Excerpted *USP-NF* and *FCC* Standards: A Hand Sanitizer Resource

A collection of standards provided as a resource to assist with the challenges posed by COVID-19

Not official text. Please refer to the currently official version of the applicable USP–NF or FCC standard for compliance purposes.

Published August 17, 2020

CONTENTS

Additional documentary standards are also included in this publication to support these highlighted standards.

Published August 17, 2020

Published August 17, 2020

GENERAL NOTICES AND REQUIREMENTS

Applying to Standards, Tests, Assays, and Other Specifications of the United States Pharmacopeia

Published August 17, 2020

Published August 17, 2020

GENERAL NOTICES AND REQUIREMENTS

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

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

Change to read:

1. TITLE AND REVISION

▲The full title of this joint compendium is *The Pharmacopeia of the United States of America* and the *National Formulary* Online (*USP–NF Online*). Although *USP* and *NF* are published together and share these *General Notices*, they are separate compendia. This is the 43rd revision of the *USP* and 38th edition of the *NF*. The final print publication of the *USP–NF* is *USP 43–NF 38*, after which official standards are published only in the *USP–NF Online*. Where the terms "*USP*," "*NF*," or "*USP–NF*" are used without further qualification, they refer to the currently official standard.▲ (USP 1-Aug-2020)

Change to read:

2. OFFICIAL STATUS AND LEGAL RECOGNITION

2.10. Official Text

Official text of the *USP* and *NF* is published in the *USP–NF Online* (www.uspnf.com). ▲▲ (USP 1-Aug-2020)

Routine revisions are published in the *USP–NF Online* and become official on the date indicated, usually six months after publication.

▲Accelerated Revisions, published periodically in the *USP–NF Online*, are designed to make revisions official more quickly than through the routine process for publishing standards in the *USP–NF Online*. Accelerated Revisions may also be published on the *Official Text* section of USP's website (https://www.uspnf.com/official-text). Accelerated Revisions supersede previously published content and become official on the date indicated.

Interim Revision Announcements are Accelerated Revisions to *USP* and *NF* that contain revisions and their official dates.

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

Errata are Accelerated Revisions representing corrections to items erroneously published.

Periodically, a non-official volume of associated revisions and additions to the *USP–NF* is published in print and/or USB flash drive. This volume is intended to serve as a historical reference document and is not considered official text.▲ (USP 1-Aug-2020)

2.20. Official Articles

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

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

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

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

2.30. Legal Recognition

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

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

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

3. CONFORMANCE TO STANDARDS

3.10. Applicability of Standards

Standards for an article recognized in the compendia (*USP–NF*) are expressed in the article's monograph, applicable general chapters, and *General Notices*. The identity, strength, quality, and purity of an article are determined by the official

tests, procedures, and acceptance criteria, and other requirements incorporated in the monograph, in applicable general chapters, or in the *General Notices*. "Applicable general chapters" means general chapters numbered below 1000 or above 2000 that are made applicable to an article through reference in *General Notices*, a monograph, or another applicable general chapter numbered below 1000. Where the requirements of a monograph differ from the requirements specified in these *General Notices* or an applicable general chapter, the monograph requirements apply and supersede the requirements of the *General Notices* or applicable general chapters, whether or not the monograph explicitly states the difference.

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

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

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

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

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

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

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

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

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

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

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

3.10.30. Applicability of Standards to the Practice of Compounding

USP compounding practice standards, *Pharmaceutical Compounding—Nonsterile Preparations* ⟨*795*⟩ and *Pharmaceutical Compounding—Sterile Preparations* ⟨*797*⟩, as appropriate, apply to compounding practice or activity regardless of whether a monograph exists for the compounded preparation or these chapters are referenced in such a monograph. In the United States, ⟨*795*⟩ and ⟨*797*⟩ are not applicable to drugs compounded by entities registered with FDA as outsourcing facilities as defined by FDCA § 503B, because such facilities are required to comply with FDA's current good manufacturing practice requirements. Compounded preparations, including drug products compounded by outsourcing facilities, may also be subject to applicable monographs; see section *2.20 Official Articles* and section *4.10 Monographs*.

3.20. Indicating Conformance

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

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

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

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

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

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

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

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

4. MONOGRAPHS AND GENERAL CHAPTERS

4.10. Monographs

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

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

4.10.10. Applicability of Test Procedures

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

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

4.10.20. Acceptance Criteria

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

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

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

4.20. General Chapters

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

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

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

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

Change to read:

5. MONOGRAPH COMPONENTS

5.10. Molecular Formula

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

5.20. Added Substances

Added substances are presumed to be unsuitable for inclusion in an official article and therefore prohibited, if their presence impairs the bioavailability, therapeutic efficacy, or safety of the official article; or they interfere with the assays and tests prescribed for determining compliance with the

compendial standards (see section *3.20 Indicating Conformance*).

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

5.20.10. Added Substances in Official Substances

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

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

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

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

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

5.20.20.1. In Compounded Preparations

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

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

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

5.20.20.2. In Dietary Supplements

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

5.30. Description and Solubility

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

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

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

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

5.40. Identification

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

5.50. Assay

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

5.50.10. Units of Potency (Biological)

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

Where available, World Health Organization (WHO) international biological standards define the International

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

5.60. Impurities and Foreign Substances

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

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

5.60.10. Other Impurities in USP and NF Articles

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

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

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

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

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

5.60.20. Residual Solvents in USP and NF Articles

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

5.60.30. Elemental Impurities in USP Drug Products and Dietary Supplements

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

5.70. Performance Tests

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

5.80. USP Reference Standards

USP Reference Standards are authentic specimens that have been approved as suitable for use ▲▲ (USP 1-Aug-2020) in *USP* or *NF* tests and assays (see *USP Reference Standards* ⟨11⟩). Where *USP* or *NF* tests or assays call for the use of a USP Reference Standard, only those results obtained using the specified USP Reference Standard are conclusive. Where a procedure calls for the use of a compendial article rather than for a USP Reference Standard as a material standard of reference, a substance meeting all of the compendial monograph requirements for that article shall be used. If any new *USP* or *NF* standard requires the use of a new USP Reference Standard that is not yet available, that portion of the standard containing the requirement shall not be official until the specified USP reference material is available.

▲Announcements of the availability of new USP Reference Standards and announcements of tests or procedures that are held in abeyance pending availability of required USP Reference Standards are also available on the "Official Text" tab of USP's website (www.uspnf.com).▲ (USP 1-Aug-2020) Unless otherwise directed in the procedure in the individual monograph or in a general chapter, USP Reference Standards are to be used in accordance with the instructions on the label of the Reference Standard.

6. TESTING PRACTICES AND PROCEDURES

6.10. Safe Laboratory Practices

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

6.20. Automated Procedures

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

6.30. Alternative and Harmonized Methods and Procedures

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

obtained by the methods and procedures given in the compendia are conclusive.

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

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

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

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

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

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

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

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

6.40.10. Ignite to Constant Weight

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

6.40.20. Dried to Constant Weight

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

6.50. Preparation of Solutions

6.50.10. Filtration

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

6.50.20. Solutions

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

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

6.50.20.1. Adjustments to Solutions

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

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

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

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

6.50.20.2. Test Solutions

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

6.50.20.3. Indicator Solutions

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

6.60. Units Necessary to Complete a Test

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

6.60.10. Tablets

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

6.60.20. Capsules

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

6.70. Reagents

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

Listing of these reagents, including the indicators and solutions employed as reagents, in no way implies that they have therapeutic utility; furthermore, any reference to *USP* or

NF in their labeling shall include also the term "reagent" or "reagent grade." USP may supply reagents if they otherwise may not be generally commercially available.

6.80. Equipment

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

6.80.10. Apparatus for Measurement

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

6.80.10.1. Pipet/Pipette

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

6.80.10.2. Light Protection

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

6.80.20. Instrumental Apparatus

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

6.80.20.1. Chromatographic Tubes and Columns

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

6.80.20.2. Tubing

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

6.80.20.3. Steam Bath

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

6.80.20.4. Water Bath

A water bath requires vigorously boiling water unless otherwise specified.

6.80.30. Temperature Reading Devices

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

7. TEST RESULTS

7.10. Interpretation of Requirements

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

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

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

7.10.5. Nominal Concentrations in Equations

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

7.10.10. Equivalence Statements in Titrimetric Procedures

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

7.20. Rounding Rules

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

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

8. TERMS AND DEFINITIONS

8.10. Abbreviations

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

8.20. About

"About" indicates a quantity within 10%.

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

8.30. Alcohol Content

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

8.40. Atomic Weights

Atomic weights used in computing molecular weights and the factors in the assays and elsewhere are those established

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

by the IUPAC Commission on Isotopic Abundances and Atomic Weights.

8.50. Blank Determinations

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

8.60. Concomitantly

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

8.70. Desiccator

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

8.80. Logarithms

Logarithms are to the base 10.

8.90. Microbial Strain

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

8.100. Negligible

"Negligible" indicates a quantity not exceeding 0.50 mg.

8.110. NLT/NMT

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

8.120. Odor

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

8.130. Percent

"Percent" used without qualification means:

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

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

8.140. Percentage Concentrations

Percentage concentrations are expressed as follows:

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

8.150. Pressure

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

8.160. Reaction Time

Reaction time is 5 minutes unless otherwise specified.

8.170. Specific Gravity

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

8.180. Temperatures

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

8.190. Time

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

8.200. Transfer

"Transfer" indicates a quantitative manipulation.

8.210. Vacuum

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

8.220. Vacuum Desiccator

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

8.230. Water

8.230.10. Water as an Ingredient in an Official Product

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

8.230.20. Water in the Manufacture of Official Substances

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

8.230.30. Water in a Compendial Procedure

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

8.240. Weights and Measures

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

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

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

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

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

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

	Units	Symbol	Notes
Length			
	meter	m	
	centimeter	cm	
	millimeter	mm	
	micrometer	µm	Previously referred to as a micron
	nanometer	nm	Previously the symbol mµ (for millimicron) was used
	Ångström	Å	Equal to 0.1 nm
Mass			
	kilogram	kg	
	gram	g	
	milligram	mg	
	microgram	µg	The symbol µg is used in the *USP* and *NF* to represent micrograms, but micrograms may be represented as "mcg" for labeling and prescribing purposes. The term "gamma," symbolized by γ, frequently is used to represent micrograms in biochemical literature.
	nanogram	ng	
	picogram	pg	
	dalton	Da	Also referred to as the unified atomic mass unit and is equal to 1/12 times the mass of the free carbon 12 atom.
	kilodalton	kDa	
Time			
	second	s	
	minute	min	
	hour	h	
Volume			
	liter	L	1 L is equal to 1000 cm^3 (cubic centimeters)
	deciliter	dL	
	milliliter	mL	1 mL is equal to 1 cm^3, sometimes referred to as cc
	microliter	µL	
Temperature			
	Celsius	°C	
Amount of Substance			
	mole	mol	Historically referred to as gram-molecular weight or gram-atomic weight
	millimole	mmol	
	micromole	µmol	
	femtomole	fmol	
	equivalent	Eq	Also referred to as gram-equivalent weight. It is used in the calculation of substance concentration in units of normality. This unit is no longer preferred for use in analytical chemistry or metrology.
	milli equivalent	mEq	
	osmole	Osmol	Osmotic pressure of a solution, related to substance concentration.
	milliosmole	mOsmol	
Pressure			
	pascal	Pa	
	kilopascal	kPa	
	pounds per square inch	psi	
	millimeter of mercury	mmHg	Equal to 133.322 Pa

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

Selected SI Prefixes

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

9. PRESCRIBING AND DISPENSING

9.10. Use of Metric Units

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

9.20. Changes in Volume

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

10. PRESERVATION, PACKAGING, STORAGE, AND LABELING

10.10. Packaging and Storage

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

10.20. Labeling

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

USP Monographs

Alcohol

Portions of this monograph that are national *USP* text, and are not part of the harmonized text, are marked with symbols (◆,◆) to specify this fact.

C_2H_6O 46.07
Ethanol;
Ethyl alcohol [64-17-5].

DEFINITION

◆Alcohol contains NLT 92.3% and NMT 93.8%, by weight, corresponding to NLT 94.9% and NMT 96.0%, by volume, at 15.56°, of C_2H_5OH.◆

IDENTIFICATION

- **A.** It meets the requirements of the test for *Specific Gravity* ⟨841⟩.
- **B. Spectroscopic Identification Tests** ⟨197⟩**,** *Infrared Spectroscopy***:** 197F or 197S. Neat.

Add the following:

▲• ◆**C. Limit of Methanol**

[Note—This test must be performed to be in compliance with USP, in addition to *Identification A* and *B* above.]

Sample solution A, Standard solution A, Standard solution B, Chromatographic system, and **System suitability:** Proceed as directed in *Organic Impurities*.

Analysis: Proceed as directed in the *Organic Impurities* test, *Methanol calculation*.

Acceptance criteria: Meets the requirements in *Table 2* for methanol.◆▲ (RB 1-Sep-2020)

IMPURITIES

- **Limit of Nonvolatile Residue**

Sample: 100 mL of Alcohol

Analysis: Evaporate the *Sample* in a tared dish on a water bath, and dry at 100°–105° for 1 h.

Acceptance criteria: The weight of the residue is NMT 2.5 mg.

Change to read:

- **Organic Impurities**

Sample solution A: Alcohol (substance under test)

Sample solution B: 300 µL/L of 4-methylpentan-2-ol in *Sample solution A*

Standard solution A: 200 µL/L of methanol in *Sample solution A*

▲◆ [Note—To be prepared for use in *Identification C*].◆▲ (RB 1-Sep-2020)

Standard solution B: 10 µL/L of methanol and 10 µL/L of acetaldehyde in *Sample solution A*

Standard solution C: 30 µL/L of acetal in *Sample solution A*

Standard solution D: 2 µL/L of benzene in *Sample solution A*

Chromatographic system

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

Mode: GC

Detector: Flame ionization

Column: 0.32-mm × 30-m fused-silica capillary; bonded with a 1.8-µm layer of phase G43

Split ratio: 20:1

Temperatures

Injection port: 200°

Detector: 280°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
40	0	40	12
40	10	240	10

Linear velocity: 35 cm/s

Carrier gas: Helium

Injection volume: 1.0 µL

System suitability

Sample: *Standard solution B*

Suitability requirements

Resolution: NLT 1.5 between the first major peak (acetaldehyde) and the second major peak (methanol)

Analysis

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

Methanol calculation

▲◆ [Note—To be performed as a part of *Identification C*.]◆▲ (RB 1-Sep-2020)

$$\text{Result} = (r_U/r_S)$$

r_U = peak area of methanol from *Sample solution A*
r_S = peak area of methanol from *Standard solution A*

Acetaldehyde calculation (sum of acetaldehyde and acetal)

$$\text{Result} = \{[A_E/(A_T - A_E)] \times C_A\} + \{[D_E/(D_T - D_E)] \times C_D \times (M_{r1}/M_{r2})\}$$

A_E = peak area of acetaldehyde from *Sample solution A*
A_T = peak area of acetaldehyde from *Standard solution B*
C_A = concentration of acetaldehyde in *Standard solution B* (µL/L)
D_E = peak area of acetal from *Sample solution A*
D_T = peak area of acetal from *Standard solution C*
C_D = concentration of acetal in *Standard solution C* (µL/L)
M_{r1} = molecular weight of acetaldehyde, 44.05
M_{r2} = molecular weight of acetal, 118.2

Benzene calculation

$$\text{Result} = [B_E/(B_T - B_E)] \times C_B$$

B_E = peak area of benzene from *Sample solution A*
B_T = peak area of benzene from *Standard solution D*
C_B = concentration of benzene in *Standard solution D* (µL/L)

[NOTE—If necessary, the identity of benzene can be confirmed using another suitable chromatographic system (stationary phase with a different polarity).]

Any other impurity calculation

$$\text{Result} = (r_U/r_M) \times C_M$$

r_U = peak area of each impurity in *Sample solution B*
r_M = peak area of 4-methylpentan-2-ol in *Sample solution B*
C_M = concentration of 4-methylpentan-2-ol in *Sample solution B* (µL/L)

Acceptance criteria: See *Table 2*.

Table 2

Name	Acceptance Criteria
Methanol	NMT 0.5, corresponding to 200 µL/L
Acetaldehyde and acetal	NMT 10 µL/L, expressed as acetaldehyde
Benzene	NMT 2 µL/L
Sum of all other impurities[a]	NMT 300 µL/L

[a] Disregard any peaks of less than 9 µL/L (0.03 times the area of the peak corresponding to 4-methylpentan-2-ol in the chromatogram obtained with *Sample solution B*).

