 2 h, add 100 mL of water, and heat on a steam bath to evaporate the alcohol, adding water occasionally to replace the alcohol. Continue the evaporation until the odor of alcohol can no longer be detected, and transfer the saponification mixture, with the aid of 100 mL of hot water, to a 500-mL separator. Using extreme caution, neutralize to litmus paper with a mixture of equal volumes of sulfuric acid and water, noting the volume used, and add a 10% excess of the dilute acid. Allow the solution to cool. If salts appear, add sufficient water to produce a clear solution. Cautiously add 100 mL of solvent hexane, shake thoroughly, and withdraw the lower layer into a second 500-mL separator. Similarly extract with two more 100-mL portions of solvent hexane. Extract the combined hexane layers with 50-mL portions of water until neutral to litmus paper. Combine the extracts with the original aqueous phase to use for the *Assay* for *Polyols*. Evaporate the solvent hexane in a tared beaker on a steam bath nearly to dryness, dry under vacuum at 60° for 1 h, cool in a desiccator, and weigh the fatty acids.

Acceptance criteria: 72.0%–78.0%

• **POLYOLS**

Sample solution: Aqueous solution retained from the *Assay* for *Fatty Acids*

Analysis: Neutralize the *Sample solution* with a potassium hydroxide solution (1 in 10) to a pH of 7, using a pH meter. Evaporate on a steam bath to a moist residue, extract the polyols from the salts with three 150-mL portions of dehydrated alcohol, boiling the salt residue for 3 min, and

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crushing it, as necessary, with the flattened end of a stirring rod during each extraction; filtering each extract, while hot, through a medium-porosity, sintered-glass funnel provided with a sheet of retentive filter paper on which a layer of purified siliceous earth has been superimposed; and receiving the filtrates in a 1-L suction flask. Transfer the clear alcoholic polyols solution to a tared beaker, evaporate the alcohol on a steam bath, dry under vacuum at 60° for 1 h, cool in a desiccator, and weigh the polyols.

Acceptance criteria: 25.0%–31.0% (w/w)

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.5%

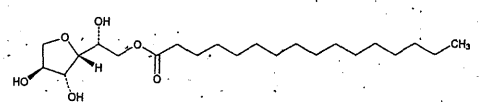
SPECIFIC TESTS

• **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 1.0%
• **FAT AND FIXED OILS,** *Acid Value* ⟨401⟩: NMT 8
• **FAT AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩: 190–215
• **FAT AND FIXED OILS,** *Iodine Value* ⟨401⟩: 62–76
• **FAT AND FIXED OILS,** *Saponification Value* ⟨401⟩: 145–160

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Isosorbide RS
USP 1,4-Sorbitan RS
$C_6H_{12}O_5$ 164.16

Sorbitan Monopalmitate



Sorbitan, esters, monohexadecanoate;
Sorbitan monopalmitate [26266-57-9].

DEFINITION

Sorbitan Monopalmitate is a partial ester of palmitic acid with Sorbitol and its mono- and dianhydrides. It yields, upon saponification, NLT 63.0% and NMT 71.0% of fatty acids, and NLT 32.0% and NMT 38.0% of polyols (w/w).

IDENTIFICATION

• **A.**
Sample: Residue obtained in the *Assay for Fatty Acids*
Acceptance criteria
Fats and Fixed Oils, Acid Value ⟨401⟩: 210–225 on 1-g sample
Fats and Fixed Oils, Iodine Value ⟨401⟩: NMT 4
• **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩
Standard solution: 33 mg/mL of USP Isosorbide RS, 25 mg/mL of USP 1,4-Sorbitan RS, and 25 mg/mL of sorbitol
Sample solution: 250 mg/mL of the polyols, obtained in the *Assay* for *Polyols*
Chromatographic system
(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
Mode: TLC
Adsorbent: 0.25-mm layer of chromatographic silica gel
Application volume: 2 µL
Developing solvent system: Acetone and glacial acetic acid (50:1)
Spray reagent: Sulfuric acid and water (50:50)
Analysis
Samples: *Standard solution* and *Sample solution*
Apply the *Standard solution* and *Sample solution*, and allow the spots to dry. Develop the chromatogram until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the developing chamber, mark the solvent front, and allow the solvent to evaporate. Spray evenly with *Spray reagent* until the surface is uniformly wet (do not overspray), and immediately place the sprayed plate on a 200° hot plate in a well-ventilated hood. Char until white fumes of sulfur trioxide cease, and cool.
Acceptance criteria: The R_F values of the spots of the *Sample solution* correspond to those of the spots of the *Standard solution*.

ASSAY

• **FATTY ACIDS**
Sample: 10 g
Analysis: Transfer the *Sample* to a 500-mL conical flask. Cautiously add 100 mL of alcohol and 3.0 g of potassium hydroxide. Connect a suitable condenser to the flask, reflux the mixture on a hot plate for 2 h, add 100 mL of water, and heat on a steam bath to evaporate the alcohol, adding water occasionally to replace the alcohol. Continue the evaporation until the odor of alcohol can no longer be detected, and transfer the saponification mixture, with the aid of 100 mL of hot water, to a 500-mL separator. Using extreme caution, neutralize to litmus paper with a mixture of equal volumes of sulfuric acid and water, noting the volume used, and add a 10% excess of the dilute acid. Allow the solution to cool. If salts appear, add sufficient water to produce a clear solution. Cautiously add 100 mL of solvent hexane, shake thoroughly, and withdraw the lower layer into a second 500-mL separator. Similarly extract with two more 100-mL portions of solvent hexane. Extract the combined hexane layers with 50-mL portions of water until neutral to litmus paper. Combine the extracts with the original aqueous phase to use for the *Assay* for *Polyols*. Evaporate the solvent hexane in a tared beaker on a steam bath nearly to dryness, dry under vacuum at 60° for 1 h, cool in a desiccator, and weigh the fatty acids.
Acceptance criteria: 63.0%–71.0%
• **POLYOLS**
Sample solution: Aqueous solution retained from the *Assay* for *Fatty Acids*
Analysis: Neutralize the *Sample solution* with a potassium hydroxide solution (1 in 10) to a pH of 7, using a pH meter. Evaporate on a steam bath to a moist residue, extract the polyols from the salts with three 150-mL portions of dehydrated alcohol, boiling the salt residue for 3 min, and crushing it, as necessary, with the flattened end of a stirring rod during each extraction; filtering each extract while hot through a medium-porosity, sintered-glass funnel provided with a sheet of retentive filter paper on which a layer of purified siliceous earth has been superimposed; and receiving the filtrates in a 1-L suction flask. Transfer the clear alcoholic polyols solution to a tared beaker, evaporate the alcohol on a steam bath, dry under vacuum at 60° for 1 h, cool in a desiccator, and weigh the polyols.
Acceptance criteria: 32.0%–38.0% (w/w)

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.5%

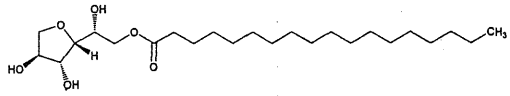
SPECIFIC TESTS

• **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 1.5%
• **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩: NMT 8
• **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩: 275–305
• **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩: 140–150

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ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Isosorbide RS
  USP 1,4-Sorbitan RS
  $C_6H_{12}O_5$ 164.16

Sorbitan Monostearate



Sorbitan, esters, monooctadecanoate;
Sorbitan Monostearate [1338-41-6].

DEFINITION
Sorbitan Monostearate is a partial ester of Stearic Acid with Sorbitol and its mono- and dianhydrides. It yields, upon saponification, NLT 68.0% and NMT 76.0% of fatty acids, and NLT 27.0% and NMT 34.0% of polyols (w/w).

IDENTIFICATION
- **A.**
  Sample: Residue obtained in the *Assay for Fatty Acids*
  Acceptance criteria
  Fats and Fixed Oils, Acid Value ⟨401⟩: 200–215 on 1-g sample
  Fats and Fixed Oils, Iodine Value ⟨401⟩: NMT 4
- **B.**
  Standard solution: 33 mg/mL of USP Isosorbide RS, 25 mg/mL of USP 1,4-Sorbitan RS, and 25 mg/mL of sorbitol
  Sample solution: 250 mg/mL of the polyols, obtained in the *Assay* for *Polyols*
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)
  Mode: TLC
  Adsorbent: 0.25-mm layer of chromatographic silica gel
  Application volume: 2 µL
  Developing solvent system: Acetone and glacial acetic acid (50:1)
  Spray reagent: Sulfuric acid and water (50:50)
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Apply the *Standard solution* and *Sample solution*, and allow the spots to dry. Develop the chromatogram until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the developing chamber, mark the solvent front, and allow the solvent to evaporate. Spray evenly with *Spray reagent* until the surface is uniformly wet (do not overspray), and immediately place the sprayed plate on a 200° hot plate in a well-ventilated hood. Char until white fumes of sulfur trioxide cease, and cool.
  Acceptance criteria: The R_F values of the spots from the *Sample solution* correspond to those of the spots from the *Standard solution*.

ASSAY
- **FATTY ACIDS**
  Sample: 10 g
  Analysis: Transfer the *Sample* to a 500-mL conical flask. Cautiously add 100 mL of alcohol, 3.0 g of potassium hydroxide, and a few glass beads. Connect a suitable condenser to the flask, reflux the mixture on a hot plate for 2 h, add 100 mL of water, and heat on a steam bath to evaporate the alcohol, adding water occasionally to replace the alcohol. Continue the evaporation until the odor of alcohol can no longer be detected, and transfer the saponification mixture, with the aid of 100 mL of hot water, to a 500-mL separator. Using extreme caution, neutralize to litmus paper with a mixture of equal volumes of sulfuric acid and water, noting the volume used, and add a 10% excess of the dilute acid. Allow the solution to cool. If salts appear, add sufficient water to produce a clear solution. Cautiously add 100 mL of solvent hexane, shake thoroughly, and withdraw the lower layer into a second 500-mL separator. Similarly extract with two more 100-mL portions of solvent hexane. Extract the combined hexane layers with 50-mL portions of water until neutral to litmus paper. Combine the extracts with the original aqueous phase to use for the *Assay* for *Polyols*. Evaporate the solvent hexane in a tared beaker on a steam bath nearly to dryness, dry under vacuum at 60° for 1 h, cool in a desiccator, and weigh the fatty acids.
  Acceptance criteria: 68.0%–76.0%
- **POLYOLS**
  Sample solution: Aqueous solution retained from the *Assay* for *Fatty Acids*
  Analysis: Neutralize the *Sample solution* with a potassium hydroxide solution (1 in 10) to a pH of 7, using a pH meter. Evaporate on a steam bath to a moist residue, extract the polyols from the salts with three 150-mL portions of dehydrated alcohol, boiling the salt residue for 3 min, and crushing it, as necessary, with the flattened end of a stirring rod, during each extraction; filtering each extract while hot through a medium-porosity, sintered-glass funnel provided with a sheet of retentive filter paper on which a layer of purified siliceous earth has been superimposed; and receiving the filtrates in a 1-L suction flask. Transfer the clear alcoholic polyols solution to a tared beaker, evaporate the alcohol on a steam bath, dry under vacuum at 60° for 1 h, cool in a desiccator, and weigh the polyols.
  Acceptance criteria: 27.0%–34.0% (w/w)

IMPURITIES
- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.5%

SPECIFIC TESTS
- **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 1.5%
- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩: NMT 10
- **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩: 235–260
- **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩: 147–157

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Isosorbide RS
  USP 1,4-Sorbitan RS
  $C_6H_{12}O_5$ 164.16

Sorbitan Sesquioleate

DEFINITION
Sorbitan Sesquioleate is a partial oleate ester of Sorbitol and its mono- and dianhydrides. It yields, upon saponification, NLT 74.0% and NMT 80.0% of fatty acids, and NLT 22.0% and NMT 28.0% of polyols (w/w).

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IDENTIFICATION

• **A. Fatty Acid Composition of Constituting Oleic Acid:** It meets the requirements of the test for *Fats and Fixed Oils, Fatty Acid Composition* ⟨401⟩.

• **B. Identification of Constituting Polyols by Thin-Layer Chromatography**

Standard solution: 33 mg/mL of USP Isosorbide RS, 25 mg/mL of USP 1,4-Sorbitan RS, and 25 mg/mL of USP Sorbitol RS

Sample solution: 250 mg/mL of polyols, obtained in the *Assay* for *Polyols*

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Mode: TLC

Adsorbent: 0.25-mm layer of chromatographic silica gel

Application volume: 2 µL

Developing solvent system: Acetone and glacial acetic acid (50:1)

Spray reagent: Sulfuric acid and water (50:50)

Analysis

Samples: *Standard solution* and *Sample solution*

Apply the *Standard solution* and *Sample solution*, and allow the spots to dry. Develop the chromatogram until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the developing chamber, mark the solvent front, and allow the solvent to evaporate. Spray evenly with *Spray reagent* until the surface is uniformly wet (do not overspray), and immediately place the sprayed plate on a 200° hot plate in a well-ventilated hood. Char until white fumes of sulfur trioxide cease, and cool.

Acceptance criteria: The R_F values of the spots from the *Sample solution* correspond to those of the spots from the *Standard solution*.

ASSAY

• **Fatty Acids**

Sample: 10 g

Analysis: Transfer the *Sample* to a 500-mL conical flask. Cautiously add 100 mL of alcohol, 3.5 g of potassium hydroxide, and a few glass beads. Connect a suitable condenser to the flask, reflux the mixture on a hot plate for 2 h, add 100 mL of water, and heat on a steam bath to evaporate the alcohol, adding water occasionally to replace the alcohol. Continue the evaporation until the odor of alcohol can no longer be detected, and transfer the saponification mixture, with the aid of 100 mL of hot water, to a 500-mL separator. Using extreme caution, neutralize to litmus paper with a mixture of equal volumes of sulfuric acid and water, noting the volume used, and add a 10% excess of the dilute acid. Allow the solution to cool. If salts appear, add sufficient water to produce a clear solution. Cautiously add 100 mL of solvent hexane, shake thoroughly, and withdraw the lower layer into a second 500-mL separator. Similarly extract with two more 100-mL portions of solvent hexane. Extract the combined hexane layers with 50-mL portions of water until neutral to litmus paper. Combine the extracts with the original aqueous phase to use for the *Assay* for *Polyols*. Evaporate the solvent hexane in a tared beaker on a steam bath nearly to dryness, dry under vacuum at 60° for 1 h, cool in a desiccator, and weigh the fatty acids.

Acceptance criteria: 74.0%–80.0%

• **Polyols**

Sample solution: Aqueous solution retained from the *Assay* for *Fatty Acids*

Analysis: Neutralize the *Sample solution* with a potassium hydroxide solution (1 in 10) to a pH of 7, using a pH meter. Evaporate on a steam bath to a moist residue, extract the polyols from the salts with three 150-mL portions of dehydrated alcohol, boiling the salt residue for 3 min and crushing it, as necessary, with the flattened end of a stirring rod during each extraction; filtering each extract while hot through a medium-porosity, sintered-glass funnel provided with a sheet of retentive filter paper on which a layer of purified siliceous earth has been superimposed; and receiving the filtrates in a 1-L suction flask. Transfer the clear alcoholic polyols solution to a tared beaker, evaporate the alcohol on a steam bath, dry under vacuum at 60° for 1 h, cool in a desiccator, and weigh the polyols.

Acceptance criteria: 22.0%–28.0% (w/w)

IMPURITIES

• **Residue on Ignition** ⟨281⟩: NMT 1.4%

SPECIFIC TESTS

• **Fats and Fixed Oils,** *Fatty Acid Composition* ⟨401⟩: It exhibits the composition profiles of fatty acids shown in *Table 1*.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
14	0	≤5.0
16	0	≤16.0
16	1	≤8.0
18	0	≤6.0
18	1	≥65.0
18	2	≤18.0
18	3	≤4.0
20, 22[a]	0	≤4.0

[a] The sum of these fatty acids should be NMT 4.0%.

• **Water Determination,** *Method I* ⟨921⟩: NMT 1.0%
• **Fats and Fixed Oils,** *Acid Value* ⟨401⟩: NMT 14
• **Fats and Fixed Oils,** *Hydroxyl Value* ⟨401⟩: 182–220
• **Fats and Fixed Oils,** *Peroxide Value* ⟨401⟩: NMT 10.0
• **Fats and Fixed Oils,** *Saponification Value* ⟨401⟩: 143–165

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Preserve in tight containers.
• **USP Reference Standards** ⟨11⟩

USP Isosorbide RS
USP 1,4-Sorbitan RS
$C_6H_{12}O_5$ 164.16
USP Sorbitol RS

Sorbitan Trioleate

DEFINITION

Sorbitan Trioleate is the triester of Oleic Acid and Sorbitol and its mono- and dianhydrides. It yields, upon saponification, NLT 85.5% and NMT 90.0% of fatty acids, and NLT 13.0% and NMT 19.0% of polyols (w/w).

IDENTIFICATION

• **A.**

Sample: Residue obtained in the *Assay for Fatty Acids*

Acceptance criteria

Fats and Fixed Oils, Acid Value ⟨401⟩: 192–204 on 1-g sample

Fats and Fixed Oils, Iodine Value ⟨401⟩: 75–95

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• **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩

Standard solution: 33 mg/mL of USP Isosorbide RS, 25 mg/mL of USP 1,4-Sorbitan RS, and 25 mg/mL of sorbitol

Sample solution: 250 mg/mL of polyols, obtained in the *Assay* for *Polyols*

Chromatographic system

(See *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*.)

Adsorbent: 0.25-mm layer of chromatographic silica gel

Application volume: 2 µL

Developing solvent system: Acetone and glacial acetic acid (50:1)

Spray reagent: Sulfuric acid and water (50:50)

Analysis

Samples: *Standard solution* and *Sample solution*

Apply the *Standard solution* and the *Sample solution*, and allow the spots to dry. Develop the chromatogram until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the developing chamber, mark the solvent front, and allow the solvent to evaporate. Spray evenly with *Spray reagent* until the surface is uniformly wet (do not overspray), and immediately place the sprayed plate on a 200° hot plate in a well-ventilated hood. Char until white fumes of sulfur trioxide cease, and cool.

Acceptance criteria: The R_F values of the spots of the *Sample solution* correspond to those of the spots of the *Standard solution*.

ASSAY

• **FATTY ACIDS**

Sample: 8.6 g

Analysis: Transfer the *Sample* to a 500-mL conical flask. Cautiously add 100 mL of alcohol and 3.5 g of potassium hydroxide, then add a few glass beads, and mix. Connect a suitable condenser to the flask, reflux the mixture on a hot plate for 2 h, add 100 mL of water, and heat on a steam bath to evaporate the alcohol, adding water occasionally to replace the alcohol. Continue the evaporation until the odor of alcohol can no longer be detected, and transfer the saponification mixture, with the aid of 100 mL of hot water, to a 500-mL separator. Using extreme caution, neutralize to litmus paper with a mixture of equal volumes of sulfuric acid and water, noting the volume used, and add a 10% excess of the dilute acid. Allow the solution to cool. If salts appear, add sufficient water to produce a clear solution. Cautiously add 100 mL of solvent hexane, shake thoroughly, and withdraw the lower layer into a second 500-mL separator. Similarly extract with two more 100-mL portions of solvent hexane. Extract the combined hexane layers with 50-mL portions of water until neutral to litmus paper. Combine the extracts with the original aqueous phase to use for the *Assay* for *Polyols*. Evaporate the solvent hexane in a tared beaker on a steam bath nearly to dryness, dry under vacuum at 60° for 1 h, cool in a desiccator, and weigh the fatty acids.

Acceptance criteria: 85.5%–90.0%

• **POLYOLS**

Sample solution: Aqueous solution of polyols retained from the *Assay* for *Fatty Acids*

Analysis: Neutralize the *Sample solution* with potassium hydroxide solution (1 in 10) to a pH of 7, using a pH meter. Evaporate on a steam bath to a moist residue. Extract the polyols from the salts with three 150-mL portions of dehydrated alcohol, boiling the salt residue for 3 min, and crushing it, as necessary, with the flattened end of a stirring rod during each extraction. Filter each extract while hot through a medium-porosity, sintered-glass funnel, provided with a sheet of retentive filter paper on which a layer of purified siliceous earth has been superimposed, and receiving the filtrates in a 1-L suction flask. Transfer the clear alcoholic polyols solution to a tared beaker, evaporate the alcohol on a steam bath, dry under vacuum at 60° for 1 h, cool in a desiccator, and weigh the polyols.

Acceptance criteria: 13.0%–19.0% (w/w)

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.25%

SPECIFIC TESTS

• **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 0.7%
• **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩: NMT 17
• **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩: 50–75
• **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩: 77–85
• **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩: 169–183

ADDITIONAL REQUIREMENTS

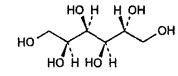
• **PACKAGING AND STORAGE:** Preserve in tight containers.
• **USP REFERENCE STANDARDS** ⟨11⟩

USP Isosorbide RS

USP 1,4-Sorbitan RS

$C_6H_{12}O_5$ 164.16

Sorbitol



$C_6H_{14}O_6$ 182.17

D-Glucitol [50-70-4].

DEFINITION

Sorbitol contains NLT 91.0% and NMT 100.5% of D-sorbitol, calculated on the anhydrous basis. The amounts of total sugars, other polyhydric alcohols, and any hexitol anhydrides, if detected, are not included in the requirements, nor in the calculated amount under *General Notices, 5.60.10 Other Impurities in USP and NF Articles*

IDENTIFICATION

• **A.**

Sample solution: 1 g of Sorbitol in 75 mL of water

Analysis: Transfer 3 mL of *Sample solution* to a 15-cm test tube, and add 3 mL of freshly prepared catechol solution (1 in 10), and mix. Add 6 mL of sulfuric acid, then gently heat the tube in a flame for 30 s.

Acceptance criteria: A deep pink or wine-red color appears.

• **B.** The retention time of the major peak of the *Sample solution* corresponds to that from the *Standard solution*, as obtained in the *Assay*.

ASSAY

• **PROCEDURE**

Mobile phase: Use degassed water.

System suitability solution: Prepare a solution containing 4.8 mg/g of each USP Sorbitol RS and mannitol

Standard solution: 4.8 mg/g of USP Sorbitol RS

Sample solution: Dissolve 0.10 g of Sorbitol in water, and dilute with water to 20 g. Record the final solution weight, and mix thoroughly.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 7.8-mm × 10-cm; packing L34

Temperature

Column: 50 ± 2°

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Detector: 35°
Flow rate: 0.7 mL/min
Injection size: 10 µL
System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The relative retention times for mannitol and sorbitol are about 0.6 and 1.0, respectively.]
Suitability requirements
Resolution: NLT 2.0 between sorbitol and mannitol, *System suitability solution*
Relative standard deviation: NMT 2.0%, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage, on the anhydrous basis, of D-sorbitol in the portion of Sorbitol taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (100/(100 - W)) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP Sorbitol RS in the *Standard solution* (mg/g)
C_U = concentration of Sorbitol in the *Sample solution* (mg/g)
W = percentage obtained in the test for *Water Determination*

Acceptance criteria: 91.0%–100.5% on the anhydrous basis

IMPURITIES

• **LIMIT OF NICKEL**
Sample solution: Dissolve 20.0 g of Sorbitol in diluted acetic acid, and dilute with diluted acetic acid to 150 mL.
Blank solution: 150 mL of diluted acetic acid
Standard solutions: Prepare three solutions by adding 0.5, 1.0, and 1.5 mL of nickel standard solution TS to 20.0 g of Sorbitol dissolved in diluted acetic acid, and dilute with the same solvent to 150 mL.
Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Analytical wavelength: 232.0 nm
Lamp: Nickel hollow-cathode
Flame: Air–acetylene
Analysis
Samples: *Standard solutions* and *Sample solution*
To each sample add 2.0 mL of a saturated ammonium pyrrolidinedithiocarbamate solution (containing 10 g/L of ammonium pyrrolidinedithiocarbamate) and 10.0 mL of methyl isobutyl ketone, and shake for 30 s. Protect from bright light. Allow the two layers to separate, and use the methyl isobutyl ketone layer. Set the instrument to zero using the organic layer from the *Blank solution*. Concomitantly determine the absorbances of the organic layer from the *Samples* at least three times each. Record the average of the steady readings for each of the *Standard solutions* and the *Sample solution*. Between each measurement, aspirate the organic layer from the *Blank solution*, and ascertain that the reading returns to zero. Plot the absorbances of the *Standard solutions* and the *Sample solution* versus the added quantity of nickel. Extrapolate the line joining the points on the graph until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of nickel in the *Sample solution*.
Acceptance criteria: NMT 1 ppm
• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%, determined on a 1.5-g portion
• **REDUCING SUGARS**
[NOTE—The amount determined in this test is not included in the calculated amount under *General Notices, 5.60.10 Other Impurities in USP and NF Articles*.]
Sample solution: Dissolve 3.3 g of Sorbitol in 3 mL of water with the aid of gentle heat. Cool, and add 20.0 mL of cupric citrate TS and a few glass beads. Heat so that boiling begins after 4 min, and maintain boiling for 3 min. Cool rapidly, and add 40 mL of diluted acetic acid, 60 mL of water, and 20.0 mL of 0.05 N iodine VS. With continuous shaking, add 25 mL of a mixture of 6 mL of hydrochloric acid and 94 mL of water.
Analysis: When the precipitate has dissolved, titrate the excess of iodine with 0.05 N sodium thiosulfate VS using 2 mL of starch TS, added toward the end of the titration, as an indicator.
Acceptance criteria: NLT 12.8 mL of 0.05 N sodium thiosulfate VS is required, corresponding to NMT 0.3% of reducing sugars, as glucose.
• **CHLORIDE AND SULFATE,** *Chloride* ⟨221⟩ (if labeled for use in preparing parenteral dosage forms)
Sample: 1.5 g
Acceptance criteria: The *Sample* shows no more chloride than corresponds to 0.10 mL of 0.020 N hydrochloric acid (NMT 0.0050%).
• **CHLORIDE AND SULFATE,** *Sulfate* ⟨221⟩ (if labeled for use in preparing parenteral dosage forms)
Sample: 1.0 g
Acceptance criteria: The *Sample* shows no more sulfate than corresponds to 0.10 mL of 0.020 N sulfuric acid (NMT 0.01%).

SPECIFIC TESTS

• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩: The total aerobic count using the *Plate Method* is NMT 1000 cfu/g, and the total combined molds and yeasts count is NMT 100 cfu/g.
• **pH** ⟨791⟩: 3.5–7.0, in a 10% (w/w) solution in carbon dioxide-free water
• **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 1.5%
• **CLARITY AND COLOR OF SOLUTION** (if labeled for use in preparing parenteral dosage forms)
Sample: 10.0 g
Analysis: Dissolve the *Sample* in 100.0 mL of carbon dioxide-free water.
Acceptance criteria: The solution is clear and colorless.
• **BACTERIAL ENDOTOXINS TEST** ⟨85⟩ (if labeled for use in preparing parenteral dosage forms): NMT 4 USP Endotoxin Units/g for parenteral dosage forms having a concentration of less than 100 g/L of sorbitol, and NMT 2.5 USP Endotoxin Units/g for parenteral dosage forms having a concentration of 100 g/L or more of sorbitol

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements are specified.
• **LABELING:** Sorbitol intended for use in preparing parenteral dosage forms is so labeled.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Sorbitol RS

Noncrystallizing Sorbitol Solution

DEFINITION

Noncrystallizing Sorbitol Solution is an aqueous solution containing NLT 45.0% of D-sorbitol ($C_6H_{14}O_6$) (w/w). The

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amounts of total sugars, other polyhydric alcohols, and any hexitol anhydrides, if detected, are not included in the requirements nor in the calculated amount under *General Notices, 5.60.10. Other Impurities in USP and NF Articles.*

IDENTIFICATION

• **A. Procedure**

Sample solution: Dissolve 1.4 g of Noncrystallizing Sorbitol Solution in 75 mL of water.

Analysis: Transfer 3 mL of *Sample solution* to a 15-cm test tube. Add 3 mL of freshly prepared catechol solution (1 in 10), and mix. Add 6 mL of sulfuric acid, mix again, and gently heat the tube in a flame for 30 s.

Acceptance criteria: A deep pink or wine-red color appears.

• **B.** The retention time of the major peak from the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

• **C. Limit of Diethylene Glycol and Ethylene Glycol**

Diluent: Acetone and water (96:4)

Standard solution: 0.08 mg/mL of USP Diethylene Glycol RS and 0.08 mg/mL of USP Ethylene Glycol RS in *Diluent*.

Sample solution: Transfer 2.0 g of Noncrystallizing Sorbitol Solution to a 25-mL volumetric flask. Add 1.0 mL of *Diluent* to the flask, and mix on a vortex mixer for 3 min. Add the remaining *Diluent* to the flask to volume in three equal portions. Mix on a vortex mixer for about 3 min after each addition of *Diluent*. Pass a portion of the supernatant layer obtained through a 0.45-µm nylon filter. Discard the first 2 mL of the filtrate, and collect the rest of the filtrate for analysis.

[Note—Acetone is used to precipitate sorbitol.]

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.32-mm × 15-m fused-silica capillary column; 0.25-µm layer of phase G46

Temperature

Detector: 300°

Injector port: 240°

Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	—	70	2
70	50	300	5

Carrier gas: Helium

Flow rate: 3.0 mL/min

Injection size: 1.0 µL

Injection type: Split injection. The split ratio is about 10:1.

[Note—A split liner, deactivated with glass wool, is used.]

System suitability

Sample: *Standard solution*

[Note—Diethylene glycol elutes after ethylene glycol in the chromatogram]

Suitability requirements

Resolution: NLT 30 between ethylene glycol and diethylene glycol

Analysis

Samples: *Standard solution* and *Sample solution*

Based on the *Standard solution*, identify the peaks of ethylene glycol and diethylene glycol. Compare the peak areas of ethylene glycol and diethylene glycol in the *Standard solution* and the *Sample solution*.

Acceptance criteria

Diethylene glycol: The peak area of diethylene glycol in the *Sample solution* is NMT the peak area of diethylene glycol in the *Standard solution*, corresponding to NMT 0.10% of diethylene glycol in Noncrystallizing Sorbitol Solution.

Ethylene glycol: The peak area of ethylene glycol in the *Sample solution* is NMT the peak area of ethylene glycol in the *Standard solution*, corresponding to NMT 0.10% of ethylene glycol in Noncrystallizing Sorbitol Solution.

ASSAY

• **Procedure**

Mobile phase: Use degassed water.

System suitability solution: 4.8 mg/g each of mannitol and USP Sorbitol RS

Standard solution: 4.8 mg/g of USP Sorbitol RS

Sample solution: Weigh 0.20 g of Noncrystallizing Sorbitol Solution, and dissolve in and dilute with water to 20 g. Record the final solution weight.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 7.8-mm × 10-cm; packing L34

Temperature

Detector: 35°

Column: 50 ± 2°

Flow rate: 0.7 mL/min

Injection size: 10 µL

System suitability

Samples: *System suitability solution* and *Standard solution*

[Note—The relative retention times for mannitol and sorbitol are about 0.6 and 1.0, respectively, *System suitability solution*.]

Suitability requirements

Resolution: NLT 2.0 between sorbitol and mannitol, *System suitability solution*

Relative standard deviation: NMT 2.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of D-sorbitol ($C_6H_{14}O_6$) in the portion of Noncrystallizing Sorbitol Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Sorbitol RS in the *Standard solution* (mg/g)

C_U = concentration of Noncrystallizing Sorbitol Solution in the *Sample solution* (mg/g)

Acceptance criteria: NLT 45.0%

IMPURITIES

Inorganic Impurities

• **Residue on Ignition** ⟨281⟩: NMT 0.1%, calculated on the anhydrous basis, determined on a 2-g portion

• **Limit of Nickel**

Solution A: A saturated ammonium pyrrolidine dithiocarbamate solution (containing 10 mg/mL of ammonium pyrrolidine dithiocarbamate)

Sample solution: Dissolve 20.0 g of Noncrystallizing Sorbitol Solution in diluted acetic acid, and dilute with diluted acetic acid to 100.0 mL. Add 2.0 mL of *Solution A* and 10.0 mL of methyl isobutyl ketone, and shake for 30 s. Protect from bright light. Allow the two layers to separate, and use the methyl isobutyl ketone layer.

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Blank solution: Prepare as directed for the *Sample solution*, except omit the use of the Noncrystallizing Sorbitol Solution.
Standard solutions: Prepare as directed for the *Sample solution*, except prepare three solutions by adding 0.5, 1.0, and 1.5 mL of nickel standard solution TS.
Spectrometric conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Analytical wavelength: 232.0 nm (maximum absorbance)
Lamp: Nickel hollow-cathode
Flame: Air–acetylene
Analysis
Samples: *Standard solutions*, *Sample solution*, and *Blank solution*
Set the instrument to zero using the *Blank solution*. Concomitantly determine the absorbances of the *Standard solutions* and the *Sample solution* at least three times each. Record the average of the steady readings for each of the *Standard solutions* and the *Sample solution*. Between each measurement, aspirate the *Blank solution*, and ascertain that the reading returns to zero. Plot the absorbances of the *Standard solutions* and the *Sample solution* versus the added quantity of nickel. Extrapolate the line joining the points on the graph until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of nickel in the *Sample solution*.
Acceptance criteria: NMT 1 µg/g on the anhydrous basis

ORGANIC IMPURITIES
• **Procedure: Reducing Sugars**
Sample: An amount of Noncrystallizing Sorbitol Solution, equivalent to 3.3 g on the anhydrous basis.
Analysis: To the *Sample*, add 3 mL of water, 20.0 mL of cupric citrate TS, and a few glass beads. Heat so that boiling begins after 4 min, and maintain boiling for 3 min. Cool rapidly, and add 40 mL of diluted acetic acid, 60 mL of water, and 20.0 mL of 0.05 N iodine VS. With continuous shaking, add 25 mL of a mixture of 6 mL of hydrochloric acid and 94 mL of water. When the precipitate has dissolved, titrate the excess of iodine with 0.05 N sodium thiosulfate VS using 2 mL of starch TS, added towards the end of the titration, as an indicator.
Acceptance criteria: NLT 12.8 mL of 0.05 N sodium thiosulfate VS is required corresponding to NMT 0.3% of reducing sugars, on the anhydrous basis, as glucose. The amount determined in this test is not included in the calculated amount under *General Notices, 5.60.10. Other Impurities in USP and NF Articles*.

SPECIFIC TESTS
• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic microbial count using the *Plate Method* is NMT 1000 cfu/mL, and the total combined molds and yeasts count is NMT 100 cfu/mL.
• **pH** ⟨791⟩**:** 5.0–7.5, in a 14% (w/w) solution of Noncrystallizing Sorbitol Solution in carbon dioxide-free water
• **WATER DETERMINATION,** *Method I* ⟨921⟩**:** 28.5%–31.5%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements are specified.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Sorbitol RS
USP Diethylene Glycol RS
USP Ethylene Glycol RS

Sorbitol Sorbitan Solution

Former Title: Anhydrized Liquid Sorbitol

DEFINITION
Sorbitol Sorbitan Solution is a water solution containing, on the anhydrous basis, NLT 25.0% of D-sorbitol ($C_6H_{14}O_6$) and NLT 15.0% of 1,4-sorbitan ($C_6H_{12}O_5$). The amounts of total sugars, other polyhydric alcohols, and any other hexitol anhydrides, if detected, are not included in the requirements or in the calculated amount under *General Notices, 5.60.10. Other Impurities in USP and NF Articles*.

IDENTIFICATION
• **A. PROCEDURE**
Sample: 1.4 g of Sorbitol Sorbitan Solution in 75 mL of water
Analysis: Transfer 3 mL of *Sample* to a 15-cm test tube. Add 3 mL of freshly prepared catechol solution (1 in 10), and mix. Add 6 mL of sulfuric acid, mix again, then gently heat the tube in a flame for about 30 s.
Acceptance criteria: A deep pink or wine-red color appears.
• **B.** The retention times of the *Sample solution* correspond to those of the *Standard solution*, as obtained in the *Assay*.
• **C. LIMIT OF DIETHYLENE GLYCOL AND ETHYLENE GLYCOL**
Diluent: Acetone and water (96:4)
Standard solution: 0.08 mg/mL of USP Diethylene Glycol RS and 0.08 mg/mL of USP Ethylene Glycol RS in *Diluent*
Sample solution: Transfer 2.0 g of Sorbitol Sorbitan Solution to a 25-mL volumetric flask. Add 1.0 mL of *Diluent* to the flask, and mix on a vortex mixer for about 3 min. Add the remaining *Diluent* to the flask to volume in three equal portions. Mix on a vortex mixer for about 3 min after each addition of *Diluent*. Pass a portion of the supernatant layer obtained through a 0.45-µm nylon filter. Discard the first 2 mL of the filtrate, and collect the rest of the filtrate for analysis. [NOTE—Acetone is used to precipitate sorbitol.]
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.32-mm × 15-m fused-silica capillary column; 0.25-µm layer of phase G46
Temperature
Detector: 300°
Injector port: 240°
Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	—	70	2
70	50	300	5

Carrier gas: Helium
Flow rate: 3.0 mL/min
Injection size: 1.0 µL
Injection type: Split injection. The split ratio is about 10:1. [NOTE—A split liner, deactivated with glass wool, is used.]
System suitability
Sample: *Standard solution*
[NOTE—Diethylene glycol elutes after ethylene glycol in the chromatogram.]
Suitability requirements
Resolution: NLT 30 between ethylene glycol and diethylene glycol

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Analysis
Samples: *Standard solution* and *Sample solution*
Based on the *Standard solution*, identify the peaks of ethylene glycol and diethylene glycol. Compare peak areas of ethylene glycol and diethylene glycol in the *Standard solution* and the *Sample solution.*
Acceptance criteria
Diethylene glycol: The peak area of diethylene glycol in the *Sample solution* is NMT the peak area of diethylene glycol in the *Standard solution*, corresponding to NMT 0.10% of diethylene glycol in Sorbitol Sorbitan Solution.
Ethylene glycol: The peak area of ethylene glycol in the *Sample solution* is NMT the peak area of ethylene glycol in the *Standard solution*, corresponding to NMT 0.10% of ethylene glycol in Sorbitol Sorbitan Solution.

ASSAY

• **PROCEDURE**
Mobile phase: Water
System suitability solution: 10 mg/g of sorbitol, 4 mg/g of 1,4-sorbitan, 4 mg/g of isosorbide, and 1 mg/g of mannitol in water
Standard solution: 10 mg/g of USP Sorbitol RS and 4 mg/g of USP 1,4-Sorbitan RS in water
Sample solution: Dissolve 0.40 g of Sorbitol Sorbitan Solution in water, and dilute with water to about 20 g. Record the weight of the final solution, and mix thoroughly.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Refractive index
Column: 7.8-mm × 10-cm; packing L34
Temperature
Detector: 35°
Column: 50 ± 2°
Flow rate: 0.6 mL/min
Injection size: 10 µL
System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—The relative retention times for 1,4-sorbitan, isosorbide, mannitol, and sorbitol are about 0.35, 0.43, 0.7, and 1.0, respectively.]
Suitability requirements
Resolution: NLT 2.0 between 1,4-sorbitan and isosorbide, *System suitability solution*
Relative standard deviation: NMT 2.0% for each analyte, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Separately calculate the percentages, on the anhydrous basis, of 1,4-sorbitan and D-sorbitol in the portion of Sorbitol Sorbitan Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times [100/(100 - W)] \times 100$$

r_U = peak responses of the corresponding analyte from the *Sample solution*
r_S = peak responses of the corresponding analyte from the *Standard solution*
C_S = concentration of the appropriate USP Reference Standard in the *Standard solution* (mg/g)
C_U = concentration of Sorbitol Sorbitan Solution in the *Sample solution* (mg/g)
W = percentage from the test for *Water Determination*

Acceptance criteria: NLT 25.0% of $C_6H_{14}O_6$ and NLT 15.0% of $C_6H_{12}O_5$ on the anhydrous basis

IMPURITIES

INORGANIC IMPURITIES

• **Residue on Ignition** ⟨281⟩: NMT 0.20%, calculated on the anhydrous basis on a 2-g portion
• **Limit of Nickel**
Solution A: A saturated ammonium pyrrolidine dithiocarbamate solution (10 mg/mL of ammonium pyrrolidine dithiocarbamate)
Sample solution: 200 mg/mL of Sorbitol Sorbitan Solution in diluted acetic acid. To 100 mL of this solution add 2.0 mL of *Solution A* and 10.0 mL of methyl isobutyl ketone, and shake for 30 s. Protect from bright light. Allow the two layers to separate, and use the methyl isobutyl ketone layer.
Standard solutions: Prepare as directed for the *Sample solution,* except to prepare three solutions by adding 0.5, 1.0, and 1.5 mL of nickel standard solution TS.
Blank solution: Prepare as directed for the *Sample solution,* except to omit the use of Sorbitol Sorbitan Solution. Quantities should be increased five fold to ensure that a sufficient volume of *Blank solution* is available.
Spectrometric conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption spectrophotometry
Analytical wavelength: 232.0 nm (maximum absorbance)
Lamp: Nickel hollow-cathode
Flame: Air–acetylene
Analysis
Samples: *Blank solution, Standard solutions,* and *Sample solution*
Set the instrument to zero, using the *Blank solution.* Concomitantly determine the absorbances of the *Standard solutions* and the *Sample solution* at least three times each. Record the average of the steady readings for each of the *Standard solutions* and the *Sample solution.* Between each measurement, aspirate the *Blank solution,* and ascertain that the reading returns to zero. Plot the absorbances of the *Standard solutions* and the *Sample solution* versus the added quantity of nickel. Extrapolate the line joining the points on the graph until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of nickel in the *Sample solution.*
Acceptance criteria: NMT 1 ppm, calculated on the anhydrous basis

ORGANIC IMPURITIES

• **Procedure: Reducing Sugars**
Sample: An amount of Sorbitol Sorbitan Solution equivalent to 3.3 g, on the anhydrous basis.
Analysis: To the *Sample*, add 3 mL of water, 20.0 mL of cupric citrate TS, and a few glass beads. Heat so that boiling begins after 4 min, and maintain boiling for 3 min. Cool rapidly, and add 40 mL of diluted acetic acid, 60 mL of water, and 20.0 mL of 0.05 N iodine VS. With continuous shaking add 25 mL of a mixture of 6 mL of hydrochloric acid and 94 mL of water. When the precipitate has dissolved, titrate the excess of iodine with 0.05 N sodium thiosulfate VS, using 2 mL of starch TS, added toward the end of the titration, as an indicator.
Acceptance criteria: NLT 12.8 mL of 0.05 N sodium thiosulfate VS is required, corresponding to NMT 0.3% of reducing sugars, on the anhydrous basis, as glucose.
[NOTE—The amount determined in this test is not included in the calculated amount under *General Notices, 5.60.10. Other Impurities in USP and NF Articles.*]

SPECIFIC TESTS

• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED ORGANISMS** ⟨62⟩: The total aerobic microbial count using the *Plate Method* is NMT 1000 cfu/mL. The

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total combined molds and yeasts count is NMT 100 cfu/mL.
- **pH** ⟨791⟩: 4.0–7.0, in a 14% (w/w) solution of Sorbitol Sorbitan Solution in carbon dioxide-free water
- **Water Determination,** *Method I* ⟨921⟩: NMT 31.5%

ADDITIONAL REQUIREMENTS
- **Packaging and Storage:** Preserve in well-closed containers. No storage requirements specified.
- **Labeling:** The labeling indicates the percentage content, on the anhydrous basis, of D-sorbitol and 1,4-sorbitan.
- **USP Reference Standards** ⟨11⟩
  USP Diethylene Glycol RS
  USP Ethylene Glycol RS
  USP 1,4-Sorbitan RS
  $C_6H_{12}O_5$ 164.16
  USP Sorbitol RS

Sorbitol Solution—*see Sorbitol Solution General Monographs*

Soybean Oil—*see Soybean Oil General Monographs*

Hydrogenated Soybean Oil

[8016-70-4].

DEFINITION
Hydrogenated Soybean Oil is the product obtained by refining, bleaching, hydrogenation, and deodorization of oil obtained from seeds of the soya plant *Glycine max* Merr. (Fabaceae). The product consists mainly of triglycerides of palmitic and stearic acids.

IDENTIFICATION
- **A.** It meets the requirements in *Specific Tests* for *Fats and Fixed Oils, Fatty Acid Composition* ⟨401⟩.
- **B.** It meets the requirements in *Specific Tests* for *Melting Range or Temperature, Class II* ⟨741⟩.

IMPURITIES
- **Limit of Nickel**

Nickel standard solution: Immediately before use, prepare the equivalent of 0.2 µg/g of nickel by diluting 10 mL of nickel standard solution TS with water to 500 mL.

Sample solution: Weigh 5.0 g of Hydrogenated Soybean Oil into a previously tared platinum or silica crucible. Cautiously heat, and introduce into the substance a wick formed from twisted ashless filter paper. Ignite the wick. When the substance ignites, stop heating. After combustion, ignite in a muffle furnace at 600°. Continue the incineration until white ash is obtained. After cooling, transfer the residue, with the aid of two 2-mL portions of diluted hydrochloric acid, to a 25-mL volumetric flask, add 0.3 mL of nitric acid, and dilute with water to volume.

Standard solutions: Into three identical 10-mL volumetric flasks introduce 1.0, 2.0, and 4.0 mL of *Nickel standard solution*. To each flask add a 2.0-mL portion of the *Sample solution*, and dilute with water to volume.

Instrumental conditions
(See *Atomic Absorption Spectroscopy* ⟨852⟩.)
Mode: Atomic absorption, equipped with a graphite furnace
Analytical wavelength: 232.0 nm
Lamp: Nickel hollow-cathode

Analysis
Samples: *Sample solution* and *Standard solutions*
Determine the absorbances of the *Samples* at least three times each. Record the average of the steady readings for each of the *Samples*. Plot the absorbances of the *Standard solutions* and the *Sample solution* versus the added quantity of nickel, and draw the straight line best fitting the three plotted points. Extrapolate the line until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of nickel in the *Sample solution*.
Acceptance criteria: NMT 1 µg/g

- **Alkaline Impurities**

Sample: 2.0 g
Analysis: Dissolve the *Sample* by gently heating in a mixture of 1.5 mL of alcohol and 3.0 mL of toluene. Add 0.05 mL of bromophenol blue TS, and titrate with 0.01 N hydrochloric acid VS to a yellow endpoint.
Acceptance criteria: NMT 0.4 mL of 0.01 N hydrochloric acid VS is required.

SPECIFIC TESTS
- **Melting Range or Temperature,** *Class II* ⟨741⟩: 66°–72°
- **Fats and Fixed Oils,** *Acid Value* ⟨401⟩

Sample: 10 g of Hydrogenated Soybean Oil
Analysis: Dissolve the *Sample* in 50 mL of a hot mixture of neutralized alcohol and toluene (1:1). Add 0.5 mL of phenolphthalein TS, and immediately titrate, while still hot, with 0.1 N potassium hydroxide VS to produce a permanent, faint pink color.
Acceptance criteria: NMT 0.5

- **Fats and Fixed Oils,** *Fatty Acid Composition* ⟨401⟩: Hydrogenated Soybean Oil exhibits the composition profile of fatty acids in *Table 1*.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
<14	0	≤0.1
14	0	≤0.5
16	0	9–16
18	0	79–89
20	0	≤1.0
22	0	≤1.0
18	1	≤4.0
18	2	≤1.0
18	3	≤0.2

- **Fats and Fixed Oils,** *Peroxide Value* ⟨401⟩: NMT 5.0
- **Fats and Fixed Oils,** *Unsaponifiable Matter* ⟨401⟩

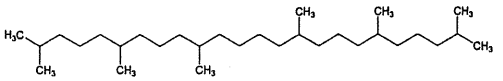
Sample: 5.0 g
Acceptance criteria: NMT 1.0%

- **Water Determination,** *Method I* ⟨921⟩: NMT 0.3%

ADDITIONAL REQUIREMENTS
- **Packaging and Storage:** Preserve in tight, light-resistant containers. No storage requirements are specified.

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Squalane



$C_{30}H_{62}$ 422.81
Tetracosane, 2,6,10,15,19,23-hexamethyl-;
2,6,10,15,19,23-Hexamethyltetracosane [111-01-3].

DEFINITION
Squalane is a saturated hydrocarbon that contains NLT 97.0% and NMT 102.0% of 2,6,10,15,19,23-hexamethyltetracosane ($C_{30}H_{62}$). It is obtained either by hydrogenation of squalene, an aliphatic triterpene derived from certain fish oils, particularly shark liver oil, or from β-farnesene, a fermentation product of plant sugars.

IDENTIFICATION

Change to read:

- **A. ▲Spectroscopic Identification Tests** ⟨197⟩, *Infrared Spectroscopy*: **197F▲** (CN 1-May-2020)
  Sample: Undried specimen
  Acceptance criteria: Meets the requirements
- **B. Chromatographic Identity**
  Analysis: Proceed as directed in the *Assay*.
  Acceptance criteria: The retention time of the major peak of the *Sample solution*, excluding the solvent peak, corresponds to that of the *Standard solution*, as obtained in the *Assay*.

ASSAY
- **Procedure**
  Diluent: *n*-Heptane
  System suitability solution: 4 mg/mL of USP Squalane RS and 4 µL/mL of methyl erucate in *Diluent*
  Standard solution: 4 mg/mL of USP Squalane RS in *Diluent*
  Sample solution: 4 mg/mL of Squalane in *Diluent*
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: GC
  Detector: Flame ionization
  Column: 0.32-mm × 30-m fused silica capillary; coated with a 1-µm film of phase G2
  Temperatures
  Injection port: 275°
  Detector: 300°
  Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
60	5.9	290	11

  Carrier gas: Helium
  Flow rate: 1.7 mL/min
  Injection volume: 1 µL
  Injection type: Split; split ratio, 12:1
  Liner: General purpose split/splitless liner with deactivated wool
  System suitability
  Samples: *System suitability solution* and *Standard solution*
  [NOTE—The relative retention times for methyl erucate and squalane are 0.9 and 1.0, respectively.]
  Suitability requirements
  Resolution: NLT 5 between the peaks due to methyl erucate and squalane, *System suitability solution*
  Relative standard deviation: NMT 2%, *Standard solution*
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Calculate the percentage of squalane ($C_{30}H_{62}$) in the portion of Squalane taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*
r_S = peak area from the *Standard solution*
C_S = concentration of USP Squalane RS in the *Standard solution* (mg/mL)
C_U = concentration of Squalane in the *Sample solution* (mg/mL)

  Acceptance criteria: 97.0%–102.0%

IMPURITIES
- **Residue on Ignition** ⟨281⟩: NMT 0.5%

SPECIFIC TESTS
- **Specific Gravity** ⟨841⟩: 0.807–0.810 at 20°
- **Fats and Fixed Oils** ⟨401⟩, *Procedures, Acid Value*: NMT 0.2
- **Fats and Fixed Oils,** *Iodine Value* ⟨401⟩: NMT 4
- **Fats and Fixed Oils,** *Saponification Value* ⟨401⟩: NMT 2
- **Refractive Index** ⟨831⟩: 1.4510–1.4525 at 20°

ADDITIONAL REQUIREMENTS
- **Packaging and Storage:** Preserve in tight containers.
- **Labeling:** Label to indicate whether it is derived from animal or plant sources.
- **USP Reference Standards** ⟨11⟩
  USP Squalane RS

Stannous Chloride

$SnCl_2 \cdot 2H_2O$ 225.65
Tin chloride ($SnCl_2$) dihydrate [10025-69-1].

DEFINITION
Stannous Chloride contains NLT 98.0% and NMT 102.0% of stannous chloride ($SnCl_2 \cdot 2H_2O$).

IDENTIFICATION
- **A.**
  Sample solution: To 0.40 g of Stannous Chloride add 1 mL of dilute hydrochloric acid solution (236 mL/L of hydrochloric acid), and dilute with water to 20 mL. [NOTE—Keep a portion for the *Limit of Sulfate* test.]
  Analysis: To 1 mL of the *Sample solution* add a mixture of 5 mL of water and 0.05 mL of mercuric chloride TS.
  Acceptance criteria: A blackish-gray precipitate forms.
- **B.**
  Sample: 1.0 g
  Analysis 1: Dissolve the *Sample* in 3.0 mL of water. Add 0.5 mL of dilute sodium hydroxide solution (85 mg/mL of sodium hydroxide) to the cloudy solution.
  Acceptance criteria 1: A yellowish, flocculent precipitate is formed.
  Analysis 2: Add 6.5 mL of water to the solution resulting from *Analysis 1*. To 1.0 mL of the previously shaken suspension add 1.0 mL of sodium hydroxide solution (420 mg/mL of sodium hydroxide).

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Acceptance criteria 2: The precipitate dissolves, and the resulting solution is clear and colorless.

• **C. IDENTIFICATION TESTS—GENERAL,** *Chloride* ⟨191⟩

Sample solution: Dissolve 10 mg of Stannous Chloride in 2 mL of 20% nitric acid.

Acceptance criteria: Meets the requirements

ASSAY

• **PROCEDURE**

Sample: 0.1 g of Stannous Chloride

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Direct titration

Titrant: 0.1 N iodine VS

Blank: 50 mL of water

Endpoint detection: Colorimetric

Analysis: Dissolve the *Sample* in 50 mL of water freed from oxygen by purging with carbon dioxide or nitrogen for 15 min prior to the addition. Add 1.5 mL of 0.8 N hydrochloric acid, 5 g of potassium sodium tartrate, 10 g of sodium bicarbonate, and 1 mL of starch TS. Titrate the resulting solution immediately with *Titrant*. Perform a blank determination.

Calculate the percentage of stannous chloride ($SnCl_2 \cdot 2H_2O$) in the *Sample*:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

V_S = *Titrant* consumed by the *Sample* (mL)
V_B = *Titrant* consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 112.8 mg/mEq
W = *Sample* weight (mg)

Acceptance criteria: 98.0%–102.0%

IMPURITIES

• **LIMIT OF SULFATE**

Acetic acid solution: Dilute 30 mL of glacial acetic acid with water to 100 mL.

30% Alcohol: Dilute 30 mL of alcohol with water to 100 mL.

Potassium sulfate solution 1: 1.8 mg/mL of potassium sulfate in *30% Alcohol*. Immediately before use, dilute with *30% Alcohol* to obtain a solution having a known concentration of about 18 µg/mL.

Potassium sulfate solution 2: 1.8 mg/mL of potassium sulfate in water. Immediately before use, dilute with water to obtain a solution having a known concentration of about 18 µg/mL.

Standard solution: Mix 3 mL of barium chloride solution (250 mg/mL) and 4.5 mL of *Potassium sulfate solution 1*. Shake, and let stand for 1 min. To 2.5 mL of this solution add 15 mL of *Potassium sulfate solution 2* and 0.5 mL of *Acetic acid solution*. Allow to stand for 5 min.

Sample solution: Use 15 mL of the solution prepared in *Identification* test *A*.

Analysis: Mix 3 mL of barium chloride solution (250 mg/mL) and 4.5 mL of *Potassium sulfate solution 1*. Shake, and let stand for 1 min. To 2.5 mL of this solution add the *Sample solution* and 0.5 mL of *Acetic acid solution*. Allow to stand for 5 min.

Acceptance criteria: Any opalescence in the *Sample solution* is not more intense than that in the *Standard solution* (500 ppm).

• **LIMIT OF IRON**

Standard iron solution: Prepare as directed in *Iron* ⟨241⟩, *Special Reagents*.

Standard solution: Immediately before use, dilute 1 mL of *Standard iron solution* with water to 10 mL. This solution contains the equivalent of 1 µg/mL of iron.

Sample solution: Dilute 5 mL of the *Sample solution*, prepared as directed in *Substances Not Precipitated by Thioacetamide*, with water to 10 mL.

Analysis: To 10 mL each of the *Standard solution* and the *Sample solution* add 2 mL of dilute citric acid (200 mg/mL) and 0.1 mL of thioglycolic acid, and mix. Make alkaline with a dilute ammonia solution (620 mL/L of ammonium hydroxide). Dilute with water to 20 mL. Allow the treated *Standard solution* and the treated *Sample solution* to stand for 5 min.

Acceptance criteria: Any pink color in the *Sample solution* is not more intense than that in the *Standard solution* (100 ppm).

• **LIMIT OF LEAD**

Dilute thioacetamide solution: [NOTE—Prepare immediately before use.] To 0.2 mL of thioacetamide TS add 1 mL of a mixture of 5 mL of water, 15 mL of 1 N sodium hydroxide, and 20 mL of 85% glycerin. Heat in a water bath for 20 s.

Standard solution: On the day of use, mix 1.0 mL of standard lead solution TS, 6 mL of water, 3 mL of sodium hydroxide solution (420 mg/mL of sodium hydroxide), and 0.5 mL of *Dilute thioacetamide solution*.

Sample solution: Dissolve 1.0 g of Stannous Chloride in 2 mL of a mixture of nitric acid and hydrochloric acid (1:3). Heat the solution on a water bath until nitrous vapor is no longer evolved. Dissolve the residue in water, and dilute with water to 25 mL. To 5 mL of this solution add 3 mL of sodium hydroxide solution (420 mg/mL of sodium hydroxide) and 2 mL of water. Heat until a clear solution is obtained, and cool. Add 0.5 mL of *Dilute thioacetamide solution*, and allow to stand for 2 min.

Analysis: Compare the *Standard solution* and the *Sample solution*.

Acceptance criteria: Any color in the *Sample solution* is not more intense than that in the *Standard solution* (50 µg/g).

SPECIFIC TESTS

• **APPEARANCE OF SOLUTION**

Standard stock solution: Pipet 30.0 mL of ferric chloride CS, 30.0 mL of cobaltous chloride CS, and 24.0 mL of cupric sulfate CS into a 100-mL volumetric flask. Dilute with 1% (w/v) hydrochloric acid to volume.

Standard solution: Dilute 1.0 mL of the *Standard stock solution* with 1% (w/v) hydrochloric acid to 100 mL.

Sample solution: Dissolve 10.0 g of Stannous Chloride in dilute hydrochloric acid solution, and dilute with dilute hydrochloric acid solution to 20 mL.

Acceptance criteria: The *Sample solution* is clear and colorless, or if not, not more intensely colored than the *Standard solution*.

• **SUBSTANCES NOT PRECIPITATED BY THIOACETAMIDE**

Sample solution: Dissolve 1.0 g of Stannous Chloride in dilute hydrochloric acid solution, and dilute with the same acid to 30 mL. Heat to boiling. Add 30 mL of thioacetamide TS, and boil for 15 min to produce *Solution A*. Filter 5 mL of *Solution A*, and heat the filtrate to boiling. Add 5 mL of thioacetamide TS, and boil for 15 min. If a precipitate is formed, add the remainder of *Solution A* to the mixture to produce *Solution A1*. Add 10 mL of thioacetamide TS, and boil. Repeat the series of operations from "Filter 5 mL" until a precipitate is no longer formed on addition of thioacetamide TS to the filtrate obtained from the 5 mL of *Solution A* (*Solution A1*, *Solution A2*, and so on, respectively). If no precipitate is formed, or if no more precipitate is formed, combine the solution obtained with the remainder of *Solution A* (*Solution A1*, *Solution A2*, and so on, respectively), filter, and wash the precipitate with 10 mL of water. Heat the filtrate until the resulting vapor no longer turns a moistened piece of lead acetate test paper

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blackish-gray. Allow to cool, and dilute with water to 50 mL. [NOTE—Keep a portion for the *Limit of Iron* test.]
Analysis: Evaporate 25 mL of the *Sample solution* to dryness, and ignite at 600°.
Acceptance criteria: The residue weighs NMT 1 mg (0.2%).

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.

Add the following:

▲Star Anise Oil

[68952-43-2].

DEFINITION
Star Anise Oil is the volatile oil distilled with steam from the dried, ripe fruit of *Illicium verum* Hook. f. It contains NLT 86% and NMT 93% of *trans*-anethole.
[NOTE—If solid material has separated, carefully warm the Star Anise Oil until it is completely liquefied, and mix before using.]

IDENTIFICATION
• **A. CHROMATOGRAPHIC IDENTITY**
Peak identification and sensitivity solution: 60 mg/mL of USP Anethole RS, 1 mg/mL of USP Foeniculin RS, and 0.1 mg/mL each of USP Pseudoisoeugenyl 2-methylbutyrate RS and USP Safrole RS in *n*-hexane
Standard: USP Star Anise Oil RS
Sample: Star Anise Oil
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.25-mm × 30-m; coated with a 0.25-µm film of phase G16
Temperatures
Injection port: 200°
Detector: 220°
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
60	—	60	5
60	2	210	15

Carrier gas: Helium
Flow rate: 1.0 mL/min
Injection volume: 0.4 µL
Injection type: Split; split ratio, 100:1
System suitability
Sample: *Peak identification and sensitivity solution*
[NOTE—The relative retention times for the *trans*-anethole, safrole, foeniculin, and pseudoisoeugenyl 2-methylbutyrate peaks are 1.0, 1.04, 1.5, and 1.7, respectively.]
Suitability requirements
Signal-to-noise ratio: NLT 10 for the pseudoisoeugenyl 2-methylbutyrate and safrole peaks
Analysis
Samples: *Peak identification and sensitivity solution*, *Standard*, and *Sample*
Identify the safrole, foeniculin, and pseudoisoeugenyl 2-methylbutyrate peaks in the chromatogram of the *Sample* based on those in the chromatogram of the *Peak identification and sensitivity solution*.
Calculate the percentage of safrole, foeniculin, and pseudoisoeugenyl 2-methylbutyrate in the portion of Star Anise Oil taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak area of safrole or foeniculin or pseudoisoeugenyl 2-methylbutyrate from the *Sample*
r_T = sum of all the peak areas, excluding the solvent peak

Acceptance criteria
Chromatographic similarity: Examine the chromatograms of the *Sample* and the *Standard*. The chromatogram of the *Sample* is similar to that of the *Standard*. [NOTE—The chromatogram of the *Standard* is similar to the reference chromatogram provided with the lot of USP Star Anise Oil RS being used.]
Safrole: NMT 0.01%
Foeniculin: NLT 0.10%
Pseudoisoeugenyl 2-methylbutyrate: NMT 0.01%
• **B. REFRACTIVE INDEX:** Meets the requirements for *Refractive Index* in *Specific Tests*

ASSAY
• **CONTENT OF *trans*-ANETHOLE**
Peak identification and sensitivity solution, Sample, and **Chromatographic system:** Proceed as directed in *Identification A*.
Analysis
Samples: *Peak identification and sensitivity solution* and *Sample*
Identify the *trans*-anethole peak in the chromatogram of the *Sample* based on that in the chromatogram of the *Peak identification and sensitivity solution*.
Calculate the percentage of *trans*-anethole in the portion of Star Anise Oil taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak area of *trans*-anethole from the *Sample*
r_T = sum of all the peak areas, excluding the solvent peak

Acceptance criteria: 86%–93%

IMPURITIES
• **LIMIT OF PHENOLS**
Sample solution: Recently distilled Star Anise Oil in 90% alcohol (1 in 3)
Acceptance criteria: The *Sample solution* is neutral to moistened neutral litmus paper, and no blue or brownish color develops upon the addition of 1 drop of ferric chloride TS to 5 mL of the *Sample solution*.

SPECIFIC TESTS
• **SOLUBILITY IN 90% ALCOHOL:** 1 volume dissolves in 3 volumes of 90% alcohol.
• **REFRACTIVE INDEX** ⟨831⟩: 1.553–1.556 at 20°

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ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-filled, tight containers, and protect from light. Avoid exposure to excessive heat.
- **LABELING:** The label states the Latin binomial name and, following the official name, the part of the plant source from which the article was derived. The label also states that if solid material has separated, carefully warm the oil until it is completely liquefied, and mix before using.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Anethole RS
  USP Foeniculin RS
  USP Pseudoisoeugenyl 2-methylbutyrate RS
  USP Safrole RS
  USP Star Anise Oil RS▲ (NF 1-Aug-2019)

Corn Starch

Portions of the monograph text that are national *USP* text, and are not part of the harmonized text, are marked with symbols (•,) to specify this fact.

DEFINITION

Corn Starch consists of the starch granules separated from the mature grain of corn [*Zea mays* L. (Fam. Gramineae)].

IDENTIFICATION

- **A.**
  Analysis: Examine under a microscope, using a mixture of glycerin and water (1:1) as a mounting agent.
  Acceptance criteria: It appears either as angular polyhedral granules of irregular sizes with diameters ranging from 2–23 µm, or as rounded or spheroidal granules of irregular sizes with diameters ranging from 25–35 µm. The central hilum consists of a distinct cavity or two- to five-rayed cleft, and there are no concentric striations. Between orthogonally oriented polarizing plates or prisms, the starch granules show a distinct black cross intersecting at the hilum.
- **B.**
  Sample solution: 20 mg/mL in water
  Analysis: Boil for 1 min, and cool.
  Acceptance criteria: A thin, cloudy mucilage is formed.
- **C.**
  Sample solution: 1 mL of the mucilage obtained in *Identification* test *B*
  Analysis: Add 0.05 mL of iodine and potassium iodide TS 2 to the *Sample solution*.
  Acceptance criteria: An orange-red to dark blue color is produced, which disappears upon heating.

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩
  Sample: 1.0 g
  Acceptance criteria: NMT 0.6%
- **LIMIT OF IRON**
  Standard iron stock solution A: Equivalent to 10 µg/mL of iron prepared as directed in *Iron* ⟨241⟩
  Standard iron stock solution B: 1 µg/mL of iron from *Standard iron stock solution A* in water [NOTE—Prepare immediately before use.]
  Standard iron solution: Transfer 10 mL of *Standard iron stock solution B* to a test tube, and add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, and dilute with water to 20 mL.
  Sample solution: Shake 1.5 g of Corn Starch with 15 mL of 2 N hydrochloric acid, and filter. Transfer 10 mL of the filtrate to a test tube, add 2 mL of citric acid solution (2 in 10), and 0.1 mL of thioglycolic acid. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, and dilute with water to 20 mL.
  Acceptance criteria: After 5 min, any pink color in the *Sample solution* is not more intense than that in the *Standard iron solution*, corresponding to a limit of 10 ppm of iron.
- **LIMIT OF SULFUR DIOXIDE**
  Carbon dioxide: Use carbon dioxide, with a flow regulator that will maintain a flow of 100 ± 10 mL/min.
  Bromophenol blue indicator solution: 0.2 mg/mL of bromophenol blue in dilute alcohol. Filter if necessary.
  Hydrogen peroxide solution: Dilute 30% hydrogen peroxide with water to obtain a 3% solution. Just before use, add 3 drops of *Bromophenol blue indicator solution*, and neutralize to a violet-blue endpoint with 0.01 N sodium hydroxide. Do not exceed the endpoint.
  Apparatus: See *Figure 1*.

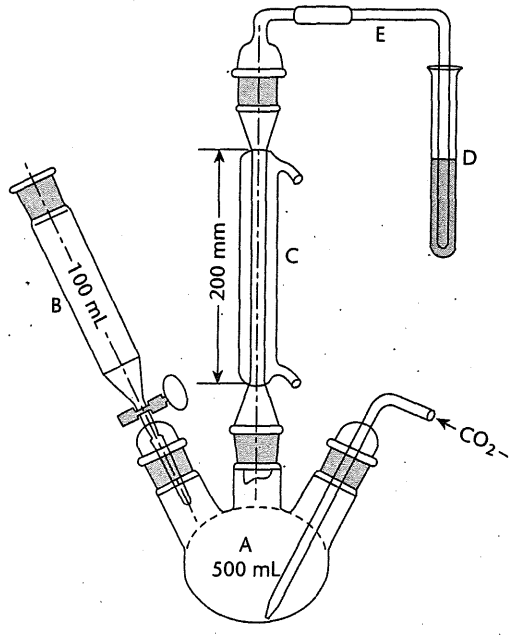


Figure 1

In this test, the sulfur dioxide is released from the sample in a boiling acid medium and is removed by a stream of carbon dioxide. The separated gas is collected in a dilute hydrogen peroxide solution where the sulfur dioxide is oxidized to sulfuric acid and titrated with standard alkali. The apparatus consists essentially of a 500-mL three-neck, round-bottom boiling flask, A; a separatory funnel, B, having a capacity of 100 mL or greater; a gas inlet tube of sufficient length to permit introduction of the carbon dioxide within 2.5 cm of the bottom of the boiling flask; a reflux condenser, C, having a jacket length of 200 mm, and a delivery tube, E, connecting the upper end of the reflux condenser to the bottom of a receiving test tube, D. Apply a thin film of stopcock grease to the sealing surfaces of all of the joints except the joint between the separatory funnel and the boiling flask, and clamp the joints to ensure tightness.

Sample: 25.0 g of Corn Starch

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Analysis: Add 150 mL of water to the boiling flask. Close the stopcock of the separatory funnel, and begin the flow of carbon dioxide at a rate of 100 ± 5 mL/min through the *Apparatus*. Start the condenser coolant flow. Add 10 mL of *Hydrogen peroxide solution* to a receiving test tube. After 15 min, without interrupting the flow of carbon dioxide, remove the separatory funnel from the boiling flask, and transfer the *Sample* into the boiling flask with the aid of 100 mL of water. Apply stopcock grease to the outer joint of the separatory funnel, and replace the separatory funnel in the boiling flask. Close the stopcock of the separatory funnel, and add 80 mL of 2 N hydrochloric acid to the separatory funnel. Open the stopcock of the separatory funnel to permit the hydrochloric acid solution to flow into the boiling flask, guarding against the escape of sulfur dioxide into the separatory funnel by closing the stopcock before the last few mL of hydrochloric acid drain out. Boil the mixture for 1 h. Remove the receiving test tube, and transfer its contents to a 200-mL wide-necked, conical flask. Rinse the receiving test tube with a small portion of water, add the rinsing to the 200-mL conical flask, and mix. Heat on a water bath for 15 min, and allow to cool.

Add 0.1 mL of *Bromophenol blue indicator solution*, and titrate the contents with 0.1 N sodium hydroxide VS until the color changes from yellow to violet-blue. Perform a blank determination, and make any necessary correction (see *Titrimetry* ⟨541⟩).

Calculate the content, in ppm, of sulfur dioxide in the *Sample* taken:

$$\text{Result} = (V \times N \times F)/W \times 1000$$

V = volume of titrant consumed (mL)
N = normality of the titrant
F = milliequivalent weight of sulfur dioxide, 32.03
W = weight of the *Sample* (g)

Acceptance criteria: NMT 50 ppm

- **LIMIT OF OXIDIZING SUBSTANCES**

Sample solution: Transfer 4.0 g to a glass-stoppered, 125-mL conical flask, and add 50.0 mL of water. Insert the stopper, and swirl for 5 min. Transfer to a glass-stoppered, 50-mL centrifuge tube, and centrifuge to clarify. Transfer 30.0 mL of the clear supernatant to a glass-stoppered, 125-mL conical flask. Add 1 mL of glacial acetic acid and 0.5–1.0 g of potassium iodide. Insert the stopper, swirl, and allow to stand for 25–30 min in the dark. Add 1 mL of starch TS.

Analysis: Titrate with 0.002 N sodium thiosulfate VS to the disappearance of the starch–iodine color. Perform a blank determination, and make any necessary correction. Each mL of 0.002 N sodium thiosulfate is equivalent to 34 µg of oxidant, calculated as hydrogen peroxide.

Acceptance criteria: NMT 1.4 mL of 0.002 N sodium thiosulfate is required (20 ppm, calculated as H_2O_2).

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic microbial count does not exceed 10^3 cfu/g; the total combined molds and yeasts count does not exceed 10^2 cfu/g; and it meets the requirements of the test for the absence of *Escherichia coli*. ‣Where it is intended for use in preparing Absorbable Dusting Powder, it also meets the requirements of the tests for absence of *Staphylococcus aureus* and *Pseudomonas aeruginosa*.‣
- **LOSS ON DRYING** ⟨731⟩

Sample: 1 g
Analysis: Dry the *Sample* at 130° for 90 min.
Acceptance criteria: NMT 15.0%

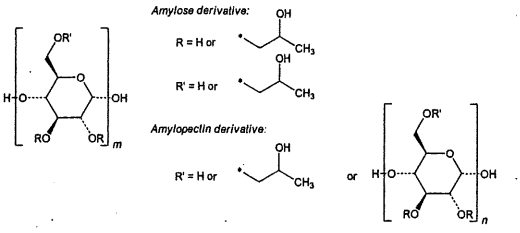
- **pH** ⟨791⟩

Sample solution: Prepare a slurry by weighing 5.0 g of Corn Starch, transferring to a suitable nonmetallic container, and adding 25.0 mL of freshly boiled and cooled water.
Analysis: Agitate continuously at a moderate rate for 1 min. Stop the agitation, and allow to stand for 15 min. Determine the pH to the nearest 0.1 unit.
Acceptance criteria: 4.0–7.0

ADDITIONAL REQUIREMENTS

- ‣**PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.‣
- ‣**LABELING:** Where Corn Starch is intended for use in preparing Absorbable Dusting Powder, it is so labeled, and the label states that it must be subjected to further processing during the preparation of Absorbable Dusting Powder.‣

Hydroxypropyl Corn Starch



For the Amylose derivative, m is about 300–1000.

DEFINITION

Hydroxypropyl Corn Starch is partially substituted 2-hydroxypropylether obtained from corn starch by a chemical modification of etherification with propylene oxide. In addition, this starch may be partially hydrolyzed using acids or enzymes to obtain thinned starch. It contains NLT 2.0% and NMT 7.0% of hydroxypropyl groups on the dried basis.

IDENTIFICATION

- **A. PROCEDURE**

Analysis: Examine under a microscope, using NLT 20× magnification and a mixture of glycerin and water (1:1) as a mounting agent.
Acceptance criteria: It presents either as angular polyhedral granules of irregular sizes with diameters of 2–23 µm, or as rounded or spheroidal granules of irregular sizes with diameters of 25–35 µm. The central hilum consists of a distinct cavity or 2- to 5-rayed cleft, and there are no concentric striations. Between crossed nicol prisms, the Hydroxypropyl Corn Starch granules show a distinct black cross intersecting at the hilum.

- **B. PROCEDURE**

Sample solution: Suspend 1 g of Hydroxypropyl Corn Starch in 50 mL of water, boil for 1 min, and cool.
Acceptance criteria: A translucent or clear mucilage is formed.

- **C. PROCEDURE**

Analysis: To 1 mL of the *Sample solution* obtained in *Identification* test *B* add 0.05 mL of iodine and potassium iodide TS 2.
Acceptance criteria: An orange-red to dark blue color is produced, which disappears upon heating.

- **D. PROCEDURE**

Ninhydrin solution: Dissolve 3 g of ninhydrin in 100 mL of a 45.5-g/L solution of sodium metabisulfite.

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Diluted sulfuric acid: 98 g/L of H_2SO_4
Sample: 100 mg of Hydroxypropyl Corn Starch
Analysis: Transfer the *Sample* to a 100-mL volumetric flask, and add 12.5 mL of *Diluted sulfuric acid*. Place the flask in a water bath, and heat until the *Sample* is dissolved. Cool, and dilute with water to 100 mL. [**CAUTION**—When sulfuric acid is miscible with water, it produces intense heat.]
Pipet 1 mL of this solution to a glass-stoppered, 25-mL graduated test tube and, with the tube immersed in cold water, add drop-wise 8 mL of sulfuric acid. Mix well, and place the tube in a boiling water bath for exactly 3 min. Immediately transfer the tube to an ice bath until the solution is chilled. Add 0.6 mL of *Ninhydrin solution*, carefully allowing the reagent to run down the walls of the test tube. Immediately shake the tube well, and place it in a water bath at 25° for 100 min. Dilute with sulfuric acid to 25 mL [**CAUTION**—Use sulfuric acid cautiously.], and mix by inverting the tube several times. Do not shake.
Acceptance criteria: A violet color develops within 5 min due to the presence of hydroxypropyl groups (starch ether).

ASSAY

• **PROCEDURE FOR HYDROXYPROPYL GROUPS**
Deuterium chloride solution: Dilute 1 mL of deuterium chloride (38% w/w) with 5 mL of deuterium oxide.
Internal standard solution: Disperse 50.0 mg of sodium 3-trimethylsilyl-1-propane sulfonate in about 5 g of deuterium oxide, weighed to the nearest 0.1 mg. Store in a sealed bottle.
Sample solution: Disperse 20 g of Hydroxypropyl Corn Starch in 200.0 mL of carbon dioxide-free water at room temperature. Agitate for 15 min, and filter. Repeat the operation two more times. If poor dispersibility or slow filtration is observed, use refrigerated carbon dioxide-free water for the washing operation. Dry the washed starch for NLT 4 h in vacuum at 30 ± 5°. Determine the moisture content (B) on 5 g of the washed and dried starch following the *Loss on Drying* test. Weigh 12.0 mg of the washed and dried starch in a 5-mm NMR tube. Add 0.75 mL of deuterium oxide and 0.1 mL of *Deuterium chloride solution*. Cap the tube, mix, and place it in a boiling water bath until a clear solution is obtained. [NOTE—This may take 3 min to 1 h.] When a clear solution is obtained, allow to cool to room temperature. Dry the exterior of the tube, and weigh to the nearest 0.1 mg. Add 0.05 mL of *Internal standard solution*, and weigh to the nearest 0.1 mg. Determine the mass of the *Internal standard solution* added. Mix thoroughly.
Nuclear magnetic resonance spectrometry
(See *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩, *Quantitative Applications*.)
Apparatus: FT-NMR spectrometer at minimum 300 MHz
Acquisition of ^{1}H NMR spectra: The following parameters may be used.
Sweep width: 8 ppm (about –1.0 to +7 ppm)
Irradiation frequency offset: None
Time domain: NLT 64 K
Pulse width: 90 degree
Pulse delay: 10 s
Dummy scans: 0
Number of scans: 8
Use the CH_3 signal of the internal standard for shift referencing. Set the shift of the peak of the singlet to 0 ppm. Record the FID signal.
Analysis
Samples: *Internal standard solution* and *Sample solution*
Call the integration sub-routine after phase corrections and baseline correction between –0.5 and +6 ppm.
Measure the peak areas of the doublet from the methyl groups of the hydroxypropyl function at +1.2 ppm (A_2), and of the methyl groups at 0 ppm of the internal standard (A_1) without ^{13}C-satellites.
Measure the signal coming from the 3 protons of the methyl group in the hydroxypropyl function.
Calculate the content of hydroxypropyl groups as a percentage (w/w, dried basis):

$$\text{Result} = (N \times A_2/A_1) \times (C_i \times W_i/W) \times (M_{r2}/M_{r1}) \times [100/(100 - B)] \times 100$$

N	= numerical value representing the 3 methyl groups in the internal standard (sodium 3-trimethylsilyl-1-propane sulfonate), 3
A_2	= area of the methyl groups of hydroxypropyl in Hydroxypropyl Corn Starch
A_1	= area of the methyl groups in the internal standard (sodium 3-trimethylsilyl-1-propane sulfonate)
C_i	= concentration of the internal standard in the *Internal standard solution* (mg/g)
W_i	= weight of the *Internal standard solution* in the NMR tube (g)
W	= weight of the washed and dried Hydroxypropyl Corn Starch in the NMR tube (mg)
M_{r1}	= molecular weight of the internal standard, 218.32 g/mol
M_{r2}	= molar mass of hydroxypropyl group, 59.09 g/mol
B	= moisture content of the washed and dried Hydroxypropyl Corn Starch used in the *Sample solution*, as a percentage (w/w)

Acceptance criteria: The content of hydroxypropyl groups is 2.0%–7.0% on the dried basis.

IMPURITIES

INORGANIC IMPURITIES

• **Residue on Ignition** ⟨281⟩: NMT 0.6%, determined on a 1.0-g test specimen
• **Limit of Iron**
Standard iron stock solution: Prepare a solution containing the equivalent of 10 µg/mL of iron, as directed under *Iron* ⟨241⟩.
Diluted standard iron solution: Immediately before use, dilute an accurately measured volume of *Standard iron stock solution* quantitatively with water to obtain a solution containing the equivalent of 1 µg/mL of iron.
Standard solution: Transfer 10 mL of the *Diluted standard iron solution* to a test tube. Add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid, and mix. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, dilute with water to 20 mL, and mix.
Sample solution: Shake 1.0 g of Hydroxypropyl Corn Starch with 20 mL of 2 N hydrochloric acid, and filter. Transfer 10 mL of the filtrate to a test tube. Add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid, and mix. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, dilute with water to 20 mL, and mix.
Acceptance criteria: After 5 min, any pink color in the *Sample solution* is not more intense than that in the *Standard solution*, corresponding to a limit of 20 µg/g of iron.
• **Limit of Sulfur Dioxide,** *Method IV* ⟨525⟩: NMT 50 ppm

ORGANIC IMPURITIES

• **Procedure 1: Limit of Oxidizing Substances**
Sample: 4.0 g of Hydroxypropyl Corn Starch
Analysis: Transfer the *Sample* to a glass-stoppered, 125-mL conical flask, and add 50.0 mL of water. Insert the stopper, and swirl for 5 min. Transfer to a glass-stoppered, 50-mL centrifuge tube, and centrifuge to clarify. Transfer 30.0 mL

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of the clear supernatant to a glass-stoppered, 125-mL conical flask. Add 1 mL of glacial acetic acid and 0.5–1.0 g of potassium iodide. Insert the stopper, swirl, and allow to stand for 25–30 min in the dark. Add 1 mL of starch TS, and titrate with 0.002 N sodium thiosulfate VS to the disappearance of the starch-iodine color. Perform a blank determination, and make any necessary correction. Each mL of 0.002 N sodium thiosulfate VS is equivalent to 34 µg of oxidant, calculated as hydrogen peroxide.

Acceptance criteria: NMT 1.4 mL of 0.002 N sodium thiosulfate VS is required (20 µg/g, calculated as H_2O_2).

• **Procedure 2: Foreign Matter**

Sample: 50 mg/mL of Hydroxypropyl Corn Starch in a mixture of glycerin and water (1:1)

Analysis: Examine under a microscope, using NLT 20× magnification and a mixture of glycerin and water (1:1) as a mounting agent.

Acceptance criteria: NMT traces of matter other than Hydroxypropyl Corn Starch granules are present.

SPECIFIC TESTS

• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩: The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g. It meets the requirements of the test for the absence of *Escherichia coli*.

• **pH** ⟨791⟩

Sample solution: Suspend 5.0 g of Hydroxypropyl Corn Starch in 25.0 mL of carbon dioxide-free water, and shake for 60 s. Allow to stand for 15 min.

Acceptance criteria: 4.5–8.0

• **LOSS ON DRYING** ⟨731⟩: Dry about 1 g at 130° for 90 min: it loses NMT 15.0% of its weight.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers. Store at room temperature.

Pregelatinized Hydroxypropyl Corn Starch

DEFINITION

Pregelatinized Hydroxypropyl Corn Starch is prepared from Hydroxypropyl Corn Starch by mechanical processing in the presence of water, with or without heat, to rupture all or some of the starch granules, and is subsequently dried. It contains NLT 2.0% and NMT 7.0% of hydroxypropyl groups on the dried basis.

IDENTIFICATION

• **A. TEST FOR PREGELATINIZED STATE**

Sample: 1 g

Analysis: Disperse the *Sample* in 50 mL of water at a temperature NMT 25°. Shake vigorously until lumps completely disperse/solubilize or until lumps disappear. Allow to stand for 20 min.

Acceptance criteria: A translucent or clear mucilage without precipitate is formed.

• **B. TEST FOR STARCH**

Analysis: Disperse 0.5 g in 2 mL of water without heating, and add 0.05 mL of iodine and potassium iodide TS2.

Acceptance criteria: A reddish-violet or blue color is produced.

• **C. NINHYDRIN TEST**

Ninhydrin solution: Dissolve 3 g of ninhydrin in 100 mL of a 45.5-g/L solution of sodium metabisulfite.

Diluted sulfuric acid: 98 g/L of H_2SO_4

Sample: 100 mg

Analysis: Transfer the *Sample* to a 100-mL volumetric flask, and add 12.5 mL of *Diluted sulfuric acid*. Place the flask in a water bath, and heat until the *Sample* is dissolved. Cool, and dilute with water to 100 mL. [**CAUTION**—When sulfuric acid is miscible with water, it produces intense heat.] Pipet 1 mL of this solution to a glass-stoppered 25-mL graduated test tube and, with the tube immersed in cold water, add dropwise 8 mL of sulfuric acid. Mix well, and place the tube in a boiling water bath for exactly 3 min. Immediately transfer the tube to an ice bath until the solution is chilled. Add 0.6 mL of *Ninhydrin solution*, carefully allowing the reagent to run down the walls of the test tube. Immediately shake the tube well, and place it in a water bath at 25° for 100 min. Dilute with sulfuric acid to 25 mL. [**CAUTION**—Use sulfuric acid cautiously.] Mix by inverting the tube several times. Do not shake.

Acceptance criteria: A violet color develops within 5 min due to the presence of hydroxypropyl groups (starch ether).

ASSAY

• **ASSAY FOR HYDROXYPROPYL GROUPS**

Deuterium chloride solution: Dilute 1 mL of deuterium chloride (38% w/w) with 5 mL of deuterium oxide.

Internal standard solution: Disperse 50.0 mg of sodium 3-trimethylsilyl-1-propane sulfonate in about 5 g of deuterium oxide, weighed to the nearest 0.1 mg. Store in a sealed bottle.

Sample solution: Determine the moisture content (*B*) of 5 g of Pregelatinized Hydroxypropyl Corn Starch following the *Loss on Drying* test. Weigh 12.0 mg of Pregelatinized Hydroxypropyl Corn Starch in a 5-mm NMR tube. Add 0.75 mL of deuterium oxide and 0.1 mL of *Deuterium chloride solution*. Cap the tube, mix, and place it in a boiling water bath until a clear solution is obtained. [NOTE—This may take from 3 min to 1 h.] When a clear solution is obtained, allow it to cool to room temperature. Dry the exterior of the tube, and weigh to the nearest 0.1 mg. Add 0.05 mL of *Internal standard solution*. Weigh to the nearest 0.1 mg. Determine the mass of the *Internal standard solution* added. Mix thoroughly.

Instrumental conditions

(See *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩, *Quantitative Applications*.)

Mode: Nuclear magnetic resonance spectrometry

Apparatus: FT-NMR spectrometer at minimum 300 MHz

Acquisition of ^{1}H NMR spectra: The following parameters may be used:

Sweep width: 8 ppm (about −1.0 to +7 ppm)
Irradiation frequency offset: None
Time domain: NLT 64 K
Pulse width: 90°
Pulse delay: 10 s
Dummy scans: 0
Number of scans: 8

Use the CH_3 signal of the internal standard for shift referencing. Set the shift of the peak of the singlet to 0 ppm. Record the FID signal.

Analysis

Samples: *Internal standard solution* and *Sample solution*

Call the integration subroutine after phase corrections and baseline correction between −0.5 and +6 ppm.

Measure the peak areas of the doublet from the methyl groups of the hydroxypropyl function at +1.2 ppm (A_2), and of the methyl groups at 0 ppm of the internal standard (A_1) without ^{13}C-satellites.

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Measure the signal originating from the 3 protons of the methyl group in the hydroxypropyl function.
Calculate the content of hydroxypropyl groups as a percentage (w/w, dried basis):

$$\text{Result} = (N \times A_2/A_1) \times (C_I \times W_I/W) \times (M_{r2}/M_{r1}) \times [100/(100 - B)] \times 100$$

N = numerical value representing the 3 methyl groups in the internal standard (sodium 3-trimethylsilyl-1-propane sulfonate), 3
A_2 = area of the methyl groups of hydroxypropyl in Pregelatinized Hydroxypropyl Corn Starch
A_1 = area of the methyl groups in the internal standard (sodium 3-trimethylsilyl-1-propane sulfonate)
C_I = concentration of the internal standard in the *Internal standard solution* (mg/g)
W_I = weight of the *Internal standard solution* in the NMR tube (g)
W = weight of the Pregelatinized Hydroxypropyl Corn Starch in the NMR tube (mg)
M_{r2} = molar mass of hydroxypropyl groups, 59.09 g/mol
M_{r1} = molecular weight of the internal standard, 218.32 g/mol
B = moisture content of the Pregelatinized Hydroxypropyl Corn Starch used in the *Sample solution*, as a percentage (w/w)

Acceptance criteria: 2.0%–7.0% of hydroxypropyl groups on the dried basis

IMPURITIES

- **Residue on Ignition** ⟨281⟩: NMT 0.6%, determined on a 1.0-g test specimen
- **Limit of Iron**

Standard iron stock solution: Prepare a solution containing the equivalent of 10 µg/mL of iron, as directed under *Iron* ⟨241⟩.
Diluted standard iron solution: Immediately before use, dilute an accurately measured volume of the *Standard iron stock solution* quantitatively with water to obtain a solution containing the equivalent of 1 µg/mL of iron.
Sample solution: Shake the residue obtained from the test for *Residue on Ignition* with 20 mL of 2 N hydrochloric acid, and filter. Transfer 10 mL of the filtrate to a test tube. Add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid, and mix. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, dilute with water to 20 mL, and mix.
Standard solution: Transfer 10 mL of the *Diluted standard iron solution* to a test tube. Add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid, and mix. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, dilute with water to 20 mL, and mix.
Acceptance criteria: After 5 min, any pink color in the *Sample solution* is not more intense than that in the *Standard solution*, corresponding to a limit of 20 ppm of iron.

- **Limit of Sulfur Dioxide,** *Method IV* ⟨525⟩: NMT 50 ppm
- **Limit of Propylene Glycol**

Internal standard solution: 0.5 mg/mL of 1,3-propanediol in anhydrous pyridine
Standard stock solution: 0.5 mg/mL of USP Propylene Glycol RS in *Internal standard solution*
Standard solution: Transfer 0.1 mL of the *Standard stock solution* to a 2-mL vessel with a screw cap fitted with a septum. Add 0.9 mL of anhydrous pyridine, 0.2 mL of hexamethyldisilazane, and 0.1 mL of trimethylchlorosilane. Close, and mix. Allow to stand for 15 min before injection.
Sample stock solution: Transfer 200 mg of Pregelatinized Hydroxypropyl Corn Starch to a 100-mL volumetric flask. Add 1.0 mL of the *Internal standard solution* and 9.0 mL of anhydrous pyridine. Boil under reflux using a water bath for 20 min. Allow to cool to room temperature.
Sample solution: Transfer 1.0 mL of the *Sample stock solution* to a 2-mL vessel with a screw cap fitted with a septum. Add 0.2 mL of hexamethyldisilazane and 0.1 mL of trimethylchlorosilane. Close, and mix. Allow to stand for 15 min before injection.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.32-mm × 30-m fused-silica capillary column; 0.25-µm layer of phase G1
Temperature
Detector: 250°
Injection port: 250°
Column: 70°. [NOTE—The column must be desorbed regularly. Conditions: Program from 70° to 300° at 7°/min, and maintain 10 min at 300°.]
Carrier gas: Helium
Flow rate: 3 mL/min
Injection type: Split ratio of 1:30
Injection size: 1 µL
System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for the trimethylsylilated derivative of propylene glycol and the trimethyl sylilated derivative of 1,3-propanediol are 1.0 and 1.4, respectively.]
Suitability requirements
Resolution: NLT 2.0 between the peaks due to the trimethylsylilated derivative of propylene glycol and the trimethylsylilated derivative of 1,3-propanediol
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of propylene glycol in the portion of Pregelatinized Hydroxypropyl Corn Starch taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = internal standard ratio (peak response of propylene glycol/peak response of 1,3-propanediol) from the *Sample solution*
R_S = internal standard ratio (peak response of propylene glycol/peak response of 1,3-propanediol) from the *Standard solution*
C_S = concentration of USP Propylene Glycol RS in the *Standard solution* (mg/mL)
C_U = concentration of Pregelatinized Hydroxypropyl Corn Starch in the *Sample solution* (mg/mL)

Acceptance criteria: NMT 0.1%

- **Limit of Oxidizing Substances**

Sample: 4.0 g
Analysis: Transfer the *Sample* to a glass-stoppered 125-mL conical flask, and add 50.0 mL of a mixture of water and methanol (1:1). Insert the stopper, and swirl for 5 min. Transfer to a glass-stoppered 50-mL centrifuge tube, and centrifuge to clarify. Transfer 30.0 mL of the clear supernatant to a glass-stoppered 125-mL conical flask. Add 1 mL of glacial acetic acid and 0.5–1.0 g of potassium iodide. Insert the stopper, swirl, and allow to stand for 25–30 min in the dark. Add 1 mL of starch TS, and titrate with 0.002 N sodium thiosulfate VS to the disappearance of the starch-iodide color. Perform a blank determination, and make any necessary correction. Each mL of 0.002 N sodium

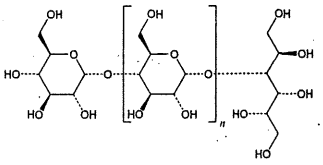
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thiosulfate VS is equivalent to 34 µg of oxidant, calculated as hydrogen peroxide.
Acceptance criteria: NMT 1.4 mL of 0.002 N sodium thiosulfate VS is required (20 ppm, calculated as H_2O_2).

SPECIFIC TESTS
- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic microbial count does not exceed 10^3 cfu/g, the total combined molds and yeasts count does not exceed 10^2 cfu/g, and it meets the requirements of the test for the absence of *Escherichia coli.*
- **pH** ⟨791⟩
  Sample solution: Progressively suspend 3.0 g of Pregelatinized Hydroxypropyl Corn Starch in 100.0 mL of carbon dioxide-free water, stirring continuously. Determine the pH when all the solid is wetted.
  Acceptance criteria: 4.5–8.0
- **LOSS ON DRYING** ⟨731⟩**:** Dry about 1 g at 130° for 90 min: it loses NMT 15.0% of its weight.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers. Store at room temperature.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Propylene Glycol RS

Hydrogenated Starch Hydrolysate



Hydrogenated Polysaccharides

$C_{12}H_{24}O_{11}(C_6H_{10}O_5)_n$
Polyglucitol;
Polyglycitol syrup [68425-17-2].

DEFINITION
Hydrogenated Starch Hydrolysate is a mixture that contains NLT 50% of hydrogenated polysaccharides containing more than 3 D-glucopyranosyl units terminated with a D-glucityl unit, calculated on the anhydrous basis. Other ingredients can include sorbitol, maltitol, and other sugar polyols.

IDENTIFICATION
- **A.** It meets the requirements of the test for *Content of Maltitol and Sorbitol.*
- **B.** It meets the requirements of the test for *Content of Hydrogenated Polysaccharides.*
- **C. LIMIT OF DIETHYLENE GLYCOL AND ETHYLENE GLYCOL**
  [NOTE—Perform this test for liquid products of Hydrogenated Starch Hydrolysate.]
  Diluent: Acetone and water (96:4)
  Standard stock solution: 0.5 mg/mL of USP Diethylene Glycol RS and 0.5 mg/mL of USP Ethylene Glycol RS in *Diluent*
  Internal standard stock solution: 0.5 mg/mL of 1,3-butanediol (internal standard) in *Diluent*
  Standard solution: 0.04 mg/mL of USP Diethylene Glycol RS, 0.04 mg/mL of USP Ethylene Glycol RS, and 0.04 mg/mL of 1,3-butanediol (internal standard), in *Diluent,* prepared from the *Standard stock solution* and the *Internal standard stock solution*
  Sample solution: Transfer 1.0 g of Hydrogenated Starch Hydrolysate to a 25-mL volumetric flask. Add 1.0 mL of water to the flask, and mix on a vortex mixer for 3 min. Add 2.0 mL of the *Internal standard stock solution,* and add the remaining *Diluent* to the flask in three equal portions to volume. Mix the contents for about 3 min after each addition of *Diluent.* Pass a portion of the supernatant layer through a nylon filter of 0.45-µm pore size. Discard the first 2 mL of the filtrate, and collect the rest of the filtrate for analysis. [NOTE—Acetone is used to precipitate sugar alcohols.]
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability.*)
  Mode: GC
  Detector: Flame ionization
  Column: 0.32-mm × 15-m fused-silica capillary; 0.25-µm layer of phase G46
  Temperature
  Detector: 300°
  Injection port: 240°
  Column: See *Table 1.*

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	—	70	2
70	50	300	5

  Carrier gas: Helium
  Flow rate: 3.0 mL/min
  Injection size: 1.0 µL
  Injection type: Split injection. The split ratio is about 10:1. [NOTE—A general-purpose split/splitless, tapered, glass wool, deactivated liner is used.]
  System suitability
  Sample: *Standard solution*
  [NOTE—See *Table 2.* Relative retention times are provided for information only, and the standards should be used to ensure appropriate peak identification.]

Table 2

Name	Relative Retention Time
Ethylene glycol	1.0
1,3-Butanediol (internal standard)	2.0
Diethylene glycol	2.5

  Suitability requirements
  Resolution: NLT 15 between ethylene glycol and 1,3-butanediol
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Based on the *Standard solution,* identify the peaks of ethylene glycol, 1,3-butanediol (internal standard), and diethylene glycol. Compare peak area ratios of ethylene glycol to the internal standard and of diethylene glycol to the internal standard in the *Standard solution* and *Sample solution,* respectively.
  Acceptance criteria
  Diethylene glycol: The peak area ratio of diethylene glycol to the internal standard in the *Sample solution* is NMT the peak area ratio of diethylene glycol to the internal standard in the *Standard solution,* corresponding to NMT

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0.10% of diethylene glycol in Hydrogenated Starch Hydrolysate.