SPECIFIC TESTS

- **•SPECIFIC GRAVITY** ⟨841⟩**:** 0.812–0.816 at 15.56°, indicating 92.3%–93.8%, by weight, or 94.9%–96.0%, by volume, of C_2H_5OH.•
- **ULTRAVIOLET ABSORPTION**

  Analytical wavelength: 235–340 nm
  Cell: 5 cm
  Reference: Water
  Acceptance criteria
  Absorbance: NMT 0.40 at 240 nm; NMT 0.30 between 250 nm and 260 nm; NMT 0.10 between 270 nm and 340 nm
  Curve: The spectrum shows a steadily descending curve with no observable peaks or shoulders.
- **•CLARITY OF SOLUTION**

  [NOTE—The *Sample solution* is to be compared to *Standard suspension A* and to water in diffused daylight 5 min after preparation of *Standard suspension A*.]
  Hydrazine solution: 10 mg/mL of hydrazine sulfate in water. Allow to stand for 4–6 h.
  Methenamine solution: Transfer 2.5 g of methenamine to a 100-mL glass-stoppered flask, add 25.0 mL of water, insert the glass stopper, and mix to dissolve.
  Primary opalescent suspension: Transfer 25.0 mL of *Hydrazine solution* to the *Methenamine solution* in the 100-mL glass-stoppered flask. Mix, and allow to stand for 24 h. This suspension is stable for 2 months, provided it is stored in a glass container free from surface defects. The suspension must not adhere to the glass and must be well mixed before use.
  Opalescence standard: Transfer 15.0 mL of the *Primary opalescent suspension* to a 1000-mL volumetric flask, and dilute with water to volume. This suspension should not be used beyond 24 h after preparation.
  Standard suspension A: *Opalescence standard* and water (1 in 20)
  Standard suspension B: *Opalescence standard* and water (1 in 10)
  Sample solution A: Substance to be examined
  Sample solution B: Dilute 1.0 mL of *Sample solution A* with water to 20 mL, and allow to stand for 5 min before testing.
  Blank: Water
  Analysis: Transfer a sufficient portion of *Sample solution A* and *Sample solution B* to separate test tubes of colorless, transparent, neutral glass with a flat base and an internal diameter of 15–25 mm to obtain a depth of 40 mm. Similarly transfer portions of *Standard suspension A, Standard suspension B*, and *Blank* to separate matching test tubes. Compare *Sample solution A*, *Sample solution B*, *Standard suspension A*, *Standard suspension B*, and *Blank* in diffused daylight, viewing vertically against a black background (see *Visual Comparison* ⟨630⟩). The diffusion of light must be such that *Standard suspension A* can readily be distinguished from water, and *Standard suspension B* can readily be distinguished from *Standard suspension A*.
  Acceptance criteria: *Sample solution A* and *Sample solution B* show the same clarity as that of water or their opalescence is not more pronounced than that of *Standardsuspension A*.•
- **ACIDITY OR ALKALINITY**

  Phenolphthalein solution: Dissolve 0.1 g of phenolphthalein in 80 mL of alcohol, and dilute with water to 100 mL.
  Sample: 20 mL of Alcohol
  Analysis: To the *Sample* add 20 mL of freshly boiled and cooled water and 0.1 mL of *Phenolphthalein solution*. The solution is colorless. Add 1.0 mL of 0.01 N sodium hydroxide.
  Acceptance criteria: The solution is pink (30 µL/L, expressed as acetic acid).
- **•COLOR OF SOLUTION**

  Standard stock solution: Combine 3.0 mL of ferric chloride CS, 3.0 mL of cobaltous chloride CS, 2.4 mL of cupric sulfate CS, and 1.6 mL of dilute hydrochloric acid (10 g/L).
  Standard solution: Transfer 1.0 mL of *Standard stock solution* to a 100-mL volumetric flask, and dilute with dilute hydrochloric acid (10 g/L). Prepare the *Standard solution* immediately before use.
  Sample solution: Substance to be examined
  Blank: Water
  Analysis: Transfer a sufficient portion of the *Sample solution* to a test tube of colorless, transparent, neutral glass with a flat base and an internal diameter of 15–25 mm to obtain a depth of 40 mm. Similarly transfer portions of the *Standard solution* and *Blank* to separate, matching test tubes. Compare the *Sample solution, Standard solution*, and *Blank* in diffused daylight, viewing vertically against a white background (see *Visual Comparison* ⟨630⟩).
  Acceptance criteria: The *Sample solution* has the appearance of water or is not more intensely colored than the *Standard solution*.•

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light.
- **USP REFERENCE STANDARDS** ⟨11⟩

  USP Alcohol RS

Dehydrated Alcohol

Portions of this monograph that are national *USP* text, and are not part of the harmonized text, are marked with symbols (♦,♦) to specify this fact.

C_2H_6O 46.07
Ethanol;
Ethyl alcohol [64-17-5].

DEFINITION

♦Dehydrated Alcohol contains NLT 99.2% by weight, corresponding to NLT 99.5% by volume, at 15.56°, of C_2H_5OH.♦

IDENTIFICATION

- **A.** It meets the requirements of the test for *Specific Gravity* ⟨841⟩.
- **B. Spectroscopic Identification Tests** ⟨197⟩**,** *Infrared Spectroscopy***:** 197F or 197S. Neat.

Add the following:

▲• ♦C. Limit of Methanol

[Note—This test must be performed to be in compliance with USP, in addition to *Identification A* and *B* above.]

Sample solution A, Standard solution A, Standard solution B, Chromatographic system, and **System suitability:** Proceed as directed in *Organic Impurities*.

Analysis: Proceed as directed in the *Organic Impurities* test, *Methanol calculation*.

Acceptance criteria: Meets the requirements in *Table 2* for methanol.♦▲ (RB 1-Sep-2020)

IMPURITIES

- **Limit of Nonvolatile Residue**

Sample: 100 mL of Dehydrated Alcohol

Analysis: Evaporate the *Sample* in a tared dish on a water bath, and dry at 100°–105° for 1 h.

Acceptance criteria: The weight of the residue is NMT 2.5 mg.

Change to read:

- **Organic Impurities**

Sample solution A: Substance to be examined

Sample solution B: 300 µL/L of 4-methylpentan-2-ol in *Sample solution A*

Standard solution A: 200 µL/L of methanol in *Sample solution A*

▲♦ [Note—To be prepared for use in *Identification* C.]♦▲ (RB 1-Sep-2020)

Standard solution B: 10 µL/L of methanol and 10 µL/L of acetaldehyde in *Sample solution A*

Standard solution C: 30 µL/L of acetal in *Sample solution A*

Standard solution D: 2 µL/L of benzene in *Sample solution A*

Chromatographic system

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

Mode: GC

Detector: Flame ionization

Column: 0.32-mm × 30-m fused-silica capillary; bonded with a 1.8-µm layer of phase G43

Split ratio: 20:1

Temperatures

Injection port: 200°

Detector: 280°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
40	0	40	12
40	10	240	10

Flow rate: 35 cm/s

Carrier gas: Helium

Injection volume: 1.0 µL

System suitability

Sample: *Standard solution B*

Suitability requirements

Resolution: NLT 1.5 between the first major peak (acetaldehyde) and the second major peak (methanol)

Analysis

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

Methanol calculation

▲♦ [Note—To be performed as a part of *Identification* C.]♦▲ (RB 1-Sep-2020)

$$\text{Result} = r_U/r_S$$

r_U = peak area of methanol from *Sample solution A*
r_S = peak area of methanol from *Standard solution A*

Acetaldehyde calculation (sum of acetaldehyde and acetal)

$$\text{Result} = \{[A_E/(A_T - A_E)] \times C_A\} + \{[D_E/(D_T - D_E)] \times C_D \times (M_{r1}/M_{r2})\}$$

A_E = peak area of acetaldehyde from *Sample solution A*
A_T = peak area of acetaldehyde from *Standard solution B*
C_A = concentration of acetaldehyde in *Standard solution B* (µL/L)
D_E = peak area of acetal from *Sample solution A*
D_T = peak area of acetal from *Standard solution C*
C_D = concentration of acetal in *Standard solution C* (µL/L)
M_{r1} = molecular weight of acetaldehyde, 44.05
M_{r2} = molecular weight of acetal, 118.2

Benzene calculation

$$\text{Result} = [B_E/(B_T - B_E)] \times C_B$$

B_E = peak area of benzene from *Sample solution A*
B_T = peak area of benzene from *Standard solution D*
C_B = concentration of benzene in *Standard solution D* (µL/L)

[Note—If necessary, the identity of benzene can be confirmed using another suitable chromatographic system (stationary phase with a different polarity).]

Any other impurity calculation

$$\text{Result} = (r_U/r_M) \times C_M$$

r_U = peak area of each impurity from *Sample solution B*
r_M = peak area of 4-methylpentan-2-ol from *Sample solution B*
C_M = concentration of 4-methylpentan-2-ol in *Sample solution B* (µL/L)

Acceptance criteria: See *Table 2*.

Table 2

Name	Acceptance Criteria
Methanol	NMT 0.5, corresponding to 200 µL/L
Acetaldehyde and acetal	NMT 10 µL/L, expressed as acetaldehyde
Benzene	NMT 2 µL/L
Sum of all other impurities[a]	NMT 300 µL/L

[a] Disregard any peaks of less than 9 µL/L (0.03 times the area of the peak corresponding to 4-methylpentan-2-ol in the chromatogram obtained with *Sample solution B*).

SPECIFIC TESTS

- **•Specific Gravity ⟨841⟩:** NMT 0.7962 at 15.56°, indicating NLT 99.2% of C_2H_5OH by weight.•
- **Ultraviolet Absorption**

  Analytical wavelength: 235–340 nm

  Cell: 5 cm

  Reference: Water

  Acceptance criteria

  Absorbance: NMT 0.40 at 240 nm; NMT 0.30 between 250 and 260 nm; NMT 0.10 between 270 and 340 nm

  Curve: The spectrum shows a steadily descending curve with no observable peaks or shoulders.
- **•Clarity of Solution**

  [Note—The *Sample solution* is to be compared to *Standard suspension A* and to water in diffused daylight 5 min after preparation of *Standard suspension A*.]

  Hydrazine solution: 10 mg/mL of hydrazine sulfate in water. Allow to stand for 4–6 h.

  Methenamine solution: Transfer 2.5 g of methenamine to a 100-mL glass-stoppered flask, add 25.0 mL of water, insert the glass stopper, and mix to dissolve.

  Primary opalescent suspension: Transfer 25.0 mL of *Hydrazine solution* to the *Methenamine solution* in the 100-mL glass-stoppered flask. Mix, and allow to stand for 24 h. This suspension is stable for 2 months, provided it is stored in a glass container free from surface defects. The suspension must not adhere to the glass and must be well mixed before use.

  Opalescence standard: Transfer 15.0 mL of the *Primary opalescent suspension* to a 1000-mL volumetric flask, and dilute with water to volume. This suspension should not be used beyond 24 h after preparation.

  Standard suspension A: Dilute 5.0 mL of the *Opalescence standard* with water to 100.0 mL.

  Standard suspension B: Dilute 10.0 mL of the *Opalescence standard* with water to 100.0 mL.

  Sample solution A: Substance to be examined

  Sample solution B: 1.0 mL of *Sample solution A* diluted with water to 20 mL. Allow to stand for 5 min before testing.

  Blank: Water

  Analysis

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

  Transfer a sufficient portion of *Sample solution A* and *Sample solution B* to separate test tubes of colorless, transparent, neutral glass with a flat base and an internal diameter of 15–25 mm to obtain a depth of 40 mm. Similarly transfer portions of *Standard suspension A*, *Standard suspension B*, and *Blank* to separate matching test tubes. Compare samples in diffused daylight, viewing vertically against a black background (see *Visual Comparison* ⟨630⟩). The diffusion of light must be such that *Standard suspension A* can be readily distinguished from water, and *Standard suspension B* can be readily distinguished from *Standard suspension A*.

  Acceptance criteria: *Sample solution A* and *Sample solution B* show the same clarity as that of water, or their opalescence is not more pronounced than that of *Standard suspension A*.•
- **Acidity or Alkalinity**

  Phenolphthalein solution: Dissolve 0.1 g of phenolphthalein in 80 mL of alcohol, and dilute with water to 100 mL.

  Sample: 20 mL of Dehydrated Alcohol

  Analysis: To the *Sample* add 20 mL of freshly boiled and cooled water and 0.1 mL of *Phenolphthalein solution*. The solution is colorless. Add 1.0 mL of 0.01 N sodium hydroxide.

  Acceptance criteria: The solution is pink (30 µg/g, expressed as acetic acid).
- **•Color of Solution**

  Standard stock solution: Combine 3.0 mL of ferric chloride CS, 3.0 mL of cobaltous chloride CS, 2.4 mL of cupric sulfate CS, and 1.6 mL of dilute hydrochloric acid (10 mg/mL).

  Standard solution: 1.0 mL of *Standard stock solution*, diluted with dilute hydrochloric acid (10 mg/mL) to 100 mL. Prepare the *Standard solution* immediately before use.

  Sample solution: Substance to be examined

  Blank: Water

  Analysis

  Samples: *Standard solution*, *Sample solution*, and *Blank*

  Transfer a sufficient portion of each of the *Samples* to individual test tubes of colorless, transparent, neutral glass with a flat base and an internal diameter of 15–25 mm to obtain a depth of 40 mm. Compare the *Samples* in diffused daylight, viewing vertically against a white background (see *Visual Comparison* ⟨630⟩).

  Acceptance criteria: The *Sample solution* has the appearance of water or is not more intensely colored than the *Standard solution*.•

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in tight containers, protected from light.
- **USP Reference Standards** ⟨11⟩

  USP Dehydrated Alcohol RS

Azeotropic Isopropyl Alcohol

DEFINITION

Azeotropic Isopropyl Alcohol contains NLT 91.0% and NMT 93.0% of isopropyl alcohol, by volume, the remainder consisting of water.

IDENTIFICATION

- **A. Infrared Absorption:** The IR absorption spectrum of a thin film of it exhibits a strong broad band at 3.0 µm; a strong region of absorption between 3.35 and 3.5 µm, with its highest peak at 3.36 µm, and others at 3.41 and 3.47 µm; many weak peaks between 3.6 and 6.0 µm, among the most noticeable being those at 3.68, 3.77, 3.97, 4.17, and 5.26 µm; a broad band at 6.2 µm; a strong region of absorption between 6.7 and 7.8 µm, the most prominent features being the peaks at 6.80, 7.09, 7.25 (the highest), 7.46, and 7.63 µm; a strong region of absorption between

8.5 and 9.2 µm, peaking at 8.6, 8.85, and 9.0 µm; and strong peaks at 10.5 and 12.3 µm.

IMPURITIES

- **Limit of Nonvolatile Residue**

Sample: 50 mL

Analysis: Evaporate the *Sample* in a tared porcelain dish on a steam bath to dryness, and heat at 105° for 1 h.

Acceptance criteria: The weight of the residue does not exceed 2.5 mg (0.005%).

- **Volatile Impurities**

System suitability solution: USP 2-Propanol System Suitability RS

Sample solution: Azeotropic Isopropyl Alcohol (Neat)

Chromatographic system

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

Mode: GC

Detector: Thermal conductivity

Column: 0.25-mm × 60-m, coated with a 1.4-µm film of phase G43

Temperature

Injector: 150°

Detector: 200°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
35	0	35	5
35	1	45	2
45	10	100	1

Carrier gas: Helium

Linear velocity: 35 cm/s

Injection size: 1 µL

Split ratio: 10:1

Run time: 30 min

System suitability

Sample: *System suitability solution*

[NOTE—Approximate relative retention times for ethyl ether, acetone, isopropyl alcohol, diisopropyl ether, 1-propanol, and 2-butanol are 0.6, 0.7, 1.0, 1.1, 1.3, and 1.5, respectively.]

Suitability requirements

Resolution: NLT 1.5 between acetone and isopropyl alcohol

Signal-to-noise ratio: NLT 10 for any of the following peaks: ethyl ether, acetone, isopropyl alcohol, diisopropyl ether, 1-propanol, and 2-butanol

Tailing factor: NMT 2.0 for the isopropyl alcohol peak

Relative standard deviation: NMT 2.0% for the isopropyl alcohol peak

Analysis

Samples: *Sample solution*

Calculate the ratio of isopropyl alcohol (C_3H_8O) in the portion of Azeotropic Isopropyl Alcohol taken:

$$\text{Result} = (r_I/r_T)$$

r_I = peak area for isopropyl alcohol

r_T = sum of all of the peak areas excluding the peak for water

Acceptance criteria: NLT 0.99

SPECIFIC TESTS

- **Specific Gravity** ⟨841⟩**:** 0.815–0.810, indicating 91.0%–93.0% of isopropyl alcohol (C_3H_8O) by volume
- **Refractive Index** ⟨831⟩**:** 1.376–1.378 at 20°
- **Acidity**

Sample: 50 mL

Analysis: Place the *Sample* in a suitable flask, and add 100 mL of carbon dioxide-free water. Add 2 drops of phenolphthalein TS, and titrate with 0.020 N sodium hydroxide to a pink color that persists for 30 s.

Acceptance criteria: NMT 0.70 mL of 0.020 N sodium hydroxide is required for neutralization.

ADDITIONAL REQUIREMENTS

- **Packaging and Storage:** Preserve in tight containers, remote from heat.

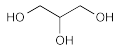
Change to read:

- **USP Reference Standards** ⟨11⟩

USP 2-Propanol System Suitability RS

▲It is a mixture of the following: ethyl ether (0.1%), acetone (0.1%), diisopropyl ether (0.1%), 1-propanol (0.1%), 2-butanol (0.1%), and isopropyl alcohol (99.5%).▲ (ERR 1-Dec-2018)

Glycerin



$C_3H_8O_3$ 92.09

1,2,3-Propanetriol;
Glycerol [56-81-5].

DEFINITION

Glycerin contains NLT 99.0% and NMT 101.0% of $C_3H_8O_3$, calculated on the anhydrous basis.

IDENTIFICATION

[NOTE—Compliance is determined by meeting the requirements for *Identification* tests *A, B,* and *C*.]