Ethylene glycol: The peak area ratio of ethylene glycol to the internal standard in the *Sample solution* is NMT the peak area ratio of ethylene glycol to the internal standard in the *Standard solution*, corresponding to NMT 0.10% of ethylene glycol in Hydrogenated Starch Hydrolysate.

ASSAY

• **CONTENT OF MALTITOL AND SORBITOL**

Mobile phase: Degassed water

Standard solution: Dissolve accurately weighed quantities of USP Maltose Monohydrate RS, USP Maltitol RS, USP Dextrose RS, and USP Sorbitol RS in water to obtain a solution having known concentrations of about 1.0 mg/mL for each, calculated on the anhydrous basis.

Sample solution: Transfer a quantity of Hydrogenated Starch Hydrolysate, equivalent to 100 mg on the anhydrous basis, to a 100-mL volumetric flask. Dilute with water to volume, and mix. Transfer approximately 10 mL of the solution into a separate container, shake the solution for 30 s, pass through a filter of 0.45-µm or finer pore size into a suitable autosampler vial, and seal.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 7.7-mm × 30-cm; packing L58

Temperature

Detector: 40°

Column: 80°, controlled within ±2°

Flow rate: 0.3 mL/min

Injection size: 50 µL

System suitability

Sample: *Standard solution*

[NOTE—See *Table 3* for relative retention times.]

Table 3

Name	Relative Retention Time
Maltose	0.81
Maltitol	0.84
Dextrose	0.94
Sorbitol	1.00

[NOTE—Sorbitol is the last peak to elute.]

System suitability requirements

Relative standard deviation: NMT 2.0% for the maltitol peak

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of each component, maltose (P_{M1}), maltitol (P_{M2}), dextrose (P_D), and sorbitol (P_S), in the solid portion of Hydrogenated Starch Hydrolysate taken:

$$\text{Result} = V \times (r_U/r_S) \times (C/W) \times 100$$

V = volume of the *Sample solution*, 100 mL

r_U = peak response for the respective component (maltose, maltitol, dextrose, sorbitol) from the *Sample solution*

r_S = peak response for the respective component (maltose, maltitol, dextrose, sorbitol) from the *Standard solution*

C = concentration of the respective component (maltose, maltitol, dextrose, sorbitol) in the *Standard solution* (mg/mL)

W = weight of Hydrogenated Starch Hydrolysate that was taken to prepare the *Sample solution* (mg), calculated on the anhydrous basis

Acceptance criteria: Less than 50% of total maltitol and sorbitol and less than 1% of total maltose and dextrose, on the anhydrous basis

• **CONTENT OF HYDROGENATED POLYSACCHARIDES**

Mobile phase, Standard solution, Sample solution, and **Chromatographic system:** Prepare as directed in the test for *Content of Maltitol and Sorbitol*.

Analysis

Samples: *Standard solution* and *Sample solution*

Inject a volume (about 50 µL) of the *Sample solution*, and measure all the peak areas. The elution pattern includes the higher-molecular-weight hydrogenated polysaccharides containing more than 11 D-gluco pyranosyl units, followed by some individual peaks representing hydrogenated polysaccharides containing NMT 10 D-glucopyranosyl units, if this hydrogenated species is present. The higher-molecular-weight hydrogenated polysaccharides containing more than 11 D-glucopyranosyl units can be integrated into one peak; the relative retention time is about 0.29 relative to the peak of sorbitol. The relative retention times for hydrogenated polysaccharides containing NMT 10 D-glucopyranosyl units are given in *Table 4*.

Table 4

Hydrogenated Species for Degree of Polymerization (DP)	Relative Retention Time
Sorbitol (HDP 1)	1.00
Maltitol (HDP 2)	0.84
Maltotriitol (HDP 3)	0.72
Maltotetraitol (HDP 4)	0.64
Maltopentaitol (HDP 5)	0.58
Maltohexaitol (HDP 6)	0.53
Maltoheptaitol (HDP 7)	0.48
Maltooctaitol (HDP 8)	0.45
Maltononaitol (HDP 9)	0.42
Maltodecaitol (HDP 10)	0.40

Calculate the percentage of hydrogenated polysaccharides containing more than 3 D-glucopyranosyl units in the solid portion of Hydrogenated Starch Hydrolysate taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = sum of peak areas of hydrogenated polysaccharides containing more than 3 D-glucopyranosyl units from the *Sample solution*

r_T = sum of all the peak areas from the *Sample solution*

Acceptance criteria: NLT 50% of hydrogenated polysaccharides containing more than 3 D-glucopyranosyl units, on the anhydrous basis

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 0.15%, ignition of a quantity of Hydrogenated Starch Hydrolysate equivalent to 1.0 g of solid, on the anhydrous basis

• **LIMIT OF CHLORIDE**

Sample solution: Transfer a quantity of Hydrogenated Starch Hydrolysate, equivalent to 25 g on the anhydrous

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basis, to a beaker, add 100 mL of water, and stir until the Hydrogenated Starch Hydrolysate is completely dissolved.

Analysis: Add 1.0 mL of potassium chromate indicator solution (1 in 20) to the *Sample solution*. Slowly titrate with 0.1 N silver nitrate VS until a reddish-orange color persists. Calculate the quantity, in µg, of chloride in each g of Hydrogenated Starch Hydrolysate taken:

$$\text{Result} = (F \times M_r \times N \times V)/W$$

F = factor converting mg to µg, 10^3 µg/mg
M_r = molar mass of chloride, 35.45
N = exact normality of the silver nitrate solution
V = volume of the silver nitrate solution consumed in the titration (mL)
W = weight of Hydrogenated Starch Hydrolysate taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 50 µg/g (ppm) of chloride

• **CHLORIDE AND SULFATE,** *Sulfate* ⟨221⟩**:** 1.0 g of the solid portion of Hydrogenated Starch Hydrolysate shows no more sulfate than corresponds to 0.10 mL of 0.020 N sulfuric acid: NMT 100 µg/g (ppm) of sulfate is found.

• **LIMIT OF NICKEL**

[NOTE—When water is specified as the diluent, use deionized ultra-filtered water.]

Digester solution (aqua regia): Add 360 mL of hydrochloric acid and 240 mL of nitric acid to 1200 mL of water.

Blank solution: Add 40 mL of nitric acid to a 2-L volumetric flask, dilute with water to volume, and mix well.

Internal standard solution: Transfer 2.0 mL of commercially prepared yttrium reference standard solution (1000 ppm) to a 1-L volumetric flask, dilute with *Blank solution* to volume, and mix well. The *Internal standard solution* contains 2 µg/mL of yttrium.

Standard stock solution: [NOTE—Prepare this solution fresh every two months.] Quantitatively dilute an accurately measured volume of a commercially prepared nickel ICP standard (1000 ppm) with *Blank solution* to obtain a solution containing 10 µg/mL of nickel (*Standard stock solution 10 ppm*).

Standard solutions: [NOTE—Prepare these solutions fresh weekly.] Separately pipet 1.0, 2.0, and 4.0 mL of *Standard stock solution*, respectively, into three 200-mL volumetric flasks. Dilute the content in each flask with *Blank solution* to volume, and mix well. These are, respectively, the *Standard nickel solution 50 ppb*, *Standard nickel solution 100 ppb*, and *Standard nickel solution 200 ppb*.

Sample solution: Transfer a quantity of Hydrogenated Starch Hydrolysate, equivalent to 10.0 g on the anhydrous basis, into a 125-mL conical flask. Add 40 mL of *Digester solution*, and place on a hot plate. Heat the solution for about 20 min, being careful to prevent the solution from boiling over. Pull the sample off the hot plate just before it turns a dark caramel color. [NOTE—Do not overburn the sample.] Transfer the flask's contents into a clean, dry, 50-mL volumetric flask with washings of *Blank solution*. Dilute with *Blank solution* to volume. Filter the sample into a 15-mL centrifuge tube, using a 10-mL BD syringe fitted with a syringe filter of 0.45-µm pore size.

Instrumental conditions

(See *Plasma Spectrochemistry* ⟨730⟩.)

Mode: Inductively coupled plasma–optical emission spectroscopy (ICP–OES) configured in an axial optical alignment

Detector: Set a UV detector to scan nickel at 232.005 nm and yttrium at 371.029 nm, and set the sample read time to 10 s minimum and 50 s maximum.

Analysis

Samples: *Blank solution*, *Standard solutions*, and *Sample solution*

Take three replicate scans with the integration set to one point per peak. Set forward power from the RF generator to 1500 watts. The argon plasma feed gas flows at 15 L/min with the auxiliary gas (shear gas) set to flow at 0.5 L/min. Use a gem cone nebulizer with a nebulization gas flow rate of 0.55 L/min. Deliver the sample to the spray chamber with a multichannel peristaltic pump set to deliver sample at a rate of 1.00 mL/min. Add the *Internal standard solution* in-line via a mixing block between the sample probe and the spray chamber. Flush the samples through the system for 30 s at a rate of 4.0 mL/min before analysis. Program a 60-s read delay into the sampling routine to allow for fluid flow equilibration after the high-speed flush, before the first analytical read of the sample. Between samples, wash the pumping system by flushing the *Blank solution* for 30 s at a rate of 4.0 mL/min.

Instrument performance must be verified to conform to the manufacturer's specifications for resolution and sensitivity. Before analyzing samples, the instrument must pass a suitable performance check.

Generate the calibration curve using the *Blank solution*, *Standard nickel solution 50 ppb*, *Standard nickel solution 100 ppb*, and *Standard nickel solution 200 ppb* as follows. Scan the *Internal standard solution* while running the *Blank solution* to measure the intensity of the yttrium emission. Hold this value constant throughout the remainder of the test. Separately scan the *Blank solution*, *Standard nickel solution 50 ppb*, *Standard nickel solution 100 ppb*, and *Standard nickel solution 200 ppb* for nickel and yttrium. [NOTE—Add the *Internal standard solution* via an in-line mixing chamber.] Normalize the yttrium intensity to the value of the *Internal standard solution*. Apply this normalization factor to the nickel intensity, which is then referred to as the corrected nickel intensity. Construct a calibration curve by plotting the corrected nickel intensity versus the known concentrations, in ng/mL, of the nickel: the linear regression coefficient is NLT 0.999.

Similarly, analyze the *Sample solution* on the ICP. Plot the intensity of the emission of the *Sample solution* on the calibration curve. Obtain the concentration of nickel, C, in ng/mL, in the *Sample solution* through the calibration curve.

Calculate the content, in µg/g, of nickel in the solid portion of Hydrogenated Starch Hydrolysate taken:

$$\text{Result} = F \times V \times (C/W)$$

F = factor converting ng to µg, 10^{-3} µg/ng
V = volume of the *Sample solution*, 50 mL
C = concentration of nickel in the *Sample solution* (ng/mL)
W = weight of Hydrogenated Starch Hydrolysate calculated on the anhydrous basis (g)

Acceptance criteria: Nickel content is NMT 1 µg/g (ppm).

• **LIMIT OF REDUCING SUGARS**

Analysis: Dissolve a quantity of Hydrogenated Starch Hydrolysate, equivalent to 1.0 g on the anhydrous basis, in 6 mL of water with the aid of gentle heat, if necessary. Cool, and add 20.0 mL of cupric citrate TS and a few glass beads. Heat so that boiling begins after 4 min, and maintain boiling for 3 min. Cool rapidly, and add 40 mL of diluted acetic acid, 60 mL of water, and 20.0 mL of 0.05 N iodine VS. With continuous shaking, add 25 mL of a mixture of 6 mL of hydrochloric acid and 94 mL of water. When the

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precipitate has dissolved, titrate the excess of iodine with 0.05 N sodium thiosulfate VS, using 2 mL of starch TS as an indicator, added toward the end of the titration.
Acceptance criteria: NLT 12.8 mL of 0.05 N sodium thiosulfate, corresponding to NMT 1% of reducing sugars

SPECIFIC TESTS
- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g. It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli.*
- **pH** ⟨791⟩**:** 3.0–7.0, in a 20% (w/w) solution in freshly boiled and cooled water
- **WATER DETERMINATION,** *Method I* ⟨921⟩
  For dried powder product: NMT 6.0%
  For liquid product: Within ±1.5 units of the labeled value

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified for liquid product; protect from moisture for dried powder product.
- **LABELING:** Label it to indicate *Water* content, as the percentage of water, for liquid product.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Dextrose RS
  USP Diethylene Glycol RS
  USP Ethylene Glycol RS
  USP Maltitol RS
  USP Maltose Monohydrate RS
  USP Sorbitol RS

Modified Starch

DEFINITION
Modified Starch is Starch modified by chemical means. Food Starch may be acid-modified, bleached, oxidized, esterified, etherified, or treated enzymatically to change its functional properties (21 CFR 172.892).

IDENTIFICATION
- **A.**
  Corn starch: Polygonal, rounded, or spheroidal granules up to 35 µm in diameter and usually having a circular or several-rayed central cleft
  Tapioca starch: Spherical granules with one truncated side, typically 5–35 µm in diameter and usually having a circular or several-rayed central cleft
  Potato starch: Irregularly shaped, ovoid, or pear-shaped granules, usually 30–100 µm in size but occasionally exceeding 100 µm; or rounded, 10–35 µm in size. There are occasional compound granules having two to four components. The ovoid and pear-shaped granules have an eccentric hilum, and the rounded granules have an accentric or slightly eccentric hilum. All granules show clearly visible concentric striations.
  Wheat starch: Large and small granules, usually 10–60 µm in diameter. The central hilum and striations are visible or barely visible.
- **B.**
  Sodium hydroxide solution: 2% (w/w)
  Sample: 0.6 g
  Analysis: Transfer the *Sample* to a 25-mL glass vial with a plastic cap. Add 9.4 g of water, cap, and shake vigorously to evenly disperse the starch. Add 10 g of the *Sodium hydroxide solution,* cap, and shake vigorously for 1 min to create a smooth mixture. Evaluate within 1 min.
  Acceptance criteria: The final solution is translucent to opaque with a fluid consistency. A yellow tint of the final solution is acceptable.
- **C.** A water slurry of the Modified Starch is colored orange-red to deep blue by iodine TS.

IMPURITIES
- **RESIDUE ON IGNITION** ⟨281⟩
  Sample: 2.0 ± 0.1 g
  Analysis: Proceed as directed in the chapter.
  Acceptance criteria: NMT 1.5%
- **IRON** ⟨241⟩
  Test preparation: Dissolve the residue obtained in the test for *Residue on Ignition* in 8 mL of hydrochloric acid with the aid of gentle heating. Dilute with water to 100 mL in a volumetric flask, and mix. Dilute 25 mL of this solution with water to 47 ± 1 mL.
  Analysis: Proceed as directed in the chapter.
  Acceptance criteria: NMT 20 ppm
- **LIMIT OF SULFUR DIOXIDE**
  Sample: 20.0 ± 0.1 g
  Analysis: Mix the *Sample* with 200 mL of 5% alcohol until a smooth suspension is obtained, and vacuum-filter through paper (Whatman No.1 or equivalent). To 100 mL of the filtrate add 3 mL of starch TS, and titrate with 0.01 N iodine VS to the first permanent blue color.
  Acceptance criteria: NMT 1.7 mL of 0.01 N iodine VS is consumed, which corresponds to NMT 50 ppm of sulfur dioxide being found.
- **OXIDIZING SUBSTANCES**
  Sample: 4.0 g
  Titrimetric system
  Mode: Direct titration
  Titrant: 0.002 N sodium thiosulfate VS
  Blank: 30.0 mL of water, accurately measured
  Endpoint detection: Visual
  Analysis: Transfer the *Sample* to a glass-stoppered, 125-mL conical flask, and add 50.0 mL of water. Insert the stopper, and swirl for 5 min. Transfer to a glass-stoppered, 50-mL centrifuge tube, and centrifuge to clarify. Transfer 30.0 mL of the clear supernatant to a glass-stoppered, 125-mL conical flask. Add 1 mL of glacial acetic acid and 0.5–1.0 g of potassium iodide. Insert the stopper, swirl, and allow to stand for 25–30 min in the dark. Add 1 mL of starch TS, and titrate with *Titrant* to the disappearance of the starch–iodine color. Perform a blank determination, and make any necessary correction. Each mL of 0.002 N sodium thiosulfate is equivalent to 34 µg of oxidant, calculated as hydrogen peroxide.
  Acceptance criteria: NMT 12.6 mL of 0.002 N sodium thiosulfate is required (180 ppm, calculated as H_2O_2), which corresponds to NMT 0.018% of oxidizing substances

SPECIFIC TESTS
- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g. It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli.*
- **pH** ⟨791⟩
  Sample: 20.0 ± 0.1 g
  Analysis: Transfer the *Sample* to a suitable nonmetallic container, and add 100 mL of water to obtain a slurry. Stir using a magnetic stirrer at a moderate rate for 5 min, and determine the pH to the nearest 0.1 unit.
  Acceptance criteria: 3.0–9.0
- **LOSS ON DRYING** ⟨731⟩
  Analysis: Dry a sample at 120° for 4 h.

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Acceptance criteria
Corn starch and **Wheat starch:** NMT 15.0%
Tapioca starch: NMT 18.0%
Potato starch: NMT 21.0%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.

Pea Starch

DEFINITION
Pea Starch is obtained from the seeds of *Pisum sativum L.*

IDENTIFICATION
- **A.** Examined under a microscope using a mixture of equal volumes of glycerin and water, it presents a majority of large elliptical granules, 25-45 µm in size, sometimes irregular, or reniform. It also presents a minority of small rounded granules, 5-8 µm in size. Granules can present cracks or irregularities. Sometimes, granules show barely visible concentric striations. Exceptionally, granules show a slit along the main axis. Between orthogonally oriented polarizing plates or prisms, the granules show a distinct black cross.
- **B.** Suspend 1 g of it in 50 mL of water, boil for 1 min, and cool: a thin, cloudy mucilage is formed.
- **C.** To 1 mL of the mucilage obtained in *Identification* test *B* add 0.05 mL of iodine and potassium iodide TS 2: an orange-red to dark blue color is produced, which disappears on heating.

IMPURITIES
INORGANIC IMPURITIES
- **Residue on Ignition** ⟨281⟩: NMT 0.6%, determined on a 1.0-g sample
- **Limit of Iron**

Standard iron stock solution: Prepare a solution containing the equivalent of 10 µg/mL of iron, as directed under *Iron* ⟨241⟩.
Diluted standard iron solution: Immediately before use, dilute a measured volume of *Standard iron stock solution* quantitatively with water to obtain a solution containing the equivalent of 1 µg/mL of iron.
Standard solution: Transfer 10 mL of the *Diluted standard iron solution* to a test tube. Add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid, and mix. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, dilute with water to 20 mL, and mix.
Sample solution: Shake 1.0 g of Pea Starch with 50 mL of 2 N hydrochloric acid, and filter. Transfer 10 mL of the filtrate to a test tube. Add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid, and mix. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, dilute with water to 20 mL, and mix.
Acceptance criteria: After 5 min, any pink color in the *Sample solution* is not more intense than that in the *Standard solution*, corresponding to a limit of 50 µg/g of iron.
- **Limit of Sulfur Dioxide**

[NOTE—Perform either *Test 1* or *Test 2*.]
Test 1
Carbon dioxide: Use carbon dioxide with a flow regulator that will maintain a flow of 100 ± 10 mL/min.
Hydrogen peroxide solution: Dilute 30% hydrogen peroxide with water to obtain a 3% solution. Neutralize the solution with 0.01 N sodium hydroxide to a pH of 4.1, determined potentiometrically.
Potassium metabisulfite solution: Transfer 0.87 g of potassium metabisulfite ($K_2S_2O_5$) and 0.2 g of edetate disodium to a 1000-mL volumetric flask. Dilute with water to volume before mixing. [NOTE—Edetate disodium is used to protect the sulfite ion from oxidation.]
Apparatus: In this test, the sulfur dioxide is released from the sample in a boiling acid medium and is removed by a stream of carbon dioxide. The separated gas is collected in a dilute hydrogen peroxide solution where the sulfur dioxide is oxidized to sulfuric acid and titrated with standard alkali. A suitable apparatus for sulfur dioxide determination is shown in the accompanying diagram (*Figure 1*).

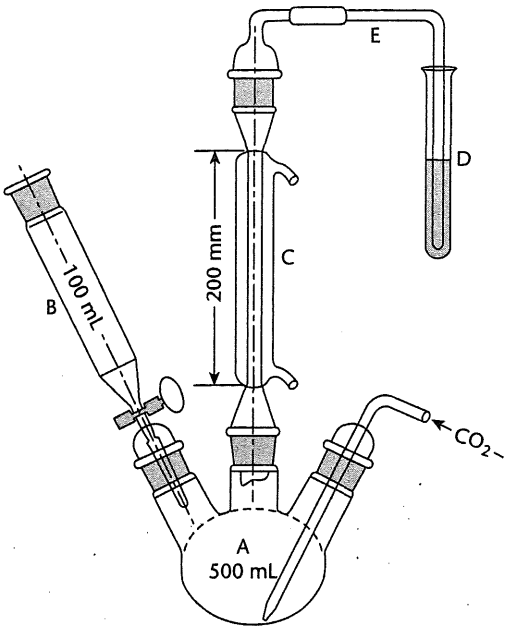


Figure 1

The apparatus consists of a 500-mL three-neck, round-bottom boiling flask, *A*; a separatory funnel, *B*, having a capacity of 100 mL or greater; a gas inlet tube of sufficient length to permit introduction of the carbon dioxide within 2.5 cm of the bottom of the boiling flask; a reflux condenser, *C*, having a jacket length of 200 mm; and a delivery tube, *E*, connecting the upper end of the reflux condenser to the bottom of a receiving test tube, *D*. Apply a thin film of stopcock grease to the sealing surfaces of all the joints except the joint between the separatory funnel and the boiling flask, and clamp the joints to ensure tightness.
System suitability test
Test A: Using the *Potassium metabisulfite solution* as the standard, proceed as directed in *Analysis*, except for replacing the 25.0 g of Pea Starch with 20 mL of the *Potassium metabisulfite solution*.
Calculate the content, in µg/mL, of sulfur dioxide in the *Potassium metabisulfite solution* taken:

$$\text{Result} = (F \times MW \times V \times N)/V_P$$

F	= factor for conversion of mg to µg, 1000
MW	= milliequivalent weight of sulfur dioxide, 32.03
V	= volume of titrant consumed (mL)
N	= normality of the titrant
V_P	= volume of the *Potassium metabisulfite solution* taken for the test (mL)

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Test B: In a 100-mL conical flask, add 20 mL of 0.02 N iodine solution and 5 mL of 2 N hydrochloric acid. Add 1 mL of starch TS, and titrate with *Potassium metabisulfite solution* until the first discoloration is observed.
Calculate the content, in µg/mL, of sulfur dioxide in *Potassium metabisulfite solution*:

$$\text{Result} = (F \times MW \times V_I \times N_I)/V_P$$

F	= factor for conversion of mg to µg, 1000
MW	= milliequivalent weight of sulfur dioxide, 32.03
V_I	= the volume of the iodine solution used in the test (mL)
N_I	= normality of the iodine solution
V_P	= volume of the *Potassium metabisulfite solution* consumed (mL)

The difference between the sulfur dioxide contents obtained from *Test A* and *Test B* is NMT 5% of their mean value. *Test B* shall be performed within 15 min after the completion of *Test A*. [NOTE—This time limit avoids potential variation in the sulfur dioxide content of the *Potassium bisulfite solution* when stored at room temperature.]

Analysis: Add 150 mL of water to the boiling flask (*A*) (see *Figure 1*). Close the stopcock of the separatory funnel, and begin the flow of carbon dioxide through the apparatus at a rate of 100 ± 5 mL/min. Start the condenser coolant flow. Place 10 mL of *Hydrogen peroxide solution* in the receiving test tube (*D*). After 15 min, without interrupting the flow of carbon dioxide, remove the separatory funnel (*B*) from the boiling flask, and transfer 25.0 g of Pea Starch to the boiling flask with the aid of 100 mL of water. Apply stopcock grease to the outer joint of the separatory funnel, and replace the separatory funnel in the boiling flask. Close the stopcock of the separatory funnel, and add 80 mL of 2 N hydrochloric acid to the separatory funnel. Open the stopcock of the separatory funnel to permit the hydrochloric acid solution to flow into the boiling flask, guarding against the escape of sulfur dioxide into the separatory funnel by closing the stopcock before the last few mL of hydrochloric acid drain out. Boil the mixture for 1 h. Open the stopcock of the funnel, stop the flow of carbon dioxide, discontinue heating the flask, and turn off the cooling water in the condenser. Remove the receiving test tube, and transfer its contents to a 200-mL wide-necked conical flask. Rinse the receiving test tube with a small portion of water, add the rinsing to the 200-mL conical flask, and mix. Heat on a water bath for 15 min, and allow to cool. Titrate the contents with 0.1 N sodium hydroxide VS until the pH reaches 4.1, determined potentiometrically. Perform a blank determination, and make any necessary correction (see *Titrimetry* ⟨541⟩).
Calculate the content, in µg/g, of sulfur dioxide in the Pea Starch taken:

$$\text{Result} = (F \times MW \times V \times N)/W$$

F	= factor for conversion of mg to µg, 1000
MW	= milliequivalent weight of sulfur dioxide, 32.03
V	= volume of titrant consumed (mL)
N	= normality of the titrant
W	= weight of the Pea Starch taken (g)

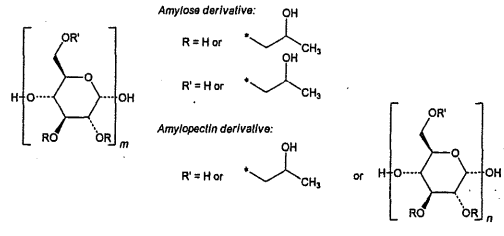
Test 2: Determine the content of sulfur dioxide as directed under *Sulfur Dioxide* ⟨525⟩, *Method I*. Use 200 mL of water as a solvent. Then, to 100 mL of the clear filtrate, add 3 mL of starch TS, and titrate with 0.01 N iodine VS to the first permanent blue color.
Acceptance criteria: NMT 50 µg/g of sulfur dioxide

ORGANIC IMPURITIES
• **Procedure 1: Limit of Oxidizing Substances**
Sample: 4.0 g of Pea Starch
Analysis: Transfer the *Sample* to a glass-stoppered, 125-mL conical flask, and add 50.0 mL of water. Insert the stopper, and swirl for 5 min. Transfer to a glass-stoppered 50-mL centrifuge tube, and centrifuge to clarify. Transfer 30.0 mL of the clear supernatant to a glass-stoppered 125-mL conical flask. Add 1 mL of glacial acetic acid and 0.5–1.0 g of potassium iodide. Insert the stopper, swirl, and allow to stand for 25–30 min in the dark. Add 1 mL of starch TS, and titrate with 0.002 N sodium thiosulfate VS to the disappearance of the starch-iodine color. Perform a blank determination, and make any necessary correction. Each mL of 0.002 N sodium thiosulfate VS is equivalent to 34 µg of oxidant, calculated as hydrogen peroxide.
Acceptance criteria: NMT 1.4 mL of 0.002 N sodium thiosulfate VS is required (20 µg/g, calculated as H_2O_2).
• **Procedure 2: Foreign Matter**
Analysis: Examine under a microscope, using a mixture of equal volumes of glycerin and water.
Acceptance criteria: NMT traces of matter other than starch granules are present. No starch grains of any other origin are present.

SPECIFIC TESTS
• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic microbial count does not exceed 1000 cfu/g, the total combined molds and yeasts count does not exceed 100 cfu/g, and it meets the requirements of the test for the absence of *Escherichia coli*.
• **pH** ⟨791⟩
Sample solution: Prepare a slurry by weighing 5.0 g of Pea Starch, transferring to a suitable nonmetallic container, and adding 25.0 mL of freshly boiled and cooled water.
Analysis: Agitate continuously at a moderate rate for 1 min. Stop the agitation, allow to stand for 15 min, and shake again. Determine the pH to the nearest 0.1 unit: the pH is determined potentiometrically.
Acceptance criteria: 5.0–8.0
• **LOSS ON DRYING** ⟨731⟩**:** Dry about 1 g at 130° for 90 min: it loses NMT 16.0% of its weight.

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers. Store at room temperature.

Hydroxypropyl Pea Starch



For the Amylose derivative, *m* is about 300–1000.

DEFINITION
Hydroxypropyl Pea Starch is partially substituted 2-hydroxy propylether obtained from pea starch by a chemical modification of etherification with propylene oxide. In addition, this starch may be partially hydrolyzed using acids

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or enzymes to obtain thinned starch. It contains NLT 2.0% and NMT 7.0% of hydroxypropyl groups, on the dried basis.

IDENTIFICATION

• **A. Procedure**

Analysis: Examine under a microscope, using NLT 20× magnification and a mixture of glycerin and water (1:1) as a mounting agent.

Acceptance criteria: It presents a majority of large elliptical granules 25–45 µm in size, sometimes irregular or reniform. It also presents a minority of small rounded granules 5–8 µm in size. Granules can present cracks or irregularities. Sometimes, granules show barely visible concentric striations. Exceptionally, granules show a slit along the main axis. Between orthogonally oriented polarizing plates or prisms, the granules show a distinct black cross.

• **B. Procedure**

Sample solution: Suspend 1 g of Hydroxypropyl Pea Starch in 50 mL of water, boil for 1 min, and cool.

Acceptance criteria: A translucent or clear mucilage is formed.

• **C. Procedure**

Analysis: To 1 mL of the *Sample solution* obtained in *Identification* test *B* add 0.05 mL of iodine and potassium iodide TS 2.

Acceptance criteria: An orange-red to dark blue color is produced, which disappears upon heating.

• **D. Procedure**

Ninhydrin solution: Dissolve 3 g of ninhydrin in 100 mL of a 45.5-g/L solution of sodium metabisulfite.

Diluted sulfuric acid: 98 g/L of H_2SO_4

Sample: 100 mg of Hydroxypropyl Pea Starch

Analysis: Transfer the *Sample* to a 100-mL volumetric flask, and add 12.5 mL of *Diluted sulfuric acid*. Place the flask in a water bath, and heat until the *Sample* is dissolved. Cool, and dilute with water to 100 mL. [**Caution**—When sulfuric acid is miscible with water, it produces intense heat.] Pipet 1 mL of this solution to a glass-stoppered, 25-mL graduated test tube and, with the tube immersed in cold water, add drop-wise 8 mL of sulfuric acid. Mix well, and place the tube in a boiling water bath for exactly 3 min. Immediately transfer the tube to an ice bath until the solution is chilled. Add 0.6 mL of *Ninhydrin solution*, carefully allowing the reagent to run down the walls of the test tube. Immediately shake the tube well, and place it in a water bath at 25° for 100 min. Dilute with sulfuric acid to 25 mL [**Caution**—Use sulfuric acid cautiously.], and mix by inverting the tube several times. Do not shake.

Acceptance criteria: A violet color develops within 5 min due to the presence of hydroxypropyl groups (starch ether).

ASSAY

• **Procedure for Hydroxypropyl Groups**

Deuterium chloride solution: Dilute 1 mL of deuterium chloride (38% w/w) with 5 mL of deuterium oxide.

Internal standard solution: Dissolve 50.0 mg of sodium 3-trimethylsilyl-1-propane sulfonate in about 5 g of deuterium oxide, weighed to the nearest 0.1 mg. Store in a sealed bottle.

Sample solution: Disperse 20 g of Hydroxypropyl Pea Starch in 200.0 mL of carbon dioxide-free water at room temperature. Agitate for 15 min, and filter. Repeat the operation two more times. If poor dispersibility or slow filtration is observed, use refrigerated carbon dioxide-free water for the washing operation. Dry the washed starch for NLT 4 h in vacuum at 30 ± 5°. Weigh 12.0 mg of this sample in a 5-mm NMR tube. Add 0.75 mL of deuterium oxide and 0.1 mL of *Deuterium chloride solution*. Cap the tube, mix, and place it in a boiling water bath until a clear solution is obtained. [Note—This may take 3 min to 1 h.] When a clear solution is obtained, allow to cool to room temperature. Dry the exterior of the tube, and weigh to the nearest 0.1 mg. Add 0.05 mL of *Internal standard solution*, and weigh to the nearest 0.1 mg. Determine the mass of the *Internal standard solution* added. Mix thoroughly.

Nuclear magnetic resonance spectrometry

(See *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩, *Quantitative Applications* .)

Apparatus: FT-NMR spectrometer at minimum 300 MHz

Acquisition of ^{1}H NMR spectra: The following parameters may be used.

Sweep width: 8 ppm (about −1.0 to +7 ppm)
Irradiation frequency offset: None
Time domain: NLT 64 K
Pulse width: 90 degree
Pulse delay: 10 s
Dummy scans: 0
Number of scans: 8

Use the CH_3 signal of the internal standard for shift referencing. Set the shift of the peak of the singlet to 0 ppm. Record the FID signal.

Analysis

Samples: *Internal standard solution* and *Sample solution*

Call the integration sub-routine after phase corrections and baseline correction between −0.5 and +6 ppm.

Measure the peak areas of the doublet from the methyl groups of the hydroxypropyl function at +1.2 ppm (A_2), and of the methyl groups at 0 ppm of the internal standard (A_1) without ^{13}C-satellites.

Measure the signal coming from the 3 protons of the methyl group in the hydroxypropyl function.

Calculate the content of hydroxypropyl groups as a percentage (w/w, dried basis):

$$\text{Result} = (N \times A_2/A_1) \times (C_i \times W_i/W) \times (M_{r2}/M_{r1}) \times [100/(100 - B)] \times 100$$

N	= numerical value representing the 3 methyl groups in the internal standard (sodium 3-trimethylsilyl-1-propane sulfonate), 3
A_2	= area of the methyl groups of hydroxypropyl in Hydroxypropyl Pea Starch
A_1	= area of the methyl groups in the internal standard (sodium 3-trimethylsilyl-1-propane sulfonate)
C_i	= concentration of the internal standard in the *Internal standard solution* (mg/g)
W_i	= weight of the *Internal standard solution* in the NMR tube (g)
W	= weight of the washed and dried Hydroxypropyl Pea Starch in the NMR tube (mg)
M_{r1}	= molecular weight of the internal standard, 218.32 g/mol
M_{r2}	= molar mass of hydroxypropyl group, 59.09 g/mol
B	= moisture content of the washed and dried Hydroxypropyl Pea Starch used in the *Sample solution*, as a percentage (w/w)

Acceptance criteria: The content of hydroxypropyl groups is 2.0%–7.0%.

IMPURITIES

Inorganic Impurities

• **Residue on Ignition** ⟨281⟩: NMT 0.6%, determined on a 1.0-g test specimen

• **Limit of Iron**

Standard iron stock solution: Prepare a solution containing the equivalent of 10 µg/mL of iron, as directed under *Iron* ⟨241⟩.

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Diluted standard iron solution: Immediately before use, dilute an accurately measured volume of *Standard iron stock solution* quantitatively with water to obtain a solution containing the equivalent of 1 µg/mL of iron.

Standard solution: Transfer 10 mL of the *Diluted standard iron solution* to a test tube. Add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid, and mix. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, dilute with water to 20 mL, and mix.

Sample solution: Shake 1.0 g of Hydroxypropyl Pea Starch with 50 mL of 2 N hydrochloric acid, and filter. Transfer 10 mL of the filtrate to a test tube. Add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid, and mix. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, dilute with water to 20 mL, and mix.

Acceptance criteria: After 5 min, any pink color in the *Sample solution* is not more intense than that in the *Standard solution*, corresponding to a limit of 50 µg/g of iron.

• **Limit of Sulfur Dioxide,** *Method IV* ⟨525⟩: NMT 50 ppm

ORGANIC IMPURITIES

• **Procedure 1: Limit of Oxidizing Substances**

Sample: 4.0 g of Hydroxypropyl Pea Starch

Analysis: Transfer the *Sample* to a glass-stoppered, 125-mL conical flask, and add 50.0 mL of water. Insert the stopper, and swirl for 5 min. Transfer to a glass-stoppered, 50-mL centrifuge tube, and centrifuge to clarify. Transfer 30.0 mL of the clear supernatant to a glass-stoppered, 125-mL conical flask. Add 1 mL of glacial acetic acid and 0.5–1.0 g of potassium iodide. Insert the stopper, swirl, and allow to stand for 25–30 min in the dark. Add 1 mL of starch TS, and titrate with 0.002 N sodium thiosulfate VS to the disappearance of the starch-iodine color. Perform a blank determination, and make any necessary correction. Each mL of 0.002 N sodium thiosulfate VS is equivalent to 34 µg of oxidant, calculated as hydrogen peroxide.

Acceptance criteria: NMT 1.4 mL of 0.002 N sodium thiosulfate VS is required (20 µg/g, calculated as H_2O_2).

• **Procedure 2: Foreign Matter**

Sample: 50 mg/mL of Hydroxypropyl Pea Starch in a mixture of glycerin and water (1:1)

Analysis: Examine under a microscope, using NLT 20× magnification and a mixture of glycerin and water (1:1) as a mounting agent.

Acceptance criteria: NMT traces of matter other than Hydroxypropyl Pea Starch granules are present.

SPECIFIC TESTS

• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩: The total aerobic microbial count does not exceed 10^3 cfu/g, the total combined molds and yeasts count does not exceed 10^2 cfu/g, and it meets the requirements of the test for the absence of *Escherichia coli*.

• **pH** ⟨791⟩

Sample solution: Suspend 5.0 g of Hydroxypropyl Pea Starch in 25.0 mL of carbon dioxide-free water, and shake for 60 s. Allow to stand for 15 min.

Acceptance criteria: 4.5–8.0

• **LOSS ON DRYING** ⟨731⟩: Dry about 1 g at 130° for 90 min: it loses NMT 15.0% of its weight.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers. Store at room temperature.

Pregelatinized Hydroxypropyl Pea Starch

DEFINITION

Pregelatinized Hydroxypropyl Pea Starch is prepared from Hydroxypropyl Pea Starch by mechanical processing in the presence of water, with or without heat, to rupture all or some of the starch granules, and is subsequently dried. It contains NLT 2.0% and NMT 7.0% of hydroxypropyl groups on the dried basis.

IDENTIFICATION

• **A. TEST FOR PREGELATINIZED STATE**

Sample: 1 g

Analysis: Disperse the *Sample* in 50 mL of water at a temperature NMT 25°. Shake vigorously until lumps completely disperse/solubilize or until lumps disappear. Allow to stand for 20 min.

Acceptance criteria: A translucent or clear mucilage without precipitate is formed.

• **B. TEST FOR STARCH**

Analysis: Disperse 0.5 g in 2 mL of water without heating, and add 0.05 mL of iodine and potassium iodide TS2.

Acceptance criteria: A reddish-violet or blue color is produced.

• **C. NINHYDRIN TEST**

Ninhydrin solution: Dissolve 3 g of ninhydrin in 100 mL of a 45.5-g/L solution of sodium metabisulfite.

Diluted sulfuric acid: 98 g/L of H_2SO_4

Sample: 100 mg

Analysis: Transfer the *Sample* to a 100-mL volumetric flask and add 12.5 mL of *Diluted sulfuric acid*. Place the flask in a water bath, and heat until the *Sample* is dissolved. Cool, and dilute with water to 100 mL. [**CAUTION**—When sulfuric acid is miscible with water, it produces intense heat.] Pipet 1 mL of this solution to a glass-stoppered 25-mL graduated test tube and, with the tube immersed in cold water, add dropwise 8 mL of sulfuric acid. Mix well, and place the tube in a boiling water bath for exactly 3 min. Immediately transfer the tube to an ice bath until the solution is chilled. Add 0.6 mL of *Ninhydrin solution*, carefully allowing the reagent to run down the walls of the test tube. Immediately shake the tube well, and place it in a water bath at 25° for 100 min. Dilute with sulfuric acid to 25 mL. [**CAUTION**—Use sulfuric acid cautiously.] Mix by inverting the tube several times. Do not shake.

Acceptance criteria: A violet color develops within 5 min due to the presence of hydroxypropyl groups (starch ether).

ASSAY

• **ASSAY FOR HYDROXYPROPYL GROUPS**

Deuterium chloride solution: Dilute 1 mL of deuterium chloride (38% w/w) with 5 mL of deuterium oxide.

Internal standard solution: Disperse 50.0 mg of sodium 3-trimethylsilyl-1-propane sulfonate in about 5 g of deuterium oxide, weighed to the nearest 0.1 mg. Store in a sealed bottle.

Sample solution: Determine the moisture content (*B*) on 5 g of Pregelatinized Hydroxypropyl Pea Starch following the *Loss on Drying* test. Weigh 12.0 mg of the Pregelatinized Hydroxypropyl Pea Starch in a 5-mm NMR tube. Add 0.75 mL of deuterium oxide and 0.1 mL of *Deuterium chloride solution*. Cap the tube, mix, and place it in a boiling water bath until a clear solution is obtained. [NOTE—This may take from 3 min to 1 h.] When a clear solution is obtained, allow to cool to room temperature. Dry the exterior of the tube, and weigh to the nearest 0.1 mg. Add 0.05 mL of the *Internal standard solution*. Weigh to the

Supplements

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nearest 0.1 mg. Determine the mass of the *Internal standard solution* added. Mix thoroughly.

Instrumental conditions

(See *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩, *Quantitative Applications* .)

Mode: Nuclear magnetic resonance spectrometry

Apparatus: FT-NMR spectrometer at minimum 300 MHz

Acquisition of ^{1}H NMR spectra: The following parameters may be used:

Sweep width: 8 ppm (about –1.0 to +7 ppm)

Irradiation frequency offset: None

Time domain: NLT 64 K

Pulse width: 90°

Pulse delay: 10 s

Dummy scans: 0

Number of scans: 8

Use the CH_3 signal of the internal standard for shift referencing. Set the shift of the peak of the singlet to 0 ppm. Record the FID signal.

Analysis

Samples: *Internal standard solution* and *Sample solution*

Call the integration subroutine after phase corrections and baseline correction between –0.5 and +6 ppm.

Measure the peak areas of the doublet from the methyl groups of the hydroxypropyl function at +1.2 ppm (A_2), and of the methyl groups at 0 ppm of the internal standard (A_1) without ^{13}C-satellites.

Measure the signal originating from the 3 protons of the methyl group in the hydroxypropyl function.

Calculate the content of hydroxypropyl groups as a percentage (w/w, dried basis):

$$\text{Result} = (N \times A_2/A_1) \times (C_I \times W_I/W) \times (M_{r2}/M_{r1}) \times [100/(100 - B)] \times 100$$

N = numerical value representing the 3 methyl groups in the internal standard (sodium 3-trimethylsilyl-1-propane sulfonate), 3

A_2 = area of the methyl groups of hydroxypropyl in Pregelatinized Hydroxypropyl Pea Starch

A_1 = area of the methyl groups in the internal standard (sodium 3-trimethylsilyl-1-propane sulfonate)

C_I = concentration of the internal standard in the *Internal standard solution* (mg/g)

W_I = weight of the *Internal standard solution* in the NMR tube (g)

W = weight of the Pregelatinized Hydroxypropyl Pea Starch in the NMR tube (mg)

M_{r2} = molar mass of hydroxypropyl groups, 59.09 g/mol

M_{r1} = molecular weight of the internal standard, 218.32 g/mol

B = moisture content of the Pregelatinized Hydroxypropyl Pea Starch used in the *Sample solution*, as a percentage (w/w)

Acceptance criteria: 2.0%–7.0% of hydroxypropyl groups on the dried basis

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 0.6%, determined on a 1.0-g test specimen
- **LIMIT OF IRON**

Standard iron stock solution: Prepare a solution containing the equivalent of 10 µg/mL of iron, as directed under *Iron* ⟨241⟩.

Diluted standard iron solution: Immediately before use, dilute an accurately measured volume of the *Standard iron stock solution* quantitatively with water to obtain a solution containing the equivalent of 1 µg/mL of iron.

Sample solution: Shake the residue obtained from the test for *Residue on Ignition* with 50 mL of 2 N hydrochloric acid, and filter. Transfer 10 mL of the filtrate to a test tube. Add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid, and mix. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, dilute with water to 20 mL, and mix.

Standard solution: Transfer 10 mL of the *Diluted standard iron solution* to a test tube. Add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid, and mix. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, dilute with water to 20 mL, and mix.

Acceptance criteria: After 5 min, any pink color in the *Sample solution* is not more intense than that in the *Standard solution*, corresponding to a limit of 50 µg/g (ppm) of iron.

- **LIMIT OF SULFUR DIOXIDE,** *Method IV* ⟨525⟩**:** NMT 50 ppm
- **LIMIT OF PROPYLENE GLYCOL**

Internal standard solution: 0.5 mg/mL of 1,3-propanediol in anhydrous pyridine

Standard stock solution: 0.5 mg/mL of USP Propylene Glycol RS in *Internal standard solution*

Standard solution: Transfer 0.1 mL of the *Standard stock solution* to a 2-mL vessel with a screw cap fitted with a septum. Add 0.9 mL of anhydrous pyridine, 0.2 mL of hexamethyldisilazane, and 0.1 mL of trimethylchlorosilane. Close, and mix. Allow to stand for 15 min before injection.

Sample stock solution: Transfer 200 mg of Pregelatinized Hydroxypropyl Pea Starch to a 100-mL volumetric flask. Add 1.0 mL of the *Internal standard solution* and 9.0 mL of anhydrous pyridine. Boil under reflux using a water bath for 20 min. Allow to cool to room temperature.

Sample solution: Transfer 1.0 mL of the *Sample stock solution* to a 2-mL vessel with a screw cap fitted with a septum. Add 0.2 mL of hexamethyldisilazane and 0.1 mL of trimethylchlorosilane. Close, and mix. Allow to stand for 15 min before injection.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*).

Mode: GC

Detector: Flame ionization

Column: 0.32-mm × 30-m fused-silica capillary column; 0.25-µm layer of phase G1

Temperature

Detector: 250°

Injection port: 250°

Column: 70°. [NOTE—The column must be desorbed regularly. Conditions: Program from 70° to 300° at 7°/min, and maintain 10 min at 300°.]

Carrier gas: Helium

Flow rate: 3 mL/min

Injection type: Split ratio of 1:30

Injection size: 1 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for the trimethylsylilated derivative of propylene glycol and the trimethylsylilated derivative of 1,3-propanediol are 1.0 and 1.4, respectively.]

Suitability requirements

Resolution: NLT 2.0 between the peaks due to the trimethylsylilated derivative of propylene glycol and the trimethylsylilated derivative of 1,3-propanediol

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of propylene glycol in the portion of Pregelatinized Hydroxypropyl Pea Starch taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

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R_U = internal standard ratio (peak response of propylene glycol/peak response of 1,3-propanediol) from the *Sample solution*
R_S = internal standard ratio (peak response of propylene glycol/peak response of 1,3-propanediol) from the *Standard solution*
C_S = concentration of USP Propylene Glycol RS in the *Standard solution* (mg/mL)
C_U = concentration of Pregelatinized Hydroxypropyl Pea Starch in the *Sample solution* (mg/mL)

Acceptance criteria: NMT 0.1%

• **LIMIT OF OXIDIZING SUBSTANCES**
Sample: 4.0 g
Analysis: Transfer the *Sample* to a glass-stoppered 125-mL conical flask, and add 50.0 mL of a mixture of water and methanol (1:1). Insert the stopper, and swirl for 5 min. Transfer to a glass-stoppered 50-mL centrifuge tube, and centrifuge to clarify. Transfer 30.0 mL of the clear supernatant to a glass-stoppered 125-mL conical flask. Add 1 mL of glacial acetic acid and 0.5–1.0 g of potassium iodide. Insert the stopper, swirl, and allow to stand for 25–30 min in the dark. Add 1 mL of starch TS, and titrate with 0.002 N sodium thiosulfate VS to the disappearance of the starch-iodine color. Perform a blank determination, and make any necessary correction. Each mL of 0.002 N sodium thiosulfate VS is equivalent to 34 µg of oxidant, calculated as hydrogen peroxide.
Acceptance criteria: NMT 1.4 mL of 0.002 N sodium thiosulfate VS is required (20 ppm, calculated as H_2O_2).

SPECIFIC TESTS

• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic microbial count does not exceed 10^3 cfu/g, the total combined molds and yeasts count does not exceed 10^2 cfu/g, and it meets the requirements of the test for the absence of *Escherichia coli*.

• **pH** ⟨791⟩
Sample solution: Progressively suspend 3.0 g of Pregelatinized Hydroxypropyl Pea Starch in 100.0 mL of carbon dioxide-free water, stirring continuously. Determine the pH when all the solid is wetted.
Acceptance criteria: 4.5–8.0

• **LOSS ON DRYING** ⟨731⟩**:** Dry about 1 g at 130° for 90 min: it loses NMT 15.0% of its weight.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers. Store at room temperature.

• **USP REFERENCE STANDARDS** ⟨11⟩
USP Propylene Glycol RS

Potato Starch

Portions of the monograph text that are national *USP* text, and are not part of the harmonized text, are marked with symbols (‣) to specify this fact.

DEFINITION

Potato Starch is obtained from the tuber of *Solanum tuberosum* L.

IDENTIFICATION

• **A. PROCEDURE**
Analysis: Examine under a microscope using a mixture of equal volumes of glycerol and water.
Acceptance criteria: It presents granules, either irregularly shaped, ovoid or pear-shaped, usually 30–100 µm in size but occasionally exceeding 100 µm, or rounded, 10–35 µm in size. There are occasional compound granules having 2–4 components. The ovoid and pear-shaped granules have an eccentric hilum and the rounded granules acentric or slightly eccentric hilum. All granules show clearly visible concentric striations. Between orthogonally oriented polarizing plates or prisms, the granules show a distinct black cross intersecting at the hilum.

• **B. PROCEDURE**
Sample solution: 20 mg/mL in water
Analysis: Boil for 1 min, and cool.
Acceptance criteria: A thick, opalescent mucilage is formed.

• **C. PROCEDURE**
Sample solution: 1 mL of the mucilage obtained in *Identification* test *B*
Analysis: Add 0.05 mL of iodine and potassium iodide TS 2 to the *Sample solution*.
Acceptance criteria: An orange-red to dark blue color is produced, which disappears upon heating.

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 0.6%, determined on a 1.0-g sample

• **LIMIT OF IRON**
Standard iron stock solution A: Equivalent of 10 µg/mL of iron prepared as directed under *Iron* ⟨241⟩
Standard iron stock solution B: 1 µg/mL of iron from *Standard iron stock solution A* in water [NOTE—Prepare immediately before use.]
Standard iron solution: Transfer 10 mL of *Standard iron stock solution B* to a test tube, and add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, and dilute with water to 20 mL.
Sample solution: Shake 1.5 g of Potato Starch with 15 mL of 2 N hydrochloric acid, and filter. Transfer 10 mL of the filtrate to a test tube, and add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, and dilute with water to 20 mL.
Acceptance criteria: After 5 min, any pink color in the *Sample solution* is not more intense than that in the *Standard iron solution*, corresponding to a limit of 10 ppm of iron.

• **LIMIT OF SULFUR DIOXIDE**
Carbon dioxide: Use carbon dioxide, with a flow regulator that will maintain a flow of 100 ± 5 mL/min.
Bromophenol blue indicator solution: 0.2 mg/mL of bromophenol blue in dilute alcohol. Filter if necessary.
Hydrogen peroxide solution: Dilute 30% hydrogen peroxide with water to obtain a 3% solution. Just before use, add three drops of *Bromophenol blue indicator solution*, and neutralize to a violet-blue endpoint with 0.01 N sodium hydroxide. Do not exceed the endpoint.
Apparatus: *Figure 1*

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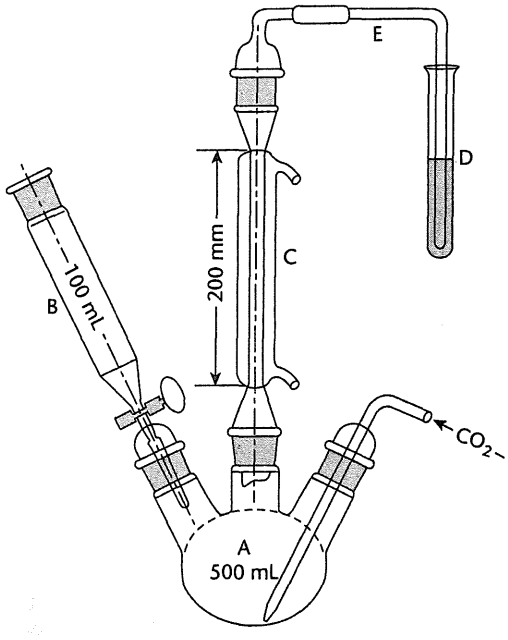


Figure 1

In this test, the sulfur dioxide is released from the sample in a boiling acid medium and is removed by a stream of carbon dioxide. The separated gas is collected in a dilute hydrogen peroxide solution where the sulfur dioxide is oxidized to sulfuric acid and titrated with standard alkali. The apparatus consists essentially of a 500-mL three-neck, round-bottom boiling flask, A; a separatory funnel, B, having a capacity of 100 mL or greater; a gas inlet tube of sufficient length to permit introduction of the carbon dioxide within 2.5 cm of the bottom of the boiling flask; a reflux condenser, C, having a jacket length of 200 mm; and a delivery tube, E, connecting the upper end of the reflux condenser to the bottom of a receiving test tube, D. Apply a thin film of stopcock grease to the sealing surfaces of all of the joints except the joint between the separatory funnel and the boiling flask, and clamp the joints to ensure tightness.

Sample: 25.0 g of Potato Starch

Analysis: Add 150 mL of water to the boiling flask. Close the stopcock of the separatory funnel, and begin the flow of carbon dioxide at a rate of 100 ± 5 mL/min through the *Apparatus*. Start the condenser coolant flow. Add 10 mL of *Hydrogen peroxide solution* to a receiving test tube. After 15 min, without interrupting the flow of carbon dioxide, remove the separatory funnel from the boiling flask, and transfer the *Sample* into the boiling flask with the aid of 100 mL of water. Apply stopcock grease to the outer joint of the separatory funnel, and replace the separatory funnel in the boiling flask. Close the stopcock of the separatory funnel, and add 80 mL of 2 N hydrochloric acid to the separatory funnel. Open the stopcock of the separatory funnel to permit the hydrochloric acid solution to flow into the boiling flask, guarding against the escape of sulfur dioxide into the separatory funnel by closing the stopcock before the last few mL of hydrochloric acid drain out. Boil the mixture for 1 h. Remove the receiving test tube, and transfer its contents to a 200-mL wide-necked, conical flask. Rinse the receiving test tube with a small portion of water, add the rinsing to the 200-mL conical flask, and mix. Heat on a water bath for 15 min, and allow to cool. Add 0.1 mL of *Bromophenol blue indicator solution*, and titrate the contents with 0.1 N sodium hydroxide VS until the color changes from yellow to violet-blue. Perform a blank determination, and make any necessary correction (see *Titrimetry* ⟨541⟩).

Calculate the content, in ppm, of sulfur dioxide in the *Sample* taken:

$$\text{Result} = 1000 \times 32.03 \times (VN/W)$$

32.03 = milliequivalent weight of sulfur dioxide
V = volume of titrant consumed (mL)
N = normality of the titrant
W = weight of the *Sample* (g)

Acceptance criteria: NMT 50 ppm

• **Limit of Oxidizing Substances**

Sample solution: Transfer 4.0 g to a glass-stoppered, 125-mL conical flask, and add 50.0 mL of water. Insert the stopper, and swirl for 5 min. Transfer to a glass-stoppered, 50-mL centrifuge tube, and centrifuge to clarify. Transfer 30.0 mL of the clear supernatant to a glass-stoppered, 125-mL conical flask. Add 1 mL of glacial acetic acid and 0.5–1.0 g of potassium iodide. Insert the stopper, swirl, and allow to stand for 25–30 min in the dark. Add 1 mL of starch TS.

Analysis: Titrate with 0.002 N sodium thiosulfate VS to the disappearance of the starch–iodine color. Perform a blank determination, and make any necessary correction. Each mL of 0.002 N sodium thiosulfate is equivalent to 34 µg of oxidant, calculated as hydrogen peroxide.

Acceptance criteria: NMT 1.4 mL of 0.002 N sodium thiosulfate is required (20 ppm, calculated as H_2O_2).

SPECIFIC TESTS

• **Microbial Enumeration Tests** ⟨61⟩ and **Tests for Specified Microorganisms** ⟨62⟩**:** The total aerobic microbial count does not exceed 10^3 cfu/g; the total combined molds and yeasts count does not exceed 10^2 cfu/g; and it meets the requirements of the test for the absence of *Escherichia coli*.

• **Loss on Drying** ⟨731⟩

Sample: 1 g

Analysis: Dry the *Sample* at 130° for 90 min.

Acceptance criteria: NMT 20.0%

• **pH** ⟨791⟩

Sample solution: Prepare a slurry by weighing 5.0 g of Potato Starch, transferring to a suitable nonmetallic container, and adding 25.0 mL of freshly boiled and cooled water.

Analysis: Agitate continuously at a moderate rate for 1 min. Stop the agitation, and allow to stand for 15 min. Determine the pH to the nearest 0.1 unit.

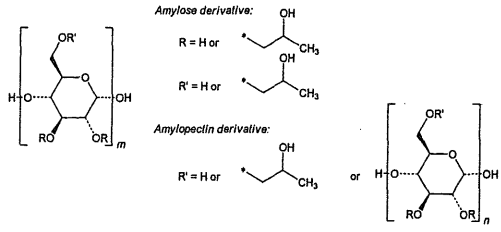
Acceptance criteria: 5.0–8.0

ADDITIONAL REQUIREMENTS

• **‘Packaging and Storage:** Preserve in well-closed containers. No storage requirements specified.,

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Hydroxypropyl Potato Starch



For the Amylose derivative, *m* is about 300–1000.

DEFINITION

Hydroxypropyl Potato Starch is partially substituted 2-hydroxypropylether obtained from potato starch by a chemical modification of etherification with propylene oxide. In addition, this starch may be partially hydrolyzed using acids or enzymes to obtain thinned starch. It contains NLT 2.0% and NMT 7.0% of hydroxypropyl groups, on the dried basis.

IDENTIFICATION

• **A. Procedure**

Analysis: Examine under a microscope, using NLT 20× magnification and a mixture of glycerin and water (1:1) as a mounting agent.

Acceptance criteria: It presents granules, either irregularly shaped, ovoid or pear-shaped, usually 30–100 µm in size, but occasionally exceeding 100 µm, or rounded 10–35 µm in size. There are occasional compound granules having 2–4 components. The ovoid and pear-shaped granules have an eccentric hilum, and the rounded granules have a centric or slightly eccentric hilum. All granules show clearly visible concentric striations. Between crossed nicol prisms, the Hydroxypropyl Potato Starch granules show a distinct black cross intersecting at the hilum.

• **B. Procedure**

Sample solution: Suspend 1 g of Hydroxypropyl Potato Starch in 50 mL of water, boil for 1 min, and cool.

Acceptance criteria: A translucent or clear mucilage is formed.

• **C. Procedure**

Analysis: To 1 mL of the *Sample solution* obtained in *Identification* test *B* add 0.05 mL of iodine and potassium iodide TS 2.

Acceptance criteria: An orange-red to dark blue color is produced, which disappears upon heating.

• **D. Procedure**

Ninhydrin solution: Dissolve 3 g of ninhydrin in 100 mL of a 45.5-g/L solution of sodium metabisulfite.

Diluted sulfuric acid: 98 g/L of H_2SO_4

Sample: 100 mg of Hydroxypropyl Potato Starch

Analysis: Transfer the *Sample* to a 100-mL volumetric flask, and add 12.5 mL of *Diluted sulfuric acid*. Place the flask in a water bath, and heat until the *Sample* is dissolved. Cool, and dilute with water to 100 mL. [**Caution**—When sulfuric acid is miscible with water, it produces intense heat.] Pipet 1 mL of this solution to a glass-stoppered, 25-mL graduated test-tube and, with the tube immersed in cold water, add drop-wise 8 mL of sulfuric acid. Mix well, and place the tube in a boiling water bath for exactly 3 min. Immediately transfer the tube to an ice bath until the solution is chilled. Add 0.6 mL of *Ninhydrin solution*, carefully allowing the reagent to run down the walls of the test tube. Immediately shake the tube well, and place it in a water bath at 25° for 100 min. Dilute with sulfuric acid to 25 mL [**Caution**—Use sulfuric acid cautiously.], and mix by inverting the tube several times. Do not shake.

Acceptance criteria: A violet color develops within 5 min due to the presence of hydroxypropyl groups (starch ether).

ASSAY

• **Procedure for Hydroxypropyl Groups**

Deuterium chloride solution: Dilute 1 mL of deuterium chloride (38% w/w) with 5 mL of deuterium oxide.

Internal standard solution: Dissolve 50.0 mg of sodium 3-trimethylsilyl-1-propane sulfonate in about 5 g of deuterium oxide, weighed to the nearest 0.1 mg. Store in a sealed bottle.

Sample solution: Disperse 20 g of Hydroxypropyl Potato Starch in 200.0 mL of carbon dioxide-free water at room temperature. Agitate for 15 min, and filter. Repeat the operation two more times. If poor dispersibility or slow filtration is observed, use refrigerated carbon dioxide-free water for the washing operation. Dry the washed starch for NLT 4 h in vacuum at 30 ± 5°. Determine the moisture content (B) on 5 g of the washed and dried starch following the *Loss on Drying* test. Weigh 12.0 mg of the washed and dried starch in a 5-mm NMR tube. Add 0.75 mL of deuterium oxide and 0.1 mL of *Deuterium chloride solution*. Cap the tube, mix, and place it in a boiling water bath until a clear solution is obtained. [Note—It may take 3 min–1 h.] When a clear solution is obtained, allow to cool to room temperature. Dry the exterior of the tube, and weigh to the nearest 0.1 mg. Add 0.05 mL of *Internal standard solution*, and weigh to the nearest 0.1 mg. Determine the mass of the *Internal standard solution* added. Mix thoroughly.

Nuclear magnetic resonance spectrometry

(See *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩, *Quantitative Applications*.)

Apparatus: FT-NMR spectrometer at minimum 300 MHz

Acquisition of ^{1}H NMR spectra: The following parameters may be used.

Sweep width: 8 ppm (about −1.0 to +7 ppm)
Irradiation frequency offset: None
Time domain: NLT 64 K
Pulse width: 90 degree
Pulse delay: 10 *s*
Dummy scans: 0
Number of scans: 8

Use the CH_3 signal of the internal standard for shift referencing. Set the shift of the peak of the singlet to 0 ppm. Record the FID signal.

Analysis

Samples: *Internal standard solution* and *Sample solution*

Call the integration sub-routine after phase corrections and baseline correction between −0.5 and +6 ppm.

Measure the peak areas of the doublet from the methyl groups of the hydroxypropyl function at +1.2 ppm (A_2), and of the methyl groups at 0 ppm of the internal standard (A_1) without ^{13}C-satellites.

Measure the signal coming from the 3 protons of the methyl group in the hydroxypropyl function.

Calculate the content of hydroxypropyl groups as a percentage (w/w, dried basis):

$$\text{Result} = (N \times A_2/A_1) \times (C_i \times W_i/W) \times (M_{r2}/M_{r1}) \times [100/(100 - B)] \times 100$$

N = numerical value representing the 3 methyl groups in the internal standard (sodium 3-trimethylsilyl-1-propane sulfonate), 3

A_2 = area of the methyl groups of hydroxypropyl in Hydroxypropyl Potato Starch

A_1 = area of the methyl groups in the internal standard (sodium 3-trimethylsilyl-1-propane sulfonate)

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C_i = concentration of the internal standard in the *Internal standard solution* (mg/g)
W_i = weight of the *Internal standard solution* in the NMR tube (g)
W = weight of the washed and dried Hydroxypropyl Potato Starch in the NMR tube (mg)
M_{r1} = molecular weight of the internal standard, 218.32 g/mol
M_{r2} = molar mass of hydroxypropyl group, 59.09 g/mol
B = moisture content of the washed and dried Hydroxypropyl Potato Starch used in the *Sample solution*, as a percentage (w/w)

Acceptance criteria: The content of hydroxypropyl groups is 2.0%–7.0% on the dried basis.

IMPURITIES

INORGANIC IMPURITIES

- **Residue on Ignition** ⟨281⟩: NMT 0.6%, determined on a 1.0-g test specimen
- **Limit of Iron**

Standard iron stock solution: Prepare a solution containing the equivalent of 10 µg/mL of iron, as directed under *Iron* ⟨241⟩.

Diluted standard iron solution: Immediately before use, dilute an accurately measured volume of *Standard iron stock solution* quantitatively with water to obtain a solution containing the equivalent of 1 µg/mL of iron.

Standard solution: Transfer 10 mL of the *Diluted standard iron solution* to a test tube. Add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid, and mix. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, dilute with water to 20 mL, and mix.

Sample solution: Shake 1.0 g of Hydroxypropyl Potato Starch with 20 mL of 2 N hydrochloric acid, and filter. Transfer 10 mL of the filtrate to a test tube. Add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid, and mix. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, dilute with water to 20 mL, and mix.

Acceptance criteria: After 5 min, any pink color in the *Sample solution* is not more intense than that in the *Standard solution*, corresponding to a limit of 20 µg/g of iron.

- **Limit of Sulfur Dioxide,** *Method IV* ⟨525⟩: NMT 50 ppm

ORGANIC IMPURITIES

- **Procedure 1: Limit of Oxidizing Substances**

Sample: 4.0 g of Hydroxypropyl Potato Starch

Analysis: Transfer the *Sample* to a glass-stoppered, 125-mL conical flask, and add 50.0 mL of water. Insert the stopper, and swirl for 5 min. Transfer to a glass-stoppered, 50-mL centrifuge tube, and centrifuge to clarify. Transfer 30.0 mL of the clear supernatant to a glass-stoppered, 125-mL conical flask. Add 1 mL of glacial acetic acid and 0.5–1.0 g of potassium iodide. Insert the stopper, swirl, and allow to stand for 25–30 min in the dark. Add 1 mL of starch TS, and titrate with 0.002 N sodium thiosulfate VS to the disappearance of the starch-iodine color. Perform a blank determination, and make any necessary correction. Each mL of 0.002 N sodium thiosulfate VS is equivalent to 34 µg of oxidant, calculated as hydrogen peroxide.

Acceptance criteria: NMT 1.4 mL of 0.002 N sodium thiosulfate VS is required (20 µg/g, calculated as H_2O_2).

- **Procedure 2: Foreign Matter**

Sample: 50 mg/mL of Hydroxypropyl Potato Starch in a mixture of glycerin and water (1:1)

Analysis: Examine under a microscope, using NLT 20× magnification and a mixture of glycerin and water (1:1) as a mounting agent.

Acceptance criteria: NMT traces of matter other than Hydroxypropyl Potato Starch granules are present.

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩: The total aerobic microbial count does not exceed 10^3 cfu/g, the total combined molds and yeasts count does not exceed 10^2 cfu/g, and it meets the requirements of the test for the absence of *Escherichia coli*.
- **pH** ⟨791⟩

Sample solution: Suspend 5.0 g of Hydroxypropyl Potato Starch in 25.0 mL of carbon dioxide-free water, and shake for 60 s. Allow to stand for 15 min.

Acceptance criteria: 4.5–8.0

- **LOSS ON DRYING** ⟨731⟩: Dry about 1 g at 130° for 90 min: it loses NMT 20.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. Store at room temperature.

Pregelatinized Hydroxypropyl Potato Starch

DEFINITION

Pregelatinized Hydroxypropyl Potato Starch is prepared from Hydroxypropyl Potato Starch by mechanical processing in the presence of water, with or without heat, to rupture all or some of the starch granules, and is subsequently dried. It contains NLT 2.0% and NMT 7.0% of hydroxypropyl groups on the dried basis.

IDENTIFICATION

- **A. TEST FOR PREGELATINIZED STATE**

Sample: 1 g

Analysis: Disperse the *Sample* in 50 mL of water at a temperature NMT 25°. Shake vigorously until lumps completely disperse/solubilize or until lumps disappear. Allow to stand for 20 min.

Acceptance criteria: A translucent or clear mucilage without precipitate is formed.

- **B. TEST FOR STARCH**

Analysis: Disperse 0.5 g in 2 mL of water without heating, and add 0.05 mL of iodine and potassium iodide TS2.

Acceptance criteria: A reddish-violet or blue color is produced.

- **C. NINHYDRIN TEST**

Ninhydrin solution: Dissolve 3 g of ninhydrin in 100 mL of a 45.5-g/L solution of sodium metabisulfite.

Diluted sulfuric acid: 98 g/L of H_2SO_4

Sample: 100 mg

Analysis: Transfer the *Sample* to a 100-mL volumetric flask, and add 12.5 mL of *Diluted sulfuric acid*. Place the flask in a water bath, and heat until the *Sample* is dissolved. Cool, and dilute with water to 100 mL. [**CAUTION**—When sulfuric acid is miscible with water, it produces intense heat.] Pipet 1 mL of this solution to a glass-stoppered 25-mL graduated test tube and, with the tube immersed in cold water, add dropwise 8 mL of sulfuric acid. Mix well, and place the tube in a boiling water bath for exactly 3 min. Immediately transfer the tube to an ice bath until the solution is chilled. Add 0.6 mL of *Ninhydrin solution*, carefully allowing the reagent to run down the walls of the test tube. Immediately shake the tube well, and place it in a water bath at 25° for 100 min. Dilute with sulfuric acid to 25 mL. [**CAUTION**—Use sulfuric acid cautiously.] Mix by inverting the tube several times. Do not shake.

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Acceptance criteria: A violet color develops within 5 min due to the presence of hydroxypropyl groups (starch ether).

ASSAY

• **ASSAY FOR HYDROXYPROPYL GROUPS**

Deuterium chloride solution: Dilute 1 mL of deuterium chloride (38% w/w) with 5 mL of deuterium oxide.

Internal standard solution: Disperse 50.0 mg of sodium 3-trimethylsilyl-1-propane sulfonate in about 5 g of deuterium oxide, weighed to the nearest 0.1 mg. Store in a sealed bottle.

Sample solution: Determine the moisture content (*B*) on 5 g of Pregelatinized Hydroxypropyl Potato Starch following the *Loss on Drying* test. Weigh 12.0 mg of the Pregelatinized Hydroxypropyl Potato Starch in a 5-mm NMR tube. Add 0.75 mL of deuterium oxide and 0.1 mL of *Deuterium chloride solution*. Cap the tube, mix, and place it in a boiling water bath until a clear solution is obtained. [NOTE—This may take from 3 min to 1 h.] When a clear solution is obtained, allow it to cool to room temperature. Dry the exterior of the tube, and weigh to the nearest 0.1 mg. Add 0.05 mL of the *Internal standard solution*. Weigh to the nearest 0.1 mg. Determine the mass of the *Internal standard solution* added. Mix thoroughly.

Instrumental conditions

(See *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩, *Quantitative Applications*.)

Mode: Nuclear magnetic resonance spectrometry

Apparatus: FT-NMR spectrometer at minimum 300 MHz

Acquisition of ^{1}H NMR spectra: The following parameters may be used:

Sweep width: 8 ppm (about −1.0 to +7 ppm)
Irradiation frequency offset: None
Time domain: NLT 64 K
Pulse width: 90°
Pulse delay: 10 s
Dummy scans: 0
Number of scans: 8

Use the CH_3 signal of the internal standard for shift referencing. Set the shift of the peak of the singlet to 0 ppm. Record the FID signal.

Analysis

Samples: *Internal standard solution* and *Sample solution*

Call the integration subroutine after phase corrections and baseline correction between −0.5 and +6 ppm.

Measure the peak areas of the doublet from the methyl groups of the hydroxypropyl function at +1.2 ppm (A_2), and of the methyl groups at 0 ppm of the internal standard (A_1) without ^{13}C-satellites.

Measure the signal originating from the 3 protons of the methyl group in the hydroxypropyl function.

Calculate the content of hydroxypropyl groups as a percentage (w/w, dried basis):

$$\text{Result} = (N \times A_2/A_1) \times (C_I \times W_I/W) \times (M_{r2}/M_{r1}) \times [100/(100 - B)] \times 100$$

N = numerical value representing the 3 methyl groups in the internal standard (sodium 3-trimethylsilyl-1-propane sulfonate), 3
A_2 = area of the methyl groups of hydroxypropyl in Pregelatinized Hydroxypropyl Potato Starch
A_1 = area of the methyl groups in the internal standard (sodium 3-trimethylsilyl-1-propane sulfonate)
C_I = concentration of the internal standard in the *Internal standard solution* (mg/g)
W_I = weight of the *Internal standard solution* in the NMR tube (g)
W = weight of the Pregelatinized Hydroxypropyl Potato Starch in the NMR tube (mg)
M_{r2} = molar mass of hydroxypropyl groups, 59.09 g/mol
M_{r1} = molecular weight of the internal standard, 218.32 g/mol
B = moisture content of the Pregelatinized Hydroxypropyl Potato Starch used in the *Sample solution*, as a percentage (w/w)

Acceptance criteria: 2.0%–7.0% of hydroxypropyl groups on the dried basis

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 0.6%, determined on a 1.0-g test specimen

• **LIMIT OF IRON**

Standard iron stock solution: Prepare a solution containing the equivalent of 10 µg/mL of iron, as directed under *Iron* ⟨241⟩.

Diluted standard iron solution: Immediately before use, dilute an accurately measured volume of the *Standard iron stock solution* quantitatively with water to obtain a solution containing the equivalent of 1 µg/mL of iron.

Sample solution: Shake the residue obtained from the test for *Residue on Ignition* with 20 mL of 2 N hydrochloric acid, and filter. Transfer 10 mL of the filtrate to a test tube. Add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid, and mix. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, dilute with water to 20 mL, and mix.

Standard solution: Transfer 10 mL of the *Diluted standard iron solution* to a test tube. Add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid, and mix. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, dilute with water to 20 mL, and mix.

Acceptance criteria: After 5 min, any pink color in the *Sample solution* is not more intense than that in the *Standard solution*, corresponding to a limit of 20 ppm of iron.

• **LIMIT OF SULFUR DIOXIDE,** *Method IV* ⟨525⟩**:** NMT 50 ppm

• **LIMIT OF PROPYLENE GLYCOL**

Internal standard solution: 0.5 mg/mL of 1,3-propanediol in anhydrous pyridine

Standard stock solution: 0.5 mg/mL of USP Propylene Glycol RS in *Internal standard solution*

Standard solution: Transfer 0.1 mL of the *Standard stock solution* to a 2-mL vessel with a screw cap fitted with a septum. Add 0.9 mL of anhydrous pyridine, 0.2 mL of hexamethyldisilazane, and 0.1 mL of trimethylchlorosilane. Close, and mix. Allow to stand for 15 min before injection.

Sample stock solution: Transfer 200 mg of Pregelatinized Hydroxypropyl Potato Starch to a 100-mL volumetric flask. Add 1.0 mL of the *Internal standard solution* and 9.0 mL of anhydrous pyridine. Boil under reflux using a water bath for 20 min. Allow to cool to room temperature.

Sample solution: Transfer 1.0 mL of the *Sample stock solution* to a 2-mL vessel with a screw cap fitted with a septum. Add 0.2 mL of hexamethyldisilazane and 0.1 mL of trimethylchlorosilane. Close, and mix. Allow to stand for 15 min before injection.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.32-mm × 30-m fused-silica capillary column; 0.25-µm layer of phase G1

Temperature

Detector: 250°
Injection port: 250°

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Column: 70°. [NOTE—The column must be desorbed regularly. Conditions: Program from 70° to 300° at 7°/min, and maintain 10 min at 300°.]
Carrier gas: Helium
Flow rate: 3 mL/min
Injection type: Split ratio of 1:30
Injection size: 1 µL
System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for the trimethylsylilated derivative of propylene glycol and the trimethylsylilated derivative of 1,3-propanediol are 1.0 and 1.4, respectively.]
Suitability requirements
Resolution: NLT 2.0 between the peaks due to the trimethylsylilated derivative of propylene glycol and the trimethylsylilated derivative of 1,3-propanediol
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of propylene glycol in the portion of Pregelatinized Hydroxypropyl Potato Starch taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = internal standard ratio (peak response of propylene glycol/peak response of 1,3-propanediol) from the *Sample solution*
R_S = internal standard ratio (peak response of propylene glycol/peak response of 1,3-propanediol) from the *Standard solution*
C_S = concentration of USP Propylene Glycol RS in the *Standard solution* (mg/mL)
C_U = concentration of Pregelatinized Hydroxypropyl Potato Starch in the *Sample solution* (mg/mL)

Acceptance criteria: NMT 0.1%
• **LIMIT OF OXIDIZING SUBSTANCES**
Sample: 4.0 g
Analysis: Transfer the *Sample* to a glass-stoppered 125-mL conical flask, and add 50.0 mL of a mixture of water and methanol (1:1). Insert the stopper, and swirl for 5 min. Transfer to a glass-stoppered 50-mL centrifuge tube, and centrifuge to clarify. Transfer 30.0 mL of the clear supernatant to a glass-stoppered 125-mL conical flask. Add 1 mL of glacial acetic acid and 0.5–1.0 g of potassium iodide. Insert the stopper, swirl, and allow to stand for 25–30 min in the dark. Add 1 mL of starch TS, and titrate with 0.002 N sodium thiosulfate VS to the disappearance of the starch-iodine color. Perform a blank determination, and make any necessary correction. Each mL of 0.002 N sodium thiosulfate VS is equivalent to 34 µg of oxidant, calculated as hydrogen peroxide.
Acceptance criteria: NMT 1.4 mL of 0.002 N sodium thiosulfate VS is required (20 ppm, calculated as H_2O_2).

SPECIFIC TESTS
• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩: The total aerobic microbial count does not exceed 10^3 cfu/g, the total combined molds and yeasts count does not exceed 10^2 cfu/g, and it meets the requirements of the test for the absence of *Escherichia coli*.
• **PH** ⟨791⟩
Sample solution: Progressively suspend 3.0 g of Pregelatinized Hydroxypropyl Potato Starch in 100.0 mL of carbon dioxide-free water, stirring continuously. Determine the pH when all the solid is wetted.
Acceptance criteria: 4.5–8.0
• **LOSS ON DRYING** ⟨731⟩: Dry about 1 g at 130° for 90 min: it loses NMT 20.0% of its weight.

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers. Store at room temperature.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Propylene Glycol RS

Pregelatinized Starch

DEFINITION
Pregelatinized Starch is Starch that has been chemically and/or mechanically processed to rupture all or part of the granules in the presence of water and subsequently dried. Some types of Pregelatinized Starch may be modified to render them compressible and flowable in character.

IDENTIFICATION
• A water slurry of it is colored orange-red to deep blue by iodine TS.

IMPURITIES
INORGANIC IMPURITIES
• **Residue on Ignition** ⟨281⟩: NMT 0.5%, determined on a 2.0-g test specimen
• **Iron** ⟨241⟩: NMT 20 ppm
Analysis: Dissolve the residue obtained in the test for *Residue on Ignition* in 8 mL of hydrochloric acid with the aid of gentle heating, and dilute with water to 100 mL. Dilute 25 mL of this solution with water to 47 mL.
• **LIMIT OF SULFUR DIOXIDE**
Sample solution: Mix 20 g with 200 mL of a 1-in-5 solution of anhydrous sodium sulfate, and filter.
Analysis: To 100 mL of the clear filtrate add 3 mL of starch TS, and titrate with 0.01 N iodine VS to the first permanent blue color.
Acceptance criteria: NMT 2.7 mL is consumed (80 ppm).

SPECIFIC TESTS
• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩: It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*. The total aerobic microbial count does not exceed 1000 cfu/g; and the total combined molds and yeasts count does not exceed 100 cfu/g.
• **PH** ⟨791⟩: 4.5–7.0
Prepare a slurry by weighing 10.0 ± 0.1 g in 10 mL of alcohol and by diluting with water to 100 mL. Agitate continuously at a moderate rate for 5 min, then cease agitation and immediately potentiometrically determine the pH to the nearest 0.1 unit.
• **LOSS ON DRYING** ⟨731⟩: Dry a sample at 120° for 4 h: it loses NMT 14.0% of its weight.
• **OXIDIZING SUBSTANCES**
Sample: 5 g
Analysis: To the *Sample* add 20 mL of a mixture of equal volumes of methanol and water, then add 1 mL of 6 N acetic acid, and stir until a homogeneous suspension is obtained. Add 0.5 mL of a freshly prepared, saturated solution of potassium iodide, and allow to stand for 5 min.
Acceptance criteria: No distinct blue, brown, or purple color is observed.

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.
• **LABELING:** Label it to indicate the botanical source from which it was derived.

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Pregelatinized Modified Starch

DEFINITION

Pregelatinized Modified Starch is Modified Starch that has been chemically or mechanically processed, or both, to rupture all or part of the granules to produce a product that swells in cold water.

IDENTIFICATION

• **A.**

Sample: 0.6 g

Analysis: Transfer the *Sample* to a 25-mL glass vial with a plastic cap. Add 9.4 g of water, cap, and shake vigorously to evenly disperse the starch. Add 10 g of 2% (w/w) NaOH solution, cap, and shake vigorously for 1 min to create a smooth mixture. Evaluate within 1 min.

Acceptance criteria: The final solution is translucent to opaque with a fluid consistency. A yellow tint of the final solution is acceptable.

• **B.** An aqueous dispersion of Pregelatinized Modified Starch is colored orange-red to deep blue by iodine TS.

IMPURITIES

• **Residue on Ignition** ⟨281⟩

Sample: 2.0 ± 0.1 g

Acceptance criteria: NMT 1.5%

• **Limit of Sulfur Dioxide**

Sample solution: Mix 20.0 ± 0.1 g of Pregelatinized Modified Starch with 100 mL of 95% alcohol, and stir for several min to completely wet the starch.

Analysis: Slowly add 100 mL of water to the *Sample solution*, and stir until a smooth suspension is obtained. Allow the starch mixture to set undisturbed until most of the starch has settled, and filter the aqueous portion through paper (Whatman No. 1 or equivalent). To 100 mL of the clear filtrate add 100 mL of water. Add 3 mL of starch TS, and titrate with 0.01 N iodine VS to the first permanent blue or purple color.

Acceptance criteria: NMT 1.7 mL of 0.010 N iodine is consumed (NMT 0.005%).

SPECIFIC TESTS

• **pH** ⟨791⟩

Sample: 10.0 ± 0.1 g

Analysis: Wet the *Sample* with 10 mL of alcohol, then dilute with water to 300 mL to obtain an aqueous dispersion. Stir continuously at a moderate rate for 5 min, and determine the pH to the nearest 0.1 unit.

Acceptance criteria: 3.0–9.0

• **Loss on Drying** ⟨731⟩

Analysis: Dry at 120° for 4 h.

Acceptance criteria: NMT 15%

• **Microbial Enumeration Tests** ⟨61⟩ and **Tests for Specified Microorganisms** ⟨62⟩: The total aerobic microbial count does not exceed 1×10^3 cfu/g, and the total combined molds and yeasts count does not exceed 1×10^2 cfu/g. It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

• **Iron** ⟨241⟩

Sample: The residue obtained in the test for *Residue on Ignition* ⟨281⟩

Analysis: Dissolve the *Sample* in 8 mL of hydrochloric acid with the aid of gentle heating. Dilute with water to 100 mL in a volumetric flask. Dilute 25 mL of this solution with water to 47 ± 1 mL.

Acceptance criteria: NMT 20 µg/g

• **Oxidizing Substances**

Sample solution: To 5 g of Pregelatinized Modified Starch add 20 mL of a mixture of methanol and water (1:1).

Analysis: To the *Sample solution* add 1 mL of 6 N acetic acid, and stir until a homogeneous suspension is obtained. Add 0.5 mL of a freshly prepared saturated solution of potassium iodide, and allow to stand for 5 min.

Acceptance criteria: No distinct blue, brown, or purple color is observed.

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Preserve in well-closed containers. No storage requirements specified.

Rice Starch

Portions of this monograph that are national *USP* text, and are not part of the harmonized text, are marked with symbols (•) to specify this fact.

DEFINITION

Rice Starch is obtained from the caryopsis of *Oryza sativa L.*

IDENTIFICATION

• **A. Procedure**

Analysis: Examine under a microscope, using NLT 20× magnification and using a mixture of glycerin and water (1:1) as a mounting agent.

Acceptance criteria: It presents polyhedral, simple grains 1–10 µm, mostly 4–6 µm in size. These simple grains often gather in ellipsoidal, compound grains 50–100 µm in diameter. The granules have a poorly visible central hilum and there are no concentric striations. Between orthogonally orientated polarising plates or prisms, the starch granules show a distinct black cross intersecting at the hilum.

• **B. Procedure**

Sample solution: 20 mg/mL in water

Analysis: Boil for 1 min, and cool.

Acceptance criteria: A thin, cloudy mucilage is formed.

• **C. Procedure**

Sample solution: 1 mL of the mucilage obtained in *Identification* test *B*

Analysis: Add 0.05 mL of iodine and potassium iodide TS 2 to the *Sample solution*.

Acceptance criteria: An orange-red to dark blue color is produced, which disappears upon heating.

IMPURITIES

Inorganic Impurities

• **Residue on Ignition** ⟨281⟩: NMT 0.6%, determined on a 1.0-g sample

• **Limit of Iron**

Standard iron stock solution A: Equivalent to 10 µg/mL of iron prepared as directed in *Iron* ⟨241⟩

Standard iron stock solution B: 1 µg/mL of iron from *Standard iron stock solution A* in water. [Note—Prepare immediately before use.]

Standard iron solution: Transfer 10 mL of *Standard iron stock solution B* to a test tube, and add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, and dilute with water to 20 mL.

Sample solution: Shake 1.5 g of Rice Starch with 15 mL of 2 N hydrochloric acid, and filter. Transfer 10 mL of the filtrate to a test tube, and add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, and dilute with water to 20 mL.

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Acceptance criteria: After 5 min, any pink color in the *Sample solution* is not more intense than that in the *Standard iron solution*, corresponding to a limit of 10 ppm of iron.

• **Limit of Sulfur Dioxide**

Carbon dioxide: Use carbon dioxide, with a flow regulator that will maintain a flow of 100 ± 5 mL/min.

Hydrogen peroxide solution: Dilute 30% hydrogen peroxide with water to obtain a 3% solution. Just before use, add 3 drops of bromophenol blue TS, and neutralize to a violet-blue endpoint with 0.1 N sodium hydroxide. Do not exceed the endpoint.

Apparatus: See *Figure 1*.

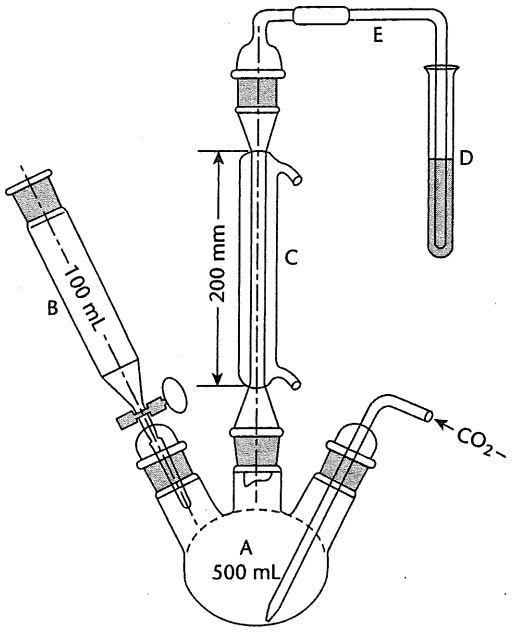


Figure 1

In this test, the sulfur dioxide is released from the sample in a boiling acid medium and is removed by a stream of carbon dioxide. The separated gas is collected in a dilute hydrogen peroxide solution where the sulfur dioxide is oxidized to sulfuric acid and titrated with standard alkali. The apparatus consists essentially of a 500-mL three-neck, round-bottom boiling flask, A; a separatory funnel, B, with a capacity of 100 mL or greater; a gas inlet tube of sufficient length to permit introduction of the carbon dioxide within 2.5 cm of the bottom of the boiling flask; a reflux condenser, C, with a jacket length of 200 mm; and a delivery tube, E, connecting the upper end of the reflux condenser to the bottom of a receiving test tube, D. Apply a thin film of stopcock grease to the sealing surfaces of all of the joints except the joint between the separatory funnel and the boiling flask, and clamp the joints to ensure tightness.

Sample: 25.0 g of Rice Starch

Analysis: Add 150 mL of water to the boiling flask. Close the stopcock of the separatory funnel, and begin the flow of carbon dioxide at a rate of 100 ± 5 mL/min through the *Apparatus*. Start the condenser coolant flow. Add 10 mL of *Hydrogen peroxide solution* to a receiving test tube. After 15 min, without interrupting the flow of carbon dioxide, remove the separatory funnel from the boiling flask, and transfer the *Sample* into the boiling flask with the aid of 100 mL of water. Apply stopcock grease to the outer joint of the separatory funnel, and replace the separatory funnel in the boiling flask. Close the stopcock of the separatory funnel, and add 80 mL of 2 N hydrochloric acid to the separatory funnel. Open the stopcock of the separatory funnel to permit the hydrochloric acid solution to flow into the boiling flask, guarding against the escape of sulfur dioxide into the separatory funnel by closing the stopcock before the last few mL of hydrochloric acid drain out. Boil the mixture for 1 h. Remove the receiving test tube, and transfer its contents to a 200-mL wide-necked, conical flask. Rinse the receiving test tube with a small portion of water, add the rinsing to the 200-mL conical flask, and mix. Heat on a water bath for 15 min, and allow to cool.

Add 0.1 mL of bromophenol blue TS, and titrate the contents with 0.1 N sodium hydroxide VS until the color changes from yellow to violet-blue. Perform a blank determination, and make any necessary correction (see *Titrimetry* ⟨541⟩).

Calculate the content, in ppm, of sulfur dioxide in the *Sample* taken:

$$\text{Result} = (V \times N \times F)/W \times 1000$$

V = volume of titrant consumed (mL)
N = normality of the titrant
F = milliequivalent weight of sulfur dioxide, 32.03
W = weight of the *Sample* (g)

Acceptance criteria: NMT 50 ppm

• **Limit of Oxidizing Substances**

Sample solution: Transfer 4.0 g to a glass-stoppered, 125-mL conical flask, and add 50.0 mL of water. Insert the stopper, and swirl for 5 min. Transfer to a glass-stoppered, 50-mL centrifuge tube, and centrifuge to clarify. Transfer 30.0 mL of the clear supernatant to a glass-stoppered, 125-mL conical flask. Add 1 mL of glacial acetic acid and 0.5–1.0 g of potassium iodide. Insert the stopper, swirl, and allow to stand for 25–30 min in the dark. Add 1 mL of starch TS.

Analysis: Titrate with 0.002 N sodium thiosulfate VS to the disappearance of the starch–iodine color. Perform a blank determination, and make any necessary correction. Each mL of 0.002 N sodium thiosulfate is equivalent to 34 µg of oxidant, calculated as hydrogen peroxide.

Acceptance criteria: NMT 1.4 mL of 0.002 N sodium thiosulfate is required [20 ppm, calculated as hydrogen peroxide (H_2O_2)]

SPECIFIC TESTS

• **Microbial Enumeration Tests** ⟨61⟩ and **Tests for Specified Microorganisms** ⟨62⟩**:** The total aerobic microbial count does not exceed 10^3 cfu/g; the total combined molds and yeasts count does not exceed 10^2 cfu/g; and it meets the requirements of the test for the absence of *Escherichia coli*.

• **Loss on Drying** ⟨731⟩

Sample: 1 g

Analysis: Dry the *Sample* at 130° for 90 min.

Acceptance criteria: NMT 15.0%

• **pH** ⟨791⟩

Sample solution: Prepare a slurry by weighing 5.0 g of Rice Starch, transferring to a suitable nonmetallic container, and adding 25.0 mL of freshly boiled and cooled water.

Analysis: Agitate continuously at a moderate rate for 1 min. Stop the agitation, and allow to stand for 15 min. Determine the pH to the nearest 0.1 unit.

Acceptance criteria: 5.0–8.0

ADDITIONAL REQUIREMENTS

• **•Packaging and Storage:** Preserve in well-closed containers. No storage requirements specified.•

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Tapioca Starch

DEFINITION

Tapioca Starch consists of starch granules separated from the tubers of tapioca (cassava) [*Manihot utilissima* Pohl (Fam. Euphorbiaceae)].

IDENTIFICATION

- **A.**

  Analysis: Examine Tapioca Starch under a microscope, using not less than 20× magnification and using glycerin as the mounting agent.

  Acceptance criteria: It appears as spherical granules, each having one truncated side, typically having a 5- to 35-µm diameter, and having circular or several-rayed central clefts.

- **B.**

  Sample suspension: 1 g of Tapioca Starch in 50 mL of water

  Analysis: Boil the *Sample suspension* for 1 min, and cool.

  Acceptance criteria: A thin, cloudy mucilage is formed.

- **C.**

  Sample: The mucilage obtained in *Identification* test *B*

  Analysis: To 1 mL of the *Sample* add 0.05 mL of iodine and potassium iodide TS 2.

  Acceptance criteria: An orange-red to dark blue color is produced, which disappears on heating.

IMPURITIES

- **Residue on Ignition** ⟨281⟩

  Sample: 1.0 g

  Acceptance criteria: NMT 0.6%

- **Iron** ⟨241⟩

  Test preparation: Shake 0.75 g of Tapioca Starch with 15 mL of 0.1 N hydrochloric acid, filter, and use 10 mL.

  Acceptance criteria: NMT 20 µg/g

- **Limit of Oxidizing Substances**

  Sample: 4.0 g

  Blank: 50 mL of water

  Titrimetric system

  (See *Titrimetry* ⟨541⟩.)

  Mode: Direct titration

  Titrant: 0.002 N sodium thiosulfate VS

  Endpoint detection: Visual

  Analysis: Transfer the *Sample* to a glass-stoppered, 125-mL conical flask, and add 50.0 mL of water. Insert the stopper, and swirl for 5 min. Decant into a glass-stoppered, 50-mL centrifuge tube, and centrifuge to clarify. Transfer 30.0 mL of the clear supernatant to a glass-stoppered, 125-mL conical flask. Add 1 mL of glacial acetic acid and 0.5–1.0 g of potassium iodide. Insert the stopper, swirl, and allow to stand for 25–30 min in the dark. Add 1 mL of starch TS, and titrate with *Titrant* to the disappearance of the starch–iodine color. Perform a blank determination, and make any necessary correction. Each mL of 0.002 N sodium thiosulfate VS is equivalent to 34 µg of oxidant, calculated as hydrogen peroxide.

  Acceptance criteria: NMT 1.4 mL of 0.002 N sodium thiosulfate VS is required (0.002%).

- **Limit of Sulfur Dioxide**

  Sample solution: Mix 20 g of Tapioca Starch with 200 mL of water until a smooth suspension is obtained, and filter.

  Analysis: To 100 mL of the clear filtrate from the *Sample solution* add 3 mL of starch TS, and titrate with 0.01 N iodine solution VS to the first permanent blue color.

  Acceptance criteria: NMT 1.7 mL of 0.01 N iodine solution VS is required (0.005%).

SPECIFIC TESTS

- **Microbial Enumeration Tests** ⟨61⟩ and **Tests for Specified Microorganisms** ⟨62⟩**:** The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined yeasts and molds count does not exceed 10^2 cfu/g. Tapioca Starch meets the requirements of the test for absence of *Escherichia coli*.

- **pH** ⟨791⟩

  Sample: 20.0 ± 0.1 g

  Analysis: Transfer the *Sample* to a suitable nonmetallic container, and add 100 mL of water to obtain a slurry. Agitate continuously at a moderate rate for 5 min, then stop agitation, and immediately determine the pH.

  Acceptance criteria: 4.5–7.0

- **Loss on Drying** ⟨731⟩

  Analysis: Dry at 130° for 90 min.

  Acceptance criteria: NMT 16.0%

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in well-closed containers. No storage requirements specified.

Topical Starch—*see Topical Starch General Monographs*

Wheat Starch

Portions of the monograph text that are national *USP* text, and are not part of the harmonized text, are marked with symbols (‣) to specify this fact.

DEFINITION

Wheat starch is obtained from the caryopsis of *Triticum aestivum* L. (*T. vulgare* Vill.).

IDENTIFICATION

- **A. Procedure**

  Analysis: Examine under a microscope using equal volumes of glycerol and water.

  Acceptance criteria: It presents large and small granules, and, very rarely, intermediate sizes. The large granules, usually 10–60 µm in diameter, are discoid or, more rarely, reniform when seen face-on. The central hilum and striations are invisible or barely visible, and the granules sometimes show cracks on the edges. Seen in profile, the granules are elliptical and fusiform and the hilum appears as a slit along the main axis. The small granules, rounded or polyhedral, are 2–10 µm in diameter. Between orthogonally oriented polarizing plates or prisms, the granules show a distinct black cross intersecting at the hilum.

- **B. Procedure**

  Sample solution: 20 mg/mL in water

  Analysis: Boil for 1 min, and cool.

  Acceptance criteria: A thin, cloudy mucilage is formed.

- **C. Procedure**

  Sample solution: 1 mL of the mucilage obtained in *Identification* test *B*

  Analysis: Add 0.05 mL of iodine and potassium iodide TS 2 to the *Sample solution*.