Change to read:

- **A. ▲Spectroscopic Identification Tests** ⟨197⟩**,** *Infrared Spectroscopy*: **197F▲** (CN 1-May-2020)
- **B. Limit of Diethylene Glycol and Ethylene Glycol**

Standard solution: 2.0 mg/mL of USP Glycerin RS, 0.050 mg/mL of USP Ethylene Glycol RS, 0.050 mg/mL of USP Diethylene Glycol RS, and 0.10 mg/mL of 2,2,2-trichloroethanol (internal standard) in methanol

Sample solution: 50 mg/mL of Glycerin and 0.10 mg/mL of 2,2,2-trichloroethanol (internal standard) in methanol

Chromatographic system

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

Mode: GC

Detector: Flame ionization

Column: 0.53-mm × 30-m fused-silica analytical column coated with 3.0-µm G43 stationary phase, and a deactivated split liner with glass wool

Temperature

Injector: 220°

Detector: 250°

Column: See the temperature program table.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
100	—	100	4
100	50	120	10
120	50	220	6

Carrier gas: Helium
Injection size: 1.0 µL
Flow rate: 4.5 mL/min
Injection type: Split ratio, about 10:1
System suitability
Sample: *Standard solution*
[NOTE—The relative retention times for ethylene glycol, 2,2,2-trichloroethanol, diethylene glycol, and glycerin are about 0.3, 0.6, 0.8 and 1.0, respectively.]
Suitability requirements
Resolution: NLT 1.5 between diethylene glycol and glycerin
Analysis
Sample: *Sample solution*
Acceptance criteria: If a peak at the retention times for the diethylene glycol or ethylene glycol is present in the *Sample solution,* the peak response ratio relative to 2,2,2-trichloroethanol is NMT the peak response ratio for diethylene glycol or ethylene glycol relative to 2,2,2-trichloroethanol in the *Standard solution;* NMT 0.10% each for diethylene glycol and ethylene glycol is found.

• **C.** Examine the chromatograms obtained in *Identification* test *B*. The retention time of the glycerin peak of the *Sample solution* corresponds to that obtained in the *Standard solution*.

ASSAY

• **PROCEDURE**

Sodium periodate solution: Dissolve 60 g of sodium metaperiodate in sufficient water containing 120 mL of 0.1 N sulfuric acid to make 1000 mL. Do not heat to dissolve the periodate. If the solution is not clear, pass through a sintered-glass filter. Store the solution in a glass-stoppered, light-resistant container. Test the suitability of this solution as follows. Pipet 10 mL into a 250-mL volumetric flask, and dilute with water to volume. To 550 mg of Glycerin dissolved in 50 mL of water, add 50 mL of the diluted periodate solution with a pipet. For a blank, pipet 50 mL of the solution into a flask containing 50 mL of water. Allow the solutions to stand for 30 min, then to each add 5 mL of hydrochloric acid and 10 mL of potassium iodide TS, and rotate to mix. Allow to stand for 5 min, add 100 mL of water, and titrate with 0.1 N sodium thiosulfate, shaking continuously and adding 3 mL of starch TS as the endpoint is approached. The ratio of the volume of 0.1 N sodium thiosulfate required for the glycerin–periodate mixture to that required for the blank should be between 0.750 and 0.765.

Analysis: Transfer 400 mg of Glycerin to a 600-mL beaker, dilute with 50 mL of water, add bromothymol blue TS, and acidify with 0.2 N sulfuric acid to a definite green or greenish yellow color. Neutralize with 0.05 N sodium hydroxide to a definite blue endpoint, free from green color. Prepare a blank containing 50 mL of water, and neutralize in the same manner. Pipet 50 mL of the *Sodium periodate solution* into each beaker, mix by swirling gently, cover with a watch glass, and allow to stand for 30 min at room temperature (not exceeding 35°) in the dark or in subdued light. Add 10 mL of a mixture of equal volumes of ethylene glycol and water, and allow to stand for 20 min. Dilute each solution with water to 300 mL, and titrate with 0.1 N sodium hydroxide VS to a pH of 8.1 ± 0.1 for the specimen under assay and 6.5 ± 0.1 for the blank, using a pH meter. Each mL of 0.1 N sodium hydroxide, after correction for the blank, is equivalent to 9.210 mg of $C_3H_8O_3$.

Acceptance criteria: 99.0%–101.0% on the anhydrous basis

IMPURITIES

INORGANIC IMPURITIES

• **Chloride and Sulfate,** *Chloride* ⟨221⟩**:** A 7.0-g portion shows no more chloride than corresponds to 0.10 mL of 0.020 N hydrochloric acid (NMT 10 ppm).

• **Chloride and Sulfate,** *Sulfate* ⟨221⟩**:** A 10-g portion shows no more sulfate than corresponds to 0.20 mL of 0.020 N sulfuric acid (NMT 20 ppm).

• **RESIDUE ON IGNITION** ⟨281⟩**:** Heat 50 g in an open, shallow 100-mL porcelain dish until it ignites, and allow it to burn without further application of heat in a place free from drafts. Cool, moisten the residue with 0.5 mL of sulfuric acid, and ignite to constant weight: the weight of the residue does not exceed 5 mg (0.01%).

ORGANIC IMPURITIES

• **Procedure 1: Related Compounds**

System suitability solution: 0.5 mg/mL each of USP Diethylene Glycol RS and USP Glycerin RS
Sample solution: 50 mg/mL of Glycerin
Chromatographic system
(See *Chromatography* ⟨621⟩*, System Suitability*.)
Mode: GC
Detector: Flame ionization
Column: 0.53-mm × 30-m fused-silica analytical column coated with 3.0-µm G43 stationary phase, and an inlet liner having an inverted cup or spiral structure
Temperature
Injector: 220°
Detector: 250°
Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
100	—	100	—
100	7.5	220	4

Carrier gas: Helium
Injection size: 0.5 µL
Linear velocity: 38 cm/s
Injection type: Split ratio, about 10:1
System suitability
Sample: *System suitability solution*
Suitability requirements
Resolution: NLT 7.0 between diethylene glycol and glycerin
Analysis
Sample: *Sample solution*
Calculate the percentage of each impurity, excluding any solvent peaks and diethylene glycol, in the portion of Glycerin taken:

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

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

Acceptance criteria
Individual impurities: NMT 0.1%
Total impurities: NMT 1.0%
• **Procedure 2: Limit of Chlorinated Compounds**
Sample: 5 g of Glycerin
Analysis: Transfer the *Sample* into a dry, round-bottom, 100-mL flask. Add 15 mL of morpholine, and connect the flask by a ground joint to a reflux condenser. Reflux gently for 3 h. Rinse the condenser with 10 mL of water, receiving the washings in the flask, and cautiously acidify with nitric acid. Transfer the solution to a suitable comparison tube, add 0.50 mL of silver nitrate TS, and dilute with water to 50.0 mL.
Acceptance criteria: The turbidity is not greater than that of a blank to which 0.20 mL of 0.020 N hydrochloric acid has been added, the refluxing being omitted (NMT 30 ppm of Cl).
• **Procedure 3: Fatty Acids and Esters**
Sample solution: Mix 50 g of Glycerin with 50 mL of freshly boiled water and 5 mL of 0.5 N sodium hydroxide VS. Boil the mixture for 5 min, cool, and add phenolphthalein TS.
Analysis: Titrate the excess alkali with 0.5 N hydrochloric acid VS. Perform a blank determination (see *Titrimetry* ⟨541⟩, *Residual Titrations*).
Acceptance criteria: NMT 1 mL of 0.5 N sodium hydroxide is consumed.

SPECIFIC TESTS

• **Color:** When viewed downward against a white surface in a 50-mL color-comparison tube, the color is not darker than the color of a standard made by diluting 0.40 mL of ferric chloride CS with water to 50 mL and similarly viewed in a color-comparison tube of approximately the same diameter and color as that containing the Glycerin.
• **Specific Gravity** ⟨841⟩**:** NLT 1.249
• **Water Determination,** *Method I* ⟨921⟩**:** NMT 5.0%

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Preserve in tight containers.
• **USP Reference Standards** ⟨11⟩
USP Diethylene Glycol RS
USP Ethylene Glycol RS
USP Glycerin RS
1,2,3-Propanetriol.
$C_3H_8O_3$ 92.10

Hydrogen Peroxide Concentrate

H_2O_2 34.01
Hydrogen peroxide [7722-84-1].

DEFINITION

Hydrogen Peroxide Concentrate contains NLT 29.0% and NMT 32.0%, by weight, of hydrogen peroxide (H_2O_2). It contains NMT 0.05% of a suitable preservative or preservatives.
[**Caution**—Hydrogen Peroxide Concentrate is a strong oxidant.]

IDENTIFICATION

• **A.**
Sample: 1 mL of Concentrate
Analysis: Shake the *Sample* with 10 mL of water containing 1 drop of 2 N sulfuric acid, and add 2 mL of ether. Subsequently add a drop of potassium dichromate TS.
Acceptance criteria: The addition of potassium dichromate TS produces an evanescent blue color in the water layer, which upon agitation and standing passes into the ether layer.

ASSAY

• **Procedure**
Sample solution: Weigh about 1 mL of Concentrate in a 100-mL volumetric flask, and dilute with water to volume.
Titrimetric system
Mode: Direct titration
Titrant: 0.1 N potassium permanganate VS
Endpoint detection: Visual
Analysis: To 20.0 mL of the *Sample solution* add 20 mL of 2 N sulfuric acid, and titrate with *Titrant*. Each mL of 0.1 N potassium permanganate is equivalent to 1.701 mg of hydrogen peroxide (H_2O_2).
Acceptance criteria: 29.0%–32.0%, by weight

IMPURITIES

• **Chloride and Sulfate,** *Chloride* ⟨221⟩
Sample solution: 1.5 g of Concentrate, diluted to 25 mL
Acceptance criteria: 50 ppm; the *Sample solution* shows no more chloride than 0.10 mL of 0.020 N hydrochloric acid.
• **Limit of Nonvolatile Residue**
Sample: 20 mL of Concentrate, previously shaken
Analysis: Evaporate the *Sample* on a steam bath to dryness, and dry the residue at 105° for 1 h.
Acceptance criteria: NMT 30 mg of residue

SPECIFIC TESTS

• **Acidity**
Sample solution: 25 g of Concentrate, diluted to 250 mL
Analysis: To 25 mL of the *Sample solution* add phenolphthalein TS, and titrate with 0.10 N sodium hydroxide.
Acceptance criteria: NMT 2.5 mL is required for neutralization.
• **Limit of Preservative**
Sample: 90 mL of Concentrate, well mixed
Analysis: Extract the *Sample* in a separator with a mixture of chloroform and ether (3:2), using 50, 25, and 25 mL, respectively. Evaporate the combined extracts at room temperature in a tared glass dish to dryness, and dry over silica gel for 2 h.
Acceptance criteria: 0.05%; the residue, if any, weighs NMT 50 mg.

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Preserve in partially filled containers having a small vent in the closure, and store in a cool place.
• **Labeling:** Label it to indicate the name and amount of any added preservative. Label it to state that this article is not intended for direct administration to humans or animals.

Hydrogen Peroxide Topical Solution

H_2O_2 34.01
Hydrogen peroxide [7722-84-1].

DEFINITION

Hydrogen Peroxide Topical Solution contains, in each 100 mL, NLT 2.5 g and NMT 3.5 g of hydrogen peroxide (H_2O_2). It contains NMT 0.05% of a suitable preservative or preservatives.

IDENTIFICATION

• **A.**
Sample: 1 mL
Analysis: Shake the *Sample* with 10 mL of water containing 1 drop of 2 N sulfuric acid, and add 2 mL of ether. Subsequently add a drop of potassium dichromate TS.

Acceptance criteria: The addition of potassium dichromate TS produces an evanescent blue color in the water layer, which upon agitation and standing passes into the ether layer.

ASSAY

• **PROCEDURE**

Sample: 2 mL

Titrimetric system

Mode: Direct titration

Titrant: 0.1 N potassium permanganate VS

Endpoint detection: Visual

Analysis: Pipet the *Sample* into a suitable flask containing 20 mL of water. Add 20 mL of 2 N sulfuric acid, and titrate with *Titrant*. Each mL of 0.1 N potassium permanganate is equivalent to 1.701 mg of hydrogen peroxide (H_2O_2).

Acceptance criteria: 2.5–3.5 g in 100 mL

IMPURITIES

• **BARIUM**

Sample: 10 mL

Analysis: To the *Sample* add two drops of 2 N sulfuric acid.

Acceptance criteria: No turbidity or precipitate is produced within 10 min.

• **LIMIT OF NONVOLATILE RESIDUE**

Sample: 20 mL, previously shaken

Analysis: Evaporate the *Sample* on a steam bath to dryness, and dry the residue at 105° for 1 h.

Acceptance criteria: NMT 30 mg of the residue

SPECIFIC TESTS

• **ACIDITY**

Sample: 25 mL

Analysis: To the *Sample* add phenolphthalein TS, and titrate with 0.10 N sodium hydroxide.

Acceptance criteria: NMT 2.5 mL is required for neutralization.

• **LIMIT OF PRESERVATIVE**

Sample: 100 mL, well mixed

Diluent: Chloroform and ether (3:2)

Analysis: Extract the *Sample* in a separator with *Diluent*, using 50, 25, and 25 mL, respectively. Evaporate the combined extracts at room temperature in a tared glass dish to dryness, and dry over silica gel for 2 h.

Acceptance criteria: 0.05%; the residue, if any, weighs NMT 50 mg.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, at controlled room temperature.

Isopropyl Alcohol

C_3H_8O 60.10

2-Propanol;
Isopropanol [67-63-0].

DEFINITION

Isopropyl Alcohol contains NLT 99.0% of isopropyl alcohol (C_3H_8O).

IDENTIFICATION

Change to read:

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

• **B.** The retention time of the major peak of the *Sample solution* corresponds to the 2-propanol peak of the *System suitability solution*, as obtained in the *Assay*.

ASSAY

• **PROCEDURE**

System suitability solution: USP 2-Propanol System Suitability RS

Sample solution: Isopropyl Alcohol (neat)

Chromatographic system

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

Mode: GC

Detector: Flame ionization

Column: 0.25-mm × 60-m fused silica column, coated with a 1.4-µm film of phase G43

Temperatures

Detector: 200°

Injection port: 150°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
35	—	35	5
35	1	45	—
45	10	100	1

Carrier gas: Helium

Flow rate: 2.3 mL/min

Injection volume: 1 µL

Injection type: Split injection; split ratio is about 50:1. [NOTE—A 4-mm straight liner is suitable.]

Run time: 22 min

System suitability

Sample: *System suitability solution*

[NOTE—See *Table 2*.]

Table 2

Name	Relative Retention Time
Ethyl ether	0.7
Acetone	0.9
Isopropyl alcohol	1.0
Diisopropyl ether	1.4
n-Propyl alcohol (1-propanol)	1.5
2-Butanol	2.0

Suitability requirements

Resolution: NLT 1.5 between acetone and isopropyl alcohol

Relative standard deviation: NMT 2.0% for the isopropyl alcohol peak

Tailing factor: NMT 2.0 for the isopropyl alcohol peak

Signal-to-noise ratio: NLT 10 for any of the following peaks: ethyl ether, acetone, isopropyl alcohol, diisopropyl ether, 1-propanol, and 2-butanol

Analysis
Sample: *Sample solution*
Calculate the percentage of isopropyl alcohol (C_3H_8O) in the portion of Isopropyl Alcohol taken:

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

r_U = peak response of isopropyl alcohol
r_T = sum of all the peak responses

Acceptance criteria: NLT 99.0%

IMPURITIES

• **LIMIT OF VOLATILE IMPURITIES**
System suitability solution, Sample solution, Chromatographic system, and **System suitability:** Proceed as directed in the *Assay*.
Analysis
Samples: *System suitability solution* and *Sample solution*
Identify each individual impurity peak in the *Sample solution* based on that in the *System suitability solution*.
Calculate the percentage of each individual impurity in the portion of Isopropyl Alcohol taken:

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

r_U = peak response of each individual impurity in the *Sample solution*
r_T = sum of all the peaks in the *Sample solution*

Acceptance criteria: See *Table 3*.

Table 3

Impurity	Percentage (%)
Each individual	NMT 0.1
Total	NMT 1.0

• **LIMIT OF NONVOLATILE RESIDUE**
Sample: 50 mL
Analysis: Evaporate the *Sample* in a tared porcelain dish on a steam bath to dryness, and heat at 105° for 1 h.
Acceptance criteria: NMT 2.5 mg (0.005%)

SPECIFIC TESTS

• **SPECIFIC GRAVITY** ⟨841⟩**:** 0.783–0.787
• **REFRACTIVE INDEX** ⟨831⟩**:** 1.376–1.378 at 20°
• **ACIDITY**
Sample solution: To 50 mL of Isopropyl Alcohol add 100 mL of carbon dioxide-free water.
Analysis: To the *Sample solution* add 2 drops of phenolphthalein TS, and titrate with 0.020 N sodium hydroxide to a pink color that persists for 30 s.
Acceptance criteria: NMT 0.70 mL of 0.020 N sodium hydroxide is required for neutralization.
• **WATER DETERMINATION,** *Method I* ⟨921⟩
Sample: 5.0 g
Acceptance criteria: NMT 0.5%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers, and prevent exposure to excessive heat. Protect from light.