  Acceptance criteria: An orange-red to dark blue color is produced, which disappears upon heating.

IMPURITIES

- **Residue on Ignition** ⟨281⟩**:** NMT 0.6%, determined on a 1.0-g sample

- **Limit of Iron**

  Standard iron stock solution A: Equivalent to 10 µg/mL of iron prepared as directed under *Iron* ⟨241⟩

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Standard iron stock solution B: 1 µg/mL of iron from *Standard iron stock solution A* in water [NOTE—Prepare immediately before use.]

Standard iron solution: Transfer 10 mL of *Standard iron stock solution B* to a test tube, and add 2 mL of citric acid solution (2 in 10), and 0.1 mL of thioglycolic acid. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, and dilute with water to 20 mL.

Sample solution: Shake 1.5 g of Wheat Starch with 15 mL of 2 N hydrochloric acid, and filter. Transfer 10 mL of the filtrate to a test tube, and add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, and dilute with water to 20 mL.

Acceptance criteria: After 5 min, any pink color in the *Sample solution* is not more intense than that in the *Standard iron solution*, corresponding to a limit of 10 ppm of iron.

• **LIMIT OF SULFUR DIOXIDE**

Carbon dioxide: Use carbon dioxide, with a flow regulator that will maintain a flow of 100 ± 5 mL/min.

Bromophenol blue indicator solution: 0.2 mg/mL of bromophenol blue in dilute alcohol. Filter if necessary.

Hydrogen peroxide solution: Dilute 30% hydrogen peroxide with water to obtain a 3% solution. Just before use, add 3 drops of *Bromophenol blue indicator solution*, and neutralize to a violet-blue endpoint with 0.01 N sodium hydroxide. Do not exceed the endpoint.

Apparatus: *Figure 1*

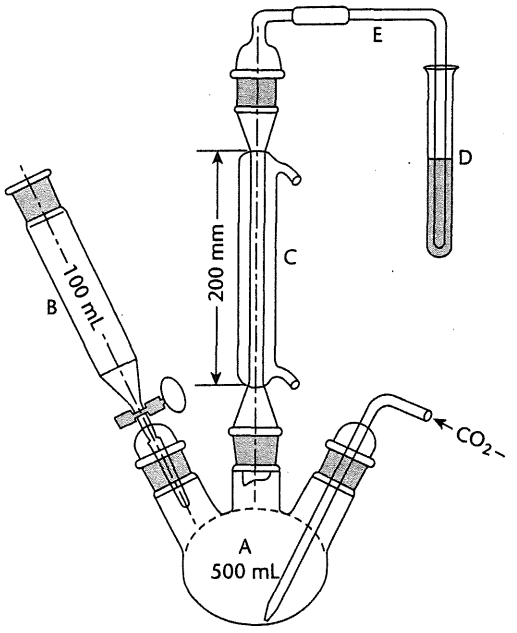


Figure 1

In this test, the sulfur dioxide is released from the sample in a boiling acid medium and is removed by a stream of carbon dioxide. The separated gas is collected in a dilute hydrogen peroxide solution where the sulfur dioxide is oxidized to sulfuric acid and titrated with standard alkali. The apparatus consists essentially of a 500-mL three-neck, round-bottom boiling flask, A; a separatory funnel, B, having a capacity of 100 mL or greater; a gas inlet tube of sufficient length to permit introduction of the carbon dioxide within 2.5 cm of the bottom of the boiling flask; a reflux condenser, C, having a jacket length of 200 mm; and a delivery tube, E, connecting the upper end of the reflux condenser to the bottom of a receiving test tube, D. Apply a thin film of stopcock grease to the sealing surfaces of all of the joints except the joint between the separatory funnel and the boiling flask, and clamp the joints to ensure tightness.

Sample: 25.0 g of Wheat Starch

Analysis: Add 150 mL of water to the boiling flask. Close the stopcock of the separatory funnel, and begin the flow of carbon dioxide at a rate of 100 ± 5 mL/min through the *Apparatus*. Start the condenser coolant flow. Add 10 mL of *Hydrogen peroxide solution* to a receiving test tube. After 15 min, without interrupting the flow of carbon dioxide, remove the separatory funnel from the boiling flask, and transfer the *Sample* into the boiling flask with the aid of 100 mL of water. Apply stopcock grease to the outer joint of the separatory funnel, and replace the separatory funnel in the boiling flask. Close the stopcock of the separatory funnel, and add 80 mL of 2 N hydrochloric acid to the separatory funnel. Open the stopcock of the separatory funnel to permit the hydrochloric acid solution to flow into the boiling flask, guarding against the escape of sulfur dioxide into the separatory funnel by closing the stopcock before the last few mL of hydrochloric acid drain out. Boil the mixture for 1 h. Remove the receiving test tube, and transfer its contents to a 200-mL wide-necked, conical flask. Rinse the receiving test tube with a small portion of water, add the rinsing to the 200-mL conical flask, and mix. Heat on a water bath for 15 min, and allow to cool. Add 0.1 mL of *Bromophenol blue indicator solution*, and titrate the contents with 0.1 N sodium hydroxide VS until the color changes from yellow to violet-blue. Perform a blank determination, and make any necessary correction (see *Titrimetry* ⟨541⟩).

Calculate the content, in ppm, of sulfur dioxide in the *Sample* taken:

$$\text{Result} = 1000 \times 32.03 \times (VN/W)$$

32.03	= milliequivalent weight of sulfur dioxide
V	= volume of titrant consumed (mL)
N	= normality of the titrant
W	= weight of the *Sample* (g)

Acceptance criteria: NMT 50 ppm

• **LIMIT OF OXIDIZING SUBSTANCES**

Sample solution: Transfer 4.0 g to a glass-stoppered, 125-mL conical flask, and add 50.0 mL of water. Insert the stopper, and swirl for 5 min. Transfer to a glass-stoppered, 50-mL centrifuge tube, and centrifuge to clarify. Transfer 30.0 mL of the clear supernatant to a glass-stoppered, 125-mL conical flask. Add 1 mL of glacial acetic acid and 0.5–1.0 g of potassium iodide. Insert the stopper, swirl, and allow to stand for 25–30 min in the dark. Add 1 mL of starch TS.

Analysis: Titrate with 0.002 N sodium thiosulfate VS to the disappearance of the starch–iodine color. Perform a blank determination, and make any necessary correction. Each mL of 0.002 N sodium thiosulfate is equivalent to 34 µg of oxidant, calculated as hydrogen peroxide.

Acceptance criteria: NMT 1.4 mL of 0.002 N sodium thiosulfate is required (20 ppm, calculated as H_2O_2).

SPECIFIC TESTS

• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩: The total aerobic microbial count does not exceed 10^3 cfu/g; the total combined molds and yeasts count does not exceed 10^2 cfu/g; and it meets the requirements of the test for the absence of *Escherichia coli*.

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• **Loss on Drying** ⟨731⟩
Sample: 1 g
Analysis: Dry the *Sample* at 130° for 90 min.
Acceptance criteria: NMT 15.0%
• **pH** ⟨791⟩
Sample solution: Prepare a slurry by weighing 5.0 g of Wheat Starch, transferring to a suitable nonmetallic container, and adding 25.0 mL of freshly boiled and cooled water.
Analysis: Agitate continuously at a moderate rate for 1 min. Stop the agitation, and allow to stand for 15 min. Determine the pH to the nearest 0.1 unit.
Acceptance criteria: 4.5–7.0
• **Total Protein**
Analysis: Weigh 6.0 g of sample containing 2 mg of nitrogen; transfer to a combustion flask; add 4 g of a powdered mixture consisting of 100 g of potassium sulfate, 5 g of cupric sulfate, and 2.5 g of selenium; and add three glass beads. Wash any adhering particles from the neck into the flask with 5 mL of sulfuric acid, allowing it to run down the sides of the flask, and mix the contents by rotation. Close the mouth of the flask loosely, for example by means of a glass bulb with a short stem, to avoid excessive loss of sulfuric acid. Heat gradually at first, then increase the temperature until there is vigorous boiling with condensation of sulfuric acid in the neck of the flask; precautions should be taken to prevent the upper part of the flask from becoming overheated. Continue the heating for 30 min, unless otherwise prescribed. Cool, dissolve the solid material by cautiously adding to the mixture 25 mL of water, cool again, and place in a steam distillation apparatus. Add 30 mL of sodium hydroxide solution (42 in 100), and distill immediately by passing steam through the mixture. Collect 40 mL of distillate in 20.0 mL of 0.01 N hydrochloric acid and enough water to cover the tip of the condenser. Toward the end of the distillation, lower the receiver so that the tip of the condenser is above the surface of the acid. Take precautions to prevent any water on the outer surface of the condenser from reaching the contents of the receiver. Titrate the distillate with 0.01 N sodium hydroxide, using methyl purple TS as the indicator (n_1 mL of 0.01 N sodium hydroxide).
Repeat the test using 50 mg of glucose in place of the substance to be examined (n_2 mL of 0.01 N sodium hydroxide).

$$\text{Content of nitrogen} = [0.01401 \times (n_2 - n_1)]/m$$

m = amount of test substance weighed (g)

Acceptance criteria: NMT 0.3% (corresponding to 0.048% N_2, conversion factor: 6.25)

ADDITIONAL REQUIREMENTS
• **•Packaging and Storage:** Preserve in well-closed containers. No storage requirements specified.•

Stearic Acid

Portions of this monograph that are national *USP* text, and are not part of the harmonized text, are marked with symbols (• •) to specify this fact.

Octadecanoic acid;
Stearic acid [57-11-4].

DEFINITION
Mixture consisting of stearic (octadecanoic) acid ($C_{18}H_{36}O_2$; M_r 284.5) and palmitic (hexadecanoic) acid ($C_{16}H_{32}O_2$; M_r 256.4) obtained from fats or oils of vegetable or animal origin.
Content:

Stearic acid 50	Stearic acid: 40.0%–60.0%. Sum of the contents of stearic acid and palmitic acids: NLT 90.0%.
Stearic acid 70	Stearic acid: 60.0%–80.0%. Sum of the contents of stearic and palmitic acids: NLT 90.0%.
Stearic acid 95	Stearic acid: NLT 90.0%. Sum of the contents of stearic acid and palmitic acids: NLT 96.0%.

•
[Note—Stearic Acid labeled solely for external use is exempt from the requirement that it be prepared from edible sources.•]

IDENTIFICATION
• **A.** It meets the requirements of the test for *Freezing Point*.
• **B. Acid Value**
Light petroleum: Use a sample that has the following properties: a clear, colorless, liquid without fluorescence; practically insoluble in water; miscible with alcohol; density at 20° about 0.720; distillation range 100°– 120°; water content NMT 0.03%.[1]
Sample solution: Dissolve 0.5 g of Stearic Acid in 50 mL of a mixture of equal volumes of alcohol and *Light petroleum* previously neutralized with 0.1 N potassium hydroxide or 0.1 N sodium hydroxide, using 0.5 mL of phenolphthalein TS as indicator. If necessary, heat to about 90° to dissolve the substance to be examined.
Analysis: Titrate the *Sample solution* with 0.1 N potassium hydroxide or 0.1 N sodium hydroxide until the pink color persists for at least 15 s. When heating has been applied to aid dissolution, maintain the temperature at about 90° during the titration.
Calculate the acid value (I_A) of the portion of Stearic Acid taken:

$$\text{Result} = (n/m) \times N \times M_r$$

n = amount of titrant used (mL)
m = amount of Stearic Acid taken to prepare the *Sample solution* (g)
N = normality of the potassium hydroxide solution
M_r = molecular weight of potassium hydroxide, 56.11

Acceptance criteria: 194–212
• **C.** The retention times of the major peaks of the *Sample solution* correspond to those of the *Standard solution*, as obtained in the *Assay*.

ASSAY
• **Procedure**
Boron trifluoride–methanol solution: 140 g/L of boron trifluoride in methanol
Sample solution: Dissolve 100 mg of Stearic Acid in a small conical flask fitted with a suitable reflux attachment with 5 mL of *Boron trifluoride–methanol solution*. Boil under reflux for 10 min. Add 4.0 mL of heptane through the condenser, and boil again under reflux for 10 min. Allow to cool. Add 20 mL of a saturated solution of sodium chloride. Shake, and allow the layers to separate. Remove about 2 mL of the organic layer, and dry it over 0.2 g of anhydrous sodium sulfate. Dilute 1.0 mL of this solution with heptane to 10.0 mL.

[1] Petroleum ether; boiling range 100°–140°; CAS 64742-49-0 from Fisher Scientific; catalog number AC23302-0025 is suitable.

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Standard solution: Prepare as directed in the *Sample solution* using 50 mg of USP Stearic Acid RS and 50 mg of USP Palmitic Acid RS.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 30-m × 0.32-mm fused silica coated with a 0.5-µm layer of stationary phase G16
Temperatures
Injection port: 220°
Detector: 260°
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	—	70	2
70	5	240	5

Carrier gas: Helium, passed through a bed of molecular sieve for drying, if necessary
Flow rate: 2.4 mL/min
Injection volume: 1 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Resolution: NLT 5.0 between the methyl palmitate and methyl stearate peaks determined on six injections
Relative standard deviation: NMT 3.0% for the methyl stearate and methyl palmitate peaks, from six replicate injections; NMT 1.0% for the ratio of the peak areas of methyl palmitate to the peak areas of methyl stearate, from six replicate injections
Analysis
Sample: *Sample solution*
Calculate the percentage of stearic acid ($C_{18}H_{36}O_2$) in the portion of Stearic Acid taken:

$$\text{Result} = (A_S/A_T) \times 100$$

A_S = peak area due to methyl stearate
A_T = sum of the peak areas of all the fatty acid esters

Calculate the percentage of palmitic acid ($C_{16}H_{32}O_2$) in the portion of Palmitic Acid taken:

$$\text{Result} = (A_P/A_T) \times 100$$

A_P = peak area due to methyl palmitate
A_T = sum of the peak areas of all the fatty acid esters

Acceptance criteria
For Stearic acid 50: 40.0–60.0% of stearic (octadecanoic) acid ($C_{18}H_{36}O_2$), and the sum of the stearic acid and palmitic acid is NLT 90.0%
For Stearic acid 70: 60.0–80.0% of stearic (octadecanoic) acid ($C_{18}H_{36}O_2$), and the sum of the stearic acid and palmitic acid is NLT 90.0%
For Stearic acid 95: NLT 90.0% of stearic (octadecanoic) acid ($C_{18}H_{36}O_2$), and the sum of the stearic acid and palmitic acid is NLT 96.0%

IMPURITIES

• ‣ **RESIDUE ON IGNITION** ⟨281⟩: NMT 4 mg, determined on a 4-g portion (0.1%).

SPECIFIC TESTS

• **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩
Sample: 1 g
Analysis: Proceed as directed in *Method I*, except use 15 mL of chloroform.
Acceptance criteria: See *Table 2*.

Table 2

Type	Iodine Value
Stearic acid 50	NMT 4.0
Stearic acid 70	NMT 4.0
Stearic acid 95	NMT 1.5

• ‣**COLOR OF SOLUTION**
Standard stock solution Y (yellow): 2.4 mL of ferric chloride CS, 0.6 mL of cobaltous chloride CS, and 7.0 mL of hydrochloric acid solution (10 g/L)
Standard stock solution BY (brownish-yellow): 2.4 mL of ferric chloride CS, 1.0 mL of cobaltous chloride CS, 0.4 mL of cupric sulfate CS, and 6.2 mL of hydrochloric acid solution (10 g/L)
Standard solution Y: 2.5 mL of *Standard stock solution Y* and 97.5 mL of hydrochloric acid solution (10 g/L)
Standard solution BY: 2.5 mL of *Standard stock solution BY* and 97.5 mL of hydrochloric acid solution (10 g/L)
Analysis: Heat Stearic Acid to 75°.
Acceptance criteria: The resulting liquid is not more intensely colored than *Standard solution Y* or *Standard solution BY*.‣

• **ACIDITY**
Sample: 5.0 g of Stearic Acid
Analysis: Melt the *Sample*, shake for 2 min with 10 mL of hot carbon dioxide-free water, cool slowly, and filter. To the filtrate add 0.05 mL of methyl orange TS.
Acceptance criteria: No red color develops.

• **FREEZING POINT**
Apparatus: Consists of a test tube about 25 mm in diameter and 150 mm long placed inside a test tube about 40 mm in diameter and 160 mm long. The inner tube is closed by a stopper which carries a thermometer about 175 mm long and graduated in 0.2°, fixed so that the bulb is about 15 mm above the bottom of the tube. The stopper has a hole allowing the passage of the stem of a stirrer made from a glass rod or other suitable material formed at one end into a loop of about 18 mm overall diameter at right angles to the rod. The inner tube with its jacket is supported centrally in a 1-L beaker containing a suitable cooling liquid to within 20-mm of the top. A thermometer is supported in the cooling bath. Place in the inner tube sufficient quantity of the liquid or previously melted substance to be examined, to cover the thermometer bulb, and determine the approximate freezing point by cooling rapidly.
Analysis: Place the inner tube in a bath about 5° above the approximate freezing point until all but the last traces of crystals are melted. Fill the beaker with water or a saturated solution of sodium chloride; at a temperature about 5° lower than the expected freezing point, insert the inner tube into the outer tube, ensuring that some seed crystals are present, and stir thoroughly until solidification takes place. Note the highest temperature observed during solidification.
Acceptance criteria: See *Table 3*.

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Table 3

Type	Freezing Point (°)
Stearic acid 50	53–59
Stearic acid 70	57–64
Stearic acid 95	64–69

ADDITIONAL REQUIREMENTS

- ‣**PACKAGING AND STORAGE:** Preserve in well-closed containers.‣
- **LABELING:** ‣If it is for external use only, the labeling so indicates.‣ The label states the type of stearic acid (50, 70, or 95).
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Palmitic Acid RS
  USP Stearic Acid RS

Purified Stearic Acid

(This monograph will be omitted after May 1, 2019 due to inclusion of the article of commerce in another compendial standard, Stearic Acid monograph, as Stearic Acid 95.) (Prior to May 1, 2019, the current practice of labeling the article of commerce with the name Purified Stearic Acid may be continued. The use of Stearic Acid 95 will be mandatory upon May 1, 2019. The 60-month extension will provide the time needed by manufacturers and users to make necessary changes.)

DEFINITION

Purified Stearic Acid is a mixture consisting of stearic (octadecanoic) acid ($C_{18}H_{36}O_2$) and palmitic (hexadecanoic) acid ($C_{16}H_{32}O_2$) obtained from fats or oils of vegetable or animal origin, which together constitute NLT 96.0% of the total content. The content of stearic acid ($C_{18}H_{36}O_2$) is NLT 90.0% of the total.

[NOTE—Purified Stearic Acid labeled solely for external use is exempt from the requirement that it be prepared from edible sources.]

IDENTIFICATION

- **A.** It meets the requirements of the test for *Freezing Point*.
- **B.** It meets the requirements of the test for *Acid Value*.
- **C.** The retention times of the major peaks of the *Sample solution* correspond to those of the *Standard solution*, as obtained in the *Assay*.

ASSAY

- **PROCEDURE**

Boron trifluoride–methanol solution: 140 g/L of boron trifluoride in methanol

Standard solution: Prepare as directed in the *Sample solution* using 50 mg of USP Stearic Acid RS and 50 mg of USP Palmitic Acid RS.

Sample solution: Dissolve 100 mg of Purified Stearic Acid in a small conical flask fitted with a suitable reflux attachment with 5 mL of *Boron trifluoride–methanol solution*. Boil under reflux for 10 min. Add 4.0 mL of heptane through the condenser, and boil again under reflux for 10 min. Allow to cool. Add 20 mL of a saturated solution of sodium chloride. Shake, and allow the layers to separate. Remove about 2 mL of the organic layer, and dry it over 0.2 g of anhydrous sodium sulfate. Dilute 1.0 mL of this solution with heptane to 10.0 mL.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC
Detector: Flame ionization
Column: 30-m × 0.32-mm fused silica coated with a 0.5-µm layer of stationary phase G16
Temperatures
Injector: 220°
Detector: 260°
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	—	70	2
70	5	240	5

Carrier gas: Helium, passed through a bed of molecular sieve for drying, if necessary
Flow rate: 2.4 mL/min
Injection volume: 1 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Resolution: NLT 5.0 between the methyl palmitate and methyl stearate peaks determined from six injections [NOTE—The relative retention times for methyl palmitate and methyl stearate are about 0.9 and 1.0, respectively.]
Relative standard deviation: NMT 3.0% for the methyl stearate and methyl palmitate peaks, from six replicate injections; NMT 1.0% for the ratio of the peak areas of methyl palmitate to the peak areas of methyl stearate, from six replicate injections
Analysis
Sample: *Sample solution*
Calculate the percentage of stearic acid ($C_{18}H_{36}O_2$) in the portion of the sample taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak area due to methyl stearate
r_T = sum of the peak areas of all the fatty acid esters

Similarly, calculate the percentage of palmitic acid ($C_{16}H_{32}O_2$) in the portion of the sample taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak area due to methyl palmitate
r_T = sum of the peak areas of all the fatty acid esters

Acceptance criteria: NLT 90.0% of stearic acid ($C_{18}H_{36}O_2$), and the sum of the stearic acid and palmitic acid is NLT 96.0%.

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩

Sample: 4 g
Acceptance criteria: NMT 4 mg (0.1%)

SPECIFIC TESTS

- **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩

Sample: 1 g
Analysis: Proceed as directed in *Method I*, except use 15 mL of chloroform.
Acceptance criteria: NMT 1.5

NF Monographs

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• **ACID VALUE**

Light petroleum: Use a sample that has the following properties: a clear, colorless, liquid without fluorescence; practically insoluble in water; miscible with alcohol; density at 20° about 0.720; distillation range 100°–120°; water content NMT 0.03%.[1]

Sample solution: Dissolve 0.5 g of Purified Stearic Acid in 50 mL of a mixture of equal volumes of alcohol and *Light petroleum* previously neutralized with 0.1 N potassium hydroxide or 0.1 N sodium hydroxide, using 0.5 mL of phenolphthalein TS as indicator. If necessary, heat to about 90° to dissolve the substance to be examined.

Analysis: Titrate the *Sample solution* with 0.1 N potassium hydroxide or 0.1 N sodium hydroxide until the pink color persists for at least 15 s. When heating has been applied to aid dissolution, maintain the temperature at about 90° during the titration.

Calculate the acid value of the portion of Purified Stearic Acid taken:

$$\text{Result} = V/W \times N \times 56.11$$

V = volume of the potassium hydroxide VS consumed in the titration (mL)
W = sample weight of Purified Stearic Acid taken to prepare the *Sample solution* (g)
N = normality of the potassium hydroxide VS
56.11 = molecular weight of potassium hydroxide

Acceptance criteria: 194–212

• **FREEZING POINT**

Apparatus: Consists of a test tube about 25 mm in diameter and 150 mm long placed inside a test tube about 40 mm in diameter and 160 mm long. The inner tube is closed by a stopper which carries a thermometer about 175 mm long and graduated in 0.2°, fixed so that the bulb is about 15 mm above the bottom of the tube. The stopper has a hole allowing the passage of the stem of a stirrer made from a glass rod or other suitable material formed at one end into a loop of about 18 mm overall diameter at right angles to the rod. The inner tube with its jacket is supported centrally in a 1-L beaker containing a suitable cooling liquid to within 20-mm of the top. A thermometer is supported in the cooling bath. Place in the inner tube sufficient quantity of the liquid or previously melted substance to be examined, to cover the thermometer bulb, and determine the approximate freezing point by cooling rapidly.

Analysis: Place the inner tube in a bath about 5° above the approximate freezing point until all but the last traces of crystals are melted. Fill the beaker with water or a saturated solution of sodium chloride at a temperature about 5° lower than the expected freezing point. Insert the inner tube into the outer tube ensuring that some seed crystals are present, and stir thoroughly until solidification takes place. Note the highest temperature observed during solidification.

Acceptance criteria: 64°–69°

• **COLOR OF SOLUTION**

Standard stock solution Y (yellow): 2.4 mL of ferric chloride CS, 0.6 mL of cobaltous chloride CS, and 7.0 mL of hydrochloric acid solution (10 g/L)

Standard stock solution BY (brownish-yellow): 2.4 mL of ferric chloride CS, 1.0 mL of cobaltous chloride CS, 0.4 mL of cupric sulfate CS, and 6.2 mL of hydrochloric acid solution (10 g/L)

Standard solution Y: 2.5 mL of *Standard stock solution Y* and 97.5 mL of hydrochloric acid solution (10 g/L)

Standard solution BY: 2.5 mL of *Standard stock solution BY* and 97.5 mL of hydrochloric acid solution (10 g/L)

Analysis: Heat Purified Stearic Acid to 75°.

Acceptance criteria: The resulting liquid is not more intensely colored than *Standard solution Y* or *Standard solution BY*.

• **ACIDITY**

Analysis: Melt 5.0 g of Purified Stearic Acid, shake for 2 min with 10 mL of hot carbon dioxide-free water, cool slowly, and filter. To the filtrate add 0.05 mL of methyl orange TS.

Acceptance criteria: No red color develops.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

• **LABELING:** If it is for external use only, the labeling so indicates.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Palmitic Acid RS
USP Stearic Acid RS

[1] Petroleum ether; boiling range 100°–140°; CAS 64742-49-0 from Fisher Scientific; catalog number AC23302-0025 is suitable.

Stearoyl Polyoxylglycerides

DEFINITION

Stearoyl Polyoxylglycerides is a mixture of monoesters, diesters, and triesters of glycerol and monoesters and diesters of polyethylene glycols. The polyethylene glycols used have a mean molecular weight between 300 and 4000. It is produced by partial alcoholysis of saturated oils, mainly containing triglycerides of stearic acid, with polyethylene glycol, by esterification of glycerol and polyethylene glycol with fatty acids, or as a mixture of glycerol esters and ethylene oxide condensate with the fatty acids of the hydrogenated oils. The hydroxyl value is NLT 85% and NMT 115% of the labeled nominal value, and the saponification value is NLT 90% and NMT 110% of the labeled nominal value. Stearoyl Polyoxylglycerides may contain free polyethylene glycols.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

• **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** ⟨201⟩

Standard solution: 50 mg/mL of USP Stearoyl Polyoxylglycerides in methylene chloride

Sample solution: 50 mg/mL of Stearoyl Polyoxylglycerides in methylene chloride

Chromatographic system

Application volume: 10 µL

Developing solvent system: Ether and hexanes (70:30)

Spray reagent: 0.1 mg/mL of rhodamine B in alcohol

Analysis

Samples: *Standard solution* and *Sample solution*

Proceed as directed in the chapter. Then spray the plate with *Spray reagent*, and locate the spots on the plate by examination under UV light at a wavelength of 365 nm.

Acceptance criteria: The R_F values of the principal spots of the *Sample solution* correspond to those of the *Standard solution*.

• **C.** It meets the requirements in *Specific Tests* (see *Table 1*) for *Fats and Fixed Oils, Fatty Acid Composition* ⟨401⟩.

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IMPURITIES

- **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩: NMT 0.2%
- **ALKALINE IMPURITIES**
  Sample: 5.0 g
  Analysis: Heat the *Sample* slightly until the test substance melts, add 10 mL of alcohol and 0.05 mL of bromophenol blue TS, and mix well. While the solution is still warm, titrate with 0.01 N hydrochloric acid VS to change the color to yellow.
  Acceptance criteria: NMT 1.0 mL of 0.01 N hydrochloric acid VS is required.
- **LIMIT OF FREE ETHYLENE OXIDE AND DIOXANE**
  Analysis: Proceed as directed in *Ethylene Oxide and Dioxane* ⟨228⟩, *Method I*.
  Acceptance criteria
  Ethylene oxide: NMT 1 µg/g
  Dioxane: NMT 10 µg/g
- **LIMIT OF FREE GLYCEROL**
  Sample: 1.20 g
  Periodic acetic acid solution: Dissolve 0.446 g of sodium periodate in 2.5 mL of a 25% (v/v) solution of sulfuric acid, diluting with glacial acetic acid to 100.0 mL.
  Potassium iodide solution: 75 mg/mL of potassium iodide
  Analysis: Dissolve the *Sample* in 25 mL of methylene chloride, heating if necessary. Cool, and add 100 mL of water and 25.0 mL of the *Periodic acetic acid solution*. Shake, and allow to stand for 30 min. Add 40 mL of the *Potassium iodide solution*, and allow to stand for 1 min. Add 1 mL of starch TS, and titrate the liberated iodine with 0.1 M sodium thiosulfate VS. Perform a blank determination, and make any necessary correction (see *Titrimetry* ⟨541⟩). Calculate the percentage of glycerol in the sample taken:

$$\text{Result} = \{[(V_B - V_S) \times N \times F]/W\} \times 100$$

V_B = *Titrant* volume consumed by the *Blank* (mL)
V_S = *Titrant* volume consumed by the *Sample* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 23.0 mg/mEq
W = *Sample* weight (mg)

Acceptance criteria: NMT 5.0%

SPECIFIC TESTS

- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩
  Sample: 2.0 g
  Acceptance criteria: NMT 2.0
- **FATS AND FIXED OILS,** *Fatty Acid Composition* ⟨401⟩: Stearoyl Polyoxylglycerides exhibits the following composition profile of fatty acids, as determined in the chapter (see *Table 1*).

Table 1

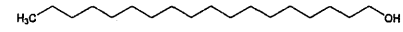
Carbon-Chain Length	Number of Double Bonds	Percentage (%)
12	0	≤5.0
14	0	≤5.0
16	0	40.0–50.0
18	0	48.0–58.0

- **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩
  Sample: 1.0 g
  Acceptance criteria: 25–56, 85%–115% of the labeled nominal value
- **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩: NMT 2.0
- **FATS AND FIXED OILS,** *Peroxide Value* ⟨401⟩
  Sample: 2.0 g
  Acceptance criteria: NMT 6.0
- **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩
  Sample: 2.0 g
  Acceptance criteria: 67–112, 90%–110% of the labeled nominal value
- **WATER DETERMINATION,** *Method I* ⟨921⟩
  Sample: 1.0 g
  Solvent: Anhydrous pyridine or a mixture of methylene chloride and anhydrous methanol (70:30)
  Acceptance criteria: NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light and moisture. Store at controlled room temperature.
- **LABELING:** Label it to indicate the type and the average nominal molecular weight of polyethylene glycols used as part of the official title. The label also indicates the hydroxyl value and the saponification value.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Stearoyl Polyoxylglycerides RS

Stearyl Alcohol



$C_{18}H_{38}O$ 270.49
1-Octadecanol;
Octadecan-1-ol [112-92-5].

DEFINITION

Stearyl Alcohol contains NLT 90.0% and NMT 102.0% of stearyl alcohol ($C_{18}H_{38}O$), the remainder consisting chiefly of related alcohols. It is obtained from sources of vegetable, animal, or synthetic origin.

IDENTIFICATION

- **A. CHROMATOGRAPHIC IDENTITY**
  Analysis: Proceed as directed in the *Assay*.
  Acceptance criteria: The retention time of the major peak of the *Sample solution*, excluding the solvent and internal standard peaks, corresponds to the stearyl alcohol peak of the *System suitability solution*.

ASSAY

- **PROCEDURE**
  Internal standard solution: 1 mg/mL of 1-pentadecanol (internal standard) in ethanol
  System suitability solution: Prepare 1 mg/mL each of USP Cetyl Alcohol RS, USP Stearyl Alcohol RS, and USP Oleyl Alcohol RS in *Internal standard solution*. Heat the solution in a sealed container in a 50° water bath until all fatty alcohols are dissolved. Allow the solution to cool to room temperature, and mix well.
  Standard solution: Prepare 1.0 mg/mL of USP Stearyl Alcohol RS in *Internal standard solution*, and heat the solution in a sealed container in a 50° water bath until stearyl alcohol is dissolved. Allow the solution to cool to room temperature, and mix well.
  Sample solution: Prepare 1.0 mg/mL of Stearyl Alcohol in *Internal standard solution*. Heat the solution in a sealed container in a 50° water bath until stearyl alcohol is dissolved. Allow the solution to cool to room temperature, and mix well.
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)

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Mode: GC
Detector: Flame ionization
Column: 0.25-mm × 30-m fused silica capillary; coated with a 0.25-µm layer of phase G7
Temperatures
Detector: 280°
Injection port: 270°
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
60	20	180	—
180	10	220	5

Carrier gas: Hydrogen
Flow rate: 2.0 mL/min, constant flow mode
Injection volume: 1 µL
Injection type: Split injection; split ratio is 100:1
Liner: Single taper, low pressure drop liner with deactivated wool
Run time: 15 min
System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—See *Table 2* for the relative retention times.]

Table 2

Component	Relative Retention Time
1-Pentadecanol (internal standard)	1.00
Cetyl alcohol	1.09
Stearyl alcohol	1.25
Oleyl alcohol	1.28

Suitability requirements
Resolution: NLT 30 between the cetyl alcohol and stearyl alcohol peaks; NLT 2.0 between the stearyl and oleyl alcohol peaks, *System suitability solution*
Tailing factor: 0.8–1.8 for the stearyl alcohol and 1-pentadecanol peaks, *Standard solution*
Relative standard deviation: NMT 1%, using the area ratio of stearyl alcohol to 1-pentadecanol, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate the percentage of stearyl alcohol ($C_{18}H_{38}O$) in the portion of Stearyl Alcohol taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of stearyl alcohol to the internal standard (peak response of stearyl alcohol/peak response of the internal standard) from the *Sample solution*
R_S = peak response ratio of stearyl alcohol to the internal standard (peak response of stearyl alcohol/peak response of the internal standard) from the *Standard solution*
C_S = concentration of USP Stearyl Alcohol RS in the *Standard solution* (mg/mL)
C_U = concentration of Stearyl Alcohol in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–102.0%

IMPURITIES
• **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 0.1%, determined on 2 g
• **LIMIT OF RELATED FATTY ALCOHOLS**
Solution A: 1 mg/mL of 1-pentadecanol in ethanol
Resolution solution: Prepare 1 mg/mL each of USP Lauryl Alcohol RS, USP Myristyl Alcohol RS, USP Cetyl Alcohol RS, USP Stearyl Alcohol RS, USP Oleyl Alcohol RS, USP Linolenyl Alcohol RS, and USP Arachidyl Alcohol RS in *Solution A*. Heat the solution in a sealed container in a 50° water bath until all fatty alcohols are dissolved. Allow the solution to cool to room temperature, and mix well. Dilute the solution with ethanol to obtain a solution containing 0.05 mg/mL each of USP Lauryl Alcohol RS, USP Myristyl Alcohol RS, USP Cetyl Alcohol RS, 1-pentadecanol, USP Stearyl Alcohol RS, USP Oleyl Alcohol RS, USP Linolenyl Alcohol RS, and USP Arachidyl Alcohol RS.
Sample solution: 1 mg/mL of Stearyl Alcohol in ethanol. Heat the solution in a sealed container in a 50° water bath until stearyl alcohol is dissolved. Allow the solution to cool to room temperature, and mix well.
Chromatographic system: Proceed as directed in the *Assay*, except for the split ratio.
Injection type: Split injection; the split ratio is 5:1.
System suitability
Sample: *Resolution solution*
[NOTE—See *Table 3* for the relative retention times.]

Table 3

Component	Relative Retention Time
Lauryl alcohol	0.79
Myristyl alcohol	0.93
1-Pentadecanol	1.00
Cetyl alcohol	1.09
Stearyl alcohol	1.25
Oleyl alcohol	1.28
Linolenyl alcohol	1.36
Arachidyl alcohol	1.44

Suitability requirements
Resolution: NLT 15 between myristyl alcohol and 1-pentadecanol peaks; NLT 30 between the cetyl alcohol and stearyl alcohol peaks; NLT 2.0 between the stearyl and oleyl alcohol peaks
Analysis
Samples: *Resolution solution* and *Sample solution*
Identify each related fatty alcohol peak in the *Sample solution* based on that in the *Resolution solution*.
Calculate the percentage of each related fatty alcohol or any unspecified impurity in the portion of Stearyl Alcohol taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of each related fatty alcohol (or any unspecified impurity) from the *Sample solution*
r_T = sum of all the peak responses excluding peak responses due to solvent from the *Sample solution*

Acceptance criteria: Disregard peaks that are less than 0.05% for any unspecified impurities, and any peaks due to solvent.
Sum of unspecified impurities: NMT 1%

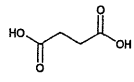
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Sum of related fatty alcohols and unspecified impurities: NMT 10.0%

SPECIFIC TESTS
- **FATS AND FIXED OILS,** *Acid Value (Free Fatty Acids)* ⟨401⟩**:** NMT 2
- **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩**:** 195–220
- **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩**:** NMT 2
- **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 0.5%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **LABELING:** Label it to indicate whether it is derived from vegetable, animal, or synthetic sources.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Arachidyl Alcohol RS
  USP Cetyl Alcohol RS
  USP Lauryl Alcohol RS
  USP Linolenyl Alcohol RS
  USP Myristyl Alcohol RS
  USP Oleyl Alcohol RS
  USP Stearyl Alcohol RS

Succinic Acid



$C_4H_6O_4$ 118.09
Butanedioic acid [110-15-6].

DEFINITION
Succinic Acid contains NLT 99.0% and NMT 100.5% of succinic acid ($C_4H_6O_4$).

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
- **B. CHROMATOGRAPHIC IDENTITY**
  The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

ASSAY
- **PROCEDURE**
  Diluent: Dissolve 6.8 g of potassium phosphate monobasic in 2 L of water. Adjust the pH of the solution to 2.3 with drops of phosphoric acid. This solution is 25 mM potassium phosphate buffer, pH 2.3.
  Mobile phase: Methanol and *Diluent* (8:92)
  System suitability solution: 1.0 mg/mL of USP Succinic Acid RS and 0.003 mg/mL of USP Fumaric Acid RS in *Diluent*
  Standard solution: 1.0 mg/mL of USP Succinic Acid RS in *Diluent*
  Sample solution: 1.0 mg/mL of Succinic Acid in *Diluent*
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: LC
  Detector: UV 204 nm
  Column: 4.6-mm × 15-cm; 3-µm packing L1
  Flow rate: 1.0 mL/min
  Injection volume: 20 µL
  System suitability
  Sample: *System suitability solution*
  [NOTE—The relative retention times for succinic acid and fumaric acid are 1.0 and 1.2, respectively.]
  Suitability requirements
  Resolution: NLT 2.0 between succinic acid and fumaric acid
  Relative standard deviation: NMT 0.5% for the succinic acid peak
  Tailing factor: 0.8–2.0
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Calculate the percentage of succinic acid ($C_4H_6O_4$) in the portion of the sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP Succinic Acid RS in the *Standard solution* (mg/mL)
C_U = concentration of Succinic Acid in the *Sample solution* (mg/mL)

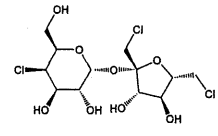
Acceptance criteria: 99.0%–100.5%

IMPURITIES
- **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 0.025%

SPECIFIC TESTS
- **MELTING RANGE OR TEMPERATURE** ⟨741⟩**:** 185.0°–190.0°

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in a well-closed container. No storage requirements specified.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Fumaric Acid RS
  USP Succinic Acid RS

Sucralose



$C_{12}H_{19}Cl_3O_8$ 397.63
1,6-Dichloro-1,6-dideoxy-β-D-fructofuranosyl-4-chloro-4-deoxy-α-D-galactopyranoside;
1′,4,6′-Trichlorogalactosucrose [56038-13-2].

DEFINITION
Sucralose contains NLT 98.0% and NMT 102.0% of $C_{12}H_{19}Cl_3O_8$, calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
- **B.** The retention time of the principal peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.
- **C.** The R_F value of the principal spot of the *Sample solution* corresponds to that of *Standard solution A*, as obtained in the test for *Related Compounds*.

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ASSAY

• **PROCEDURE**

Mobile phase: Acetonitrile and water (3:17)

Standard solution: 1 mg/mL of USP Sucralose RS in *Mobile phase*

Sample solution: 1 mg/mL of Sucralose in *Mobile phase*

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 8-mm × 10-cm; packing L1

Flow rate: 1.5 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

[NOTE—The retention time of sucralose is about 9 min.]

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of sucralose ($C_{12}H_{19}Cl_3O_8$) in the portion of Sucralose taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of the *Sample solution*

r_S = peak response of the *Standard solution*

C_S = concentration of USP Sucralose RS in the *Standard solution* (mg/mL)

C_U = concentration of Sucralose in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–102.0% on the anhydrous basis

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 0.7%

• **LIMIT OF METHANOL**

Internal standard solution: 0.1 µL/mL of *n*-propyl alcohol in pyridine

Standard solution: 0.2 µL/mL of methanol in *Internal standard solution*

Sample solution: 0.2 g/mL of Sucralose in *Internal standard solution*

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 4-mm × 2-m glass column; packed with 80- to 100-mesh silanized support S6

Temperature

Column: 150°

Detector: 250°

Injector: 200°

Carrier gas: Helium

Flow rate: 20 mL/min

Injection size: 1 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of methanol in the portion of Sucralose taken:

$$\text{Result} = (R_U/R_S) \times [(C_S/C_U) \times F_1] \times F_2 \times 100$$

R_U = peak response ratio of methanol to *n*-propyl alcohol, from the *Sample solution*

R_S = peak response ratio of methanol to *n*-propyl alcohol, from the *Standard solution*

C_S = concentration of methanol in the *Standard solution* (µL/mL)

C_U = concentration of Sucralose in the *Sample solution* (g/mL)

F_1 = conversion factor from µL to mL

F_2 = specific gravity of methanol, 0.79 g/cm³

Acceptance criteria: NMT 0.1%

• **RELATED COMPOUNDS**

Adsorbent: 0.20-mm layer of octadecylsilanized chromatographic silica gel. The thin-layer chromatographic plate also has a preadsorbent zone.

Detection reagent: Sulfuric acid in methanol (3 in 20)

Standard solution A: 10.0 mg/mL of USP Sucralose RS in methanol

Standard solution B: 0.5 mL *Standard solution A* diluted to 10.0 mL with methanol

Sample solution: 100.0 mg/mL of Sucralose in methanol

Developing solvent system: Acetonitrile and sodium chloride solution (1 in 20) (3:7)

Application volume: 5 µL

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Proceed as directed under *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*. Spray the plate with *Detection reagent*. Heat the plate for 10 min at 125°.

Acceptance criteria: The R_F value of the principal spot from the *Sample solution* corresponds to that obtained from *Standard solution A*, and the color of any other single spot from the *Sample solution* is not more intense than that of the principal spot from *Standard solution B* (0.5%).

• **LIMIT OF HYDROLYSIS PRODUCTS**

[NOTE—This test does not require a developing solvent.]

Adsorbent: 0.25-mm layer of chromatographic silica gel

Spray reagent: 12.3 mg/mL of *p*-anisidine and 16.6 mg/mL of phthalic acid in methanol. Store the solution in the dark and refrigerate to prevent discoloration. Discard if the solution becomes discolored. [**CAUTION**—*p*-Anisidine is toxic if inhaled or if absorbed through the skin.]

Standard solution A: 100 mg/mL of mannitol

Standard solution B: 0.4 mg/mL of fructose and 100 mg/mL of mannitol

Sample solution: 250 mg/mL of Sucralose in methanol

Application volume: 5-µL portions separately applied in 1-µL increments, allowing the plate to dry between applications

Analysis

Samples: *Standard solution* and *Sample solution*

Proceed as directed under *Chromatography* ⟨621⟩, *Thin-Layer Chromatography*. Spray the plate with *Spray reagent*, and heat the plate at 100 ± 2° for 15 min. If the spot from *Standard solution A* has darkened, repeat the test, heating for a shorter period of time. Immediately after heating, view the plate against a dark background.

Acceptance criteria: The color of the spot from the *Sample solution* is not more intense than that from *Standard solution B* (0.1%).

SPECIFIC TESTS

• **OPTICAL ROTATION,** *Specific Rotation* ⟨781S⟩**:** +84.0° to +87.5° at 20°

Sample solution: 10 mg/mL of Sucralose

• **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 2.0%

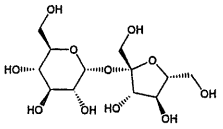
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ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, in a cool, dry place, at a temperature not exceeding 21°.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Sucralose RS

Sucrose

Portions of the monograph text that are national *USP* text, and are not part of the harmonized text, are marked with symbols (• •) to specify this fact.



$C_{12}H_{22}O_{11}$ 342.30

α-D-Glucopyranoside, β-D-fructofuranosyl-sucrose [57-50-1].

DEFINITION

Sucrose is a sugar obtained from *Saccharum officinarum* Linné (Fam. Gramineae), *Beta vulgaris* Linné (Fam. Chenopodiaceae), and other sources. It contains no added substances.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

IMPURITIES

- **SULFITE**

Sample solution: 400 mg/mL of Sucrose in freshly prepared distilled water

Sulfite standard solution (80 ppm SO_2): 0.1575 mg/mL of anhydrous sodium sulfite in freshly prepared distilled water

Reference solution: Dissolve 4.0 g of Sucrose in freshly prepared distilled water, add 0.5 mL of *Sulfite standard solution (80 ppm SO_2)*, and dilute with freshly prepared distilled water to 10.0 mL.

Blank: Freshly prepared distilled water

Analysis: Determine the sulfite content by a suitable enzymatic method based on the following reactions. Sulfite is oxidized by sulfite oxidase to sulfate and hydrogen peroxide, which in turn is reduced by nicotinamide–adenine dinucleotide–peroxidase in the presence of reduced nicotinamide–adenine dinucleotide (NADH). The amount of NADH oxidized is proportional to the amount of sulfite. Separately introduce 2.0 mL each of the *Sample solution*, *Reference solution*, and *Blank* in 10-mm cuvettes, and add the reagents as described in the kit instructions.[1] Measure the absorbance at the maximum at about 340 nm before and at the end of the reaction time, and subtract the value obtained with the *Blank*.

Acceptance criteria: The absorbance difference of the *Sample solution* is NMT half the absorbance difference of the *Reference solution*.

[1] Test kit for sulfite determination may be ordered from Boehringer Mannheim Roche/R-Biopharm, Catalog #10 725 854 035.

SPECIFIC TESTS

- **APPEARANCE OF SOLUTION**

Sample solution: 500 mg/mL of Sucrose in water. [NOTE—Set a portion of this solution aside for the tests for *Dextrins* and *Reducing Sugars*.]

Hydrazine sulfate solution: 10 mg/mL of hydrazine sulfate in water. Allow to stand for 4–6 h.

Hexamethylenetetramine solution: In a 100-mL ground glass-stoppered flask dissolve 2.5 g of hexamethylenetetramine in 25.0 mL of water.

Primary opalescent suspension: To the *Hexamethylenetetramine solution* in the flask add 25.0 mL of *Hydrazine sulfate solution*. Mix, and allow to stand for 24 h. This suspension is stable for 2 months, provided it is stored in a glass container free from surface defects. The suspension must not adhere to the glass and must be well mixed before use.

Standard of opalescence: *Primary opalescent suspension* in water (3 in 200). This suspension is freshly prepared and may be stored for up to 24 h.

Reference suspension I: *Standard of opalescence* and water (5:95)

Acceptance criteria: The clarity of the *Sample solution* is the same as that of water or its opalescence is not more pronounced than that of *Reference suspension I*.

- **CONDUCTIVITY**

Sample solution: 313 mg/mL of Sucrose in freshly boiled and cooled water

Apparatus: Use a conductivity meter or resistivity meter that measures the resistance of the column of liquid between the electrodes of the immersed measuring device. The apparatus is supplied with alternating current to avoid the effects of electrode polarization. It is equipped with a temperature compensation device or a precision thermometer.

Calibration: Choose a conductivity cell that is appropriate for the conductivity of the solution to be examined. The higher the expected conductivity, the higher the cell constant that must be chosen so that the value measured, *R*, is as large as possible for the apparatus used. Commonly used conductivity cells have cell constants on the order of 0.1 cm^{-1}, 1 cm^{-1}, and 10 cm^{-1}. Use a standard solution of potassium chloride that is appropriate for the measurement. Rinse the cell several times with water that has been previously boiled and cooled to room temperature and at least twice with the potassium chloride solution used for the determination of the cell constant of the conductivity cell. Measure the resistance of the conductivity cell using the potassium chloride solution at 20 ± 0.1°.

The constant, *C* (in cm^{-1}), of the conductivity cell is given by the expression:

$$C = R_{KCl} \times K_{KCl}$$

R_{KCl} = measured resistance (MΩ)

K_{KCl} = conductivity of the standard solution of potassium chloride used (μS · cm^{-1})

The measured constant, *C*, of the conductivity cell must be within 5% of the given value.

Analysis

Sample: *Sample solution*

Measure the conductivity of the *Sample solution* (C_1), while gently stirring with a magnetic stirrer, and that of the water used for preparing the *Sample solution* (C_2). The readings must be stable within 1% over a period of 30 s.

Calculate the conductivity of the *Sample solution* from the expression:

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$$\text{Result} = C_1 - (0.35 \times C_2)$$

C_1 = conductivity of the *Sample solution*
C_2 = water used for preparing the *Sample solution*

Acceptance criteria: NMT 35 µS · cm^{-1} at 20°

• **OPTICAL ROTATION,** *Specific Rotation* ⟨781S⟩
Sample solution: 260 mg/mL
Acceptance criteria: +66.3° to +67.0° at 20°

• **•COLOR VALUE•**
Sample solution: Dissolve 50.0 g in 50.0 mL of water. Mix, filter (diameter of pores, 0.45 µm), and degas.
Analysis: Measure the absorbance at 420 nm, using a cell of at least 4 cm (a cell length of 10 cm or more is preferred). Calculate the *Color Value* using the expression:

$$\text{Result} = (A \times 1000)/(b \times c)$$

A = absorbance measured at 420 nm
b = cell path length (cm)
c = concentration of the solution (g/mL), calculated from the refractive index of the solution. Use *Table 1*, and interpolate the values if necessary.

Suitability requirements
Repeatability: The absolute difference between two results is NMT 3.

Table 1

n^{20}_D	c (g/mL)
1.4138	0.570
1.4159	0.585
1.4179	0.600
1.4200	0.615
1.4221	0.630
1.4243	0.645
1.4264	0.661

Acceptance criteria: NMT 45 if labeled as parenteral grade; NMT 75 for nonparenteral grade.•

• **DEXTRINS**
[NOTE—If intended for use in the preparation of large-volume infusions, it complies with the test for *Dextrins*.]
Sample solution: Prepare as directed in the test for *Appearance of Solution*.
Analysis: To 2 mL of the *Sample solution* add 8 mL of water, 0.05 mL of dilute hydrochloric acid (73 g/L of HCl), and 0.05 mL of 0.05 M iodine.
Acceptance criteria: The solution remains yellow.

• **REDUCING SUGARS**
Sample solution: Prepare as directed in the test for *Appearance of Solution*.
Analysis: To 5 mL of the *Sample solution* in a test tube, about 150-mm long and 16-mm in diameter, add 5 mL of water, 1.0 mL of 1 M sodium hydroxide, and 1.0 mL of a 1-g/L solution of methylene blue. Mix, and place in a water bath. After exactly 2 min, take the tube out of the bath, and examine the solution immediately.
Acceptance criteria: The blue color does not disappear completely, ignoring any blue color at the air/solution interface.

• **LOSS ON DRYING** ⟨731⟩
Sample: 2.000 g
Analysis: Dry the *Sample* at 105° for 3 h.
Acceptance criteria: NMT 0.1%

• **BACTERIAL ENDOTOXINS TEST** ⟨85⟩**:** Less than 0.25 IU/mg
[NOTE—If intended for use in the preparation of large-volume infusions, it complies with the test for *Bacterial Endotoxins*.]

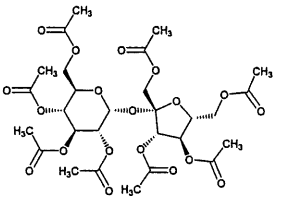
ADDITIONAL REQUIREMENTS

• **•PACKAGING AND STORAGE:** Preserve in well-closed containers.•

• **LABELING:** The label states, where applicable, that the substance is suitable for use in the manufacture of large-volume parenteral dosage forms.

• **USP REFERENCE STANDARDS** ⟨11⟩
USP Sucrose RS

Sucrose Octaacetate



$C_{28}H_{38}O_{19}$ 678.59
α-D-Glucopyranoside, 1,3,4,6-tetra-*O*-acetyl-β-D-fructofuranosyl, tetraacetate;
Octaacetyl sucrose [126-14-7].

DEFINITION
Sucrose Octaacetate contains NLT 98.0% and NMT 100.5% of sucrose octaacetate ($C_{28}H_{38}O_{19}$), calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K▲** (CN 1-May-2020)

ASSAY

• **PROCEDURE**
Sample: 100 mg in a 500-mL conical flask
Blank: 50 mL of 70% alcohol
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Residual titration
Titrant: 0.1 N sodium hydroxide VS
Back-titrant: 0.1 N sulfuric acid VS
Endpoint detection: Visual
Analysis: Dissolve the *Sample* in 50 mL of 70% alcohol. Neutralize the solution with 0.1 N sodium hydroxide VS using phenolphthalein TS as the indicator. Add 25.0 mL of 0.1 N sodium hydroxide VS, attach an air condenser to the flask, protect from absorption of carbon dioxide, and reflux on a steam bath for 1 h. Remove from the steam bath, cool quickly, and titrate the excess alkali with 0.1 N sulfuric acid VS using phenolphthalein TS as the indicator. Perform a blank determination.
Calculate the percentage of sucrose octaacetate ($C_{28}H_{38}O_{19}$) in the portion of Sucrose Octaacetate taken. Each mL of 0.1 N sodium hydroxide is equivalent to 8.483 mg of sucrose octaacetate ($C_{28}H_{38}O_{19}$).
Acceptance criteria: 98.0%–100.5% on the anhydrous basis

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IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%

SPECIFIC TESTS

• **MELTING RANGE OR TEMPERATURE** ⟨741⟩: NLT 78°

• **ACIDITY**

Sample: 1 g

Analysis: Dissolve the *Sample* in 20 mL of neutralized alcohol, and add 2 drops of phenolphthalein TS.

Acceptance criteria: NMT 2 drops of 0.1 N sodium hydroxide are required to produce a red color.

• **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 1.0%

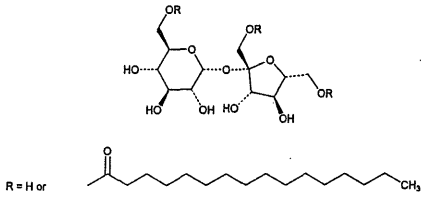
ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Sucrose Octaacetate RS

Sucrose Palmitate



$C_{28}H_{52}O_{12}$ 580.71

$C_{44}H_{82}O_{13}$ 819.11

$C_{60}H_{112}O_{14}$ 1057.52

Sucrose monopalmitate;
Sucrose hexadecanoate [26446-38-8].

DEFINITION

Sucrose Palmitate is a mixture of sucrose monoesters, mainly sucrose monopalmitate, obtained by transesterification of palmitic acid methyl esters of vegetable origin with sucrose. The manufacture of the fatty acid methyl esters includes a distillation step. It contains variable quantities of mono- and diesters as set forth in the following table:

Content of Monoesters (%)	Content of Diesters (%)	Sum of Triesters and Polyesters (%)
NLT 55.0	NMT 40.0	NMT 20.0

IDENTIFICATION

• **A.** It meets the requirements of the *Fatty Acid Composition* test.

• **B.** It meets the requirements of the *Content of Monesters, Diesters, Triesters, and Polyesters.*

ASSAY

• **CONTENT OF MONOESTERS, DIESTERS, TRIESTERS, AND POLYESTERS**

Mobile phase: Tetrahydrofuran

Sample solution: 15 mg/mL of Sucrose Palmitate in tetrahydrofuran

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability.*)

Mode: LC, size-exclusion

Detector: Differential refractometer

Column: 7-mm × 60-cm; packing L21, 100 Å

[NOTE—Two 7-mm × 30-cm L21 columns may be used in place of one 60-cm column, provided system suitability requirements are met.]

Flow rate: 1.2 mL/min

Injection size: 20 µL

Analysis

Sample: *Sample solution*

[NOTE—The relative retention time with reference to the monoester peak (retention time is approximately 10 min) is about 0.92 for diesters, and about 0.90 for triesters and polyesters.]

[NOTE—Disregard solvent peaks and peaks having a signal-to-noise ratio less than 10.]

Calculate the percentage of monoesters in the portion of Sucrose Palmitate taken:

$$\text{Result} = A \times (100 - D - S - E)/100$$

A = percentage of monoesters determined by peak normalization

D = percentage of free fatty acids, using the following formula:

$$\text{Result} = AV \times 256/561.1$$

AV = acid value

S = percentage of free sucrose (see *Free Sucrose* in *Organic Impurities*)

E = percentage of water (see *Water Determination, Ia* in *Specific Tests*)

Calculate the percentage of diesters in the portion of Sucrose Palmitate taken:

$$\text{Result} = B \times (100 - D - S - E)/100$$

B = percentage of diesters determined by peak normalization

D = percentage of free fatty acids (above)

S = percentage of free sucrose (see *Free Sucrose* in *Organic Impurities*)

E = percentage of water (see *Water Determination, Ia* in *Specific Tests*)

Calculate the percentage of triesters and polyesters in the portion of Sucrose Palmitate taken:

$$\text{Result} = C \times (100 - D - S - E)/100$$

C = percentage of triesters and polyesters determined by peak normalization

D = percentage of free fatty acids (above)

S = percentage of free sucrose (see *Free Sucrose* in *Organic Impurities*)

E = percentage of water (see *Water Determination, Ia* in *Specific Tests*)

• **FATTY ACID COMPOSITION:** Sucrose Palmitate exhibits the following composition profiles of fatty acids, as determined under *Fats and Fixed Oils, Fatty Acid Composition* ⟨401⟩.

Fatty Acid	Percentage (%)
Lauric acid	NMT 3.0
Myristic acid	NMT 3.0
Palmitic acid	70.0–85.0
Stearic acid	10.0–25.0

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Fatty Acid	Percentage (%)
Sum of the contents of palmitic acid and stearic acid	NLT 90.0

IMPURITIES

INORGANIC IMPURITIES

• **Fats and Fixed Oils,** *Acid Value* ⟨401⟩: NMT 6.0%, determined on a 3-g sample. Use a freshly neutralized mixture of 2-propanol and water (2:1), and gently heat.

ORGANIC IMPURITIES

• **Procedure: Free Sucrose**

Solution A: 10 µg/mL of ammonium acetate in acetonitrile

Solution B: 10 µg/mL of ammonium acetate in tetrahydrofuran and water (90:10)

Diluent: Tetrahydrofuran and water (87.5: 12.5)

System suitability solution: 500 µg/mL of USP Sucrose RS in *Diluent*

Standard solutions: 0.50, 1.0, 2.0, and 2.5 mg/mL of USP Sucrose RS in *Diluent*

Sample solution: 50 mg/mL of Sucrose Palmitate in *Diluent*

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability.*)

Mode: LC

Detector: Evaporative light-scattering. [NOTE—If the detector has different setting parameters, adjust the detector settings so that they comply with the *System suitability* requirements.]

Carrier gas: Nitrogen

Detector temperature: 45°

Nebulizer temperature: 40°

Column: 4.6-mm × 0.25-m; packing L8

Injection size: 20 µL

Mobile phase and flow rate: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)	Flow Rate (mL/min)
1	100	0	1.0
8	0	100	1.0
7	0	100	1.0
0.01	0	100	2.5
15.99	0	100	2.5
1	100	0	2.5
3	100	0	1.0

System suitability

Sample: *System suitability solution*

[NOTE—The retention time is about 26 min for sucrose palmitate.]

Suitability requirements

Signal-to-noise ratio: 10:1

Analysis

Samples: *Standard solutions* and *Sample solution*

Prepare a standard curve by plotting the peak response versus concentration of sucrose in the *Standard solutions.* Calculate the amount of free sucrose in the Sucrose Palmitate taken.

Acceptance criteria: NMT 4.0%

SPECIFIC TESTS

• **WATER DETERMINATION,** *Method Ia* ⟨921⟩: NMT 4.0% on a 0.20-g sample

• **TOTAL ASH**

Sample: 1.0 g

Analysis: Heat a silica or platinum crucible to redness for 30 min, allow to cool in a desiccator, and weigh. Transfer the *Sample* into the crucible. Dry at 100°–105° for 1 h and ignite to constant weight in a muffle furnace at 600 ± 25°, allowing the crucible to cool in a desiccator after each ignition. Flames should not be produced at any time during the procedure. If after prolonged ignition the ash still contains black particles, add hot water, filter through an ashless filter paper, and ignite the residue and the filter paper. Combine the filtrate with the ash, carefully evaporate to dryness and ignite to constant weight.

Acceptance criteria: NMT 1.5%

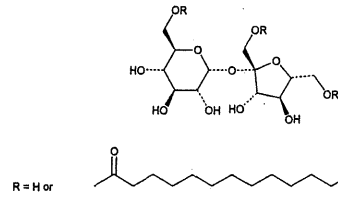
ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in a well-closed container. Protect from humidity and avoid high temperatures.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Sucrose RS

Sucrose Stearate



$C_{30}H_{56}O_{12}$ 608.76

$C_{48}H_{90}O_{13}$ 875.22

$C_{66}H_{124}O_{14}$ 1141.68

Sucrose monostearate;
Sucrose octadecanoate [25168-73-4].

DEFINITION

Sucrose Stearate is a mixture of sucrose esters, mainly sucrose stearate, obtained by transesterification of stearic acid methyl esters derived from vegetable origin with sucrose. The manufacture of the fatty acid methyl esters includes a distillation step. The mono- and diesters requirements differ for the two types of sucrose stearate as set forth in the following table.

	Content of Monoesters (%)	Content of Diesters (%)	Sum of Triesters and Polyesters (%)
Type I	NLT 50.0	NMT 40.0	NMT 25.0
Type II	20.0–45.0	30.0–40.0	NMT 30.0

IDENTIFICATION

• **A.** It meets the requirements of the *Fatty Acid Composition* test.

• **B.** It meets the requirements of *Content of Monoesters, Diesters, Triesters, and Polyesters.*

ASSAY

• **CONTENT OF MONOESTERS, DIESTERS, TRIESTERS, AND POLYESTERS**

Mobile phase: Tetrahydrofuran

Sample solution: 15 mg/mL of Sucrose Stearate in tetrahydrofuran

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability.*)

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Mode: LC, size-exclusion
Detector: Differential refractometer
Column: 7-mm × 60-cm; packing L21, 100 Å. [NOTE—Two 7-mm × 30-cm L21 columns may be used in place of one 60-cm column, provided system suitability requirements are met.]
Flow rate: 1.2 mL/min
Injection size: 20 µL
Analysis
Sample: *Sample solution*
[NOTE—The relative retention time with reference to the monoester peak (retention time is approximately 10 min) is about 0.92 for diesters, and about 0.90 for triesters and polyesters.]
[NOTE—Disregard solvent peaks and peaks having a signal-to-noise ratio less than 10.]
Calculate the percentage of monoesters in the portion of Sucrose Stearate taken:

$$\text{Result} = A \times (100 - D - S - E)/100$$

A = percentage of monoesters determined by peak normalization
D = percentage of free fatty acids, obtained by AV × 284.5/561.1, where AV is the acid value
S = percentage of free sucrose (see *Free Sucrose* in *Organic Impurities*)
E = percentage of water (see *Water Determination, Ia* in *Specific Tests*)
AV = acid value

Calculate the percentage of diesters in the portion of Sucrose Stearate taken:

$$\text{Result} = B \times (100 - D - S - E)/100$$

B = percentage of diesters determined by peak normalization
D = percentage of free fatty acids (above)
S = percentage of free sucrose (see *Free Sucrose* in *Organic Impurities*)
E = percentage of water (see *Water Determination, Ia* in *Specific Tests*)

Calculate the percentage of triesters and polyesters in the portion of Sucrose Stearate taken:

$$\text{Result} = C \times (100 - D - S - E)/100$$

C = percentage of triesters and polyesters determined by peak normalization
D = percentage of free fatty acids (above)
S = percentage of free sucrose (see *Free Sucrose* in *Organic Impurities*)
E = percentage of water (see *Water Determination, Ia* in *Specific Tests*)

• **FATTY ACID COMPOSITION:** Sucrose Stearate exhibits the following composition profiles of fatty acids, as determined in *Fats and Fixed Oils* ⟨401⟩, *Fatty Acid Composition.*

Fatty Acid	Percentage (%)
Lauric acid	NMT 3.0
Myristic acid	NMT 3.0
Palmitic acid	25.0–40.0
Stearic acid	55.0–75.0

Fatty Acid	Percentage (%)
Sum of the contents of palmitic acid and stearic acid	NLT 90.0

IMPURITIES

INORGANIC IMPURITIES

• **Fats and Fixed Oils,** *Acid Value* ⟨401⟩: NMT 6, determined on a 3-g sample. Use a freshly neutralized mixture of 2-propanol and water (2:1), and gently heat.

ORGANIC IMPURITIES

• **Procedure: Free Sucrose**
Solution A: 10 µg/mL of ammonium acetate in acetonitrile
Solution B: 10 µg/mL of ammonium acetate in tetrahydrofuran and water (90:10)
Diluent: Tetrahydrofuran and water (87.5: 12.5)
System suitability solution: 500 µg/mL of USP Sucrose RS in *Diluent*
Standard solutions: 0.50, 1.0, 2.0, and 2.5 mg/mL of USP Sucrose RS in *Diluent*
Sample solution: 50 mg/mL of Sucrose Stearate in *Diluent*
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability.*)
Mode: LC
Detector: Evaporative light-scattering. [NOTE—If the detector has different setting parameters, adjust the detector settings so as to comply with the *System Suitability* requirements.]
Carrier gas: Nitrogen
Detector temperature: 45°
Nebulizer temperature: 40°
Column: 4.6-mm × 0.25-m; packing L8
Injection size: 20 µL
Mobile phase and flow rate: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)	Flow Rate (mL/min)
1	100	0	1.0
8	0	100	1.0
7	0	100	1.0
0.01	0	100	2.5
15.99	0	100	2.5
1	100	0	2.5
3	100	0	1.0

System suitability
Sample: *System suitability solution*
[NOTE—The retention time for sucrose stearate is about 26 min.]
Suitability requirements
Signal-to-noise ratio: 10:1
Analysis
Samples: *Standard solutions* and *Sample solution*
Prepare a standard curve by plotting the peak response versus concentration of sucrose in the *Standard solution.* Calculate the quantity of free sucrose in the Sucrose Stearate taken.
Acceptance criteria: NMT 4.0%

SPECIFIC TESTS

• **WATER DETERMINATION,** *Method Ia* ⟨921⟩: NMT 4.0%, on a 0.20-g sample
• **TOTAL ASH**
Analysis: Heat a silica or platinum crucible to redness for 30 min, allow to cool in a desiccator, and weigh. Transfer a 1.0-g sample into a crucible. Dry at 100°–105° for 1 h and

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ignite to constant weight in a muffle furnace at 600 ± 25°, allowing the crucible to cool in a desiccator after each ignition. Flames should not be produced at any time during the procedure. If after prolonged ignition the ash still contains black particles, add hot water, pass through an ashless filter paper, and ignite the residue and the filter paper. Combine the filtrate with the ash, carefully evaporate to dryness, and ignite to constant weight.
Acceptance criteria: NMT 1.5%

ADDITIONAL REQUIREMENTS
- **LABELING:** Label to indicate whether it is Type I or Type II.
- **PACKAGING AND STORAGE:** Preserve in a well-closed container. Protect from humidity and avoid high temperatures.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Sucrose RS

Compressible Sugar

DEFINITION
Compressible Sugar contains NLT 95.0% and NMT 98.0% of sucrose ($C_{12}H_{22}O_{11}$) on the dried basis. It may contain starch, maltodextrin, or invert sugar and may contain a suitable lubricant.

IDENTIFICATION
- **A.** It meets the requirements of the test for *Specific Rotation* in *Specific Tests*.

Change to read:

- **B.** ▲**SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

ASSAY
- **CONTENT OF SUCROSE**

Mobile phase: Acetonitrile and water (80:20, v/v)
System suitability solution: Prepare an aqueous solution containing 20 mg/mL of sucrose, 1.0 mg/mL of dextrose (glucose), 0.6 mg/mL of fructose, 0.6 mg/mL of maltose, and 0.8 mg/mL of lactose using USP Sucrose RS, USP Dextrose RS, USP Fructose RS, USP Maltose Monohydrate RS, and USP Anhydrous Lactose RS.
Standard solution: Dissolve USP Sucrose RS in water to obtain a solution having a concentration of about 20 mg/mL of sucrose.
Sample solution: 20 mg/mL of Compressible Sugar in water. Pass the solution through a 0.2-µm nylon syringe filter.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: LC
Detector: Refractive index
Column: 4.6-mm × 15-cm; 5-µm packing L8
Temperatures
Column: 45°
Detector: 40°
Flow rate: 2.0 mL/min
Injection volume: 15 µL
System suitability
Samples: *System suitability solution* and *Standard solution*
[NOTE—For relative retention times, see *Table 1*.]

Table 1

Name	Relative Retention Time
Fructose	0.5
Dextrose (glucose)	0.6
Sucrose	1.0
Maltose	1.3
Lactose	1.5

Suitability requirements
Resolution: NLT 1.3 between all adjacent peaks, *System suitability solution*
Relative standard deviation: NMT 2.0%, *Standard solution*
Analysis
Samples: *Standard solution* and *Sample solution*
Calculate, on the dried basis, the percentage of sucrose ($C_{12}H_{22}O_{11}$) in the portion of Compressible Sugar taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP Sucrose RS in the *Standard solution* (mg/mL)
C_U = concentration of Compressible Sugar in the *Sample solution* (mg/mL)

Acceptance criteria: 95.0%–98.0% on the dried basis

IMPURITIES
- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.1%
- **CHLORIDE AND SULFATE,** *Chloride* ⟨221⟩

Standard solution: 0.40 mL of 0.020 N hydrochloric acid
Sample solution: Transfer 20 g to a 100-mL volumetric flask, add 80 mL of water, shake to dissolve the sucrose, and then add water to volume. Separate the solubilized sucrose from any insoluble matter by filtration until the filtrate is clear, and use the freshly prepared, clear filtrate.
Acceptance criteria: 0.014%; a 10-mL portion of the *Sample solution* shows no more chloride than the *Standard solution*.
- **CHLORIDE AND SULFATE,** *Sulfate* ⟨221⟩

Standard solution: 0.50 mL of 0.020 N sulfuric acid
Sample solution: 25 mL of the *Sample solution* from the test for *Chloride and Sulfate* ⟨221⟩, *Chloride*
Acceptance criteria: 0.010%; the *Sample solution* shows no more sulfate than the *Standard solution*.

Change to read:

- **LIMIT OF CALCIUM**▲ (ERR 1-Jun-2018)

Sample solution: 5 mL of the *Sample solution* from the test for *Chloride and Sulfate* ⟨221⟩, *Chloride*
Analysis: To the *Sample solution* add 1 mL of ammonium oxalate TS.
Acceptance criteria: The solution remains clear for NLT 1 min.
- **LIMIT OF DEXTROSE (GLUCOSE), FRUCTOSE, MALTOSE, AND LACTOSE**

Mobile phase, System suitability solution, Sample solution, and **Chromatographic system:** Proceed as directed in the test for *Content of Sucrose* in the *Assay*.
System suitability
Sample: *System suitability solution*

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[NOTE—The relative retention times for fructose, dextrose (glucose), sucrose, maltose, and lactose are 0.5, 0.6, 1.0, 1.3, and 1.5, respectively.]

Suitability requirements

Resolution: NLT 1.3 between all adjacent peaks

Acceptance criteria: The sum of the peak areas for dextrose, fructose, maltose, and lactose from the *Sample solution* is less than one third of the sum of the peak areas for dextrose, fructose, maltose, and lactose from the *System suitability solution*, corresponding to NMT 5% for the sum of dextrose, fructose, maltose, and lactose.

SPECIFIC TESTS

- **SPECIFIC ROTATION**

Sample solution: Transfer 26.0 g of Compressible Sugar, previously dried, to a 100-mL volumetric flask. Add 0.3 mL of a saturated aqueous solution of lead acetate, shake with 90 mL of water, and dilute with water to volume. Distribute evenly on the surface of a sheet of medium-fast filter paper 8 g of chromatographic siliceous earth suitable for column partition chromatography (see *Reagents, Indicators, and Solutions—Reagents*), and filter the solution, with the aid of vacuum, discarding the first 20 mL of the filtrate.

Instrumental conditions

(See *Optical Rotation* ⟨781⟩.)

Mode: Specific rotation

Temperature: 20°

Analysis

Uninverted solution: Pipet 25 mL of the *Sample solution* into a 50-mL volumetric flask. Cool to 20°, and dilute with water to volume at 20°. Maintain a temperature of 20° for 30 min.

Acid-inverted solution: Pipet 25 mL of the *Sample solution* into a 50-mL volumetric flask. Slowly add 6 mL of dilute hydrochloric acid (1 in 2) while rotating it, dilute with water nearly to volume, and mix. Place the flask in a water bath maintained at a temperature of 60°, continuously shake the flask in the bath for 3 min, and allow the flask to remain in the bath for a total of 10 min. Immediately cool to 20° by plunging the flask into a cold bath, and dilute with water to volume at 20°. Maintain the flask at a temperature of 20° for 30 min.

Determine the specific rotation of the *Uninverted solution* and *Acid-inverted solution* at 20°.

Acceptance criteria

The specific rotation determined from the *Uninverted solution*: 62.6°–73.4°

The specific rotation determined from the *Acid-inverted solution*: Levorotatory

- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g. It meets the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli*.
- **LOSS ON DRYING** ⟨731⟩

Analysis: Dry at 105° for 4 h.

Acceptance criteria: NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **LABELING:** Label it to indicate the name and amount of any added lubricant.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Anhydrous Lactose RS
USP Compressible Sugar RS
USP Dextrose RS
USP Fructose RS
USP Maltose Monohydrate RS
USP Sucrose RS

Confectioner's Sugar

DEFINITION

Confectioner's Sugar is Sucrose ground together with corn starch to a fine powder. It contains NLT 95.0% of sucrose ($C_{12}H_{22}O_{11}$), calculated on the dried basis.

IDENTIFICATION

- **A.**

Sample solution: Transfer 20 g to a 100-mL volumetric flask, add 80 mL of water, shake to dissolve the sucrose, and then add water to volume. Separate the solubilized sucrose from the insoluble starch component by filtration until the filtrate is clear. Use the insoluble portion for the *Identification* test, and use the freshly prepared, clear filtrate in the *Impurities* tests for *Chloride and Sulfate* ⟨221⟩, *Chloride* and *Chloride and Sulfate* ⟨221⟩, *Sulfate* and for *Calcium*; and in *Specific Tests* for *Optical Rotation* ⟨781⟩, *Specific Rotation*.

Analysis: To the water slurry of the insoluble portion add iodine TS.

Acceptance criteria: A reddish-violet to deep blue color is produced.

- **B.** It meets the requirements of the test for *Optical Rotation* ⟨781⟩, *Specific Rotation* in *Specific Tests*.

ASSAY

- **CONTENT OF SUCROSE**

Mobile phase: Acetonitrile and water (80:20, v/v)

System suitability solution: Prepare an aqueous solution containing 20 mg/mL of sucrose, 1.0 mg/mL of dextrose (glucose), 0.6 mg/mL of fructose, 0.6 mg/mL of maltose, and 0.8 mg/mL of lactose using USP Sucrose RS, USP Dextrose RS, USP Fructose RS, USP Maltose Monohydrate RS, and USP Anhydrous Lactose RS.

Standard solution: Dissolve USP Sucrose RS in water to obtain a solution having a concentration of about 20 mg/mL of sucrose.

Sample solution: 20 mg/mL of Confectioner's Sugar in water. Pass the solution through a 0.2-µm nylon syringe filter.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 4.6-mm × 15-cm; 5-µm packing L8

Temperatures

Column: 45°

Detector: 40°

Flow rate: 2.0 mL/min

Injection volume: 15 µL

System suitability

Samples: *System suitability solution* and *Standard solution*

[NOTE—For relative retention times, see *Table 1*.]

Table 1

Name	Relative Retention Time
Fructose	0.5
Dextrose (glucose)	0.6
Sucrose	1.0
Maltose	1.3

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Table 1 *(continued)*

Name	Relative Retention Time
Lactose	1.5

Suitability requirements
Resolution: NLT 1.3 between all adjacent peaks, *System suitability solution*
Relative standard deviation: NMT 2.0%, *Standard solution*

Analysis
Samples: *Standard solution* and *Sample solution*
Calculate, on the dried basis, the percentage of sucrose ($C_{12}H_{22}O_{11}$) in the portion of Confectioner's Sugar taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP Sucrose RS in the *Standard solution* (mg/mL)
C_U = concentration of Confectioner's Sugar in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 95.0% of sucrose on the dried basis

IMPURITIES

- **Residue on Ignition** ⟨281⟩**:** NMT 0.08%
- **Chloride and Sulfate,** *Chloride* ⟨221⟩
  Standard solution: 0.40 mL of 0.020 N hydrochloric acid
  Sample solution: 10 mL of the clear filtrate from *Identification* test *A*
  Acceptance criteria: 0.014%; the *Sample solution* shows no more chloride than the *Standard solution.*
- **Chloride and Sulfate,** *Sulfate* ⟨221⟩
  Standard solution: 0.30 mL of 0.020 N sulfuric acid
  Sample solution: 25 mL of the clear filtrate from *Identification* test *A*
  Acceptance criteria: 0.006%; the *Sample solution* shows no more sulfate than the *Standard solution.*
- **Calcium**
  Sample solution: 5 mL of the clear filtrate from *Identification* test *A*
  Analysis: To the *Sample solution* add 5 mL of water and 1 mL of ammonium oxalate TS.
  Acceptance criteria: The *Sample solution* remains clear for NLT 1 min.

SPECIFIC TESTS

- **Microbial Enumeration Tests** ⟨61⟩ and **Tests for Specified Microorganisms** ⟨62⟩**:** The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g. It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli.*
- **Loss on Drying** ⟨731⟩
  Analysis: Dry at 105° for 4 h.
  Acceptance criteria: NMT 1.0%
- **Optical Rotation,** *Specific Rotation* ⟨781S⟩
  Sample solution: Use the clear filtrate from *Identification* test *A.*
  Acceptance criteria: NLT +62.6° corresponding to NLT 95.0% of sucrose ($C_{12}H_{22}O_{11}$), calculated on the dried basis

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in well-closed containers.
- **USP Reference Standards** ⟨11⟩
  USP Anhydrous Lactose RS
  USP Dextrose RS
  USP Fructose RS
  USP Maltose Monohydrate RS
  USP Sucrose RS

Sugar Spheres

DEFINITION

Sugar Spheres contain NLT 62.5% and NMT 91.5% of sucrose ($C_{12}H_{22}O_{11}$), calculated on the dried basis, the remainder consisting chiefly of starch. They consist of approximately spherical particles of a labeled nominal size range. They may contain color additives permitted by the FDA for use in drugs.

IDENTIFICATION

- **A.**
  Sample suspension: 1:10
  Analysis: Add iodine TS to the *Sample suspension.*
  Acceptance criteria: A violet to deep blue color is produced.
- **B.** It meets the requirements of the test for *Optical Rotation* ⟨781S⟩, *Specific Rotation* in *Specific Tests.*

ASSAY

- **Content of Sucrose**
  Mobile phase: Acetonitrile and water (80:20, v/v)
  System suitability solution: Prepare an aqueous solution containing 20 mg/mL of sucrose, 1.0 mg/mL of dextrose (glucose), 0.6 mg/mL of fructose, 0.6 mg/mL of maltose, and 0.8 mg/mL of lactose using USP Sucrose RS, USP Dextrose RS, USP Fructose RS, USP Maltose Monohydrate RS, and USP Anhydrous Lactose RS.
  Standard solution: Dissolve USP Sucrose RS in water to obtain a solution having a concentration of about 20 mg/mL of sucrose.
  Sample solution: 20 mg/mL of Sugar Spheres in water. Pass the solution through a 0.2-µm nylon syringe filter.
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability.*)
  Mode: LC
  Detector: Refractive index
  Column: 4.6-mm × 15-cm; 5-µm packing L8
  Temperatures
  Column: 45°
  Detector: 40°
  Flow rate: 2.0 mL/min
  Injection volume: 15 µL
  System suitability
  Samples: *System suitability solution* and *Standard solution*
  [NOTE—See *Table 1.*]

Table 1

Name	Relative Retention Time
Fructose	0.5
Dextrose (glucose)	0.6
Sucrose	1.0
Maltose	1.3
Lactose	1.5

Suitability requirements
Resolution: NLT 1.3 between all adjacent peaks, *System suitability solution*

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Relative standard deviation: NMT 2.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of sucrose in the portion of Sugar Spheres taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP Sucrose RS in the *Standard solution* (mg/mL)
C_U = concentration of Sugar Spheres in the *Sample solution* (mg/mL)

Acceptance criteria: 62.5%–91.5% on the dried basis

IMPURITIES

• **Residue on Ignition** ⟨281⟩
Sample: 2.0 g
Analysis: Ignite at a temperature of 700 ± 25°.
Acceptance criteria: NMT 0.25%

SPECIFIC TESTS

• **Microbial Enumeration Tests** ⟨61⟩ and **Tests for Specified Microorganisms** ⟨62⟩**:** The total aerobic microbial count does not exceed 10^2 cfu/g, and the Spheres meet the requirements of the tests for absence of *Salmonella* species, *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*.

• **Optical Rotation,** *Specific Rotation* ⟨781S⟩
Sample solution: Transfer 20 g to a 200-mL volumetric flask, add 160 mL of water, shake to dissolve the sucrose, add water to volume, and mix. Pass the solubilized sucrose solution by vacuum filtration through fine filter paper.
Acceptance criteria: +41° to +61°, corresponding to 62.5%–91.5% of sucrose ($C_{12}H_{22}O_{11}$), calculated on the dried basis

• **Loss on Drying** ⟨731⟩
Analysis: Dry at 105° for 4 h.
Acceptance criteria: NMT 4.0%

• **Particle Size Distribution Estimation by Analytical Sieving** ⟨786⟩**:** NLT 90% of it passes the coarser sieve size stated in the labeling; all of it passes the next coarser sieve size listed in *Table 1* of the chapter; and NMT 10% passes the finer sieve size stated in the labeling.

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Preserve in well-closed containers.

• **Labeling:** The label states the nominal particle size range. Label it to indicate the name and amount of any added color additives.

• **USP Reference Standards** ⟨11⟩
USP Anhydrous Lactose RS
USP Dextrose RS
USP Fructose RS
USP Maltose Monohydrate RS
USP Sucrose RS

Sulfur Dioxide

SO_2 64.06
Sulfur dioxide [7446-09-5].

DEFINITION

Sulfur Dioxide contains NLT 97.0%, by volume, of sulfur dioxide (SO_2).

[**Caution**—Sulfur Dioxide is poisonous.]

ASSAY

• **Procedure**
Sample: 100.0 mL of gaseous Sulfur Dioxide
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.1 N iodine VS
Endpoint detection: Visual
Analysis: Collect the *Sample* over mercury, and note the temperature of the *Sample* and the pressure upon it. Slowly introduce 50.0 mL of 0.1 N sodium hydroxide into the air space over the mercury, and absorb the *Sample* in the solution by shaking. When absorption is complete, transfer the solution to a 250-mL conical flask, add 3 mL of starch TS, and titrate with *Titrant* until the solution is pale blue in color.
Calculate the percentage of sulfur dioxide (SO_2) at a temperature of 0° and a pressure of 760 mm of mercury in the portion of Sulfur Dioxide taken. Each mL of 0.1 N iodine is equivalent to 1.094 mL of SO_2.
Acceptance criteria: NLT 97.0%, by volume

IMPURITIES

• **Limit of Nonvolatile Residue**
Sample: 300 g (209 mL)
Analysis: Transfer the *Sample* to a tared, 250-mL conical flask, and allow the liquid to evaporate spontaneously in a well-ventilated hood. When evaporation appears complete, blow a current of dry, filtered air through the flask until the odor of sulfur dioxide is no longer apparent.
Acceptance criteria: NMT 7.5 mg (0.0025%)

• **Sulfuric Acid**
Analysis: To the flask containing the residue obtained in the test for *Limit of Nonvolatile Residue* add 25 mL of water previously neutralized to methyl red TS. Swirl the flask, and titrate with 0.10 N sodium hydroxide.
Acceptance criteria: NMT 1.3 mL is required (about 0.002%).

SPECIFIC TESTS

• **Water Determination,** *Method I* ⟨921⟩
Sample: 3 g (2.1 mL)
Analysis: Taking precautions to avoid absorption of moisture, transfer the *Sample* to a suitable flask, and add 20 mL of anhydrous pyridine.
Acceptance criteria: NMT 2.0%

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Preserve in cylinders.
[Note—Sulfur Dioxide is used most in the form of a gas in pharmaceutical applications, and is described herein for such purposes. However, it is usually packaged under pressure, hence the preceding specifications are designed for testing it in liquid form.]

Sulfuric Acid

H_2SO_4 98.08
Sulfuric acid [7664-93-9].

DEFINITION

Sulfuric Acid contains NLT 95.0% and NMT 98.0%, by weight, of sulfuric acid (H_2SO_4).

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[**CAUTION**—When sulfuric acid is to be mixed with other liquids, always add it to the diluent, and exercise great caution.]

IDENTIFICATION

• **A. IDENTIFICATION TESTS—GENERAL,** *Sulfate* ⟨191⟩**:** Meets the requirements

ASSAY

• **PROCEDURE**

Sample solution: Place 1 mL of Sulfuric Acid in a weighed, glass-stoppered flask containing 20 mL of water, and weigh again to obtain the weight of the Sulfuric Acid. Dilute with 25 mL of water, and cool.

Titrimetric system

(See *Titrimetry* ⟨541⟩.)

Mode: Direct titration

Titrant: 1 N sodium hydroxide VS

Blank: 45 mL of water

Endpoint detection: Visual

Analysis: To the *Sample solution* add methyl orange TS, and titrate with 1 N sodium hydroxide VS. Perform a blank determination. Calculate the percentage of sulfuric acid (H_2SO_4) taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

V_S = *Titrant* volume consumed by sulfuric acid in the *Sample solution* (mL)
V_B = *Titrant* volume consumed by the *Blank* (mL)
N = *Titrant* actual normality (mEq/mL)
F = equivalency factor, 49.04 mg/mEq
W = weight of sulfuric acid in the *Sample solution* (mg)

Acceptance criteria: 95.0%–98.0%

IMPURITIES

• **RESIDUE ON IGNITION** ⟨281⟩

Sample: 22 mL (40 g)

Analysis: Evaporate the *Sample* to dryness, and ignite.

Acceptance criteria: NMT 2 mg of residue remains (0.005%).

• **CHLORIDE AND SULFATE,** *Chloride* ⟨221⟩**:** A dilution of 1.1 mL (2.0 g) in water shows no more chloride than corresponds to 0.15 mL of 0.020 N hydrochloric acid (50 ppm).

• **ARSENIC** ⟨211⟩

Test preparation: Add 1.6 mL (3.0 g) to a mixture of nitric acid and water (3:20). Evaporate the solution until dense fumes of sulfur trioxide form. Cool, and cautiously wash the solution into an arsine generating flask with 50 mL of water.

Analysis: Proceed as directed in the chapter, omitting the addition of the 20 mL of 7 N sulfuric acid.

Acceptance criteria: NMT 1 ppm

SPECIFIC TESTS

• **REDUCING SUBSTANCES**

Sample: 4.4 mL (8.0 g)

Analysis: Carefully dilute the *Sample* with 50 mL of ice-cold water, keeping the solution cold during the addition. Add 0.10 mL of 0.10 N potassium permanganate.

Acceptance criteria: The solution remains pink for 5 min.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers.

Sunflower Oil

[8001-21-6].

DEFINITION

Sunflower Oil is a refined fixed oil obtained from the seeds of the sunflower plant *Helianthus annuus* L. (Fam. Asteraceae alt. Compositae). It may contain a suitable antioxidant.

IDENTIFICATION

• **A. IDENTITY BY FATTY ACID COMPOSITION**

Analysis: Proceed as directed in the test for *Fats and Fixed Oils, Fatty Acid Composition* ⟨401⟩.

Acceptance criteria: It meets the composition profile of fatty acids in *Table 2*.

• **B. IDENTITY BY TRIGLYCERIDE PROFILE**

Analysis: Perform this test for generic oil only. Proceed as directed in *Identification of Fixed Oils by Thin-Layer Chromatography* ⟨202⟩.

Acceptance criteria: It meets the requirements in the chapter.

IMPURITIES

• **ALKALINE IMPURITIES**

Sample: 10 mL of Sunflower Oil

Analysis: Mix 10 mL of freshly opened acetone and 0.3 mL of water, and add 0.05 mL of bromophenol blue TS. Add the *Sample*, shake, and allow to stand. Titrate with 0.01 N hydrochloric acid VS to change the color of the upper layer to yellow.

Acceptance criteria: NMT 0.1 mL of 0.01 N hydrochloric acid is required.

SPECIFIC TESTS

• **FATS AND FIXED OILS,** *Acid Value (Free Fatty Acids)* ⟨401⟩**:** NMT 2.5 mL of 0.020 N sodium hydroxide is required for neutralization.

• **FATS AND FIXED OILS,** *Fatty Acid Composition* ⟨401⟩

Standard solution: Prepare an ester mixture containing methyl linoleate, methyl oleate, methyl palmitate, methyl stearate, and methyl linolenate (50:35:7:5:3).[1]

Sample solution: Transfer 100 mg of Sunflower Oil to a 50-mL conical flask fitted with a suitable water-cooled reflux condenser and a magnetic stir bar. Add 4 mL of 0.5 N methanolic sodium hydroxide solution, and reflux until fat globules disappear (usually 5–10 min). Add 5 mL of a solution prepared by dissolving 14 g of boron trifluoride in methanol to make 100 mL, swirl to mix, and reflux for 2 min. Add 4 mL of chromatographic *n*-heptane through the condenser, and reflux for 1 min. Cool, remove the condenser, add 15 mL of saturated sodium chloride solution, shake, and allow the layers to separate. Pass the *n*-heptane layer through 0.1 g of anhydrous sodium sulfate (previously washed with chromatographic *n*-heptane) into a suitable flask. Transfer 1.0 mL of this solution to a 10-mL volumetric flask, dilute with chromatographic *n*-heptane to volume, and mix.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.25-mm × 30-m fused-silica capillary column bonded with a 0.25-µm layer of phase G5

Temperatures

Injector: 220°

Detector: 250°

Column: See *Table 1*.

[1] Ester mixtures are available commercially from Nu-Chek-Prep, Inc., P.O. Box 295, Elysian, MN 56028. Typical Nu-Chek-Prep ester mixtures useful in this test include Nu-Chek 15A. This mixture may contain other components.

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Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
120	—	120	2
120	4	240	5

Carrier gas: Hydrogen
Flow rate: 1 mL/min
Injection volume: 1 µL
Injection type: Splitless
System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for methyl palmitate, methyl stearate, and methyl oleate are about 0.87, 0.99, and 1.0, respectively.]
Suitability requirements
Resolution: NLT 1.5 between methyl stearate and methyl oleate
Relative standard deviation: NMT 6.0% peak areas for the palmitate and stearate peaks for replicate injections; NMT 1.0% peak area ratio of the palmitate to stearate peaks from these replicate injections
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the areas of the five major peaks for the methyl esters of the fatty acids, which elute in the following order: palmitate, stearate, oleate, linoleate, and linolenate.
Calculate the percentage of palmitate, stearate, oleate, linoleate, and linolenate in the portion of Sunflower Oil taken:

$$\text{Result} = (A/B) \times 100$$

A = peak area of palmitate, stearate, oleate, linoleate, or linolenate
B = total area of the five major peaks

Acceptance criteria: See *Table 2*.

Table 2

	Generic Oil (%)	Mid-Oleic Oil (%)	High-Oleic Oil (%)
Methyl palmitate	3–10	2–9	2–9
Methyl stearate	2–8	2–8	2–8
Methyl oleate	14–39	40–70	70–90
Methyl linoleate	48–73	15–40	5–15
Methyl linolenate	0–3	0–3	0–3

• **FATS AND FIXED OILS,** *Iodine Value, Method II* ⟨401⟩
Acceptance criteria
Generic oil: 128–148
Mid-oleic oil: 98–118
High-oleic oil: 78–98
• **FATS AND FIXED OILS,** *Unsaponifiable Matter* ⟨401⟩**:** NMT 1.0%
• **LIMIT OF PEROXIDE**
[NOTE—This test must be performed promptly after sampling to avoid oxidation of the test specimen.]
Solvent A: Chloroform and glacial acetic acid (40:60)
Potassium iodide solution: Prepare a saturated solution of potassium iodide in freshly boiled and cooled water, and store protected from light. Discard the solution if it becomes colored upon the addition of *Solvent A* and iodide-free starch TS.
Sample: 10 g of Sunflower Oil
Analysis: Transfer the *Sample* to a conical flask, add 30 mL of *Solvent A*, and swirl to dissolve. Add 0.5 mL of *Potassium iodide solution*, swirl for 1.0 min, and add 30 mL of water. Titrate with 0.01 N sodium thiosulfate VS, with vigorous agitation, to a light yellow color. Add 2.0 mL of iodide-free starch TS, and continue the titration until the blue color has disappeared. Perform a blank determination, and make any necessary correction.
Calculate the amount of peroxide, in mEq/kg, in the portion of Sunflower Oil taken:

$$\text{Result} = [V \times (N/W)] \times F$$

V = volume of sodium thiosulfate used in the titration (mL)
N = normality of sodium thiosulfate VS (mEq/mL)
W = weight of Sunflower Oil taken (g)
F = conversion factor, 1000 g/kg

Acceptance criteria: NMT 10.0 mEq/kg
• **WATER DETERMINATION,** *Method Ic* ⟨921⟩**:** NMT 0.1%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers. No storage requirement specified.
• **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant source from which the article was derived. Label it to indicate whether it is Generic Sunflower Oil, Mid-Oleic Sunflower Oil, or High-Oleic Sunflower Oil. The label also indicates the name and concentration of any additive.

Suspension Structured Vehicle

DEFINITION
Prepare Suspension Structured Vehicle as follows (see *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩).

Potassium Sorbate	0.15 g
Xanthan Gum	0.15 g
Citric Acid, Anhydrous	0.15 g
Sucrose	20 g
Purified Water, a sufficient quantity to make	100 mL

Transfer the *Potassium Sorbate* to a suitable beaker, and dissolve in 50 mL of *Purified Water*. Place the beaker on an electric hot plate and stirrer, and add the *Xanthan Gum* into the vortex while slowly stirring. Apply minimal heat, and incorporate the *Citric Acid* and the *Sucrose*. Add a sufficient quantity of *Purified Water* to obtain a final volume of 100 mL, and mix.

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Package in tight, light-resistant containers. Store at room temperature, and avoid freezing.
• **LABELING:** Label it to state that it must be well shaken before use.
• **BEYOND-USE DATE:** NMT 30 days after the date on which it was compounded

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Sugar-Free Suspension Structured Vehicle

DEFINITION
Prepare Sugar-Free Suspension Structured Vehicle as follows (see *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩).

Xanthan Gum	0.20 g
Saccharin Sodium	0.20 g
Potassium Sorbate	0.15 g
Citric Acid	0.10 g
Sorbitol	2.0 g
Mannitol	2.0 g
Glycerin	2.0 mL
Purified Water, a sufficient quantity to make	100 mL

Transfer 30 mL of *Purified Water* to a beaker, placing it on an electric hot plate and stirrer. Using moderate heat, stir to form a vortex, and slowly sprinkle the *Xanthan Gum* into the vortex. In a separate beaker, dissolve the *Saccharin Sodium*, *Potassium Sorbate*, and *Citric Acid* in 50 mL of *Purified Water*. Using moderate heat, incorporate the *Sorbitol*, *Mannitol*, and *Glycerin* into this mixture. Add to this mixture the previously prepared xanthan gum dispersion. Add a sufficient quantity of *Purified Water* to obtain a final volume of 100 mL, and mix.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Package in tight, light-resistant containers. Store at room temperature, and avoid freezing.
- **LABELING:** Label it to state that it must be well shaken before use.
- **BEYOND-USE DATE:** NMT 30 days after the date on which it was compounded

Syrup

DEFINITION
Syrup is a solution of Sucrose in Purified Water. It contains a preservative unless it is used when freshly prepared.

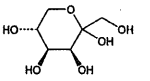
Sucrose	850 g
Purified Water, a sufficient quantity to make	1000 mL

Syrup may be prepared by the use of boiling water or, preferably, without heat, by the following process. Place the *Sucrose* in a suitable percolator, the neck of which is nearly filled with loosely packed cotton, moistened after packing with a few drops of water. Pour carefully 450 mL of *Purified Water* upon the *Sucrose*, and regulate the outflow to a steady drip of percolate. Return the percolate, if necessary, until all of the *Sucrose* has dissolved. Then wash the inside of the percolator and the cotton with sufficient *Purified Water* to bring the volume of the percolate to 1000 mL, and mix.

SPECIFIC TESTS
- **SPECIFIC GRAVITY** ⟨841⟩: NLT 1.30

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Package in tight containers, preferably in a cool place.