• **USP REFERENCE STANDARDS** ⟨11⟩
USP 2-Propanol RS
USP 2-Propanol System Suitability RS
It contains isopropyl alcohol with 0.1% each of ethyl ether, acetone, diisopropyl ether, 1-propanol, and 2-butanol.

Purified Water

[NOTE—For microbiological guidance, see general information chapter *Water for Pharmaceutical Purposes* ⟨1231⟩.]

H_2O 18.02

DEFINITION

Purified Water is water obtained by a suitable process. It is prepared from water complying with the U. S. Environmental Protection Agency National Primary Drinking Water Regulations or with the drinking water regulations of the European Union or of Japan, or with the World Health Organization's Guidelines for Drinking Water Quality. It contains no added substance.
[NOTE—Purified Water whether it is available in bulk or packaged forms, is intended for use as an ingredient of official preparations and in tests and assays unless otherwise specified (see *General Notices, 8.230. Water*). Where used for sterile dosage forms, other than for parenteral administration, process the article to meet the requirements under *Sterility Tests* ⟨71⟩, or first render the Purified Water sterile and thereafter protect it from microbial contamination. Do not use Purified Water in preparations intended for parenteral administration. For such purposes use Water for Injection, Bacteriostatic Water for Injection, or Sterile Water for Injection. In addition to the *Specific Tests*, Purified Water that is packaged for commercial use elsewhere meets the additional requirements for *Packaging and Storage* and *Labeling* as indicated under *Additional Requirements*.]

SPECIFIC TESTS

[NOTE—Required for bulk and packaged forms of *Purified Water*]
• **TOTAL ORGANIC CARBON** ⟨643⟩**:** Meets the requirements
• **WATER CONDUCTIVITY,** *Bulk Water* ⟨645⟩**:** Meets the requirements

ADDITIONAL REQUIREMENTS

[NOTE—Required for packaged forms of Purified Water]
• **PACKAGING AND STORAGE:** Where packaged, preserve in unreactive storage containers that are designed to prevent microbial entry.
• **LABELING:** Where packaged, label it to indicate the method of preparation and that it is not intended for parenteral administration.

Delete the following:

▲• **USP REFERENCE STANDARDS** ⟨11⟩
USP 1,4-Benzoquinone RS▲ (ERR 1-Nov-2018)

Published August 17, 2020

Excipients

USP and NF Excipients, Listed by Functional Category

In the following reference table, the grouping of excipients by functional category is intended to summarize commonly identified purposes that these excipients serve in drug product formulations. The association of a functional category with a particular dosage form in this table is not absolute and does not limit the use of an excipient to a single type of dosage form or delivery system.

Adhesive

Dosage Form: Transdermals and "Patches"

Dimethicone
Polyisobutylene

Air Displacement

Carbon Dioxide
Nitrogen

Alcohol Denaturant

Denatonium Benzoate
Methyl Isobutyl Ketone
Sucrose Octaacetate

Antifoaming Agent

Dimethicone
Lauric Acid
Myristic Acid
Palmitic Acid
Simethicone

Antimicrobial Preservative

Dosage Form: Oral Liquids

Alcohol
Benzalkonium Chloride
Benzalkonium Chloride Solution
Benzethonium Chloride
Benzoic Acid
Benzyl Alcohol
Boric Acid
Butylparaben
Calcium Acetate
Calcium Chloride
Calcium Lactate
Calcium Propionate
Cetrimonium Bromide
Cetylpyridinium Chloride
Chlorobutanol
Chlorocresol
Chloroxylenol
Cresol
Dehydroacetic Acid
Erythorbic Acid
Ethylparaben
Ethylparaben Sodium
Glycerin
Imidurea
Mandelic Acid
Methylparaben
Methylparaben Sodium
Monothioglycerol
Pentetic Acid
Phenol
Phenoxyethanol
Phenylethyl Alcohol
Phenylmercuric Acetate
Phenylmercuric Nitrate
Potassium Benzoate
Potassium Metabisulfite
Potassium Sorbate
Propionic Acid
Propylene Glycol
Propylparaben
Propylparaben Sodium
Sodium Acetate
Sodium Benzoate
Sodium Borate
Sodium Dehydroacetate
Sodium Lactate Solution
Sodium Metabisulfite
Sodium Propionate
Sodium Sulfite
Sorbic Acid
Sulfur Dioxide
Thimerosal
Thymol
Zinc Oxide

Dosage Form: Ophthalmic Preparations

Benzalkonium Chloride

Benzyl Alcohol
Chlorobutanol
Propylparaben
Sorbic Acid

Antioxidant

Dosage Form: Oral Liquids
Ascorbic Acid
Ascorbyl Palmitate
Butylated Hydroxyanisole
Butylated Hydroxytoluene
Citric Acid Monohydrate
Erythorbic Acid
Fumaric Acid
Hypophosphorous Acid
Lactobionic Acid
Malic Acid
Methionine
Monothioglycerol
Potassium Metabisulfite
Propionic Acid
Propyl Gallate
Racemethionine
Sodium Ascorbate
Sodium Bisulfite
Sodium Formaldehyde Sulfoxylate
Sodium Metabisulfite
Sodium Sulfite
Sodium Thiosulfate
Stannous Chloride
Sulfur Dioxide
Thymol
Tocopherol
Tocopherols Excipient
Vitamin E
Vitamin E Polyethylene Glycol Succinate

Bulking Agent

Alpha-Lactalbumin
Polydextrose
Polydextrose, Hydrogenated
Pullulan

Dosage Form: Parenterals
Creatinine
Glycine
Mannitol
Trehalose

Capsule Shell

Dosage Form: Tablets and Capsules
Gelatin
Hypromellose
Pullulan

Dosage Form: Dry Powder Inhalers
Gelatin
Hypromellose

Carrier

Dosage Form: Dry Powder Inhalers
Lactose, Anhydrous
Lactose, Monohydrate

Chelating and/or Complexing Agent

Dosage Form: Oral Liquids
Alfadex
Betadex
Betadex Sulfobutyl Ether Sodium
Citric Acid Monohydrate
Edetate Calcium Disodium
Edetate Disodium
Edetic Acid
Galactose
Gamma Cyclodextrin
Hydroxypropyl Betadex
Alpha-Lactalbumin
Malic Acid
Oxyquinoline Sulfate
Pentetic Acid
Potassium Citrate
Sodium Phosphate, Dibasic
Sodium Phosphate, Monobasic

Change to read:

Coating Agent

Dosage Form: Tablets and Capsules
Amino Methacrylate Copolymer
Ammonio Methacrylate Copolymer
Ammonio Methacrylate Copolymer Dispersion
Calcium Carbonate
Carboxymethylcellulose Calcium
Carboxymethylcellulose Sodium
Carboxymethylcellulose Sodium, Enzymatically-Hydrolyzed
Cellaburate
Cellacefate
Cellulose Acetate
Cetyl Alcohol
Chitosan
Coconut Oil
Coconut Oil, Hydrogenated
Copovidone
Corn Syrup Solids
Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion
Ethylcellulose
Ethylcellulose Aqueous Dispersion
Ethylcellulose Dispersion Type B
Ethylene Glycol and Vinyl Alcohol Graft Copolymer
Gelatin
Glaze, Pharmaceutical
Glucose, Liquid
Glyceryl Behenate
▲▲ (NF 1-Aug-2020)▲▲ (NF 1-Aug-2020)
Glyceryl Dibehenate
Hydroxyethyl Cellulose

Hydroxypropyl Cellulose
Hypromellose
Hypromellose Acetate Succinate
Hypromellose Phthalate
Isomalt
Alpha-Lactalbumin
Maltitol
Maltodextrin
Methacrylic Acid and Ethyl Acrylate Copolymer
Methacrylic Acid and Ethyl Acrylate Copolymer Dispersion
Methacrylic Acid and Ethyl Acrylate Copolymer, Partially-Neutralized
Methacrylic Acid and Methyl Methacrylate Copolymer
Methylcellulose
Palm Kernel Oil
Palm Oil
Palm Oil, Hydrogenated
Polydextrose
Polydextrose, Hydrogenated
Polyethylene Glycol
Polyethylene Glycol 3350
Polyethylene Oxide
Polyvinyl Acetate
Polyvinyl Acetate Dispersion
Polyvinyl Acetate Phthalate
Polyvinyl Alcohol
Pullulan
Rapeseed Oil, Fully Hydrogenated
Rapeseed Oil, Superglycerinated Fully Hydrogenated
Shellac
Starch, Pregelatinized Modified
Sucrose
Sugar, Confectioner's
Sunflower Oil
Titanium Dioxide
Wax, Carnauba
Wax, Microcrystalline
Xylitol
Zein
Zinc Oxide

Colloid Stabilizing Agent

Dosage Form: Radiopharmaceuticals
Gelatin

Coloring Agent

Caramel
Ferric Oxide
Ferrosoferric Oxide

Dosage Form: Tablets and Capsules
Aluminum Oxide

Desiccant

Calcium Chloride
Calcium Sulfate
Polyvinyl Acetate
Silicon Dioxide

Change to read:

Diluent

▲Sucrose Diacetate Hexaisobutyrate▲ (NF 1-Aug-2020)

Dosage Form: Tablets and Capsules
Amino Methacrylate Copolymer
Ammonio Methacrylate Copolymer
Ammonio Methacrylate Copolymer Dispersion
Calcium Carbonate
Calcium Phosphate, Dibasic, Anhydrous
Calcium Phosphate, Dibasic, Dihydrate
Calcium Phosphate, Tribasic
Calcium Sulfate
Cellaburate
Cellulose, Microcrystalline
Cellulose, Silicified Microcrystalline
Cellulose, Powdered
Cellulose Acetate
Corn Syrup
Corn Syrup Solids
Dextrates
Dextrin
Dextrose
Dextrose Excipient
Erythritol
Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion
Fructose
Invert Sugar
Isomalt
Kaolin
Alpha-Lactalbumin
Lactitol
Lactose, Anhydrous
Lactose, Monohydrate
Magnesium Carbonate
Magnesium Oxide
Maltitol
Maltodextrin
Maltose
Mannitol
Methacrylic Acid and Ethyl Acrylate Copolymer
Methacrylic Acid and Ethyl Acrylate Copolymer Dispersion
Methacrylic Acid and Methyl Methacrylate Copolymer
Polydextrose
Polyethylene Glycol
Polyethylene Glycol 3350
Propylene Glycol Monocaprylate
Pullulan
Simethicone
Sodium Chloride
Sorbitol
Starch, Pregelatinized
Starch, Pregelatinized Modified
Starch, Corn
Starch, Hydroxypropyl Corn
Starch, Pregelatinized Hydroxypropyl Corn
Starch, Pea
Starch, Hydroxypropyl Pea

Starch, Pregelatinized Hydroxypropyl Pea
Starch, Potato
Starch, Hydroxypropyl Potato
Starch, Pregelatinized Hydroxypropyl Potato
Starch, Tapioca
Starch, Wheat
Starch Hydrolysate, Hydrogenated
Sucrose
Sugar, Compressible
Sugar, Confectioner's
Sugar Spheres
Sunflower Oil
Talc
Trehalose
Xylitol

Disintegrant

Dosage Form: Tablets and Capsules

Alginic Acid
Carboxymethylcellulose Calcium
Carboxymethylcellulose Sodium
Cellulose, Microcrystalline
Cellulose, Silicified Microcrystalline
Cellulose, Powdered
Croscarmellose Sodium
Crospovidone
Glycine
Guar Gum
Hydroxypropyl Cellulose, Low-Substituted
Magnesium Aluminum Silicate
Maltose
Methylcellulose
Polacrilin Potassium
Pullulan
Silicon Dioxide, Colloidal
Sodium Alginate
Sodium Starch Glycolate
Starch, Pregelatinized Modified
Starch, Corn
Starch, Hydroxypropyl Corn
Starch, Pregelatinized Hydroxypropyl Corn
Starch, Pea
Starch, Hydroxypropyl Pea
Starch, Pregelatinized Hydroxypropyl Pea
Starch, Potato
Starch, Hydroxypropyl Potato
Starch, Pregelatinized Hydroxypropyl Potato
Starch, Tapioca
Starch, Wheat
Trehalose

Emollient

Dosage Form: Semisolids, Topicals, and Suppositories

Alkyl (C12-15) Benzoate
Almond Oil
Aluminum Monostearate
Canola Oil
Castor Oil
Cetostearyl Alcohol
Cholesterol
Coconut Oil
Cyclomethicone
Dimethicone
Ethylene Glycol Stearates
Glycerin
Glyceryl Monooleate
Glyceryl Monostearate
Isopropyl Isostearate
Isopropyl Myristate
Isopropyl Palmitate
Isostearyl Isostearate
Hydrogenated Lanolin
Lecithin
Mineral Oil
Mineral Oil, Light
Myristyl Alcohol
Octyldodecanol
Oleyl Alcohol
Oleyl Oleate
Petrolatum
Polydecene, Hydrogenated
Polypropylene Glycol 11 Stearyl Ether
Propylene Glycol Dilaurate
Propylene Glycol Monolaurate
Safflower Oil
Soybean Oil, Hydrogenated
Sunflower Oil
Wax, Cetyl Esters
Xylitol
Zinc Acetate

Emulsifying Agent

Dosage Form: Oral Liquids

Acacia
Agar
Behenoyl Polyoxylglycerides
Benzalkonium Chloride
Benzyl Benzoate
Caprylic Acid
Caprylocaproyl Polyoxylglycerides
Carbomer Copolymer
Carbomer Homopolymer
Carbomer Interpolymer
Carboxymethylcellulose Calcium
Cetostearyl Alcohol
Cetyl Alcohol
Cetylpyridinium Chloride
Cholesterol
Coconut Oil
Desoxycholic Acid
[(Title for this monograph—not to change until December 1, 2021.) (Prior to December 1, 2021, the current practice of labeling the article of commerce with the name Desoxycholic Acid may be continued. Use of the name Deoxycholic Acid will be permitted as of December 1, 2016; however, the use of this name will not be mandatory until December 1, 2021. The 60-month extension will provide the time needed by manufacturers and users to make necessary changes.)]
Deoxycholic Acid

Diethanolamine (Adjunct)
Diethylene Glycol Monoethyl Ether
Diethylene Glycol Stearates
Egg Phospholipids
Ethylene Glycol Stearates
Glyceryl Distearate
Glyceryl Mono and Dicaprylate
Glyceryl Mono and Dicaprylocaprate
Glyceryl Monocaprylate
Glyceryl Monocaprylocaprate
Glyceryl Monolinoleate
Glyceryl Monooleate
Glyceryl Monostearate
Glyceryl Tricaprylate
Glyceryl Tristearate
Hydroxypropyl Cellulose
Hypromellose
Isopropyl Isostearate
Isostearyl Isostearate
Alpha-Lactalbumin
Lanolin
Hydrogenated Lanolin
Lanolin Alcohols
Lauric Acid
Lauroyl Polyoxylglycerides
Lecithin
Linoleoyl Polyoxylglycerides
Magnesium Oxide
Medium-chain Triglycerides
Methylcellulose
Mono- and Di-glycerides
Monoethanolamine (Adjunct)
Myristic Acid
Octyldodecanol
Oleic Acid (Adjunct)
Oleoyl Polyoxylglycerides
Oleyl Alcohol (Stabilizer)
Oleyl Oleate
Palm Kernel Oil
Palm Oil
Palmitic Acid
Pectin
Poloxamer
Polycarbophil
Polyglyceryl 3 Diisostearate
Polyglyceryl Dioleate
Polyoxyl 10 Oleyl Ether
Polyoxyl 15 Hydroxystearate
Polyoxyl 20 Cetostearyl Ether
Polyoxyl 35 Castor Oil
Polyoxyl 40 Castor Oil, Hydrogenated
Polyoxyl 40 Stearate
Polyoxyl Lauryl Ether
Polyoxyl Stearate
Polyoxyl Stearyl Ether
Polysorbate 20
Polysorbate 40
Polysorbate 60
Polysorbate 80
Potassium Alginate
Propylene Glycol Alginate
Propylene Glycol Diacetate
Propylene Glycol Dicaprylate/Dicaprate
Propylene Glycol Dilaurate
Propylene Glycol Monocaprylate
Propylene Glycol Monolaurate
Propylene Glycol Monostearate
Rapeseed Oil, Superglycerinated Fully Hydrogenated
Sodium Borate
Sodium Cetostearyl Sulfate
Sodium Lauryl Sulfate
Sodium Stearate
Sorbitan Monolaurate
Sorbitan Monooleate
Sorbitan Monopalmitate
Sorbitan Monostearate
Sorbitan Sesquioleate
Sorbitan Trioleate
Stannous Chloride
Starch, Hydroxypropyl Corn
Starch, Hydroxypropyl Pea
Starch, Hydroxypropyl Potato
Stearic Acid
Stearoyl Polyoxylglycerides
Sucrose Palmitate
Sucrose Stearate
Sunflower Oil
Trolamine
Vitamin E Polyethylene Glycol Succinate
Wax, Emulsifying

Film-Forming Agent

Dosage Form: Tablets and Capsules

Alginic Acid
Amino Methacrylate Copolymer
Ammonio Methacrylate Copolymer
Ammonio Methacrylate Copolymer Dispersion
Carboxymethylcellulose Calcium
Carboxymethylcellulose Sodium
Carboxymethylcellulose Sodium, Enzymatically-Hydrolyzed
Cellaburate
Cellacefate
Cellulose Acetate
Chitosan
Copovidone
Dibutyl Phthalate
Diethyl Phthalate
Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion
Ethylcellulose
Ethylcellulose Aqueous Dispersion
Ethylcellulose Dispersion Type B
Ethylene Glycol and Vinyl Alcohol Grafted Copolymer
Gelatin
Glaze, Pharmaceutical
Hydroxyethyl Cellulose
Hydroxypropyl Cellulose
Hypromellose
Hypromellose Acetate Succinate