Tagatose



$C_6H_{12}O_6$ 180.16
D-Tagatose;
D-*lyxo*-Hexulose [87-81-0].

DEFINITION
Tagatose is a ketohexose, an epimer of D-fructose inverted at C-4. It is obtained from D-galactose by isomerization under alkaline conditions in the presence of calcium. It contains NLT 98.0% of tagatose ($C_6H_{12}O_6$), calculated on the dried basis.

IDENTIFICATION
- **A.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.
- **B.** It meets the requirements of the test for *Optical Rotation* ⟨781⟩, *Specific Rotation*.
- **C.**
  Sample solution: 200 mg/mL of Tagatose
  Analysis: Add 3 mL of the *Sample solution* to 5 mL of hot alkaline cupric tartrate TS.
  Acceptance criteria: A copious red precipitate of cuprous oxide is formed.

ASSAY
- **PROCEDURE**
  Mobile phase: 0.05 mg/mL of calcium acetate
  Standard solution: 5 mg/mL of USP Tagatose RS. Pass through a filter of 0.2-µm pore size.
  Sample solution: 5 mg/mL of Tagatose, previously dried. Pass through a filter of 0.2-µm pore size.
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: LC
  Detector: Refractive index
  Column: 7.8-mm × 30-cm; 9-µm packing L19
  Column temperature: 85°
  Flow rate: 0.6 mL/min
  Injection size: 20 µL
  System suitability
  Sample: *Standard solution*
  Suitability requirements
  Relative standard deviation: NMT 2.0% of replicate injections
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Calculate the percentage of tagatose ($C_6H_{12}O_6$) in the portion of Tagatose taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP Tagatose RS in the *Standard solution* (mg/mL)
C_U = concentration of Tagatose in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 98.0% on the dried basis

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IMPURITIES

• **LIMIT OF LEAD**

Sample solution: 2.5 g of Tagatose dissolved in a mixture of 4 mL of sulfuric acid and 5 mL of hydrochloric acid. Dilute with water to 50 mL.

Standard stock solution A: Dissolve 1.60 g of lead nitrate in diluted nitric acid (10 mL of nitric acid diluted with 20 mL water, boiled to remove nitrous fumes, and cooled), and dilute with water to 1000 mL.

Standard stock solution B: *Standard stock solution A* and water (1:50). [NOTE—This solution contains the equivalent of 20 µg/mL of lead.]

Standard solutions: To a series of 100-mL volumetric flasks pipet 0, 1, 2, 3, 4, and 5 mL of *Standard stock solution B*, and dilute with water to about 50 mL. Add 8 mL of sulfuric acid and 10 mL of hydrochloric acid to each flask, shake to dissolve, and dilute with water to volume. [NOTE—These solutions contain 0, 0.2, 0.4, 0.6, 0.8, and 1.0 µg/mL of lead, respectively.]

Instrumental conditions

(See *Atomic Absorption Spectroscopy* ⟨852⟩.)

Mode: Atomic absorption

Analytical wavelength: 283.3 nm

Analysis

Samples: *Standard solutions* and *Sample solution*

Concomitantly determine the absorbances of the *Standard solutions* and the *Sample solution*. Plot the absorbances of the *Standard solutions* versus the concentration of lead. Using this graph, determine the concentration of lead in the *Sample solution*.

Acceptance criteria: NMT 1 ppm

SPECIFIC TESTS

• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*. The total aerobic microbial count does not exceed 1000 cfu/g, and the total combined molds and yeasts count does not exceed 100 cfu/g.

• **MELTING RANGE OR TEMPERATURE,** *Class I* ⟨741⟩**:** 133°–144°

• **OPTICAL ROTATION,** *Specific Rotation* ⟨781⟩**:** –4° to –7°

Sample solution: 10 mg/mL

• **LOSS ON DRYING** ⟨731⟩**:** Dry a sample at 102° for 2 h: it loses NMT 0.5% of its weight

• **ARTICLES OF BOTANICAL ORIGIN,** *Total Ash* ⟨561⟩**:** NMT 0.1%, determined on a 1.0-g specimen

ADDITIONAL REQUIREMENTS

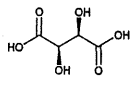
• **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at room temperature.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Tagatose RS

Talc—*see Talc General Monographs*

Tartaric Acid



$C_4H_6O_6$ 150.09

Butanedioic acid, 2,3-dihydroxy-;
Butanedioic acid, 2,3-dihydroxy-, [*R*-(*R**,*R**)]-;
Tartaric acid; L-(+)-Tartaric acid [526-83-0; 87-69-4].

DEFINITION

Tartaric Acid, dried over phosphorus pentoxide for 3 h, contains NLT 99.7% and NMT 100.5% of $C_4H_6O_6$.

IDENTIFICATION

• **A. IDENTIFICATION TESTS—GENERAL,** *Tartrate* ⟨191⟩**:** It meets the requirements.

Change to read:

• **B. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

ASSAY

• **PROCEDURE**

Sample: 2 g of Tartaric Acid, previously dried, in a conical flask

Analysis: Dissolve the *Sample* in 40 mL of water, add phenolphthalein TS, and titrate with 1 N sodium hydroxide VS. Each mL of 1 N sodium hydroxide is equivalent to 75.04 mg of $C_4H_6O_6$.

Acceptance criteria: 99.7%–100.5%

IMPURITIES

INORGANIC IMPURITIES

• **Residue on Ignition** ⟨281⟩**:** NMT 0.1%

• **Chloride and Sulfate,** *Sulfate* ⟨221⟩

Sample solution: 10 mL of a 1-in-100 solution of Tartaric Acid

Analysis: Add to the *Sample solution* 3 drops of hydrochloric acid and 1 mL of barium chloride TS.

Acceptance criteria: No turbidity is produced.

SPECIFIC TESTS

• **OPTICAL ROTATION,** *Specific Rotation* ⟨781S⟩**:** +12.0° to +13.0°

Sample solution: 200 mg/mL in water

• **LOSS ON DRYING** ⟨731⟩**:** Dry over phosphorus pentoxide for 3 h: it loses NMT 0.5% of its weight.

• **LIMIT OF OXALATE**

Sample solution: 10 mL of a 1-in-10 solution of Tartaric Acid

Analysis: Nearly neutralize the *Sample solution* with 6 N ammonium hydroxide, and add 10 mL of calcium sulfate TS.

Acceptance criteria: No turbidity is produced.

ADDITIONAL REQUIREMENTS

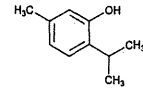
• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Tartaric Acid RS

Thimerosal—*see Thimerosal General Monographs*

Thymol



$C_{10}H_{14}O$ 150.22

Phenol, 5-methyl-2-(1-methylethyl)-;
Thymol;
p-Cymen-3-ol [89-83-8].

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DEFINITION
Thymol contains NLT 99.0% and NMT 101.0% of thymol ($C_{10}H_{14}O$).

IDENTIFICATION

Change to read:

- **A.** ▲**SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
- **B.** It meets the requirements in *Specific Tests* for *Melting Range or Temperature* ⟨741⟩.

ASSAY
- **PROCEDURE**

Sample: 100 mg

Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.1 N bromine VS
Endpoint detection: Visual

Analysis: Transfer the *Sample* to a 250-mL iodine flask, and dissolve in 25 mL of 1 N sodium hydroxide. Add 20 mL of hot dilute hydrochloric acid (1 in 2), and immediately titrate with *Titrant* to within 1–2 mL of the calculated endpoint. Warm the solution to between 70° and 80°, add 2 drops of methyl orange TS, and continue the titration slowly, swirling vigorously after each addition. When the color of the methyl orange is bleached, add 2 drops of *Titrant*, shake for 10 s, add 1 drop of methyl orange TS, and shake vigorously. If the solution is red, continue the titration, dropwise and with shaking, until the color is discharged. Repeat the alternate addition of the *Titrant* and methyl orange TS until the red color is discharged after the addition of the methyl orange TS. Each mL of *Titrant* is equivalent to 3.755 mg of thymol ($C_{10}H_{14}O$).

Acceptance criteria: 99.0%–101.0%

IMPURITIES
- **LIMIT OF NONVOLATILE RESIDUE**

Sample: 2 g
Analysis: Volatilize the *Sample* on a steam bath, and dry at 105° to constant weight.
Acceptance criteria: NMT 0.05%

SPECIFIC TESTS
- **MELTING RANGE OR TEMPERATURE** ⟨741⟩**:** 48°–51°; but when melted, Thymol remains liquid at a considerably lower temperature.

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Thymol RS

Titanium Dioxide—*see Titanium Dioxide General Monographs*

Tocopherols Excipient

DEFINITION
Tocopherols Excipient is a vegetable oil solution containing NLT 50.0% of total tocopherols, of which NLT 80.0% consists of varying amounts of beta, gamma, and delta tocopherols.

IDENTIFICATION
- **A.**

Sample solution: 50 mg of Tocopherols Excipient in 10 mL of dehydrated alcohol
Analysis: To the *Sample solution* add with swirling 2 mL of nitric acid, and heat at about 75° for 15 min.
Acceptance criteria: A bright red or orange color develops.
- **B.** The retention time of the third major peak (i.e., the peak occurring just before that of the internal standard) of the *Sample solution* corresponds to that of the *Standard solution*, both relative to that of the internal standard, as obtained in the *Assay*.

ASSAY
- **PROCEDURE**

Solution A: Pyridine and propionic anhydride (2:1)
Internal standard solution: 3 mg/mL of hexadecyl hexadecanoate in *Solution A*
Standard solutions: Using low-actinic glassware, add 12-, 25-, 37-, and 50-mg portions of USP Alpha Tocopherol RS to separate 50-mL conical flasks having 19/38 standard-taper ground-glass necks. Pipet 25 mL of the *Internal standard solution* into each flask, and reflux for 10 min under water-cooled condensers.
Sample solution: Using low-actinic glassware, add 60 mg of Tocopherols Excipient to a 50-mL conical flask similar to the flasks used in preparing the *Standard solutions*. Add 10.0 mL of *Internal standard solution*, and reflux for 10 min under a water-cooled condenser.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 4-mm × 2-m borosilicate glass; packed with 2%–5% liquid phase G2 on 80- to 100-mesh support S1AB using either a glass-lined sample introduction system or on-column injection
Temperatures
Column: 245°–265°, maintained isothermally
Injection port: 10° higher than the *Column* temperature
Detector: 10° higher than the *Column* temperature
Flow rate: Dry carrier gas is adjusted to obtain a hexadecyl hexadecanoate peak 30–32 min after sample introduction. [NOTE—Cure and condition the column as necessary.]
Injection volume: 2–5 µL
System suitability
Sample: *Sample solution*
[NOTE—The relative retention times for delta tocopheryl propionate, beta plus gamma tocopheryl propionate, and hexadecyl hexadecanoate are about 0.50, 0.63, and 1.00, respectively.]
Suitability requirements
Resolution: Chromatograph a sufficient number of injections to ensure that a resolution of NLT 2.5 between delta tocopheryl propionate and beta plus gamma tocopheryl propionate relative to hexadecyl hexadecanoate is met.
Analysis
Samples: *Standard solutions* and *Sample solution*
Calibration: Chromatograph each *Standard solution*, and calculate the relative response factor, *F*, for each concentration of the *Standard solution* taken:

$$F = (r_S/r_D) \times (C_D/C_S)$$

r_S = peak response of alpha tocopherol in the *Standard solution*
r_D = peak response of hexadecyl hexadecanoate in the *Standard solution*

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C_D = concentration of hexadecyl hexadecanoate in the *Standard solution* (mg/mL)
C_S = concentration of USP Alpha Tocopherol RS in the *Standard solution* (mg/mL)

Chromatograph a sufficient number of injections of each *Standard solution* to ensure that F is constant within a range of 2.0%. Prepare a relative response factor curve by plotting F versus the alpha tocopheryl propionate peak response.
Inject the *Sample solution*, and measure the responses for the four major peaks occurring at relative retention times of approximately 0.50, 0.63, 0.76, and 1.00, and record the values as a_δ, $a_{\beta\gamma}$, a_α, and a_D, corresponding to delta tocopheryl propionate, beta plus gamma tocopheryl propionates, alpha tocopheryl propionate, and hexadecyl hexadecanoate, respectively.
Calculate the quantity of each tocopherol form in the Tocopherols Excipient taken:

$$\text{delta tocopherol} = (V \times C_D/F) \times (a_\delta/a_D)$$

$$\text{beta plus gamma tocopherols} = (V \times C_D/F) \times (a_{\beta\gamma}/a_D)$$

$$\text{alpha tocopherol} = (V \times C_D/F) \times (a_\alpha/a_D)$$

V = volume of *Internal standard solution* used in the *Sample solution* (mL)
F = obtained from the relative response factor curve (see *Calibration*) for each of the corresponding responses for the delta, beta plus gamma, and alpha tocopheryl propionate peaks produced by the *Sample solution*

[NOTE—The relative response factor for delta tocopheryl propionate and for beta plus gamma tocopheryl propionates has been determined empirically to be the same as for alpha tocopheryl propionate.]

Acceptance criteria: NLT 50.0% of total tocopherols, of which NLT 80.0% consists of varying amounts of beta, gamma, and delta tocopherols

SPECIFIC TESTS

- **ACIDITY**

Solution A: Alcohol and ether (50%:50%). Neutralize to phenolphthalein with 0.1 N sodium hydroxide.
Sample solution: Dissolve 1.0 g of Tocopherols Excipient in 25 mL of *Solution A*.
Analysis: To the *Sample solution* add 0.5 mL of phenolphthalein TS, and titrate with 0.10 N sodium hydroxide until the solution remains faintly pink after being shaken for 30 s.
Acceptance criteria: NMT 1.0 mL of 0.10 N sodium hydroxide is required.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light. Protect with a blanket of an inert gas.
- **LABELING:** Label it to indicate the content, in mg/g, of total tocopherols and of the sum of beta, gamma, and delta tocopherols.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Alpha Tocopherol RS

Tolu Balsam Syrup

DEFINITION

Prepare Tolu Balsam Syrup as follows (see *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩).

Tolu Balsam Tincture	50 mL
Magnesium Carbonate	10 g
Sucrose	820 g
Purified Water, a sufficient quantity to make	1000 mL

Add the *Tincture* all at once to the *Magnesium Carbonate* and 60 g of the *Sucrose* in a mortar, and mix. Gradually add 430 mL of *Purified Water* with trituration, and filter. Dissolve the remainder of the *Sucrose* in the clear filtrate with gentle heating, strain the syrup while warm, and add sufficient *Purified Water* through the strainer to make the product measure 1000 mL, and mix.
Tolu Balsam Syrup may also be prepared as follows. Place 760 g of the *Sucrose* in a suitable percolator, the neck of which is nearly filled with loosely packed cotton, moistened after packing with a few drops of water. Pour the filtrate, obtained as directed in the preceding instructions, on the *Sucrose*, and regulate the outflow to a steady drip of percolate. When all of the liquid has run through, return portions of the percolate, if necessary, to dissolve all the *Sucrose*. Then pass enough *Purified Water* through the cotton to make the product measure 1000 mL, and mix.

OTHER COMPONENTS

- **ALCOHOL DETERMINATION,** *Method II* ⟨611⟩**:** 3.0%–5.0%

SPECIFIC TESTS

- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩

Sample solution: 2% of solution
Analysis: Add phenolphthalein TS, and titrate with 0.5 N alcoholic potassium hydroxide VS.
Acceptance criteria: 112–168

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Package in tight containers, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial name and, following the official name, the part of the plant source from which the article was derived.

Tolu Balsam Tincture

DEFINITION

Tolu Balsam Tincture is prepared from Tolu Balsam obtained from *Myroxylon balsamum* (L.) Harms var. balsamum (Fam. Fabaceae).
Prepare Tolu Balsam Tincture as follows (see *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩).

Tolu Balsam	200 g
Alcohol	750 mL
Alcohol, a sufficient quantity to make	1000 mL

Macerate the *Tolu Balsam* with 750 mL of *Alcohol* in a container that can be closed, and put in a warm place. Agitate it frequently during 3 days or until the soluble matter is dissolved. Transfer the mixture to a filter, and when most of the liquid has drained away, wash the residue on the filter

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with a sufficient quantity of *Alcohol*, combining the filtrates to produce 1000 mL of Tincture, and mix.

OTHER COMPONENTS

- **ALCOHOL DETERMINATION,** *Method I* ⟨611⟩: 77.0%–83.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Package in tight, light-resistant containers, and store at controlled room temperature. Avoid exposure to direct sunlight and to excessive heat.
- **LABELING:** The label states the Latin binomial name and, following the official name, the part of the plant source from which the article was derived.

Tragacanth

DEFINITION

Tragacanth is the dried gummy exudation from *Astragalus gummifer* Labill., or other Asiatic species of *Astragalus* (Fam. Leguminosae).

IDENTIFICATION

- **A.** Add 1 g to 50 mL of water: it swells and forms a smooth, nearly uniform, stiff, opalescent mucilage free from cellular fragments.

IMPURITIES

- **LEAD** ⟨251⟩: NMT 10 ppm

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩: It meets the requirements for absence of *Salmonella* species and *Escherichia coli*.
- **BOTANIC CHARACTERISTICS**

Tragacanth: It is flattened, lamellated, frequently curved fragments or straight or spirally twisted linear pieces from 0.5 to 2.5 mm in thickness. It is white to weak yellow in color, translucent, and horny in texture. Its fracture is short. It is rendered more easily pulverizable by heating to 50°. It is odorless.

Histology: Pieces of Tragacanth softened in water and mounted in water or glycerin show numerous lamellae and a few starch grains.

Powdered tragacanth: It is white to yellowish white. When examined in water mounts, it shows numerous angular fragments of mucilage with circular or irregular lamellae, and occasional starch grains up to 25 µm in diameter, mostly simple, spherical to elliptical, with occasional two- to four-compound grains, a few of the grains being swollen and more or less altered. The powder shows few or no fragments of lignified vegetable tissue (Indian gum).

- **KARAYA GUM**

Sample solution: 1 g in 20 mL of water

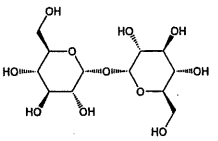
Analysis: Boil the *Sample solution* until a mucilage is formed, add 5 mL of hydrochloric acid, and again boil the mixture for 5 min.

Acceptance criteria: No pink or red color develops.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Trehalose



$C_{12}H_{22}O_{11}$ 342.30

$C_{12}H_{22}O_{11} \cdot 2H_2O$ 378.33

α-D-Glucopyranosyl α-D-glucopyranoside.

Anhydrous [99-20-7].

Dihydrate [6138-23-4].

DEFINITION

Trehalose is a stable, nonreducing disaccharide with two glucose molecules linked in an α,α-1,1 configuration. It is obtained through enzymatic conversion of food-grade starch. It contains NLT 97.0% and NMT 102.0% of trehalose ($C_{12}H_{22}O_{11}$), calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

- **A.** ▲**SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)
- **B.**

Sample solution: 400 mg/mL of Trehalose

Analysis: Add 0.4 mL of a solution containing 1-naphthol in 95% alcohol (1 in 20) to 1 mL of the *Sample solution*. Gently add 2 mL of sulfuric acid to the solution.

Acceptance criteria: A violet color develops at the interface between the two solutions.

- **C.**

Glycine solution: 40 mg/mL of glycine

Sample solution: 40 mg/mL of Trehalose

Analysis: Add 1 mL of diluted hydrochloric acid to 2 mL of the *Sample solution*. Allow to stand for 20 min at room temperature. Add 4 mL of sodium hydroxide TS and 2 mL of *Glycine solution* to the *Sample solution*. Heat the solution for 10 min in boiling water.

Acceptance criteria: A brown color does not develop.

ASSAY

- **PROCEDURE**

Mobile phase: Water

Standard solution: 10 mg/mL of USP Trehalose RS, calculated on the anhydrous basis

Sample solution: 10 mg/mL of Trehalose, calculated on the anhydrous basis

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 8-mm × 30-cm; packing L58

Temperatures

Detector: 40°

Column: 80°

Flow rate: Adjust so that the retention time of trehalose is about 15 min.

Injection volume: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

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Calculate the percentage of trehalose ($C_{12}H_{22}O_{11}$) in the portion of Trehalose taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
r_S = peak response from the *Standard solution*
C_S = concentration of USP Trehalose RS in the *Standard solution* (mg/mL)
C_U = concentration of Trehalose in the *Sample solution* (mg/mL)

Acceptance criteria: 97.0%–102.0% on the anhydrous basis

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 0.1%, determined on 2.0 g of Trehalose
- **RELATED SUBSTANCES**

Mobile phase and **Chromatographic system:** Proceed as directed in the *Assay*.
Sample solution: 10 mg/mL of Trehalose
System suitability solution: Transfer 2.5 mL of the *Sample solution*, 25 mg of maltotriose, and 25 mg of glucose to a 10-mL volumetric flask, and dilute with water to volume.
Standard solution: 0.1 mg/mL of the *Sample solution*
System suitability
Sample: *System suitability solution*
[NOTE—The relative retention times for maltotriose, trehalose, and glucose are about 0.9, 1.0, and 1.2, respectively.]
Suitability requirements
Resolution: NLT 1.5 between trehalose and maltotriose
Relative standard deviation: NMT 2.0% for the trehalose peak
Analysis
Samples: *Sample solution* and *Standard solution*
Determine the peak areas for all peaks.
Acceptance criteria: For the *Sample solution*, the areas of any peaks corresponding to maltotriose and other polysaccharrides and eluting before trehalose are NMT half of the area of the peak corresponding to trehalose in the chromatogram of the *Standard solution* (0.5%). The areas of any peaks corresponding to glucose and eluting after trehalose are NMT half of the area of the peak corresponding to trehalose in the chromatogram of the *Standard solution* (0.5%).

SPECIFIC TESTS

- **COLOR AND CLARITY OF SOLUTION**

Sample solution: 33 g of Trehalose in 67 g of recently boiled water
Analysis: Using a suitable spectrophotometer (see *Ultraviolet-Visible Spectroscopy* ⟨857⟩), measure the absorbances of the *Sample solution* at 420 and 720 nm in a 10-cm cuvette. The absorbance of the *Sample solution* at 720 nm is NMT 0.050.
Determine the absorbance difference:

$$\text{Result} = A_{420} - A_{720}$$

A_{420} = absorbance of the *Sample solution* at 420 nm
A_{720} = absorbance of the *Sample solution* at 720 nm

Acceptance criteria: The absorbance difference is NMT 0.100.

- **OPTICAL ROTATION,** *Specific Rotation* ⟨781S⟩

Sample solution: 100 mg/mL
Acceptance criteria: +197° to +201° at 20°

- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total aerobic microbial count is NMT 100 cfu/g, and the total combined molds and yeasts count is NMT 100 cfu/g. It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.
- **PH** ⟨791⟩

Sample solution: 100 mg/mL
Acceptance criteria: 4.5–6.5

- **WATER DETERMINATION,** *Method I* ⟨921⟩

Sample: 0.1 g
Acceptance criteria
Anhydrous: NMT 1.0%
Dihydrate: 9.0%–11.0%

- **BACTERIAL ENDOTOXINS TEST** ⟨85⟩**:** If labeled for use in preparing parenteral dosage forms, it also meets the following requirements. The level of bacterial endotoxins is such that the requirement in the relevant dosage form monograph(s) in which Trehalose is used can be met. Where the label states that Trehalose must be subjected to further processing during the preparation of injectable dosage forms, the level of bacterial endotoxins is such that the requirement in the relevant dosage form monograph(s) in which Trehalose is used can be met.
- **CHLORIDE AND SULFATE,** *Chloride* ⟨221⟩

Sample: 2.0 g
Acceptance criteria: No more chloride than corresponds to 0.70 mL of 0.01 M hydrochloric acid (NMT 0.0125%)

- **CHLORIDE AND SULFATE,** *Sulfate* ⟨221⟩

Sample: 2.0 g
Acceptance criteria: No more sulfate than corresponds to 0.83 mL of 0.005 M sulfuric acid (NMT 0.0200%)

- **NITROGEN DETERMINATION,** *Method II* ⟨461⟩

Sample: 5.0 g
Analysis: Proceed as directed in *Method II*, except increase the volume of sulfuric acid for digestion to 30 mL and the volume of the sodium hydroxide solution (2 in 5) to 45 mL.
Acceptance criteria: NMT 0.005%

- **SOLUBLE STARCH**

Sample solution: 10% Trehalose (w/v)
Analysis: Add several drops of iodine TS to the *Sample solution*.
Acceptance criteria: No blue color develops.

ADDITIONAL REQUIREMENTS

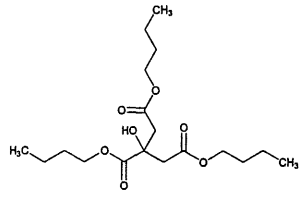
- **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.
- **LABELING:** Where Trehalose is intended for use in the manufacture of injectable dosage forms, it is so labeled. Where Trehalose must be subjected to further processing during the preparation of injectable dosage forms to ensure acceptable levels of bacterial endotoxins, it is so labeled.
- **USP REFERENCE STANDARDS** ⟨11⟩

USP Trehalose RS

Triacetin—*see Triacetin General Monographs*

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Tributyl Citrate



$C_{18}H_{32}O_7$ 360.44

DEFINITION
Tributyl Citrate contains NLT 99.0% of tributyl citrate ($C_{18}H_{32}O_7$), calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F**▲ (CN 1-May-2020)
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *System suitability solution*, as obtained in the *Assay*.

ASSAY
- **PROCEDURE**

System suitability solution: 30 mg/mL each of USP Tributyl Citrate RS and USP Acetyltributyl Citrate RS in toluene
Sample solution: 30 mg/mL of Tributyl Citrate in toluene
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC, equipped with an on-column, temperature-programmable injector
Detector: Flame ionization
Column: 0.32-mm × 30-m, bonded with a 0.5-µm layer of phase G42
Temperatures
Injector: See *Table 1*.
Detector: 275°
Column: See *Table 2*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
85	—	85	0.5
85	20	225	10

Table 2

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
80	—	80	0.5
80	20	220	10

Carrier gas: Helium
Flow rate: 2.3 mL/min
Injection volume: 1 µL
System suitability
Sample: *System suitability solution*
[NOTE—The relative retention times for tributyl citrate and acetyltributyl citrate are 0.9 and 1.0, respectively.]
Suitability requirements
Resolution: NLT 1.5 between tributyl citrate and acetyltributyl citrate
Relative standard deviation: NMT 2.0%, determined from both the tributyl citrate and acetyltributyl citrate peaks
Analysis
Sample: *Sample solution*
Calculate the percentage of tributyl citrate ($C_{18}H_{32}O_7$) in the portion of sample taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak area of tributyl citrate from the *Sample solution*
r_T = sum of all peaks excluding the solvent peak

Acceptance criteria: NLT 99.0% on the anhydrous basis

SPECIFIC TESTS
- **SPECIFIC GRAVITY** ⟨841⟩: 1.037–1.045
- **REFRACTIVE INDEX** ⟨831⟩: 1.443–1.445
- **ACIDITY**

Sample: 32.0 g
Analysis: Dissolve the *Sample* in 30 mL of isopropyl alcohol, previously neutralized to bromothymol blue. Add bromothymol blue TS, and titrate with 0.10 N sodium hydroxide to a faint blue endpoint.
Acceptance criteria: NMT 1.0 mL of 0.10 N sodium hydroxide is required.
- **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 0.2%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Acetyltributyl Citrate RS
  USP Tributyl Citrate RS

Trichloromonofluoromethane



CCl_3F 137.37
Methane, trichlorofluoro-;
Trichlorofluoromethane [75-69-4].

DEFINITION
Trichloromonofluoromethane contains NLT 99.6% and NMT 100.0% of trichloromonofluoromethane (CCl_3F), calculated on the anhydrous basis.

IDENTIFICATION
- **A.** The IR absorption spectrum, determined in a 10-cm cell with sodium chloride windows, at atmospheric pressure, exhibits maxima, among others, at the following wavelengths, in µm: 4.67 (m), 5.95 (m), 7.28 (s), 8.06 (m), 9.2 (vs), 10.7 (vs), 11.8 (vs), and 13.4 (m). The stronger maxima are best obtained at pressures less than 10 mm of mercury.

ASSAY
- **PROCEDURE**

System suitability solution: Introduce a liquid-phase mixture of dichlorodifluoromethane, dichlorotetrafluoroethane, and trichloromonofluoromethane into an evacuated headspace vial.

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Sample solution: Introduce the liquid phase of Trichloromonofluoromethane into an evacuated headspace vial.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 2-mm × 1.8-m stainless steel; 1% phase G25 on support S12
Temperatures
Injection port: 110°
Detector: 200°
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	10	170	5

Carrier gas: Helium
Flow rate: 20 mL/min
Headspace sampler: The bath temperature is 100°, the valve/loop temperature is 105°, and the sampling time is 3 s. Make adjustments as necessary to optimize peak areas to record trace-level impurities.
System suitability
Sample: Gas phase headspace of the *System suitability solution*
[NOTE—The relative retention times for dichlorodifluoromethane, dichlorotetrafluoroethane, and trichloromonofluoromethane are 0.5, 0.8, and 1.0, respectively.]
Suitability requirements
Resolution: NLT 2.0 between dichlorotetrafluoroethane and trichloromonofluoromethane
Analysis
Sample: Gas phase headspace of the *Sample solution*
Calculate the percentage of trichloromonofluoromethane (CCl_3F) in the portion of Trichloromonofluoromethane taken.
Acceptance criteria: 99.6%–100.0% on the anhydrous basis

IMPURITIES

• **INORGANIC CHLORIDES**
Sample: 7 g
Analysis: Place 5 mL of anhydrous methanol in a test tube, add 3 drops of a saturated solution of silver nitrate in anhydrous methanol, shake, and add the *Sample*.
Acceptance criteria: No opalescence or turbidity is produced.
• **CHROMATOGRAPHIC PURITY**
Analysis: In the chromatogram from the *Assay*, identify the dichlorodifluoromethane and dichlorotetrafluoroethane peaks from relative retention times of those peaks in the chromatogram of the *System suitability solution*.
Acceptance criteria
Sum of the peak areas for dichlorodifluoromethane and dichlorotetrafluoroethane: NMT 0.2% of the total of all peak areas
Sum of the areas of all peaks other than that for trichloromonofluoromethane: NMT 0.4% of the total of all peak areas.

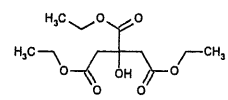
SPECIFIC TESTS

• **BOILING TEMPERATURE**
Analysis: Determine as directed in *Propellants* ⟨602⟩.
Acceptance criteria: Approximately 24°
• **WATER**
Analysis: Determine as directed in *Propellants* ⟨602⟩, and *Water Deterimination, Method Ic* ⟨921⟩.
Acceptance criteria: NMT 0.001%
• **HIGH-BOILING RESIDUES**
Analysis: Determine as directed in *Propellants* ⟨602⟩.
Acceptance criteria: NMT 0.01%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight cylinders, and avoid exposure to excessive heat.

Triethyl Citrate



$C_{12}H_{20}O_7$ 276.28

DEFINITION

Triethyl Citrate contains NLT 99.0% and NMT 100.5% of $C_{12}H_{20}O_7$, calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F**▲ (CN 1-May-2020)
• **B.** The retention time of the major peak of the *Sample solution* corresponds to that of a similar preparation of USP Triethyl Citrate RS, as obtained in the *Assay*.

ASSAY

• **PROCEDURE**
System suitability solution: 30 mg/mL each of USP Triethyl Citrate RS and USP Acetyltriethyl Citrate RS in toluene
Sample solution: 30 mg/mL of Triethyl Citrate in toluene
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.32-mm × 30-m; 0.5-µm layer of phase G42
Temperature
Injector: 225°
Detector: 275°
Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
80	—	80	0.5
80	20	220	20

Flow rate: 2.3 mL/min
Carrier gas: Helium
Injection type: Split, 30:1
Injection size: 1 µL
System suitability
Sample: *System suitability solution*
[NOTE—The relative retention times for triethyl citrate and acetyltriethyl citrate are 0.9 and 1.0, respectively.]

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Suitability requirements
Resolution: NLT 1.5 between triethyl citrate and acetyltriethyl citrate
Relative standard deviation: NMT 2.0% (determined from both the triethyl citrate and acetyltriethyl citrate peaks, based on area percentage calculation)
Analysis
Sample: *Sample solution*
[NOTE—Measure all of the peak areas, excluding the solvent peak.]
Calculate the percentage of $C_{12}H_{20}O_7$ in the portion of Triethyl Citrate taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak area for triethyl citrate
r_T = sum of the area responses of all the peaks

Acceptance criteria: 99.0%–100.5% on the anhydrous basis

SPECIFIC TESTS
- **SPECIFIC GRAVITY** ⟨841⟩: 1.135–1.139
- **REFRACTIVE INDEX** ⟨831⟩: 1.439–1.441
- **ACIDITY**

Neutralized isopropyl alcohol: To a suitable quantity of isopropyl alcohol add 2–3 drops of bromothymol blue TS and just sufficient 0.10 N sodium hydroxide dropwise to produce a faint blue color. [NOTE—Prepare *Neutralized isopropyl alcohol* just prior to use.]
Sample solution: 32.0 g of Triethyl Citrate in 30 mL of *Neutralized isopropyl alcohol*
Analysis: Add bromothymol blue TS. Titrate with 0.10 N sodium hydroxide to a faint blue endpoint.
Acceptance criteria: NMT 1.0 mL of 0.10 N sodium hydroxide is required.

- **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 0.25%

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **USP REFERENCE STANDARDS** ⟨11⟩
USP Acetyltriethyl Citrate RS
USP Triethyl Citrate RS

Medium-Chain Triglycerides

Glycerides, mixed decanoyl and octanoyl;
Caprylic and capric triglycerides.

DEFINITION
Medium-Chain Triglycerides consist of a mixture of triglycerides of saturated fatty acids, mainly of caprylic acid ($C_8H_{16}O_2$) and capric acid ($C_{10}H_{20}O_2$). The fatty acids are derived from the oil extracted from the hard, dried fraction of the endosperm of *Cocos nucifera* L. or from the dried endosperm of *Elaeis guineensis* Jacq. They contain NLT 95% of saturated fatty acids with 8 and 10 carbon atoms.

IDENTIFICATION

Delete the following:

▲• **A.** Meet the requirements in *Specific Tests* for *Fats and Fixed Oils* ⟨401⟩, *Procedures, Saponification Value*▲ (NF 1-May-2020)

Change to read:

• ▲**A.**▲ (NF 1-May-2020) *Specific TestsFats and Fixed Oils* ⟨401⟩, *Procedures, Fatty Acid Composition*

IMPURITIES
- **LIMIT OF CHROMIUM, COPPER, LEAD, AND NICKEL**

[NOTE—Use this test for Medium-Chain Triglycerides intended for use in parenteral nutrition.]
Internal standard solution: [NOTE—Prepare this solution fresh every 6 months.] Transfer 2.0 mL of a solution containing 1000 mg/L of yttrium [NOTE—Yttrium ICP standard solutions are commercially available.[1]] and 2.0 mL of a solution containing 1000 mg/L of lutetium [NOTE—Lutetium ICP standard solutions are commercially available.[2]] to a 1000-mL volumetric flask, add 10 mL of 65% ultratrace nitric acid, dilute with water to volume, and mix.
Blank standard: Transfer 1.0 mL of the *Internal standard solution* to a 100-mL volumetric flask, add 10.0 mL of 65% ultratrace nitric acid, dilute with water to volume, and mix.
Standard stock solution: Transfer 1.0 mL each of the solutions containing 1000 mg/L of chromium, copper, lead, and nickel [NOTE—Single-element ICP standard solutions are commercially available.[3]] to a 100-mL volumetric flask, add 10.0 mL of 65% ultratrace nitric acid, dilute with water to volume, and mix. [NOTE—Prepare this solution fresh monthly.] Transfer 1.0 mL of this solution to a 100-mL volumetric flask, add 10 mL of 65% ultratrace nitric acid, dilute with water to volume, and mix. The concentration of each element in this solution is 100 µg/L. [NOTE—Prepare this solution fresh weekly.]
Standard solutions: [NOTE—Prepare these solutions fresh each time.] Into two separate 100-mL volumetric flasks, transfer 1.0 mL and 5.0 mL of the *Standard stock solution*, add 1.0 mL of the *Internal standard solution* and 10.0 mL of 65% ultratrace nitric acid, dilute with water to volume, and mix well. The concentration of each element in these solutions is 1 µg/L and 5 µg/L, respectively.
Sample solution: Transfer 3.5 g of Medium-Chain Triglycerides into a suitable quartz flask. Add 5 mL of concentrated sulfuric acid[4] and heat slowly on a hot-plate in a fume hood. At the boiling heat, carefully add 5 mL of 30% hydrogen peroxide[5] in 1-mL increments. Continue heating until the solution is clear and colorless. Otherwise, add another 3 mL of concentrated sulfuric acid and continue to add more 30% hydrogen peroxide. After cooling, cautiously add about 5 mL of water dropwise. Quantitatively transfer the flask's contents into a clean, dry, 20-mL volumetric flask, dilute with water to volume, and mix. Transfer 1.0 mL of digestion solution to a 20-mL volumetric flask, add 0.2 mL of the *Internal standard solution* and 2.0 mL of 65% ultratrace nitric acid, dilute with water to volume, and mix. Retain the remaining digestion solution for use in the test for *Limit of Tin*.
Blank solution: Prepare the blank digestion solution, following the preparation procedure for the *Sample solution*, but without using Medium-Chain Triglycerides.

[1] A suitable yttrium ICP standard is available from LGC (www.lgcstandards.com) or Millipore Sigma (www.sigmaaldrich.com).
[2] A suitable lutenium ICP standard is available from LGC (www.lgcstandards.com) or Millipore Sigma (www.sigmaaldrich.com).
[3] Suitable single-element ICP standards are available from LGC (www.lgcstandards.com) or Millipore Sigma (www.sigmaaldrich.com).
[4] Suitable ultratrace concentrated sulfuric acid is available from Spectrum Chemicals (www.spectrumchemical.com) or Millipore Sigma (www.sigmaaldrich.com).
[5] Suitable ultratrace 30% hydrogen peroxide is available from Spectrum Chemicals (www.spectrumchemical.com) or Millipore Sigma (www.sigmaaldrich.com).

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Instrumental conditions
(See *Plasma Spectrochemistry* ⟨730⟩.)
Mode: ICP–MS
Spectrometer: Quadrupole mass spectrometer
Detector: Ion detector maintained under vacuum
System suitability
Samples: *Blank standard* and *Standard solutions*
Suitability requirements: Instrument performance must be verified to conform to the manufacturer's specifications for resolution and sensitivity. Before analyzing the *Sample solutions*, the instrument must pass a suitable performance check. The instrument should read all isotopes for the following elements shown in *Table 1* for the yttrium internal standard (89 amu) and the lutetium internal standard (175 amu), and should report the total element contents using the isotopes. Generate the calibration curve using the *Blank standard* and *Standard solutions* for each element. The linear regression coefficient is NLT 0.99.

Table 1

Element	Isotope (amu)
Chromium	52
Copper	63
Lead	206
Nickel	58
Tin	118

Analysis
Samples: *Blank solution* and *Sample solution*
Determine the concentration of each element in the *Blank solution* and in the *Sample solution* using the calibration curve.
Calculate the quantity, in µg/g, of each element in the portion of Medium-Chain Triglycerides taken:

$$\text{Result} = (C_U - C_B)/C_S$$

C_U = concentration of each element in the *Sample solution* (µg/L)
C_B = concentration of each element in the *Blank solution* (µg/L)
C_S = concentration of Medium-Chain Triglycerides in the *Sample solution* (g/L)

Acceptance criteria: See *Table 2*.

Table 2

Element	Acceptance Criteria, NMT (µg/g)
Chromium	0.05
Copper	0.1
Lead	0.1
Nickel	0.1

• **LIMIT OF TIN**
[NOTE—Use this test for Medium-Chain Triglycerides intended for use in parenteral nutrition.]
Internal standard solution, Sample solution, Blank solution, Instrumental conditions, System suitability, and **Analysis:** Proceed as directed in the test for *Limit of Chromium, Copper, Lead, and Nickel.*

Blank standard: Transfer 1.0 mL of the *Internal standard solution* to a 100-mL volumetric flask, add 10.0 mL of 30% hydrochloric acid,[6] dilute with water to volume, and mix.
Standard stock solution: Transfer 1.0 mL of a solution containing 1000 mg/L of tin [NOTE—Single-element ICP standard solutions are commercially available.[7]] to a 100-mL volumetric flask, add 25.0 mL of 30% hydrochloric acid, dilute with water to volume, and mix. [NOTE—Prepare this solution fresh monthly.] Transfer 1.0 mL of this solution to a 100-mL volumetric flask, add 25.0 mL of 30% hydrochloric acid, dilute with water to volume, and mix. The concentration of tin in this solution is 100 µg/L. [NOTE—Prepare this solution fresh each time.]
Standard solutions: [NOTE—Prepare these solutions fresh each time.] Into two separate 100-mL volumetric flasks, transfer 1.0 mL and 5.0 mL of the *Standard stock solution*, add 1.0 mL of the *Internal standard solution* and 25.0 mL of 30% hydrochloric acid, dilute with water to volume, and mix well. The concentration of tin in these solutions is 1 µg/L and 5 µg/L, respectively.
Acceptance criteria: NMT 0.1 µg/g

Change to read:

• **ALKALINE IMPURITIES**
Sample solution: Dissolve 2.0 g of Medium-Chain Triglycerides in a mixture of alcohol and ethyl ether (1.5:3.0).
Analysis: Add 0.05 mL of bromophenol blue TS to the *Sample solution*, and titrate with ▲0.01 N hydrochloric acid VS▲ (NF 1-May-2020) to a yellow endpoint.
Acceptance criteria: NMT 0.15 mL of 0.01 N hydrochloric acid ▲VS▲ (NF 1-May-2020) is required.

SPECIFIC TESTS

Delete the following:

▲• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Unsaponifiable Matter*
Sample: 5.0 g
Acceptance criteria: NMT 0.5%▲ (NF 1-May-2020)
• **SPECIFIC GRAVITY** ⟨841⟩: 0.93–0.96 at 20°
• **WATER DETERMINATION** ⟨921⟩, *Method I*: NMT 0.2%
• **APPEARANCE**
Diluent: Hydrochloric acid and water (2.75%:97.25%)
Sample: 10 mL
Standard solution: Prepare immediately before use by mixing 2.4 mL of ferric chloride CS and 0.6 mL of cobaltous chloride CS with *Diluent* to make 10.0 mL, and diluting 5.0 mL of the solution with *Diluent* to make 10.0 mL.
Analysis: Compare the *Sample* and the *Standard solution* by viewing them downward in matched color–comparison tubes against a white surface (see *Color and Achromicity* ⟨631⟩).
Acceptance criteria: The *Sample* is clear and not more intensely colored than the *Standard solution*.
• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Acid Value*: NMT 0.2
• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Fatty Acid Composition*: The fatty acid fraction of Medium-Chain Triglycerides exhibits the following composition as seen in *Table 3*. Disregard any peak with an area less than 0.05% of the total area.

[6] Suitable ultratrace 30% hydrochloric acid is available from Spectrum Chemicals (www.spectrumchemical.com), Millipore Sigma (www.sigmaaldrich.com), WVR (us.vwr.com/store/) or other vendors.
[7] A suitable tin ICP standard is available from LGC (www.lgcstandards.com) or Millipore Sigma (www.sigmaaldrich.com).

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Table 3

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
6	0	≤2.0
8	0	50.0–80.0
10	0	20.0–50.0
12	0	≤3.0
14	0	≤1.0

- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Hydroxyl Value*: NMT 10
- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Iodine Value*: NMT 1.0
- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Peroxide Value*: NMT 1.0

Delete the following:

- **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Saponification Value*
  Sample: 1.0 g
  Acceptance criteria: 310–360 (NF 1-May-2020)
- **VISCOSITY—CAPILLARY METHODS** ⟨911⟩
  Analysis: Determine at 20 ± 0.1° with a capillary viscometer.
  Acceptance criteria: 25–33 mPa · s

Delete the following:

- **REFRACTIVE INDEX** ⟨831⟩: 1.440–1.452 at 20° (NF 1-May-2020)
- **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Methods of Analysis, Total Ash*
  Sample: 2.0 g
  Acceptance criteria: NMT 0.1%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light. Store at temperatures not exceeding 25°.

Change to read:

- **LABELING:** Where Medium-Chain Triglycerides is intended for use in the manufacture of injectable dosage forms or parenteral nutrition therapy, it is so labeled. (NF 1-May-2020)

Trolamine

$C_6H_{15}NO_3$ 149.19
Ethanol, 2,2′,2″-nitrilotris-;
2,2′,2″-Nitrilotriethanol [102-71-6].

DEFINITION

Trolamine is a mixture of alkanolamines consisting largely of triethanolamine [$N(C_2H_4OH)_3$] containing some diethanolamine [$NH(C_2H_4OH)_2$] and monoethanolamine [$NH_2(C_2H_4OH)$]. It contains NLT 99.0% and NMT 107.4% of alkanolamines, calculated on the anhydrous basis as $N(C_2H_4OH)_3$.

IDENTIFICATION

Change to read:

- **A. SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F** (CN 1-May-2020)
- **B.**
  Analysis 1: To 1 mL add 0.1 mL of cupric sulfate TS.
  Acceptance criteria 1: A deep blue color is produced.
  Analysis 2: Add 5 mL of 1 N sodium hydroxide, and concentrate to one-third of the original volume by boiling.
  Acceptance criteria 2: The blue color remains.
- **C.**
  Analysis: To 1 mL add 0.3 mL of cobaltous chloride TS.
  Acceptance criteria: A carmine red color is produced.

ASSAY

- **PROCEDURE**
  Sample: 2 g of Trolamine
  Analysis: Transfer the *Sample* to a 300-mL conical flask. Add 75 mL of water and 2 drops of methyl red TS, and titrate with 1 N hydrochloric acid VS. Each mL of 1 N hydrochloric acid is equivalent to 149.2 mg of triethanolamine, expressed as $N(C_2H_4OH)_3$.
  Acceptance criteria: 99.0%–107.4% on the anhydrous basis

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.05%

SPECIFIC TESTS

- **SPECIFIC GRAVITY** ⟨841⟩: 1.120–1.128
- **REFRACTIVE INDEX** ⟨831⟩: 1.481–1.486 at 20°
- **WATER DETERMINATION,** *Method I* ⟨921⟩: NMT 0.5%, using a mixture of glacial acetic acid and methanol (1:4) as the solvent

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Trolamine RS

Tromethamine—*see Tromethamine General Monographs*

Tyloxapol—*see Tyloxapol General Monographs*

Vanilla

DEFINITION

Vanilla is the cured, full-grown, unripe fruit of *Vanilla planifolia* Andrews, often known in commerce as Mexican, Bourbon, or Madagascar vanilla, or of *Vanilla tahitensis* J.W. Moore, known in commerce as Tahitian vanilla (Fam. Orchidaceae). Vanilla yields NLT 12.0% of anhydrous, diluted alcohol-soluble extractive.

COMPOSITION

- **CONTENT OF ANHYDROUS, DILUTED ALCOHOL-SOLUBLE EXTRACTIVE**
  Sample: 2 g of Vanilla
  Analysis: Place the *Sample*, finely cut or coarsely powdered and accurately weighed, in a suitable flask. Add 70 mL of diluted alcohol, shake by mechanical means for 2 h or for 8 h at 30-min intervals, and allow to stand overnight. Decant the liquid into a filter, and wash the flask and residue with small portions of diluted alcohol, passing the washings through the filter until the filtrate measures 100.0 mL. Mix

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the filtrate well, evaporate a 50.0-mL portion in a suitable tared container on a steam bath to dryness, and dry the residue at 105° for 4 h. The weight obtained represents the yield of anhydrous, diluted alcohol-soluble extractive from one-half of the portion of Vanilla taken. Calculate the yield for the entire *Sample* taken.

Acceptance criteria: NLT 12.0%

CONTAMINANTS

• **ARTICLES OF BOTANICAL ORIGIN** ⟨561⟩, *Pesticide Residue Analysis*: Meets the requirements

SPECIFIC TESTS

• **BOTANICAL CHARACTERISTICS**

Unground Vanilla

Macroscopic: Linear, flattened capsules of 12–35 cm in length and 5–9 mm in width, with an apex terminating in a flat, circular scar and a gradually tapering base that is more or less curved or hooked; or in Tahitian vanilla, broad in the middle and tapering toward either end, the base closely resembling the summit. It is flexible and tough, nearly black, dusky brown to moderate brown externally, longitudinally wrinkled, moist, glossy, and occasionally has efflorescence of acicular or prismatic crystals of vanillin. Internally it is unilocular, with a brownish-black pulp and numerous minute seeds. Occasional capsules are split near the summit into three parts.