Hypromellose Phthalate
Methacrylic Acid and Ethyl Acrylate Copolymer
Methacrylic Acid and Ethyl Acrylate Copolymer Dispersion
Methacrylic Acid and Ethyl Acrylate Copolymer, Partially-Neutralized
Methacrylic Acid and Methyl Methacrylate Copolymer
Methylcellulose
Polyethylene Glycol 3350
Polyvinyl Acetate
Polyvinyl Acetate Dispersion
Polyvinyl Acetate Phthalate
Polyvinyl Alcohol
Pullulan
Pyroxylin
Shellac
Sodium Alginate

Dosage Form: Transdermals and "Patches"
Chitosan
Dextrin
Gelatin
Hydroxyethyl Cellulose
Hypromellose
Pectin
Polyethylene Glycol
Polyvinyl Alcohol
Pullulan
Sodium Alginate
Xanthan Gum

Filtering Aid

Cellulose, Powdered
Siliceous Earth, Purified

Flavors and Fragrance

Anise Oil
Eucalyptus Oil
Isobutyl Alcohol
Neohesperidin Dihydrochalcone
Sodium Succinate
Star Anise Oil

Dosage Form: Tablets and Capsules
Adipic Acid
Almond Oil
Anethole
Benzaldehyde
Denatonium Benzoate
Ethyl Acetate
Ethyl Maltol
Ethyl Vanillin
Ethylcellulose
Fructose
Fumaric Acid
L-Glutamic Acid, Hydrochloride
Lactitol
Leucine
Malic Acid
Maltol
Menthol
Methionine
Methyl Salicylate
Monosodium Glutamate
Peppermint
Peppermint Oil
Peppermint Spirit
Racemethionine
Rose Oil
Rose Water, Stronger
Sodium Acetate
Sodium Lactate Solution
Tartaric Acid
Thymol
Vanillin

Free Radical Scavenger

Dosage Form: Radiopharmaceuticals
Aminobenzoic Acid
Methylene Blue

Glidant and/or Anticaking Agent

Dosage Form: Tablets and Capsules
Calcium Phosphate, Tribasic
Calcium Silicate
Cellulose, Powdered
Magnesium Oxide
Magnesium Silicate
Magnesium Trisilicate
Silica, Dental-Type
Silica, Hydrophobic Colloidal
Silicon Dioxide, Colloidal
Sodium Stearate
Talc

Humectant

Corn Syrup Solids
Cyclomethicone
Erythritol
Glycerin
Hexylene Glycol
Inositol
Hydrogenated Lanolin
Maltitol
Polydextrose
Polydextrose, Hydrogenated
Propylene Glycol
Sodium Lactate Solution
Sorbitol
Sorbitol Sorbitan Solution
Starch Hydrolysate, Hydrogenated
Tagatose
Triacetin
Xylitol

Change to read:

Lubricant

Dosage Form: Tablets and Capsules
Behenoyl Polyoxylglycerides
Calcium Stearate

Castor Oil, Hydrogenated
Coconut Oil, Hydrogenated
Glyceryl Behenate
▲▲ (NF 1-Aug-2020)▲▲ (NF 1-Aug-2020)
Glyceryl Dibehenate
Glyceryl Mono and Dicaprylate
Glyceryl Mono and Dicaprylocaprate
Glyceryl Monocaprylate
Glyceryl Monocaprylocaprate
Glyceryl Monostearate
Glyceryl Tricaprylate
Glyceryl Tristearate
Lauric Acid
Magnesium Stearate
Mineral Oil, Light
Myristic Acid
Palm Oil, Hydrogenated
Palmitic Acid
Poloxamer
Polyethylene Glycol
Polyethylene Glycol 3350
Polyoxyl 10 Oleyl Ether
Polyoxyl 15 Hydroxystearate
Polyoxyl 20 Cetostearyl Ether
Polyoxyl 35 Castor Oil
Polyoxyl 40 Castor Oil, Hydrogenated
Polyoxyl 40 Stearate
Polysorbate 20
Polysorbate 40
Polysorbate 60
Polysorbate 80
Potassium Benzoate
Sodium Benzoate
Sodium Lauryl Sulfate
Sodium Stearate
Sodium Stearyl Fumarate
Sorbitan Monolaurate
Sorbitan Monooleate
Sorbitan Monopalmitate
Sorbitan Monostearate
Sorbitan Sesquioleate
Sorbitan Trioleate
Stearic Acid
Stearic Acid, Purified
Sucrose Stearate
Talc
Vegetable Oil, Hydrogenated, Type I
Zinc Stearate

Change to read:

Ointment Base

Dosage Form: Semisolids, Topicals, and Suppositories
Caprylocaproyl Polyoxylglycerides
Coconut Oil
Diethylene Glycol Monoethyl Ether
Lanolin
Hydrogenated Lanolin
Lanolin Alcohols
Lauroyl Polyoxylglycerides
Linoleoyl Polyoxylglycerides
Ointment, Hydrophilic
Ointment, White
Ointment, Yellow
Oleoyl Polyoxylglycerides
Paraffin
Petrolatum
Petrolatum, Hydrophilic
Petrolatum, White
Polydecene, Hydrogenated
Polyethylene Glycol
Polyethylene Glycol 3350
Polyethylene Glycol Monomethyl Ether
Polyglyceryl 3 Diisostearate
Rose Water Ointment
Squalane
Stearoyl Polyoxylglycerides
▲Sucrose Diacetate Hexaisobutyrate▲ (NF 1-Aug-2020)
Vegetable Oil, Hydrogenated, Type II
Vitamin E Polyethylene Glycol Succinate

Pharmaceutical Water

Dosage Form: Parenterals
Water for Injection
Water for Injection, Bacteriostatic
Water for Injection, Sterile
Water for Irrigation, Sterile
Water Purified
Water Purified, Sterile

pH Modifier (Acidifying Agent/ Alkalizing Agent/Buffering Agent)

Sodium Succinate

Dosage Form: Oral Liquids
Acetic Acid
Acetic Acid, Glacial
Adipic Acid
Ammonia Solution, Strong
Ammonium Carbonate
Ammonium Chloride
Ammonium Phosphate
Boric Acid
Calcium Carbonate
Calcium Hydroxide
Calcium Lactate
Calcium Phosphate, Tribasic
Citric Acid Monohydrate
Citric Acid, Anhydrous
Diethanolamine
Fumaric Acid
Glycine
Hydrochloric Acid
Hydrochloric Acid, Diluted
Alpha-Lactalbumin
Lactic Acid
Lysine Hydrochloride
Maleic Acid
Malic Acid
Methionine

Monoethanolamine
Monosodium Glutamate
Nitric Acid
Phosphoric Acid
Phosphoric Acid, Diluted
Potassium Bicarbonate
Potassium Citrate
Potassium Hydroxide
Potassium Metaphosphate
Potassium Phosphate, Dibasic
Potassium Phosphate, Monobasic
Propionic Acid
Racemethionine
Sodium Acetate
Sodium Bicarbonate
Sodium Borate
Sodium Carbonate
Sodium Citrate
Sodium Hydroxide
Sodium Lactate Solution
Sodium Phosphate, Dibasic
Sodium Phosphate, Monobasic
Succinic Acid
Sulfuric Acid
Tartaric Acid
Trolamine

Change to read:

Plasticizer

Dosage Form: Tablets and Capsules
Acetyltributyl Citrate
Acetyltriethyl Citrate
Benzyl Benzoate
Castor Oil
Chlorobutanol
Diacetylated Monoglycerides
Dibutyl Sebacate
Diethyl Phthalate
Glycerin
Mannitol
Polyethylene Glycol
Polyethylene Glycol 3350
Polyethylene Glycol Monomethyl Ether
Propylene Glycol
Pullulan
Sorbitol
Sorbitol Sorbitan Solution
▲Sucrose Diacetate Hexaisobutyrate▲ (NF 1-Aug-2020)
Triacetin
Tributyl Citrate
Triethyl Citrate
Vitamin E

Polymer Membrane

Dosage Form: Tablets and Capsules
Amino Methacrylate Copolymer
Ammonio Methacrylate Copolymer
Ammonio Methacrylate Copolymer Dispersion
Cellaburate
Cellulose Acetate
Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion
Ethylcellulose
Ethylcellulose Aqueous Dispersion
Ethylcellulose Dispersion Type B
Pullulan

Polymers for Ophthalmic Use

Dosage Form: Ophthalmic Preparations
Carbomer Copolymer
Carbomer Homopolymer
Carbomer Interpolymer
Carmellose
Guar Gum
Hydroxyethyl Cellulose
Hypromellose
Polyvinyl Alcohol
Povidone
Xanthan Gum

Propellant

Dosage Form: Aerosols
Butane
Carbon Dioxide
Dichlorodifluoromethane
Dichlorotetrafluoroethane
Isobutane
Nitrogen
Nitrous Oxide
Propane
Trichloromonofluoromethane

Reducing Agent

Dosage Form: Radiopharmaceuticals
Stannous Chloride
Stannous Fluoride

Release-Modifying Agent

Dosage Form: Tablets and Capsules
Alginic Acid
Carbomer Copolymer
Carbomer Homopolymer
Carbomer Interpolymer
Carboxymethylcellulose Sodium
Carrageenan
Cellaburate
Ethylcellulose
Ethylcellulose Aqueous Dispersion
Ethylcellulose Dispersion Type B
Glyceryl Monooleate
Glyceryl Monostearate
Guar Gum
Hydroxypropyl Betadex
Hydroxypropyl Cellulose
Hypromellose
Polyethylene Oxide
Polyvinyl Acetate Dispersion
Shellac

Sodium Alginate
Starch, Pregelatinized
Starch, Pregelatinized Modified
Xanthan Gum

Sequestering Agent

Dosage Form: Oral Liquids

Betadex
Betadex Sulfobutyl Ether Sodium
Calcium Acetate
Cyclodextrin, Gamma
Hydroxypropyl Betadex
Pentetic Acid
Pullulan
Sodium Citrate
Sodium Tartrate
Tartaric Acid

Solvent

Acetone
Alcohol
Alcohol, Diluted
Almond Oil
Amylene Hydrate
Benzyl Alcohol
Benzyl Benzoate
Butyl Alcohol
Butylene Glycol
Canola Oil
Caprylocaproyl Polyoxylglycerides
Castor Oil
Corn Oil
Cottonseed Oil
Dibutyl Phthalate
Diethyl Phthalate
Diethylene Glycol Monoethyl Ether
Dimethyl Sulfoxide
Ethyl Acetate
Ethyl Oleate
Glycerin
Hexylene Glycol
Isobutyl Alcohol
Isopropyl Alcohol
Isopropyl Myristate
Isopropyl Palmitate
Lauroyl Polyoxylglycerides
Linoleoyl Polyoxylglycerides
Medium-chain Triglycerides
Methyl Alcohol
Methyl Isobutyl Ketone
Methylene Chloride
Methylpyrrolidone
Mineral Oil
Mineral Oil, Light
Oleoyl Polyoxylglycerides
Peanut Oil
Polydecene, Hydrogenated
Polyethylene Glycol
Polyethylene Glycol 3350
Polyethylene Glycol Monomethyl Ether
Polypropylene Glycol 11 Stearyl Ether
Propylene Carbonate
Propylene Glycol
Propylene Glycol Diacetate
Safflower Oil
Sesame Oil
Soybean Oil
Stearoyl Polyoxylglycerides
Sunflower Oil
Triacetin
Triethyl Citrate
Water for Injection
Water for Injection, Sterile
Water for Irrigation, Sterile
Water, Purified

Sorbent

Cellulose, Powdered
Charcoal, Activated
Siliceous Earth, Purified

Sorbent, Carbon Dioxide

Barium Hydroxide Lime
Soda Lime

Stiffening Agent

Dosage Form: Semisolids, Topicals, and Suppositories

Castor Oil, Hydrogenated
Cetostearyl Alcohol
Cetyl Alcohol
Cetyl Palmitate
Dextrin
Hard Fat
Alpha-Lactalbumin
Paraffin
Paraffin, Synthetic
Rapeseed Oil, Fully Hydrogenated
Rapeseed Oil, Superglycerinated Fully Hydrogenated
Sodium Stearate
Stearyl Alcohol
Wax, Cetyl Esters
Wax, Emulsifying
Wax, Microcrystalline
Wax, White
Wax, Yellow

Suppository Base

Dosage Form: Semisolids, Topicals, and Suppositories

Agar
Cocoa Butter
Hard Fat
Palm Kernel Oil
Polyethylene Glycol
Polyethylene Glycol 3350

Change to read:

Suspending and/or Viscosity-Increasing Agent

Dosage Form: Semisolids, Topicals, and Suppositories

Acacia
Agar
Alamic Acid
Alginic Acid
Aluminum Monostearate
Attapulgite, Activated
Attapulgite, Colloidal Activated
Bentonite
Bentonite, Purified
Bentonite Magma
Carbomer 910
Carbomer 934
Carbomer 934P
Carbomer 940
Carbomer 941
Carbomer 1342
Carbomer Copolymer
Carbomer Homopolymer
Carbomer Interpolymer
Carboxymethylcellulose Calcium
Carboxymethylcellulose Sodium
Carboxymethylcellulose Sodium 12
Carboxymethylcellulose Sodium, Enzymatically-Hydrolyzed
Carmellose
Carrageenan
Cellulose, Microcrystalline
Cellulose, Microcrystalline, and Carboxymethylcellulose Sodium
Cellulose, Powdered
Cetostearyl Alcohol
Chitosan
Corn Syrup
Corn Syrup Solids
Cyclomethicone
Dextrin
Egg Phospholipids
Ethylcellulose
Gelatin
Gellan Gum
Glyceryl Behenate
▲▲ (NF 1-Aug-2020)▲▲ (NF 1-Aug-2020)
Glyceryl Dibehenate
Guar Gum
Hydroxyethyl Cellulose
Hydroxypropyl Cellulose
Hypromellose
Isomalt
Alpha-Lactalbumin
Kaolin
Magnesium Aluminum Silicate
Maltitol Solution
Maltodextrin
Medium-chain Triglycerides
Methylcellulose
Pectin
Polycarbophil
Polydextrose
Polydextrose, Hydrogenated
Polyethylene Oxide
Polysorbate 20
Polysorbate 40
Polysorbate 60
Polysorbate 80
Polyvinyl Alcohol
Potassium Alginate
Povidone
Propylene Glycol Alginate
Pullulan
Silica, Dental-Type
Silica, Hydrophobic Colloidal
Silicon Dioxide
Silicon Dioxide, Colloidal
Sodium Alginate
Sorbitan Monolaurate
Sorbitan Monooleate
Sorbitan Monopalmitate
Sorbitan Monostearate
Sorbitan Sesquioleate
Sorbitan Trioleate
Starch, Corn
Starch, Hydroxypropyl Corn
Starch, Pregelatinized Hydroxypropyl Corn
Starch, Pea
Starch, Hydroxypropyl Pea
Starch, Pregelatinized Hydroxypropyl Pea
Starch, Potato
Starch, Hydroxypropyl Potato
Starch, Pregelatinized Hydroxypropyl Potato
Starch, Tapioca
Starch, Wheat
Sucrose
Sucrose Palmitate
Tragacanth
Vitamin E Polyethylene Glycol Succinate
Xanthan Gum

Sweetening Agent

Dosage Form: Oral Liquids

Acesulfame Potassium
Aspartame
Aspartame Acesulfame
Corn Syrup
Corn Syrup, High Fructose
Corn Syrup Solids
Dextrates
Dextrose
Dextrose Excipient
Erythritol
Fructose
Galactose
Glucose, Liquid
Glycerin
Inulin

Invert Sugar
Isomalt
Lactitol
Maltitol
Maltitol Solution
Maltose
Mannitol
Neotame
Neohesperidin Dihydrochalcone
Saccharin
Saccharin Calcium
Saccharin Sodium
Sorbitol
Sorbitol Solution
Starch Hydrolysate, Hydrogenated
Sucralose
Sucrose
Sugar, Compressible
Sugar, Confectioner's
Syrup
Tagatose
Trehalose
Xylitol

Tonicity Agent

Dosage Form: Parenterals
Corn Syrup
Corn Syrup Solids
Dextrose
Glycerin
Mannitol
Potassium Chloride
Sodium Chloride

Transfer Ligand

Dosage Form: Radiopharmaceuticals
Edetate Disodium
Sodium Acetate
Sodium Citrate
Sodium Gluconate
Sodium Tartrate

Vehicle

Polypropylene Glycol 11 Stearyl Ether

Dosage Form: Oral Liquids

FLAVORED AND/OR SWEETENED
Aromatic Elixir
Benzaldehyde Elixir, Compound
Corn Syrup Solids
Dextrose
Ethyl Maltol
Peppermint Water
Sorbitol Solution
Syrup
Trehalose

OLEAGINOUS
Alkyl (C12–15) Benzoate
Almond Oil
Canola Oil
Castor Oil
Corn Oil
Cottonseed Oil
Ethyl Oleate
Isopropyl Myristate
Isopropyl Palmitate
Mineral Oil
Mineral Oil, Light
Myristyl Alcohol
Octyldodecanol
Olive Oil
Peanut Oil
Polydecene, Hydrogenated
Polyoxyl 15 Hydroxystearate
Safflower Oil
Sesame Oil
Soybean Oil
Squalane
Sunflower Oil

SOLID CARRIER
Chitosan
Corn Syrup Solids
Alpha-Lactalbumin
Glyceryl Tricaprylate
Propylene Glycol Dicaprylate/Dicaprate
Propylene Glycol Monocaprylate
Sugar Spheres

STERILE
rAlbumin Human
Sodium Chloride Injection, Bacteriostatic
Water for Injection, Bacteriostatic

Water-Repelling Agent

Cyclomethicone
Dimethicone
Simethicone

Change to read:

Wet Binder

Dosage Form: Tablets and Capsules
Acacia
Agar
Alginic Acid
Amino Methacrylate Copolymer
Ammonio Methacrylate Copolymer
Ammonio Methacrylate Copolymer Dispersion
Calcium Carbonate
Calcium Lactate
Carbomer Copolymer
Carbomer Homopolymer
Carbomer Interpolymer
Carboxymethylcellulose Sodium
Cellulose, Microcrystalline
Cellulose, Silicified Microcrystalline
Coconut Oil, Hydrogenated
Copovidone
Corn Syrup
Corn Syrup Solids

Dextrates
Dextrin
Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion
Ethylcellulose
Ethylene Glycol and Vinyl Alcohol Graft Copolymer
Gelatin
Glucose, Liquid
Glyceryl Behenate
▲▲ (NF 1-Aug-2020)▲▲ (NF 1-Aug-2020)
Glyceryl Dibehenate
Guar Gum
Hydroxyethyl Cellulose
Hydroxypropyl Cellulose
Hydroxypropyl Cellulose, Low-Substituted
Hypromellose
Hypromellose Acetate Succinate
Inulin
Invert Sugar
Alpha-Lactalbumin
Lactose, Monohydrate
Maltodextrin
Maltose
Methacrylic Acid and Ethyl Acrylate Copolymer
Methacrylic Acid and Ethyl Acrylate Copolymer Dispersion
Methacrylic Acid and Methyl Methacrylate Copolymer
Methylcellulose
Palm Oil, Hydrogenated
Polycarbophil
Polydextrose, Hydrogenated
Polyethylene Oxide
Polyvinyl Acetate
Povidone
Pullulan
Sodium Alginate
Starch, Pregelatinized
Starch, Pregelatinized Modified
Starch, Corn
Starch, Hydroxypropyl Corn
Starch, Pregelatinized Hydroxypropyl Corn
Starch, Pea
Starch, Hydroxypropyl Pea
Starch, Pregelatinized Hydroxypropyl Pea
Starch, Potato
Starch, Hydroxypropyl Potato
Starch, Pregelatinized Hydroxypropyl Potato
Starch, Tapioca
Starch, Wheat
Starch Hydrolysate, Hydrogenated
Sucrose
▲Sucrose Diacetate Hexaisobutyrate▲ (NF 1-Aug-2020)
Sunflower Oil
Syrup
Trehalose
Vegetable Oil, Hydrogenated
Vitamin E Polyethylene Glycol Succinate
Zein

Wetting and/or Solubilizing Agent

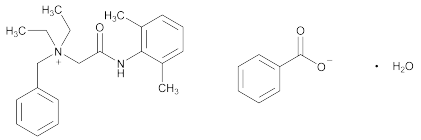
Betadex Sulfobutyl Ether Sodium

Dosage Form: Oral Liquids

Behenoyl Polyoxylglycerides
Benzalkonium Chloride
Benzethonium Chloride
Butylene Glycol
Caprylocaproyl Polyoxylglycerides
Cetylpyridinium Chloride
Docusate Sodium
Egg Phospholipids
Glycine
Lauroyl Polyoxylglycerides
Linoleoyl Polyoxylglycerides
Nonoxynol 9
Octoxynol 9
Oleoyl Polyoxylglycerides
Poloxamer
Polyoxyl 10 Oleyl Ether
Polyoxyl 15 Hydroxystearate
Polyoxyl 20 Cetostearyl Ether
Polyoxyl 35 Castor Oil
Polyoxyl 40 Castor Oil, Hydrogenated
Polyoxyl 40 Stearate
Polyoxyl Lauryl Ether
Polyoxyl Stearate
Polyoxyl Stearyl Ether
Polysorbate 20
Polysorbate 40
Polysorbate 60
Polysorbate 80
Pullulan
Sodium Lauryl Sulfate
Sorbitan Monolaurate
Sorbitan Monooleate
Sorbitan Monopalmitate
Sorbitan Monostearate
Sorbitan Sesquioleate
Sorbitan Trioleate
Stearoyl Polyoxylglycerides
Tyloxapol
Wax, Emulsifying

NF Monographs

Denatonium Benzoate



$C_{28}H_{34}N_2O_3 \cdot H_2O$ 464.60

$C_{28}H_{34}N_2O_3$ 446.59

Benzenemethanaminium, *N*-[2-[(2,6-dimethylphenyl) amino]-2-oxoethyl]-*N,N*-diethyl-, benzoate, monohydrate;
Benzyldiethyl[(2,6-xylylcarbamoyl)methyl]ammonium benzoate monohydrate [86398-53-0].
Anhydrous [3734-33-6].

DEFINITION

Denatonium Benzoate, dried at 105° for 2 h, contains one molecule of water of hydration or is anhydrous. When dried at 105° for 2 h, it contains NLT 99.5% and NMT 101.0% of denatonium benzoate ($C_{28}H_{34}N_2O_3$).

IDENTIFICATION

Change to read:

• **A. ▲Spectroscopic Identification Tests** ⟨197⟩**,** *Infrared Spectroscopy*: **197K**▲ (CN 1-May-2020)

Change to read:

• **B. ▲Spectroscopic Identification Tests** ⟨197⟩**,** *Ultraviolet-Visible Spectroscopy*: **197U**▲ (CN 1-May-2020)
Analytical wavelength: 263 nm
Sample solution: 100 µg/mL
Medium: Water
Acceptance criteria: Absorptivities, calculated on the dried basis, do not differ by more than 3.0%.

• **C.**
Sample: 150 mg
Analysis: Dissolve the *Sample* in 10 mL of water, and add 15 mL of trinitrophenol TS.
Acceptance criteria: A yellow precipitate is formed.

• **D.**
Sample: 100 mg
Analysis: Dissolve the *Sample* in 10 mL of water, and add 20 mL of 2 N sulfuric acid and 15 mL of ammonium reineckate TS. Mix, filter through a sintered-glass crucible using gentle suction, and wash thoroughly with water. Remove as much water as possible with suction, and then dry in an oven at 105° for 1 h.
Acceptance criteria: The denatonium reineckate so obtained melts at about 170° (see *Melting Range or Temperature* ⟨741⟩).

ASSAY

• **Procedure**
Sample: 900 mg, previously dried
Blank: 50 mL of glacial acetic acid
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Direct titration
Titrant: 0.1 N perchloric acid VS
Endpoint detection: Visual
Analysis: Dissolve the *Sample* in 50 mL of glacial acetic acid, and add 1 drop of crystal violet TS. Titrate with *Titrant* to a green endpoint. Perform a blank determination, and make any necessary correction.
Calculate the percentage of denatonium benzoate ($C_{28}H_{34}N_2O_3$) in the portion of sample taken:

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

V_S = *Titrant* volume consumed by the *Sample* (mL)
V_B = *Titrant* volume consumed by the *Blank* (mL)
N = actual normality of the *Titrant* (mEq/mL)
F = equivalency factor, 446.6 mg/mEq
W = *Sample* weight (mg)

Acceptance criteria: 99.5%–101.0% on the dried basis

IMPURITIES

• **Residue on Ignition** ⟨281⟩**:** NMT 0.1%
• **Chloride and Sulfate,** *Chloride* ⟨221⟩
Standard solution: 0.10 mL of 0.020 N hydrochloric acid
Sample soluton: Dissolve 350 mg in 9 mL of water, add 1 mL of nitric acid, and filter.
Analysis: Use 1.0 mL of the *Sample solution*, and proceed as directed in the chapter
Acceptance criteria: 0.2%; the *Sample solution* shows no more chloride than the *Standard solution*.

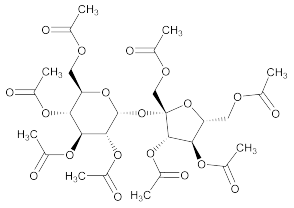
SPECIFIC TESTS

• **Melting Range or Temperature** ⟨741⟩**:** 163°–170°, on a dried specimen
• **pH** ⟨791⟩
Sample solution: 30 mg/mL
Acceptance criteria: 6.5–7.5
• **Loss on Drying** ⟨731⟩
Analysis: Dry at 105° for 2 h.
Acceptance criteria
Monohydrate form: 3.5%–4.5%
Anhydrous form: NMT 1.0%

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Preserve in tight containers.
• **Labeling:** Label it to indicate whether it is hydrous or anhydrous.
• **USP Reference Standards** ⟨11⟩
USP Denatonium Benzoate RS

Sucrose Octaacetate



$C_{28}H_{38}O_{19}$ 678.59

α-D-Glucopyranoside, 1,3,4,6-tetra-*O*-acetyl-β-D-fructofuranosyl, tetraacetate;
Octaacetyl sucrose [126-14-7].

DEFINITION

Sucrose Octaacetate contains NLT 98.0% and NMT 100.5% of sucrose octaacetate ($C_{28}H_{38}O_{19}$), calculated on the anhydrous basis.

IDENTIFICATION

Change to read:

• **A. ▲Spectroscopic Identification Tests** ⟨197⟩**,** *Infrared Spectroscopy*: **197K▲** (CN 1-May-2020)

ASSAY

• **Procedure**

Sample: 100 mg in a 500-mL conical flask
Blank: 50 mL of 70% alcohol
Titrimetric system
(See *Titrimetry* ⟨541⟩.)
Mode: Residual titration
Titrant: 0.1 N sodium hydroxide VS
Back-titrant: 0.1 N sulfuric acid VS
Endpoint detection: Visual
Analysis: Dissolve the *Sample* in 50 mL of 70% alcohol. Neutralize the solution with 0.1 N sodium hydroxide VS using phenolphthalein TS as the indicator. Add 25.0 mL of 0.1 N sodium hydroxide VS, attach an air condenser to the flask, protect from absorption of carbon dioxide, and reflux on a steam bath for 1 h. Remove from the steam bath, cool quickly, and titrate the excess alkali with 0.1 N sulfuric acid VS using phenolphthalein TS as the indicator. Perform a blank determination.
Calculate the percentage of sucrose octaacetate ($C_{28}H_{38}O_{19}$) in the portion of Sucrose Octaacetate taken. Each mL of 0.1 N sodium hydroxide is equivalent to 8.483 mg of sucrose octaacetate ($C_{28}H_{38}O_{19}$).
Acceptance criteria: 98.0%–100.5% on the anhydrous basis

IMPURITIES

• **Residue on Ignition** ⟨281⟩**:** NMT 0.1%

SPECIFIC TESTS

• **Melting Range or Temperature** ⟨741⟩**:** NLT 78°
• **Acidity**
Sample: 1 g
Analysis: Dissolve the *Sample* in 20 mL of neutralized alcohol, and add 2 drops of phenolphthalein TS.
Acceptance criteria: NMT 2 drops of 0.1 N sodium hydroxide are required to produce a red color.
• **Water Determination,** *Method I* ⟨921⟩**:** NMT 1.0%

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Preserve in tight containers.
• **USP Reference Standards** ⟨11⟩
USP Sucrose Octaacetate RS

Reference Tables

ALCOHOLOMETRIC TABLE

Based on data appearing in the National Bureau of Standards Bulletin, vol. 9, pp. 424–425 (publication of the National Institute of Standards and Technology).

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Percentage of C_2H_5OH		Specific gravity in air		Percentage of C_2H_5OH		Specific gravity in air	
By volume at 15.56°C	By weight	At 25°/25°	At 15.56°/15.56°	By weight	By volume at 15.56°C	At 25°/25°	At 15.56°/15.56°
0	0.00	1.0000	1.0000	0	0.00	1.0000	1.0000
1	0.80	0.9985	0.9985	1	1.26	0.9981	0.9981
2	1.59	0.9970	0.9970	2	2.51	0.9963	0.9963
3	2.39	0.9956	0.9956	3	3.76	0.9945	0.9945
4	3.19	0.9941	0.9942	4	5.00	0.9927	0.9928
5	4.00	0.9927	0.9928	5	6.24	0.9911	0.9912
6	4.80	0.9914	0.9915	6	7.48	0.9894	0.9896
7	5.61	0.9901	0.9902	7	8.71	0.9879	0.9881
8	6.42	0.9888	0.9890	8	9.94	0.9863	0.9867
9	7.23	0.9875	0.9878	9	11.17	0.9848	0.9852
10	8.05	0.9862	0.9866	10	12.39	0.9833	0.9839
11	8.86	0.9850	0.9854	11	13.61	0.9818	0.9825
12	9.68	0.9838	0.9843	12	14.83	0.9804	0.9812
13	10.50	0.9826	0.9832	13	16.05	0.9789	0.9799
14	11.32	0.9814	0.9821	14	17.26	0.9776	0.9787
15	12.14	0.9802	0.9810	15	18.47	0.9762	0.9774
16	12.96	0.9790	0.9800	16	19.68	0.9748	0.9763
17	13.79	0.9778	0.9789	17	20.88	0.9734	0.9751
18	14.61	0.9767	0.9779	18	22.08	0.9720	0.9738
19	15.44	0.9756	0.9769	19	23.28	0.9706	0.9726
20	16.27	0.9744	0.9759	20	24.47	0.9692	0.9714
21	17.10	0.9733	0.9749	21	25.66	0.9677	0.9701
22	17.93	0.9721	0.9739	22	26.85	0.9663	0.9688
23	18.77	0.9710	0.9729	23	28.03	0.9648	0.9675
24	19.60	0.9698	0.9719	24	29.21	0.9633	0.9662
25	20.44	0.9685	0.9708	25	30.39	0.9617	0.9648
26	21.29	0.9673	0.9697	26	31.56	0.9601	0.9635
27	22.13	0.9661	0.9687	27	32.72	0.9585	0.9620
28	22.97	0.9648	0.9676	28	33.88	0.9568	0.9605
29	23.82	0.9635	0.9664	29	35.03	0.9551	0.9590
30	24.67	0.9622	0.9653	30	36.18	0.9534	0.9574

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Percentage of C_2H_5OH		Specific gravity in air		Percentage of C_2H_5OH		Specific gravity in air	
By volume at 15.56°C	By weight	At 25°/25°	At 15.56°/15.56°	By weight	By volume at 15.56°C	At 25°/25°	At 15.56°/15.56°
31	25.52	0.9609	0.9641	31	37.32	0.9516	0.9558
32	26.38	0.9595	0.9629	32	38.46	0.9498	0.9541
33	27.24	0.9581	0.9617	33	39.59	0.9480	0.9524
34	28.10	0.9567	0.9604	34	40.72	0.9461	0.9506
35	28.97	0.9552	0.9590	35	41.83	0.9442	0.9488
36	29.84	0.9537	0.9576	36	42.94	0.9422	0.9470
37	30.72	0.9521	0.9562	37	44.05	0.9402	0.9451
38	31.60	0.9506	0.9548	38	45.15	0.9382	0.9432
39	32.48	0.9489	0.9533	39	46.24	0.9362	0.9412
40	33.36	0.9473	0.9517	40	47.33	0.9341	0.9392
41	34.25	0.9456	0.9501	41	48.41	0.9320	0.9372
42	35.15	0.9439	0.9485	42	49.48	0.9299	0.9352
43	36.05	0.9421	0.9469	43	50.55	0.9278	0.9331
44	36.96	0.9403	0.9452	44	51.61	0.9256	0.9310
45	37.87	0.9385	0.9434	45	52.66	0.9235	0.9289
46	38.78	0.9366	0.9417	46	53.71	0.9213	0.9268
47	39.70	0.9348	0.9399	47	54.75	0.9191	0.9246
48	40.62	0.9328	0.9380	48	55.78	0.9169	0.9225
49	41.55	0.9309	0.9361	49	56.81	0.9147	0.9203
50	42.49	0.9289	0.9342	50	57.83	0.9124	0.9181
51	43.43	0.9269	0.9322	51	58.84	0.9102	0.9159
52	44.37	0.9248	0.9302	52	59.85	0.9079	0.9137
53	45.33	0.9228	0.9282	53	60.85	0.9056	0.9114
54	46.28	0.9207	0.9262	54	61.85	0.9033	0.9092
55	47.25	0.9185	0.9241	55	62.84	0.9010	0.9069
56	48.21	0.9164	0.9220	56	63.82	0.8987	0.9046
57	49.19	0.9142	0.9199	57	64.80	0.8964	0.9024
58	50.17	0.9120	0.9177	58	65.77	0.8941	0.9001
59	51.15	0.9098	0.9155	59	66.73	0.8918	0.8978
60	52.15	0.9076	0.9133	60	67.79	0.8895	0.8955
61	53.15	0.9053	0.9111	61	68.64	0.8871	0.8932
62	54.15	0.9030	0.9088	62	69.59	0.8848	0.8909
63	55.17	0.9006	0.9065	63	70.52	0.8824	0.8886
64	56.18	0.8983	0.9042	64	71.46	0.8801	0.8862
65	57.21	0.8959	0.9019	65	72.38	0.8777	0.8839
66	58.24	0.8936	0.8995	66	73.30	0.8753	0.8815
67	59.28	0.8911	0.8972	67	74.21	0.8729	0.8792
68	60.33	0.8887	0.8948	68	75.12	0.8706	0.8768
69	61.38	0.8862	0.8923	69	76.02	0.8682	0.8745
70	62.44	0.8837	0.8899	70	76.91	0.8658	0.8721
71	63.51	0.8812	0.8874	71	77.79	0.8634	0.8697
72	64.59	0.8787	0.8848	72	78.67	0.8609	0.8673
73	65.67	0.8761	0.8823	73	79.54	0.8585	0.8649