Microscopic: The epidermis has a distinct cuticle and occasional stomata. The epidermal cells contain red to brown bodies and occasional prisms of calcium oxalate or crystals of vanillin. It has a collenchyma layer of one or two rows of cells, a thick sarcocarp composed of parenchyma and an interrupted circle of fibrovascular bundles, the latter leptocentric with a few vessels, and an outer circle of fibers with thin, strongly lignified walls and numerous transverse simple pits. The vessels with walls have slit-like pits or spiral thickenings; the parenchyma cells are usually thin-walled and deeply undulate, some thick-walled with oblique, slit-like pits or broad spiral bands, and contain occasional bundles of acicular crystals of calcium oxalate, up to 400 µm in length, or a thin protoplasmic layer enclosing numerous oil globules. It has an endocarp composed of placental and interplacental regions; the placental region consists of six bifid placentas extending into the cavity of the fruit and bears irregularly trianguloid, black to reddish, flattened seeds, up to about 250 µm in diameter, having a deeply reticulate seed coat; the interplacental regions show long, nearly straight hairs more or less matted together by their gummy, resinous secretion.

Powdered Vanilla

Macroscopic: Dusky brown to nearly black

Microscopic: The principal elements of identification are fragments of parenchyma of the sarcocarp with long, oblique, slit-like walls or broad spiral bands, calcium oxalate crystals of acicular outline and up to 400 µm in length, and monoclinic prisms up to 35 µm in length. It has numerous unicellular, nearly straight, glandular hairs, fragments of the seed coat with polygonal stone cells, and slender crystals of vanillin.

• **TEST FOR VANILLIN**

Analysis: Place a few of the crystals, occurring as an efflorescence on the fruit, on a microslide or watch glass, and add 1 drop of phloroglucinol TS and 1 drop of hydrochloric acid.

Acceptance criteria: The solution immediately acquires a red color.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers, and store in a cold place.

• **LABELING:** The label states the Latin binomial and, following the official name, the part(s) of the plant contained in the article. The commercial variety of Vanilla, whether Mexican, Bourbon, Madagascar, or Tahitian, is also stated on the label. The label states that Vanilla that has become brittle is not to be used.

Vanilla Tincture

DEFINITION

Prepare Vanilla Tincture as follows (see *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩).

Vanilla, cut into small pieces	100 g
Purified Water	200 mL
Alcohol	207 mL
Sucrose, in coarse granules	200 g
Diluted Alcohol, a sufficient quantity to make	1000 mL

Add *Purified Water* to the comminuted *Vanilla* in a suitable covered container, and macerate for 12 h, preferably in a warm place. Add *Alcohol* to the mixture, mix, and macerate for about 3 days. Transfer the mixture to a percolator containing *Sucrose*, and drain. Pack the drug firmly, and percolate slowly, using *Diluted Alcohol* as the menstruum.

OTHER COMPONENTS

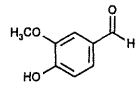
• **ALCOHOL DETERMINATION,** *Method I* ⟨611⟩: 38.0%–42.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Package in tight, light-resistant containers, and avoid exposure to direct sunlight and excessive heat.

• **LABELING:** The label states the Latin binomial name and, following the official name, the part of the plant source from which the article was derived.

Vanillin



$C_8H_8O_3$ 152.15

Benzaldehyde, 4-hydroxy-3-methoxy-;
Vanillin [121-33-5].

DEFINITION

Vanillin contains NLT 97.0% and NMT 103.0% of vanillin ($C_8H_8O_3$), calculated on the dried basis.

IDENTIFICATION

Change to read:

• **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

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Change to read:

• **B. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Ultraviolet-Visible Spectroscopy*: **197U**▲ (CN 1-May-2020)
Sample solution: 8 µg/mL in methanol
Acceptance criteria: Meets the requirements

ASSAY
• **PROCEDURE**
Standard solution: 8 µg/mL of USP Vanillin RS in methanol
Sample solution: 8 µg/mL of Vanillin in methanol
Blank: Methanol
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: UV
Analytical wavelength: 308 nm
Cell: 1 cm
Analysis
Samples: *Standard solution*, *Sample solution*, and *Blank*
Calculate the percentage of vanillin ($C_8H_8O_3$) in the portion of Vanillin taken:

$$\text{Result} = (A_U/A_S) \times (C_S/C_U) \times 100$$

A_U = absorbance of the *Sample solution*
A_S = absorbance of the *Standard solution*
C_S = concentration of USP Vanillin RS in the *Standard solution* (µg/mL)
C_U = concentration of the *Sample solution* (µg/mL)

Acceptance criteria: 97.0%–103.0% on the dried basis

IMPURITIES
• **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.05%

SPECIFIC TESTS
• **MELTING RANGE OR TEMPERATURE** ⟨741⟩: 81°–83°
• **LOSS ON DRYING** ⟨731⟩: Dry the sample over silica gel for 4 h: it loses NMT 1.0% of its weight.

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Vanillin RS

Hydrogenated Vegetable Oil

DEFINITION

Change to read:

Hydrogenated Vegetable Oil is a mixture of triglycerides of fatty acids. ▲The melting range, iodine value, and saponification value differ,▲ (ERR 1-May-2018) depending on Type, as described in the table below.

	Type I	Type II
Melting Range or Temperature ⟨741⟩, *Procedures, Procedure for Class II*	57°–85°	20°–50°
▲▲ (Official 1-Jan-2018)	▲▲ (Official 1-Jan-2018)	▲▲ (Official 1-Jan-2018)
Fats and Fixed Oils ⟨401⟩, *Procedures, Iodine Value, Method II*	0–5	55–80
Fats and Fixed Oils ⟨401⟩, *Procedures, Saponification Value*	175–200	175–200

▲If the botanical source is identified as palm or soybean and the type as Type I, compliance with Hydrogenated Palm Oil NF or Hydrogenated Soybean Oil NF is required.▲ 2S (NF36)

IDENTIFICATION

Add the following:

▲• **A.** It meets the requirements for *Fats and Fixed Oils* ⟨401⟩, *Procedures, Iodine Value*.▲ 2S (NF36)

Add the following:

▲• **B.** It meets the requirements for *Melting Range or Temperature* ⟨741⟩.▲ 2S (NF36)

SPECIFIC TESTS
• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry a sample at 105° for 4 h.
Acceptance criteria: NMT 0.1%
• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Acid Value*
Sample: 20 g in a conical flask
Analysis: Melt the *Sample* on a steam bath, add 100 mL of hot alcohol previously neutralized with 0.1 N sodium hydroxide to phenolphthalein TS, swirl, and add 1 mL of phenolphthalein TS. Titrate with 0.10 N sodium hydroxide until the solution remains faintly pink after being shaken for 15 s.
Acceptance criteria: NMT 4.0
• **FATS AND FIXED OILS** ⟨401⟩, *Procedures, Unsaponifiable Matter*: NMT 0.8%

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in tight containers, in a cool place.

Change to read:

• **LABELING:** ▲Label it to indicate the botanical source of vegetable oil.▲ 2S (NF36) Label it to state whether it is Type I or Type II.

Vitamin E Polyethylene Glycol Succinate

DEFINITION
Vitamin E Polyethylene Glycol Succinate is a mixture formed by the esterification of *d*-alpha tocopheryl acid succinate and polyethylene glycol. The ester mixture consists primarily of mono-esterified polyethylene glycol and a small amount of di-esterified polyethylene glycol. It contains NLT 25.0% of *d*-alpha tocopherol ($C_{29}H_{50}O_2$).

IDENTIFICATION
• **A. GAS CHROMATOGRAPHIC IDENTIFICATION TEST**
Analysis: Proceed as directed in the test for *Content of Alpha Tocopherol*.
Acceptance criteria: The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*.

COMPOSITION
• **CONTENT OF ALPHA TOCOPHEROL**
Solvent: 0.25 mL of phenolphthalein TS in 1 L of alcohol
Internal standard solution: 12 mg/mL of ethyl arachidate in isooctane
Standard solution: Transfer 32.5 mg of USP Alpha Tocopherol RS to a suitable reaction flask. Add 2 mL of pyridine and 0.5 mL of *N,O*-bis(trimethylsilyl)trifluor

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oacetamide with 1% trimethylchlorosilane, and heat the flask at 100° for 10 min. Cool the flask, add 5.0 mL of *Internal standard solution* followed by 20 mL of isooctane, and shake.

Sample solution: Transfer a quantity equivalent to 0.100–0.160 g of Vitamin E Polyethylene Glycol Succinate molten at 60° to a culture tube (about 20 cm long and 2.5 cm in diameter) equipped with a screw cap. Add 40–50 mg of ascorbic acid and a few boiling chips, followed by 20 mL of *Solvent*. [NOTE—Reflux the solution gently without emission of contents.] Place the tube in a heating block set at 100°–150°. When the sample is fully dissolved, add 0.25 g of potassium hydroxide, and continue to reflux for 30 min. Remove the tube from heat, and while contents are still hot, add 1–2 mL of hydrochloric acid dropwise until the pink coloration disappears. [**CAUTION**—Exothermic reaction. Allow the acid to trickle down the inside of the tube to prevent splashing.] Cool the tube, then wash the sides of the tube with 20 mL of water. Add 5.0 mL of *Internal standard solution,* cap, and shake to ensure thorough mixing. Allow the tube to stand until two distinct layers are formed. Transfer 2.5–3.5 mL of the upper layer into a suitable reaction flask, and add 2.0 mL of pyridine followed by 2.5 mL of *N,O*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane. Heat the flask at 100° for 10 min. Cool, and then add 12 mL of isooctane.

Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.25-mm × 15-m fused-silica capillary; coated with a 0.25-µm film of phase G27
Temperature
Injector: 280°
Detector: 345°
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
260	20	340	1

Carrier gas: Helium
Flow rate: 1.5 mL/min
Injection size: 1 µL
Injection type: Split ratio, 200:1

System suitability
Sample: *Standard solution*
Suitability requirements
Tailing factor: NMT 2.0 for the alpha tocopherol peak
Relative standard deviation: NMT 2.0% for the ratio of the alpha tocopherol peak area to the internal standard peak area

Analysis
Samples: *Standard solution* and *Sample Solution*
Calculate the percentage of *d*-alpha tocopherol ($C_{29}H_{50}O_2$) in the portion of Vitamin E Polyethylene Glycol Succinate taken:

$$\text{Result} = (R_U/R_S) \times (W_S/W_U) \times 100$$

R_U = internal standard ratio (peak area of alpha tocopherol/peak area of the internal standard) from the *Sample solution*
R_S = internal standard ratio (peak area of alpha tocopherol/peak area of the internal standard) from the *Standard solution*
W_S = weight of USP Alpha Tocopherol RS used to prepare the *Standard solution* (mg)
W_U = weight of Vitamin E Polyethylene Glycol Succinate taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 25.0%

SPECIFIC TESTS

• **OPTICAL ROTATION,** *Specific Rotation* ⟨781S⟩
[NOTE—This test identifies *d*-alpha tocopherol after saponification.]
Sample solution: Transfer 0.9 g of Vitamin E Polyethylene Glycol Succinate, molten at 60°, to a suitable test tube fitted with a cap, and dissolve in 10.0 mL of alcohol. Place the tube in a heating block set at 100°–105°. [NOTE—Reflux the solution gently without emission of contents.] When the sample is fully dissolved, add 2–3 pellets of sodium hydroxide, and continue to reflux for an additional 30 min. Remove the tube from the heat, and while contents are still hot, neutralize using phenolphthalein as the indicator by slowly adding 10 mL of a mixture of water and hydrochloric acid (1:1) until the pink color disappears. [**CAUTION**—Exothermic reaction. Allow the acid solution to trickle down the inside of the tube to prevent splashing.] Cool the tube, cap, and shake until contents are well mixed. Add 25.0 mL of heptane, cap, and shake for 1 min to ensure thorough mixing. Allow the tube to stand until two distinct layers are formed. Transfer the top layer to a clean, dry culture tube, then add 10.0 mL of water to the recovered solution. Cap, shake, and allow the layers to separate. Transfer the upper layer to a clean, dry tube. Add 10.0 mL of potassium ferricyanide solution, prepared by dissolving 2 g of potassium ferricyanide in 10.0 mL of 0.2 M sodium hydroxide, and replace the cap. Shake vigorously for 45 s, and allow the layers to separate for 30 min. If the top heptane layer is clear, proceed with the measurement for specific rotation; if not clear, dry over anhydrous sodium sulfate before proceeding with the test. [NOTE—Use the results of the test for *Content of Alpha Tocopherol* to calculate the specific rotation.]
Acceptance criteria: NLT +24.0°

• **SOLUBILITY IN WATER**
Sample: 20 g of melted Vitamin E Polyethylene Glycol Succinate
Analysis: Place the *Sample* in a glass container on a magnetic stirrer. Immediately add 80 mL of boiling water while stirring. Allow to cool to room temperature with constant stirring.
Acceptance criteria: The solution becomes clear within 3 h.

• **ACID VALUE**
Sample: 1 g of Vitamin E Polyethylene Glycol Succinate
Analysis: Dissolve the *Sample* in 25 mL of a mixture of alcohol and ether (1:1) that has been neutralized to phenolphthalein with 0.1 N sodium hydroxide. Add 0.5 mL of phenolphthalein TS, and titrate with 0.10 N sodium hydroxide until the solution remains faintly pink after shaking for 30 s.
Acceptance criteria: NMT 0.027 mEq/g, equivalent to NMT 0.27 mL of 0.10 N sodium hydroxide

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers, and store protected from light.
• **LABELING:** The labeling indicates the *d*-alpha tocopherol content, expressed in mg/g.
• **USP REFERENCE STANDARDS** ⟨11⟩
USP Alpha Tocopherol RS

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Water for Injection—*see Water for Injection General Monographs*

Water for Injection, Sterile—*see Sterile Water for Injection General Monographs*

Water for Irrigation, Sterile—*see Sterile Water for Irrigation General Monographs*

Water, Purified
—*see Purified Water General Monographs*

Carnauba Wax

DEFINITION
Carnauba Wax is obtained from the leaves of *Copernicia cerifera* Mart. (Fam. Palmae).

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197F** or **197A**▲ (CN 1-May-2020)

IMPURITIES
- **RESIDUE ON IGNITION** ⟨281⟩
  Sample: 2 g
  Analysis: Heat the *Sample* in an open porcelain or platinum dish over a flame; it volatilizes without emitting an acrid odor. Ignite.
  Acceptance criteria: The weight of the residue is NMT 5 mg, corresponding to NMT 0.25%.

SPECIFIC TESTS
- **MELTING RANGE OR TEMPERATURE** ⟨741⟩, *Procedures, Procedure for Class II* : 80°–86°
- **ACID VALUE**
  Sample: 3 g
  Analysis: Weigh the *Sample* into a 250-mL flask attached to a reflux condenser. Add 50 mL of a mixture of isopropyl alcohol and toluene (5:4), and boil gently until the wax is completely dissolved. Remove the flask from the condenser, add 1 mL of phenolphthalein TS, and immediately titrate with 0.5 N alcoholic potassium hydroxide VS to a faint, reddish-yellow color. [NOTE—Do not allow the solution to cool. Titrate at warm temperature after refluxing.]
  Calculate the acid value as the number of milligrams of potassium hydroxide required to neutralize the free acids in 1 g of Carnauba Wax.
  Acceptance criteria: 2–7
- **FATS AND FIXED OILS** ⟨401⟩, *Saponification Value*
  Sample: Use the solution from the test for *Acid Value*.
  Analysis: To the *Sample* add 15.0 mL of 0.5 N alcoholic potassium hydroxide VS, reflux for 3 h, and titrate the excess alkali with 0.5 N hydrochloric acid VS to a yellow-amber color. Perform a blank determination (see *Titrimetry* ⟨541⟩, *Residual Titrations*).
  Calculate the ester value. The saponification value is the sum of the ester value and the acid value.
  Acceptance criteria: 78–95

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP Carnauba Wax RS

Emulsifying Wax

DEFINITION
Emulsifying Wax is a waxy solid prepared from Cetostearyl Alcohol containing a polyoxyethylene derivative of a fatty acid ester of sorbitan.

SPECIFIC TESTS
- **MELTING RANGE OR TEMPERATURE** ⟨741⟩
  Sample: Emulsifying Wax
  Analysis: Melt a quantity of the *Sample* slowly, while stirring, until it reaches a temperature of 90°–92°. Remove the source of the heat, and allow the molten substance to cool to a temperature of 8°–10° above the expected melting point. Chill the bulb of a suitable thermometer (see *General Notices, 6.80.30. Temperature Reading Devices*) to 5°, wipe it dry, and while it is still cold, dip it into the molten substance so that the bulb is completely covered. Withdraw it immediately, and hold it vertically away from the heat until the surface dulls. Fix the thermometer securely in a test tube so that the lower point is 15 mm from the bottom of the test tube. Place the test tube in a water bath at 10°–15°, and allow it to remain at that temperature for 30 min. Raise the temperature of the bath at the rate of 2°/min to 30°, then change to a rate of 1°/min, and note the temperature at which the first drop of melted substance leaves the thermometer. Repeat the determination twice on a freshly melted portion of the sample substance. If the variation of three determinations is less than 1°, take the average of the three as the melting point. Otherwise, make two additional determinations, and take the average of the five.
  Acceptance criteria: 50°–54°
- **FATS AND FIXED OILS,** *Hydroxyl Value* ⟨401⟩: 178–192
- **FATS AND FIXED OILS,** *Iodine Value* ⟨401⟩: NMT 3.5
- **FATS AND FIXED OILS,** *Saponification Value* ⟨401⟩: NMT 14
- **PH** ⟨791⟩
  Sample dispersion: Heat 3 g of Emulsifying Wax in 100 mL of water to 55°, with stirring, followed by cooling to 25°.
  Acceptance criteria: 5.5–7.0

ADDITIONAL REQUIREMENTS
- **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Microcrystalline Wax

DEFINITION
Microcrystalline Wax is a mixture of straight-chain, branched-chain, and cyclic hydrocarbons, obtained by solvent fractionation of the still bottom fraction of petroleum by suitable dewaxing or deoiling means.

IMPURITIES
- **RESIDUE ON IGNITION** ⟨281⟩
  Sample: 2 g
  Analysis: Heat the *Sample* in an open porcelain or platinum dish over a flame.

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Acceptance criteria: It volatilizes without emitting an acrid odor and on ignition yields NMT 0.1%.

SPECIFIC TESTS

- **COLOR**

Standard solution: Mix 3.8 mL of ferric chloride CS and 1.2 mL of cobaltous chloride CS in a clear-glass, 16- × 150-mm bacteriological test tube.

Sample solution: Melt 10 g of Microcrystalline Wax on a steam bath, and pour 5 mL of the liquid into a clear-glass, 16- × 150-mm bacteriological test tube.

Analysis: Visually compare the contents of both tubes in reflected light against a white background, holding the tubes directly against the background at such an angle that there is no fluorescence.

Acceptance criteria: The *Sample solution* is not darker than the *Standard solution*.

- **MELTING RANGE OR TEMPERATURE,** *Class III* ⟨741⟩**:** 54°–102°
- **CONSISTENCY**

Sample: Microcrystalline Wax

Apparatus: Determine the consistency of the *Sample* by means of a penetrometer fitted with a polished metal needle weighing 2.5 ± 0.05 g and having a truncated symmetric tapered angle of 9°0′ ± 15′. The needle is tapered, with a length of 25.4 mm, and the shaft attached to the needle is 58 mm in length and 3.17 mm in diameter. The plunger that fits into the penetrometer and guides the path of the needle weighs 47.5 ± 0.05 g. An additional weight of 50 ± 0.05 g is added to the top of the plunger to give a total load of 100 g.

Analysis: The *Sample* is cast in a brass cylinder open at both ends. The cylinder has an inside diameter of 25.4 mm and is 31.8 mm in height. Place the cylinder on a brass plate wetted with an equal volume mixture of glycerin and water, and place the plate on two corks. Pour the wax, melted at approximately 17° above its congealing point, into the cylinder. Continue pouring the wax until a convex meniscus is formed above the cylinder. Allow the specimen to cool for 1 h at approximately 24°. Shave excess wax from the top of the cylinder, and remove the plate. With the smooth wax surface in the up position, condition the specimen in a water bath at 25° for 1 h.

Arrange the penetrometer so that the wax specimen is completely immersed in the water bath while penetration is run. Lower the needle until the tip just touches the top surface of the specimen. Release the needle for 5 s, and read the depth of penetration in tenths of millimeters. Perform four determinations, and calculate the average value of the four readings.

Acceptance criteria: 3–100 (0.3–10.0 mm)

- **ORGANIC ACIDS**

Sample solution: 20 g of Microcrystalline Wax in 100 mL of a mixture of neutralized alcohol and water (1:2). Agitate thoroughly, and heat to boiling.

Analysis: To the *Sample solution* add 1 mL of phenolphthalein TS, and titrate rapidly with 0.1 N sodium hydroxide VS, with vigorous agitation, to a sharp pink endpoint in the alcohol–water layer.

Acceptance criteria: NMT 0.4 mL of 0.1 N sodium hydroxide is required.

- **FIXED OILS, FATS, AND ROSIN**

Sample: 10 g

Sample solution: Digest the *Sample* with 50 mL of sodium hydroxide solution (1 in 5) at 100° for 30 min. Separate the water layer, and acidify it with 2 N sulfuric acid.

Acceptance criteria: No oily or solid matter separates.

- **ALKALINITY**

Sample: 35 g

Analysis: Introduce the *Sample* into a 250-mL separator, add 100 mL of boiling water, and shake vigorously for 5 min. Draw off the separated water into a casserole, wash further with two 50-mL portions of boiling water, and add the washings to the casserole. To the pooled washings add 1 drop of phenolphthalein TS, and boil.

Acceptance criteria: The solution does not acquire a pink color.

- **ACIDITY**

Analysis: If the addition of phenolphthalein TS in the test for *Alkalinity* produces no pink color, add 0.1 mL of methyl orange TS.

Acceptance criteria: No red or pink color is produced.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **LABELING:** Label it to indicate the name and proportion of any added stabilizer.

White Wax

DEFINITION

White Wax is the product of bleaching and purifying Yellow Wax that is obtained from the honeycomb of the bee [*Apis mellifera* L. (Fam. Apidae)] and that meets the requirements of the *Saponification Cloud Test*.

SPECIFIC TESTS

- **SAPONIFICATION CLOUD TEST**

Sample: 3.00 g

Alcoholic potassium hydroxide: Dissolve 40 g of potassium hydroxide in about 900 mL of aldehyde-free alcohol maintained at a temperature not exceeding 15°, and then when solution is complete, warm to room temperature, and add aldehyde-free alcohol to make 1000 mL.

Analysis: Place the *Sample* in a 100-mL round-bottom boiling flask fitted with a ground-glass joint. Add 30 mL of *Alcoholic potassium hydroxide*. Reflux the mixture gently for 2 h. At the end of this period, open the flask, insert a thermometer into the solution, and place the flask in a container of water at a temperature of 80°. Rotate the flask in the bath while both the bath and the solution cool.

Acceptance criteria: The solution shows no cloudiness or globule formation before the temperature reaches 65°.

- **MELTING RANGE OR TEMPERATURE,** *Class II* ⟨741⟩**:** 62°–65°
- **FATS OR FATTY ACIDS, JAPAN WAX, ROSIN,** and **SOAP**

Sample: 1 g

Analysis 1: Boil the *Sample* for 30 min with 35 mL of 3.5 N sodium hydroxide contained in a 100-mL beaker, maintaining the volume of solution by the occasional addition of water, and allow the mixture to cool at room temperature for about 2 h.

Acceptance criteria 1: The wax separates, leaving the liquid clear, turbid, or translucent, but not opaque.

Analysis 2: Filter the cool mixture obtained in *Analysis 1*, and acidify the clear filtrate with hydrochloric acid.

Acceptance criteria 2: The liquid remains clear or shows NMT a slight amount of turbidity or precipitate.

- **FATS AND FIXED OILS,** *Acid Value* ⟨401⟩

Sample: 3 g

Analysis: Warm the *Sample* in a 200-mL flask with 25 mL of neutralized dehydrated alcohol until melted, then shake the mixture. Add 1 mL of phenolphthalein TS, and titrate the warm liquid with 0.5 N alcoholic potassium hydroxide VS to produce a permanent, faint pink color. Calculate the acid value as directed in the chapter.

Acceptance criteria: 17–24

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• **FATS AND FIXED OILS,** *Ester Value* ⟨401⟩
Sample solution: The solution resulting from the determination of *Acid Value*
Analysis: To the *Sample solution* add 25.0 mL of 0.5 N alcoholic potassium hydroxide VS and 50 mL of aldehyde-free alcohol, and reflux the mixture for 4 h. Titrate the excess alkali with 0.5 N hydrochloric acid VS. Perform a blank determination (see *Titrimetry* ⟨541⟩, *Residual Titrations*). Calculate the ester value as directed in the chapter.
Acceptance criteria: 72–79

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Yellow Wax

DEFINITION
Yellow Wax is the purified wax from the honeycomb of the bee [*Apis mellifera* L. (Fam. Apidae)]. The crude beeswax used to prepare Yellow Wax conforms to the *Saponification Cloud Test*.

SPECIFIC TESTS
• **SAPONIFICATION CLOUD TEST**
Sample: 3.00 g
Alcoholic potassium hydroxide: Dissolve 40 g of potassium hydroxide in about 900 mL of aldehyde-free alcohol maintained at a temperature not exceeding 15°, and then when solution is complete, warm to room temperature, and add aldehyde-free alcohol to make 1000 mL.
Analysis: Place the *Sample* in a 100-mL round-bottom boiling flask fitted with a ground-glass joint. Add 30 mL of *Alcoholic potassium hydroxide*. Reflux the mixture gently for 2 h. At the end of this period, open the flask, insert a thermometer into the solution, and place the flask in a container of water at a temperature of 80°. Rotate the flask in the bath while both the bath and the solution cool.
Acceptance criteria: The solution shows no cloudiness or globule formation before the temperature reaches 65°.
• **MELTING RANGE OR TEMPERATURE,** *Class II* ⟨741⟩**:** 62°–65°
• **FATS OR FATTY ACIDS, JAPAN WAX, ROSIN,** and **SOAP**
Sample: 1 g
Analysis 1: Boil the *Sample* for 30 min with 35 mL of 3.5 N sodium hydroxide contained in a 100-mL beaker, maintaining the volume of solution by the occasional addition of water, and allow the mixture to cool at room temperature for about 2 h.
Acceptance criteria 1: The wax separates, leaving the liquid clear, turbid, or translucent, but not opaque.
Analysis 2: Filter the cool mixture obtained in *Analysis 1*, and acidify the clear filtrate with hydrochloric acid.
Acceptance criteria 2: The liquid remains clear or shows NMT a slight amount of turbidity or precipitate.
• **FATS AND FIXED OILS,** *Acid Value (Free Fatty Acids)* ⟨401⟩
Sample: 3 g
Analysis: Warm the *Sample* in a 200-mL flask with 25 mL of neutralized dehydrated alcohol until melted, then shake the mixture. Add 1 mL of phenolphthalein TS, and titrate the warm liquid with 0.5 N alcoholic potassium hydroxide VS to produce a permanent, faint pink color. Calculate the *Acid Value* as directed in the chapter.
Acceptance criteria: 17–24
• **FATS AND FIXED OILS,** *Ester Value* ⟨401⟩
Sample solution: The solution resulting from the determination of *Acid Value*
Analysis: To the *Sample solution* add 25.0 mL of 0.5 N alcoholic potassium hydroxide VS and 50 mL of aldehyde-free alcohol, and reflux the mixture for 4 h. Titrate the excess alkali with 0.5 N hydrochloric acid VS. Perform a blank determination (see *Titrimetry* ⟨541⟩, *Residual Titrations*). Calculate the *Ester Value* as directed in the chapter.
Acceptance criteria: 72–79

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Xanthan Gum

DEFINITION
Xanthan Gum is a high molecular weight polysaccharide gum produced by a pure-culture fermentation of a carbohydrate with *Xanthomonas campestris*, then purified by recovery with Isopropyl Alcohol, dried, and milled. It contains D-glucose and D-mannose as the dominant hexose units, along with D-glucuronic acid, and is prepared as the sodium, potassium, or calcium salt. It yields NLT 4.2% and NMT 5.0% of carbon dioxide, calculated on the dried basis, corresponding to NLT 91.0% and NMT 108.0% of Xanthan Gum.

IDENTIFICATION
• **A.**
Sample: Prepare a dry blend of 1.5 g of Xanthan Gum and 1.5 g of locust bean gum.
Control: 3.0 g of Xanthan Gum
Analysis
Samples: *Sample* and *Control*
To two separate 400-mL beakers add 300 mL of water, and heat to 80°. Stir rapidly by mechanical means. Add the *Sample* to one of the beakers and the *Control* to the other beaker at the point of maximum agitation. Stir until the mixtures dissolve, and then continue stirring for 30 min longer. Do not allow the temperature of the mixtures to drop below 60° during the stirring. Discontinue stirring, and allow the mixtures to cool at room temperature for NLT 2 h.
Acceptance criteria: A firm, rubbery gel forms with the *Sample* after the temperature drops below 40°, but no such gel forms with the *Control*.

ASSAY
• **PROCEDURE**
Sample: 1.2 g
Analysis: Proceed as directed in *Alginates Assay* ⟨311⟩.
Acceptance criteria: 4.2%–5.0% of carbon dioxide on the dried basis, corresponding to 91.0%–108.0% of Xanthan Gum

IMPURITIES
• **ARSENIC,** *Method II* ⟨211⟩**:** NMT 3 µg/g
• **LEAD** ⟨251⟩
Sample: Prepare a *Test Preparation* as directed in the chapter
Control: Use 5 mL of *Diluted Standard Lead Solution* (5 µg of Pb).
Analysis: Proceed as directed in the chapter.
Acceptance criteria: NMT 5 µg/g
• **LIMIT OF ISOPROPYL ALCOHOL**
Internal standard solution: 1 mg/mL of tertiary butyl alcohol
Standard stock solution: 1 mg/mL of isopropyl alcohol

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Standard solution: Pipet 4 mL of the *Standard stock solution* and 4 mL of the *Internal standard solution* into a 100-mL volumetric flask, and dilute with water to volume.
Sample solution: Disperse 1 mL of a suitable antifoam emulsion in 200 mL of water contained in a 1000-mL, round-bottom distilling flask having a 24/40 standard taper ground joint. Add 5 g of Xanthan Gum, and shake for 1 h on a wrist-action mechanical shaker. Connect the flask to a fractionating column, and distill 100 mL, adjusting the heat so that foam does not enter the column. Add by pipet 4 mL of the *Internal standard solution*.
Chromatographic system
(See *Chromatography* ⟨621⟩, *System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 3.2-mm × 1.8-m stainless steel column packed with 80- to 100-mesh surface-silanized packing S3, or equivalent
Temperatures
Column: 165°
Detector: 200°
Injection port: 200°
Carrier gas: Helium
Injection volume: 4–5 µL
Analysis
Samples: *Standard solution* and *Sample solution*
[NOTE—The retention time of tertiary butyl alcohol is 1.5 relative to that of isopropyl alcohol.]
Calculate the percentage of isopropyl alcohol in the portion of Xanthan Gum taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of isopropyl alcohol to tertiary butyl alcohol from the *Sample solution*
R_S = peak response ratio of isopropyl alcohol to tertiary butyl alcohol from the *Standard solution*
C_S = concentration of isopropyl alcohol in the *Standard solution* (mg/mL)
C_U = concentration of the *Sample solution* (mg/mL)

Acceptance criteria: NMT 0.075%

• **PYRUVIC ACID**
Solution A: 5 mg/mL of 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid
Standard stock solution: 90 µg/mL of pyruvic acid
Standard solution: Transfer 10.0 mL of the *Standard stock solution* to a glass-stoppered, 50-mL flask. Add 20.0 mL of 1 N hydrochloric acid, weigh the flask, and reflux for 3 h, taking precautions to prevent loss of vapors. Cool, and add water to make up for any weight loss during refluxing. Transfer 2.0 mL of this solution to a 30-mL separator containing 1.0 mL of *Solution A*. Mix, and allow to stand for 5 min. Extract the mixture with 5 mL of ethyl acetate, and discard the aqueous layer. Extract the hydrazone from the ethyl acetate with three 5-mL portions of sodium carbonate TS, collect the extracts in a 50-mL volumetric flask, and dilute with sodium carbonate TS to volume.
Sample stock solution: 6 mg/mL of Xanthan Gum
Sample solution: Transfer 10.0 mL of the *Sample stock solution* to a glass-stoppered, 50-mL flask. Proceed as directed in the *Standard solution*, beginning with "Add 20.0 mL of 1 N hydrochloric acid...".
Blank: Sodium carbonate TS
Instrumental conditions
(See *Ultraviolet-Visible Spectroscopy* ⟨857⟩.)
Mode: Spectrophotometry
Analytical wavelength: 375 nm
Cell: 1 cm
Analysis
Samples: *Standard solution, Sample solution,* and *Blank*
Acceptance criteria: The absorbance of the *Sample solution* is NLT that of the *Standard solution*, corresponding to NLT 1.5% of pyruvic acid.

SPECIFIC TESTS
• **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩: It meets the requirements of the tests for *Salmonella* species and *Escherichia coli*.
• **LOSS ON DRYING** ⟨731⟩
Analysis: Dry at 105° for 2.5 h.
Acceptance criteria: NMT 15.0%
• **ASH**
Sample: Weigh 3 g in a tared crucible.
Analysis: Incinerate the *Sample* at 650° until free from carbon. Cool the crucible and its contents in a desiccator, and weigh.
Acceptance criteria: The weight of the ash is between 6.5%–16.0%, calculated on the dried basis.
• **VISCOSITY—ROTATIONAL METHODS** ⟨912⟩
Sample: Prepare a dry blend of 3.0 g of Xanthan Gum and 3.0 g of potassium chloride.
Instrumental conditions
Instrument: Rotational viscometer
Spindle cylinder dimensions
Diameter: 1.27 cm
Height: 0.16 cm
Shaft diameter: 0.32 cm
Distance from top of cylinder to lower lip of shaft: 2.54 cm
Immersion depth: 5.00 cm (No. 3 spindle)
Spindle rotation speed: 60 rpm
Analysis: To a 400-mL beaker add 250 mL of water. Add the *Sample* slowly while stirring at 800 rpm, using a low-pitched, propeller-type stirrer. Add 44 mL of water, rinsing the walls of the beaker. Approximately 10 min after the addition of the *Sample* to the water, remove the beaker from the propeller-type stirrer, and vigorously stir the solution by hand to ensure that all the particles around the edge of the beaker are in solution. Return the beaker to the stirrer, and agitate at 800 rpm for a total mixing time of 2 h. Adjust the temperature to 24 ± 1°, and stir by hand in a vertical motion to eliminate any thixotropic effects or layering. Each hand mixing should be NMT 15–30 s, and the last hand mixing should occur immediately before measuring the viscosity. With the spindle rotating at 60 rpm, immediately observe and record the scale reading. Convert the scale readings to centipoises by multiplying the readings by the constant for the viscometer spindle and speed used.
Acceptance criteria: NLT 600 centipoises at 24°

ADDITIONAL REQUIREMENTS
• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Xanthan Gum Solution

DEFINITION
Prepare Xanthan Gum Solution of the designated percentage strength as follows (see *Pharmaceutical Compounding—Nonsterile Preparations* ⟨795⟩).

Xanthan Gum (for a 0.1% solution)	100 mg

NF Monographs

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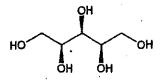
Xanthan Gum (for 1.0% solution)	1.0 g
Methylparaben	100 mg
Propylparaben	20 mg
Purified Water, a sufficient quantity to make	100 mL

Dissolve a weighed quantity of *Propylparaben* in *Purified Water* with heating to 50° and stirring. Cool, and dilute quantitatively, and stepwise if necessary, with *Purified Water* to obtain 90 mL of solution containing 20 mg of *Propylparaben*. Heat to 50°, and add the *Methylparaben*, with stirring, to dissolve. Cool, stir with a blender, slowly sift the Xanthan Gum into the vortex, and continue to blend for 2 min after the Xanthan Gum has been added. Add 10 mL of *Purified Water*, and blend for 5 min. Allow to stand for 1 h for excess foam to subside, and remove most of the remaining foam by passing the solution through a strainer. Add *Purified Water*, if necessary, to make the final volume 100 mL, and stir. [NOTE—Depending on the volume needed and the equipment available, adjust the formula proportionately.]

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at controlled room temperature.
- **LABELING:** Label it to state, as part of the official title, the percentage content of Xanthan Gum.
- **BEYOND-USE DATE:** Six weeks after the day on which it was compounded

Xylitol



$C_5H_{12}O_5$ 152.15
Xylitol.

DEFINITION

Xylitol contains NLT 98.5% and NMT 101.0% of $C_5H_{12}O_5$, calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

- **A. ▲SPECTROSCOPIC IDENTIFICATION TESTS** ⟨197⟩, *Infrared Spectroscopy*: **197K▲** (CN 1-May-2020)

  Sample: Undried
- **B.** The retention time of the xylitol peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

ASSAY

- **PROCEDURE**

  Mobile phase: Acetonitrile and water (20:80)
  System suitability solution: 2.5 mg/mL of USP Galactitol RS and 25 mg/mL of USP Xylitol RS in *Mobile phase*
  Standard solution: 25 mg/mL of USP Xylitol RS in *Mobile phase*
  Sample solution: 25 mg/mL of Xylitol in *Mobile phase*
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: LC
  Detector: UV 192 nm
  Column: 8.0-mm × 30-cm; 7-µm packing L34
  Column temperature: 80°
  Flow rate: 0.5 mL/min
  Injection size: 25 µL
  System suitability
  Sample: *System suitability solution* and *Standard solution*
  [NOTE—The relative retention times for xylitol and galactitol are about 1.0 and 1.10, respectively.]
  Suitability requirements
  Resolution: NLT 2.0 between galactitol and xylitol, *System suitability solution*
  Relative standard deviation: NMT 2.0%, *Standard solution*
  Analysis
  Samples: *Standard solution* and *Sample solution*
  Calculate the percentage of xylitol ($C_5H_{12}O_5$) in the portion of sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of xylitol from the *Sample solution*
r_S = peak response of xylitol from the *Standard solution*
C_S = concentration of USP Xylitol RS in the *Standard solution* (mg/mL)
C_U = concentration of the *Sample solution* (mg/mL)

  Acceptance criteria: 98.5%–101.0% on the anhydrous basis

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩: NMT 0.5%
- **REDUCING SUGARS**

  Sample: 500 mg
  Analysis: Dissolve the *Sample* in 2.0 mL of water in a 10-mL conical flask. Into a similar flask, pipet 2 mL of a 0.5 mg/mL dextrose solution. To each flask add 1 mL of alkaline cupric tartrate TS, heat to boiling, and cool.
  Acceptance criteria: Any turbidity in the xylitol flask is NMT that in the dextrose flask, in which a reddish-brown precipitate forms (0.2% reducing sugars, as dextrose).
- **LIMIT OF OTHER POLYOLS**

  Mobile phase: Acetonitrile and water (20:80)
  System suitability solution: 0.5 mg/mL each of USP L-Arabinitol RS, USP Galactitol RS, USP Mannitol RS, and USP Sorbitol RS, and 100 mg/mL of USP Xylitol RS in *Mobile phase*
  Standard solution: 0.5 mg/mL each of USP L-Arabinitol RS, USP Galactitol RS, USP Mannitol RS, and USP Sorbitol RS in *Mobile phase*
  Sample solution: 100 mg/mL of Xylitol in *Mobile phase*
  Chromatographic system
  (See *Chromatography* ⟨621⟩, *System Suitability*.)
  Mode: LC
  Detector: UV 192 nm
  Column: 8.0-mm × 30-cm; 7-µm packing L34
  Column temperature: 80°
  Flow rate: 0.5 mL/min
  Injection size: 25 µL
  System suitability
  Samples: *System suitability solution* and *Standard solution*
  [NOTE—The relative retention times for L-arabinitol, mannitol, xylitol, galactitol, and sorbitol are about 0.76, 0.81, 1.0, 1.12, and 1.22, respectively.]
  Suitability requirements
  Resolution: NLT 1.5 between all adjacent polyol peaks, *System suitability solution*
  Relative standard deviation: NMT 5.0% for the galactitol peak, *Standard solution*
  Analysis
  Samples: *Standard solution* and *Sample solution*

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Calculate the percentage of each polyol (L-arabinitol, galactitol, mannitol, or sorbitol) in the portion of sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of the individual polyol from the *Sample solution*
r_S = peak response of the individual polyol from the *Standard solution*
C_S = concentration of the individual polyol in the *Standard solution* (mg/mL)
C_U = concentration of the *Sample solution* (mg/mL)

Acceptance criteria: The sum of the polyols is NMT 2.0%, calculated on the anhydrous basis.

SPECIFIC TESTS

- **WATER DETERMINATION,** *Method I* ⟨921⟩**:** NMT 0.5%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **USP REFERENCE STANDARDS** ⟨11⟩
  USP L-Arabinitol RS
  USP Galactitol RS
  USP Mannitol RS
  USP Sorbitol RS
  USP Xylitol RS

Xylose—*see Xylose General Monographs*

Zein

[9010-66-6].

DEFINITION

Zein is a prolamine derived from corn [*Zea mays Linné* (Fam. Gramineae)].

IDENTIFICATION

- **A.**
  Sample solution: Dissolve 0.1 g in 10 mL of 0.1 N sodium hydroxide.
  Analysis: To the *Sample solution* add a few drops of cupric sulfate TS. Warm in a water bath.
  Acceptance criteria: A purple color develops.
- **B.**
  Sample solution: In a test tube add 1 mL of nitric acid to 25 mg of Zein.
  Analysis: Agitate the *Sample solution* vigorously.
  Acceptance criteria: The solution becomes light yellow. Further addition of about 10 mL of 6 N ammonium hydroxide produces an orange color.
- **C. IDENTIFICATION OF ALPHA-ZEIN BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS**
  Solvent: 55% Isopropyl alcohol with 2% beta mercaptoethanol
  Sample loading buffer:[1] 0.5 M tris hydrochloride pH 6.8, 20% glycerin, 4% sodium dodecyl sulfate (SDS), and 0.005% bromophenol blue
  Gel running buffer stock solution:[2] 0.25 M tris base pH 8.6, 1.92 M glycine, and 1.0% SDS
  Gel running buffer: *Gel running buffer stock solution* and water (1:9)
  Gel staining solution: A suitable Coomassie blue-based solution[3]
  Molecular weight marker: Use a suitable molecular weight marker[4] containing protein bands at 10–190 kDa. [NOTE—A molecular weight marker with protein bands at 10–100 KDa can also be used.]
  Molecular weight standard solution: Dilute the *Molecular weight marker* with the *Sample loading buffer* (1:1). Incubate the mixture in a closed microcentrifuge tube at 95° for 10 min. After incubation, allow the tube to flash-cool on ice. Place the tube in a microcentrifuge, spin at a top speed for a few seconds, and allow to stop on its own to collect any condensation on the sides and top of the tube.
  Independent standard stock solution: 10 mg/mL of β-lactoglobulin A in water
  Independent standard working solution: 1 mg/mL of β-lactoglobulin A in water
  Independent standard running solution: To the final 1 volume of *Independent standard running solution*, add 0.1 volume of *Independent standard working solution*, 0.5 volume of *Sample loading buffer*, 0.1 volume of β-mercaptoethanol, and sufficient water to obtain 0.1 mg/mL of β-lactoglobulin A and 10% of β-mercaptoethanol.
  Sample stock solution: 10 mg/mL of Zein in *Solvent*. Mix on a vortex mixer until the sample is fully dissolved. Centrifuge at 10,000–12,000 rpm in a microcentrifuge with a fixed rotor for 10 min to pellet any undissolved material.
  Sample solution: Dilute the *Sample stock solution* with the *Sample loading buffer* (1:1). Incubate the mixture in a closed microcentrifuge tube for 10 min at 95°. After incubation, allow the tube to flash-cool on ice. Place the tube in a microcentrifuge, spin at a top speed for a few seconds, and allow to stop on its own to collect any condensation on the sides and top of the tube. Dilute the solution so obtained with a mixed solution of *Sample loading buffer* and water (1:1) to 5 folds.
  Electrophoretic system
  SDS-PAGE gel and apparatus setup: Following the manufacturer's instructions, assemble and fill a precast 16% Tris-Glycine gel[5] in an appropriate electrophoresis module.
  Running buffer: *Gel running buffer*
  Voltage: 100 V
  Run time: 2.5 h or until the upper dye front is at the bottom of the gel. [NOTE—The total run time may need to be altered, depending on the molecular weight standards as well on as laboratory equipment variability, because the dye front may co-migrate with or close to the lowest bands of the set.]
  Analysis
  Samples: *Molecular weight standard solution*, *Independent standard running solution*, and *Sample solution*
  Gel loading: Load 25 µL of the *Molecular weight standard solution*. Load a volume of the *Independent standard running solution*, equal to 2 µg of β-lactoglobulin A. Load a volume of the *Sample solution*, equal to approximately 5 µg of calculated Zein. [NOTE—If the 5 µg loading amount

[1] Available from Invitrogen (Life Technologies) as Tris-Glycine SDS Sample Buffer (2X), catalog number LC2676.

[2] Available from Invitrogen as Tris-Glycine SDS Running Buffer (10X), catalog number LC2675.

[3] Available from Invitrogen as SimplyBlue Stain, catalog number LC6065.

[4] Available from Invitrogen as BenchMark Prestained Protein Ladder, catalog number 1074810.

[5] Available from Invitrogen, catalog number EC6495. However, these are readily available from several other manufacturers.

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is inadequate for the test, the 10 µg loading amount may be used. Loading a lesser quantity on the gel results in an overall higher molecular weight determination due to sharper bands. The actual amount of Zein extracted from the starting material cannot be quantified. Therefore, an estimated amount is derived.]

Gel staining: After electrophoresis, carefully remove the gel from the plates. Rinse the gel three times with water. Stain the gel by following the manufacturer's directions for the stain used.

Destaining: After staining as directed by the manufacturer, destain as directed by the manufacturer.

Gel scan procedure: Set up a gel scanner according to the manufacturer's instructions. Place the gel in the detector, and obtain a single image of all loaded lines of the gel.

Acceptance criteria: Zein has two major bands for α-zein at 15–26 kDa.

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩**:** NMT 2.0%, using an ignition temperature of 800 ± 25°
- **LIMIT OF HEXANE-SOLUBLE MATTER**

Sample: 15 g of Zein

Solvent: Alcohol and water (17:3, w/w)

Analysis: Dissolve the *Sample* in 150 mL of *Solvent*. Stir the mixture, using a magnetic stirrer, and heat the solution to 30°. Once the *Sample* is dissolved, transfer the solution to a 500-mL separatory funnel.

Add 60 mL of *n*-hexane. Shake the mixture, and allow the phases to separate. Discharge the bottom layer (alcohol) to a beaker, and transfer the top layer (hexane) to a first 500-mL flask. Weigh the first 500-mL flask, and record the weight. Pour the bottom layer of alcohol back into the separatory funnel. Repeat this step four more times.

After the five 60-mL hexane solutions have been added to the first 500-mL flask, attach it to a rotary evaporator to distill the hexane. Collect the hexane in a second 500-mL flask.

The first 500-mL flask contains a yellow to reddish oil. Record the weight of the flask containing this oil.

Calculate the percentage of hexane-soluble matter in the portion of Zein taken:

$$\text{Result} = [(W_T - W_F)/W] \times 100$$

W_T = weight of the flask (g)
W_F = weight of the first 500-mL flask (g)
W = weight of the *Sample* (g)

Acceptance criteria

For Zein from normal dent corn: NMT 12.5% for hexane-soluble matter

For Zein from waxy corn: NMT 16.0% for hexane-soluble matter

SPECIFIC TESTS

- **LOSS ON DRYING** ⟨731⟩

Analysis: Dry at 105° for 2 h.

Acceptance criteria: NMT 8.0%

- **MICROBIAL ENUMERATION TESTS** ⟨61⟩ and **TESTS FOR SPECIFIED MICROORGANISMS** ⟨62⟩**:** The total bacterial count does not exceed 10^3 cfu/g, and the tests for *Salmonella* species and *Escherichia coli* are negative.
- **PROTEIN CONTENT**

Analysis: Proceed as directed in *Nitrogen Determination* ⟨461⟩, *Method I*. Calculate the weight percentage of the protein content in Zein by multiplying the percentage of nitrogen found by 6.25.

Acceptance criteria: 81.9%–100.0% on the dried basis

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at room temperature.
- **LABELING:** Label it to indicate the corn source from which it is derived.

Zinc Stearate—*see Zinc Stearate General Monographs*

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