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Percentage of C_2H_5OH		Specific gravity in air		Percentage of C_2H_5OH		Specific gravity in air	
By volume at 15.56°C	By weight	At 25°/25°	At 15.56°/15.56°	By weight	By volume at 15.56°C	At 25°/25°	At 15.56°/15.56°
74	66.77	0.8735	0.8797	74	80.41	0.8561	0.8625
75	67.87	0.8709	0.8771	75	81.27	0.8537	0.8601
76	68.98	0.8682	0.8745	76	82.12	0.8512	0.8576
77	70.10	0.8655	0.8718	77	82.97	0.8488	0.8552
78	71.23	0.8628	0.8691	78	83.81	0.8463	0.8528
79	72.38	0.8600	0.8664	79	84.64	0.8439	0.8503
80	73.53	0.8572	0.8636	80	85.46	0.8414	0.8479
81	74.69	0.8544	0.8608	81	86.28	0.8389	0.8454
82	75.86	0.8516	0.8580	82	87.08	0.8364	0.8429
83	77.04	0.8487	0.8551	83	87.89	0.8339	0.8404
84	78.23	0.8458	0.8522	84	88.68	0.8314	0.8379
85	79.44	0.8428	0.8493	85	89.46	0.8288	0.8354
86	80.66	0.8397	0.8462	86	90.24	0.8263	0.8328
87	81.90	0.8367	0.8432	87	91.01	0.8237	0.8303
88	83.14	0.8335	0.8401	88	91.77	0.8211	0.8276
89	84.41	0.8303	0.8369	89	92.52	0.8184	0.8250
90	85.69	0.8271	0.8336	90	93.25	0.8158	0.8224
91	86.99	0.8237	0.8303	91	93.98	0.8131	0.8197
92	88.31	0.8202	0.8268	92	94.70	0.8104	0.8170
93	89.65	0.8167	0.8233	93	95.41	0.8076	0.8142
94	91.03	0.8130	0.8196	94	96.10	0.8048	0.8114
95	92.42	0.8092	0.8158	95	96.79	0.8020	0.8086
96	93.85	0.8053	0.8118	96	97.46	0.7992	0.8057
97	95.32	0.8011	0.8077	97	98.12	0.7962	0.8028
98	96.82	0.7968	0.8033	98	98.76	0.7932	0.7998
99	98.38	0.7921	0.7986	99	99.39	0.7902	0.7967
100	100.00	0.7871	0.7936	100	100.00	0.7871	0.7936

Published August 17, 2020

General Chapters

General Tests and Assays

⟨11⟩ USP REFERENCE STANDARDS

Change to read:

▲INTRODUCTION

Reference Standards provided by the United States Pharmacopeial Convention (USP Reference Standards or USP RS) are highly characterized materials demonstrated to have the appropriate qualities to support their intended use. USP RS are not for use in humans or animals.

USP RS are generally linked to relevant tests and assays in the *United States Pharmacopeia* (*USP*) or *National Formulary* (*NF*) documentary standards. They have been approved and established as suitable for use in the context of these applications. When approved as suitable for use in *USP* or *NF* tests and assays, USP RS also assume official status and legal recognition in the United States and other jurisdictions that recognize the *USP* or *NF* (see *General Notices, 2.30 Legal Recognition*). Where *USP* or *NF* tests or assays call for the use of a USP RS, only those results obtained using the specified USP RS are conclusive (see *General Notices, 5.80 USP Reference Standards*).

USP RS may also be used to support other measurements not necessarily prescribed in *USP–NF*. Assessment of the suitability for use in other applications is the responsibility of the user.

ESTABLISHMENT APPROACHES AND VALUE ASSIGNMENT

USP RS, when they are physical materials, are Reference Materials as defined in the *International Vocabulary of Metrology—Basic and General Concepts and Associated Terms (VIM)*. The value assigned to quantitative standards relies on a mass determination by a primary reference measurement procedure such as a mass balance determination. If the value has been assigned by using a primary reference measurement procedure, the USP RS in metrology terms can be considered a primary measurement standard. If the value has been assigned by comparison or calibration to another material, the USP RS should be considered a secondary measurement standard; for example, when appropriate, USP RS are calibrated relative to international reference materials such as those provided by the World Health Organization (WHO).

USP may choose to develop USP RS that follow the requirements for the development of Certified Reference Materials (CRMs) in accordance with the relevant International Organization for Standardization (ISO) Guides. Correct use of these CRMs support traceability of results to SI units and comparability of procedures. USP may also provide RS that are traceable to a CRM established by a National Metrology Institute or other official provider as required or referenced by the documentary standard.

USP may issue USP RS that are not required in a documentary standard. The material and performance attributes for these USP RS are described in the supporting documentation supplied with the USP RS.

USP RS go through a rigorous characterization as part of their establishment process. The types and extent of testing (including the number of laboratories) are primarily driven by the official uses of the standard, but material characterization goes beyond the establishment of suitability for use. Typically, a comparison to the previous lot is performed during the study when establishing a replacement lot of a USP RS as an additional verification of the suitability of the new standard lot.

USP REFERENCE STANDARDS FOR *USP* OR *NF*

Official applications of USP RS are specified in *USP* or *NF* monographs and general chapters. These applications are as follows. Some USP RS could be used for several types of applications listed below.

1. **Quantitative determinations**
   - A. The majority of USP RS for quantitative determinations support measurements for total amounts of material on a mass basis. This category includes USP RS for USP or NF articles and impurity standards labeled for quantitative use. The assigned value of the USP RS is stated on the labeling and should be included in calculations used in the monograph and applicable general chapters.
   - B. USP RS for relative determinations of potency or activity are often required for the measurement of complex materials (e.g., biologics, antibiotics, herbals, some dietary supplements) and quantitative amounts may be expressed in units or relative potency terms other than mass.
     These USP RS are established by calibration to a primary standard where the property of the material determines the unit. For these standards where an International Standard (IS) established by the WHO exist, USP RS are

National Measurement Standards. The USP RS documentation will indicate when the USP RS has been established by comparison to an International Standard (IS) established by the WHO. Results may be expressed in USP Units, Units, or International Units. Additional statements about unit/mass relationship, specific activity, or other relevant information related to the measurement may be provided in the USP RS documentation.
For antibiotics that use microbial assays to determine activity, the potency is determined in units or micrograms per milligram (µg/mg) of activity. The units or µg/mg of activity is established against a WHO IS when one exists for that antibiotic. Where no WHO IS is presently available, USP establishes and maintains the standard to which USP RS lots are calibrated. This approach may also be chosen for other complex materials for which no WHO IS exists. In these instances, the USP standard is established in such a way as to ensure long-term stability and fitness for purpose, which permits the calibration of successive lots of USP RS with increased confidence that drift in the assigned unit can be avoided.

2. **Qualitative determinations**
   A. Identification USP RS: USP RS for identification tests are typically presented as single components of high chemical purity, but may also be complex materials of natural, synthetic, or recombinant origin (e.g., biologics, natural products, botanicals, complex nonbiologicals, others). For complex materials, the identification attributes are presented in a matrix of other materials and require highly specific measurement systems (e.g., nucleic acid-based identity determination for naturally derived materials).
   B. Impurity USP RS: Impurity USP RS are typically used for system suitability or as impurity markers. They may be presented as single-component materials, as mixtures containing more than one impurity, or as drug substance(s) containing one or more impurities.
   C. Digital and Visual USP RS: Unlike chemical reference materials, these USP RS are not physical materials used in chemical analyses. Instead, these visual images are used by analysts to compare test articles to ensure that they meet compendial requirements
3. **Performance verification.** These USP RS are typically called for in general tests and assays and are provided to analyze and, where appropriate, to facilitate adjustment of the operation of an instrument to ensure the results obtained are accurate and/or precise or otherwise give acceptable results. The use of these USP RS is generally described in associated general chapters and in the supporting documentation supplied with the USP RS.

USP REFERENCE STANDARDS FOR OTHER MEASUREMENTS AND DETERMINATIONS

USP also develops Reference Standards that may not be required in official *USP–NF* tests or assays. USP provides RS specified in the current edition of the *Food Chemicals Codex*, the *Herbal Medicines Compendium*, and standards referenced in regulatory requirements.

USP RS without an official use in the *USP–NF* are developed following the same quality systems used for the characterization and release of USP RS used in official tests and assays. These USP RS are generally intended to address common quality issues and challenges inherent to technologies that cut across different types of products (e.g., system suitability samples, calibrators used to demonstrate performance of an analytical procedure, process, or equipment). Extensive characterization of the USP RS candidate is required and the testing plan takes into account the use of different methods to measure the same attribute, demonstrating broader applicability of these standards. In the absence of a companion monograph or chapter, the information generated from these studies may be disseminated to the user via other types of supporting documents including but not limited to the USP Certificate.

LABELING

The labeling material consists of the label affixed to the USP RS and the associated USP Certificate. Both must be reviewed prior to handling or using the USP RS because in some cases not all of the necessary information can fit on the affixed label. USP Certificates are lot specific and are publicly available on the USP website (www.usp.org). Additional documentation may be provided with the USP RS as needed.

The affixed USP RS label typically contains the RS name, catalog number, lot number, package size, assigned value, storage conditions, handling instructions, and country of origin information. For multi-component items, there is also an outer package and label.

The affixed label also includes hazard and precautionary statements required by the Occupational Safety and Health Administration (OSHA) under the current revision of the Hazard Communication Standard (29 CFR 1910.1200). Terms used in these statements do not necessarily reflect specific definitions in the *USP–NF*. Safety Data Sheets for all USP RS are publicly available on the USP website (www.usp.org).

In addition to the information provided on the affixed USP RS label, the USP Certificate will generally contain the RS chemical name and structure, sequence (if applicable), CAS number, molecular formula, and molecular weight. A typical chromatogram may also be included if necessary for the intended use. Additional information may be included such as special handling instructions or information needed for the use of the USP RS. The USP Certificate also includes a copy of the label text and a series of general instructions.

PACKAGING

The amount of material per individual USP RS container depends on the application of the standard. Some standards (mainly materials with significant handling requirements or materials that are available only in small amounts) are provided in single-use containers. Some single-use products may be lyophilized with content labeled in mass or activity units per container. If so labeled, the content of the container must be reconstituted in its entirety without any additional weighing. Instructions for use are given either on the label or USP Certificate, or in the monographs where the standard is used.

STORAGE

USP RS should be stored in the packaging configuration provided by USP, according to the label and USP Certificate instructions. When storage in refrigerator or freezer is stated on the label, follow the definitions given in *Packaging and Storage Requirements* ⟨659⟩. If no specific directions or limitations are provided on the label, the conditions of storage shall be room temperature and protection from moisture, light, freezing, and excessive heat.

Any unused portions remaining after the container has been opened should be carefully stored in accordance with the user's Standard Operating Procedures and good laboratory practices. Decisions concerning the proper use of previously opened USP RS are the responsibility of the user, unless otherwise specified on the labeling. The user is responsible for ensuring that the contents of opened vials continue to be suitable for their intended use.

CONTINUED SUITABILITY FOR USE

All USP RS are periodically reevaluated by USP throughout their lifecycles. The USP Continued Suitability for Use (CSU) program is designed to monitor real-time suitability for use of all current lots of USP RS. Suitability testing intervals are established based on collaborative study data, manufacturer or supplier data, testing results, and CSU data trending and projections. When and where applicable, an accelerated degradation study may be performed to provide additional information on the stability of the USP RS and to support suitability testing intervals. The goal of the CSU program is to confirm the continued suitability of the material for use of a USP RS in its compendial applications during its valid use period.

VALID USE DATE

USP RS lots are assigned a valid use date upon depletion. The valid use date is the last day upon which a particular lot of USP RS can be used. Typically, the valid use date assigned is one year from the date the last vial of a lot is sold.

It is the responsibility of the user to ascertain that a particular lot of a USP RS has official status either as a "Current Lot" or as a "Previous Lot" prior to the valid use date. Current and previous lot information, as well as the most current version of the catalog, can be found on the USP website (www.usp.org).

PROPER USE

Many compendial tests and assays are based on comparison of a sample to a USP RS. In such cases, measurements are made on preparations of both the sample and the USP RS. Where it is directed that a standard solution or a standard preparation be prepared for a quantitative determination, it is intended that the USP RS substance be accurately weighed (see *Balances* ⟨41⟩) and subsequent dilutions be performed using volumetric apparatuses with, at least, the prescribed tolerances (see *Volumetric Apparatus* ⟨31⟩). Potential errors associated with the use of volumetric apparatus of small volume should be taken into account (see also *General Notices, 6.50.20.1 Adjustments to Solutions*).

Whenever the labeled directions for use require either drying or a correction for water and/or volatiles, this should be performed at the time of use. Further experimental details should be controlled by the user's Standard Operating Procedures and good laboratory practices.

The following list of label terms and definitions is provided as guidance for the handling and use of USP RS:

- **Assigned Value (Calculation Value):** The quantity value assigned to a USP RS for its use in the quantitative compendial applications.
- **As Is:** Use the USP RS as received, without drying or additional testing and apply the assigned value to correct the concentration of the standard solution and/or preparation. This is the preferred option, and is selected whenever data indicate the moisture content is constant over time. For the USP RS to be used on the as is basis, the assigned value has already been corrected for volatiles, including moisture.
- **Anhydrous Basis, Determine Water Content Titrimetrically at Time of Use:** Use the USP RS as received, and apply the water content determined to correct the weight of the standard. After the correction for water is applied, use the assigned value to correct the concentration of the standard solution and/or preparation. At the time a USP RS is to be weighed, proceed as directed under *Water Determination* ⟨921⟩*, Method I*, and determine the water content on a separate portion of material. Instrumental or microanalytical methods are acceptable for this purpose. When using typical amounts (about 50 mg of the USP RS), titrate with a 2- to 5-fold dilution of the reagent.
- **Dried Basis, Determine Loss on Drying at Time of Use:** Use the USP RS as received, and apply the loss on drying value obtained to correct the weight of the standard. After the correction for loss on drying is applied, use the assigned value to correct the concentration of the standard solution and/or preparation. Determine the loss on drying value on a separate

portion of material, following the monograph procedure under *Loss on Drying*. Sample sizes smaller than those required in the general test chapter may be used for a USP RS provided that the user can obtain a sufficiently accurate result.

- **Dried Material, Dry Before Use, or Use Previously Dried Material:** Dry the USP RS before use. Use immediately after drying under stated conditions. Drying should not be performed in the original container. A portion of the material should be transferred to a separate drying vessel. Apply the assigned value to correct the concentration of the standard solution and/or preparation prepared with the dried USP RS.▲ (USP 1-Aug-2020)

⟨71⟩ STERILITY TESTS

•Portions of this general chapter have been harmonized with the corresponding texts of the European Pharmacopeia and/or the Japanese Pharmacopeia. Those portions that are not harmonized are marked with symbols (•,•) to specify this fact.•

These Pharmacopeial procedures are not by themselves designed to ensure that a batch of product is sterile or has been sterilized. This is accomplished primarily by validation of the sterilization process or of the aseptic processing procedures.

The test is applied to substances, preparations, or articles which, according to the Pharmacopeia, are required to be sterile. However, a satisfactory result only indicates that no contaminating microorganism has been found in the sample examined under the conditions of the test.

PRECAUTIONS AGAINST MICROBIAL CONTAMINATION

The test for sterility is carried out under aseptic conditions. In order to achieve such conditions, the test environment has to be adapted to the way in which the sterility test is performed. The precautions taken to avoid contamination are such that they do not affect any microorganisms that are to be revealed in the test. The working conditions in which the tests are performed are monitored regularly by appropriate sampling of the working area and by carrying out appropriate controls.

CULTURE MEDIA AND INCUBATION TEMPERATURES

Media for the test may be prepared as described below or equivalent commercial media may be used provided that they comply with the requirements of the *Growth Promotion Test of Aerobes, Anaerobes, and Fungi*.

The following culture media have been found to be suitable for the test for sterility. *Fluid Thioglycollate Medium* is primarily intended for the culture of anaerobic bacteria. However, it will also detect aerobic bacteria. *Soybean–Casein Digest Medium* is suitable for the culture of both fungi and aerobic bacteria.

Fluid Thioglycollate Medium

L-Cystine	0.5 g
Sodium Chloride	2.5 g
Dextrose Monohydrate/Anhydrous	5.5/5.0 g
Agar	0.75 g
Yeast Extract (water-soluble)	5.0 g
Pancreatic Digest of Casein	15.0 g
Sodium Thioglycollate	0.5 g
or Thioglycolic Acid	0.3 mL
Resazurin Sodium Solution (1 in 1000), freshly prepared	1.0 mL
Purified Water	1000 mL

pH after sterilization: 7.1±0.2.

Mix the L-cystine, agar, sodium chloride, dextrose, yeast extract, and pancreatic digest of casein with the purified water, and heat until solution is effected. Dissolve the sodium thioglycollate or thioglycolic acid in the solution and, if necessary, add 1 N sodium hydroxide so that, after sterilization, the solution will have a pH of 7.1 ± 0.2. If filtration is necessary, heat the solution again without boiling, and filter while hot through moistened filter paper. Add the resazurin sodium solution, mix, and place the medium in suitable vessels that provide a ratio of surface to depth of medium such that not more than the upper half of the medium has undergone a color change indicative of oxygen uptake at the end of the incubation period. Sterilize using a validated process. If the medium is stored, store at a temperature between 2° and 25° in a sterile, airtight container. If more than the upper one-third of the medium has acquired a pink color, the medium may be restored once by heating the containers in a water-bath or in free-flowing steam until the pink color disappears and by cooling quickly, taking care to prevent the introduction of nonsterile air into the container. Do not use the medium for a longer storage period than has been validated.

Fluid Thioglycollate Medium is to be incubated at 30°–35°. For products containing a mercurial preservative that cannot be tested by the membrane filtration method, *Fluid Thioglycollate Medium* incubated at 20°–25° may be used instead of *Soybean–Casein Digest Medium* provided that it has been validated as described in *Growth Promotion Test of Aerobes, Anaerobes, and Fungi*. Where prescribed or justified and authorized, the following alternative thioglycollate medium might be used. Prepare a mixture having the same composition as that of the *Fluid Thioglycollate Medium*, but omitting the agar and the

resazurin sodium solution. Sterilize as directed above. The pH after sterilization is 7.1 ± 0.2. Heat in a water bath prior to use and incubate at 30°–35° under anaerobic conditions.

Soybean–Casein Digest Medium

Pancreatic Digest of Casein	17.0 g
Papaic Digest of Soybean Meal	3.0 g
Sodium Chloride	5.0 g
Dibasic Potassium Phosphate	2.5 g
Dextrose Monohydrate/Anhydrous	2.5/2.3 g
Purified Water	1000 mL

pH after sterilization: 7.3±0.2.

Dissolve the solids in the Purified Water, heating slightly to effect a solution. Cool the solution to room temperature, and adjust the pH with 1 N sodium hydroxide so that, after sterilization, it will have a pH of 7.3 ± 0.2. Filter, if necessary to clarify, dispense into suitable containers, and sterilize using a validated procedure. Store at a temperature between 2° and 25° in a sterile well-closed container, unless it is intended for immediate use. Do not use the medium for a longer storage period than has been validated.

Soybean–Casein Digest Medium is to be incubated at 22.5 ± 2.5°.

♦Media for Penicillins or Cephalosporins

Where sterility test media are to be used in the *Direct Inoculation of the Culture Medium* method under *Test for Sterility of the Product to be Examined*, modify the preparation of *Fluid Thioglycollate Medium* and the *Soybean–Casein Digest Medium* as follows. To the containers of each medium, transfer aseptically a quantity of β-lactamase sufficient to inactivate the amount of antibiotic in the specimen under test. Determine the quantity of β-lactamase required to inactivate the antibiotic by using a β-lactamase preparation that has been assayed previously for its penicillin- or cephalosporin-inactivating power. [NOTE—Supplemented β-lactamase media can also be used in the membrane filtration test.]

Alternatively (in an area completely separate from that used for sterility testing), confirm that an appropriate amount of β-lactamase is incorporated into the medium, following either method under *Method Suitability Test*, using less than 100 colony-forming units (cfu) of *Staphylococcus aureus* (see *Table 1*) as the challenge. Typical microbial growth of the inoculated culture must be observed as a confirmation that the β-lactamase concentration is appropriate.♦

Table 1. Strains of the Test Microorganisms Suitable for Use in the Growth Promotion Test and the Method Suitability Test

Aerobic bacteria	
Staphylococcus aureus	ATCC 6538, CIP 4.83, NCTC 10788, NCIMB 9518, NBRC 13276
Bacillus subtilis	ATCC 6633, CIP 52.62, NCIMB 8054, NBRC 3134
Pseudomonas aeruginosa♦[1]♦	ATCC 9027, NCIMB 8626, CIP 82.118, NBRC 13275
Anaerobic bacterium	
Clostridium sporogenes♦[2]♦	ATCC 19404, CIP 79.3, NCTC 532 or ATCC 11437, NBRC 14293
Fungi	
Candida albicans	ATCC 10231, IP 48.72, NCPF 3179, NBRC 1594
Aspergillus brasiliensis (Aspergillus Niger)	ATCC 16404, IP 1431.83, IMI 149007, NBRC 9455

♦[1] An alternative microorganism is *Kocuria rhizophila (Micrococcus luteus)* ATCC 9341.♦

♦[2] An alternative to *Clostridium sporogenes,* when a nonspore-forming microorganism is desired, is *Bacteroides vulgatus* (ATCC 8482).♦

The media used comply with the following tests, carried out before, or in parallel, with the test on the product to be examined.

Sterility

Incubate portions of the media for 14 days. No growth of microorganisms occurs.

Growth Promotion Test of Aerobes, Anaerobes, and Fungi

Test each lot of ready-prepared medium and each batch of medium prepared either from dehydrated medium or from ingredients. Suitable strains of microorganisms are indicated in *Table 1*.

Inoculate portions of *Fluid Thioglycollate Medium* with a small number (not more than 100 cfu) of the following microorganisms, using a separate portion of medium for each of the following species of microorganism: *Clostridium sporogenes*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. ♦Inoculate portions of alternative thioglycollate medium with a

small number (not more than 100 cfu) of *Clostridium sporogenes*.◆ Inoculate portions of *Soybean–Casein Digest Medium* with a small number (not more than 100 cfu) of the following microorganisms, using a separate portion of medium for each of the following species of microorganism: *Aspergillus brasiliensis*, *Bacillus subtilis,* and *Candida albicans*. Incubate for not more than 3 days in the case of bacteria and not more than 5 days in the case of fungi. Seed lot culture maintenance techniques (seed-lot systems) are used so that the viable microorganisms used for inoculation are not more than five passages removed from the original master seed-lot.

The media are suitable if a clearly visible growth of the microorganisms occurs.

◆DILUTING AND RINSING FLUIDS FOR MEMBRANE FILTRATION

Fluid A

PREPARATION

Dissolve 1 g of peptic digest of animal tissue in water to make 1 L, filter or centrifuge to clarify, if necessary, and adjust to a pH of 7.1 ± 0.2. Dispense into containers, and sterilize using a validated process.

PREPARATION FOR PENICILLINS OR CEPHALOSPORINS

Aseptically add to the above *Preparation*, if necessary, a quantity of sterile β-lactamase sufficient to inactivate any residual antibiotic activity on the membranes after the solution of the test specimen has been filtered (see *Media for Penicillins or Cephalosporins*).

Fluid D

To each L of *Fluid A* add 1 mL of polysorbate 80, adjust to a pH of 7.1 ± 0.2, dispense into containers, and sterilize using a validated process. Use this fluid for articles containing lecithin or oil, or for devices labeled as "sterile pathway."

Fluid K

Dissolve 5.0 g of peptic digest of animal tissue, 3.0 g of beef extract, and 10.0 g of polysorbate 80 in water to make 1 L. Adjust the pH to obtain, after sterilization, a pH of 6.9 ± 0.2. Dispense into containers, and sterilize using a validated process.◆

METHOD SUITABILITY TEST

Carry out a test as described below under *Test for Sterility of the Product to be Examined* using exactly the same methods, except for the following modifications.

Membrane Filtration

After transferring the content of the container or containers to be tested to the membrane, add an inoculum of a small number of viable microorganisms (not more than 100 cfu) to the final portion of sterile diluent used to rinse the filter.

Direct Inoculation

After transferring the contents of the container or containers to be tested (for catgut and other surgical sutures for veterinary use: strands) to the culture medium, add an inoculum of a small number of viable microorganisms (not more than 100 cfu) to the medium.

In both cases use the same microorganisms as those described above under *Growth Promotion Test of Aerobes, Anaerobes, and Fungi*. Perform a growth promotion test as a positive control. Incubate all the containers containing medium for not more than 5 days.

If clearly visible growth of microorganisms is obtained after the incubation, visually comparable to that in the control vessel without product, either the product possesses no antimicrobial activity under the conditions of the test or such activity has been satisfactorily eliminated. The test for sterility may then be carried out without further modification.

If clearly visible growth is not obtained in the presence of the product to be tested, visually comparable to that in the control vessels without product, the product possesses antimicrobial activity that has not been satisfactorily eliminated under the conditions of the test. Modify the conditions in order to eliminate the antimicrobial activity, and repeat the *Method Suitability Test*.

This method suitability is performed (a) when the test for sterility has to be carried out on a new product; and (b) whenever there is a change in the experimental conditions of the test. The method suitability may be performed simultaneously with the *Test for Sterility of the Product to be Examined*.

TEST FOR STERILITY OF THE PRODUCT TO BE EXAMINED

♦Number of Articles to Be Tested

Unless otherwise specified elsewhere in this chapter or in the individual monograph, test the number of articles specified in *Table 3*. If the contents of each article are of sufficient quantity (see *Table 2*), they may be divided so that equal appropriate portions are added to each of the specified media. [NOTE—Perform sterility testing employing two or more of the specified media.] If each article does not contain sufficient quantities for each medium, use twice the number of articles indicated in *Table 3*.♦

Table 2. Minimum Quantity to be Used for Each Medium

Quantity per Container	Minimum Quantity to be Used (unless otherwise justified and authorized)
Liquids	
Less than 1 mL	The whole contents of each container
1–40 mL	Half the contents of each container, but not less than 1 mL
Greater than 40 mL, and not greater than 100 mL	20 mL
Greater than 100 mL	10% of the contents of the container, but not less than 20 mL
Antibiotic liquids	1 mL
Insoluble preparations, creams, and ointments to be suspended or emulsified	Use the contents of each container to provide not less than 200 mg
Solids	
Less than 50 mg	The whole contents of each container
50 mg or more, but less than 300 mg	Half the contents of each container, but not less than 50 mg
300 mg–5 g	150 mg
Greater than 5 g	500 mg
Catgut and other surgical sutures for veterinary use	3 sections of a strand (each 30-cm long)
♦Surgical dressing/cotton/gauze (in packages)	100 mg per package
Sutures and other individually packaged single-use material	The whole device
Other medical devices	The whole device, cut into pieces or disassembled♦

Table 3. Minimum Number of Articles to be Tested in Relation to the Number of Articles in the Batch

Number of Items in the Batch*	Minimum Number of Items to be Tested for Each Medium (unless otherwise justified and authorized)**
Parenteral preparations	
Not more than 100 containers	10% or 4 containers, whichever is the greater
More than 100 but not more than 500 containers	10 containers
More than 500 containers	2% or 20 containers, whichever is less
♦ *For large-volume parenterals*	2% or 10 containers, whichever is less
Antibiotic solids	
Pharmacy bulk packages (<5 g)	20 containers
Pharmacy bulk packages (≥5 g)	6 containers
Bulks and blends	See *Bulk solid products*♦
Ophthalmic and other noninjectable preparations	
Not more than 200 containers	5% or 2 containers, whichever is the greater
More than 200 containers	10 containers
If the product is presented in the form of single-dose containers, apply the scheme shown above for preparations for parenteral use.	
Catgut and other surgical sutures for veterinary use	2% or 5 packages, whichever is the greater, up to a maximum total of 20 packages
♦Not more than 100 articles	10% or 4 articles, whichever is greater

Table 3. Minimum Number of Articles to be Tested in Relation to the Number of Articles in the Batch *(continued)*

Number of Items in the Batch*	Minimum Number of Items to be Tested for Each Medium (unless otherwise justified and authorized)**
More than 100, but not more than 500 articles	10 articles
More than 500 articles	2% or 20 articles, whichever is less.
Bulk solid products	
Up to 4 containers	Each container
More than 4 containers, but not more than 50 containers	20% or 4 containers, whichever is greater
More than 50 containers	2% or 10 containers, whichever is greater

* If the batch size is unknown, use the maximum number of items prescribed.
** If the contents of one container are enough to inoculate the two media, this column gives the number of containers needed for both the media together.

The test may be carried out using the technique of *Membrane Filtration* or by *Direct Inoculation of the Culture Medium* with the product to be examined. Appropriate negative controls are included. The technique of membrane filtration is used whenever the nature of the product permits; that is, for filterable aqueous preparations, for alcoholic or oily preparations, and for preparations miscible with, or soluble in, aqueous or oily solvents, provided these solvents do not have an antimicrobial effect in the conditions of the test.

Membrane Filtration

Use membrane filters having a nominal pore size not greater than 0.45 µm, in which the effectiveness to retain microorganisms has been established. Cellulose nitrate filters, for example, are used for aqueous, oily, and weakly alcoholic solutions; and cellulose acetate filters, for example, are used for strongly alcoholic solutions. Specially adapted filters may be needed for certain products (e.g., for antibiotics).

The technique described below assumes that membranes about 50 mm in diameter will be used. If filters of a different diameter are used, the volumes of the dilutions and the washings should be adjusted accordingly. The filtration apparatus and membrane are sterilized by appropriate means. The apparatus is designed so that the solution to be examined can be introduced and filtered under aseptic conditions: it permits the aseptic removal of the membrane for transfer to the medium, or it is suitable for carrying out the incubation after adding the medium to the apparatus itself.

AQUEOUS SOLUTIONS

If appropriate, transfer a small quantity of a suitable, sterile diluent such as •*Fluid A* (see *Diluting and Rinsing Fluids for Membrane Filtration*)• onto the membrane in the apparatus and filter. The diluent may contain suitable neutralizing substances and/or appropriate inactivating substances, for example, in the case of antibiotics.

Transfer the contents of the container or containers to be tested to the membrane or membranes, if necessary, after diluting to the volume used in the *Method Suitability Test* with the chosen sterile diluent, but using not less than the quantities of the product to be examined prescribed in *Tables 2* and *3*. Filter immediately. If the product has antimicrobial properties, wash the membrane not less than three times by filtering through it each time the volume of the chosen sterile diluent used in the *Method Suitability Test.* Do not exceed a washing cycle of five times 100 mL per filter, even if during method suitability it has been demonstrated that such a cycle does not fully eliminate the antimicrobial activity. Transfer the whole membrane to the culture medium or cut it aseptically into two equal parts, and transfer one half to each of two suitable media. Use the same volume of each medium as in the *Method Suitability Test.* Alternatively, transfer the medium onto the membrane in the apparatus. Incubate the media for not less than 14 days.

SOLUBLE SOLIDS

Use for each medium not less than the quantity prescribed in *Tables 2* and *3* of the product dissolved in a suitable solvent, such as the solvent provided with the preparation, Sterile Water for Injection, sterile saline, or a suitable sterile solution such as •*Fluid A* (*Diluting and Rinsing Fluids for Membrane Filtration*),• and proceed with the test as described above for *Aqueous Solutions* using a membrane appropriate to the chosen solvent.

OILS and OILY SOLUTIONS

Use for each medium not less than the quantity of the product prescribed in *Tables 2* and *3*. Oils and oily solutions of sufficiently low viscosity may be filtered without dilution through a dry membrane. Viscous oils may be diluted as necessary with a suitable sterile diluent such as isopropyl myristate shown not to have antimicrobial activity in the conditions of the test. Allow the oil to penetrate the membrane by its own weight, and then filter, applying the pressure or suction gradually. Wash the membrane at least three times by filtering through it each time about 100 mL of a suitable sterile solution such as •*Fluid A* (see *Diluting and Rinsing Fluids for Membrane Filtration*)• containing a suitable emulsifying agent at a concentration shown to be appropriate in the *Method Suitability Test,* for example polysorbate 80 at a concentration of 10 g per L •(*Fluid K*)•. Transfer the membrane or membranes to the culture medium or media, or vice versa, as described above for *Aqueous Solutions*, and incubate at the same temperatures and for the same times.

OINTMENTS and CREAMS

Use for each medium not less than the quantities of the product prescribed in *Tables 2* and *3*. Ointments in a fatty base and emulsions of the water-in-oil type may be diluted to 1% in isopropyl myristate as described above, by heating, if necessary, to not more than 40°. In exceptional cases it may be necessary to heat to not more than 44°. Filter as rapidly as possible, and proceed as described above for *Oils and Oily Solutions*.

♦PREFILLED SYRINGES

For prefilled syringes without attached sterile needles, expel the contents of each syringe into one or two separate membrane filter funnels or into separate pooling vessels prior to transfer. If a separate sterile needle is attached, directly expel the syringe contents as indicated above, and proceed as directed for *Aqueous Solutions*. Test the sterility of the needle, using *Direct Inoculation* under *Method Suitability Test*.

SOLIDS FOR INJECTION OTHER THAN ANTIBIOTICS

Constitute the test articles as directed on the label, and proceed as directed for *Aqueous Solutions* or *Oils and Oily Solutions*, whichever applies. [NOTE—If necessary, excess diluent can be added to aid in the constitution and filtration of the constituted test article.]

ANTIBIOTIC SOLIDS FOR INJECTION

Pharmacy Bulk Packages, <5 g—From each of 20 containers, aseptically transfer about 300 mg of solids, into a sterile 500-mL conical flask, dissolve in about 200 mL of *Fluid A* (see *Diluting and Rinsing Fluids for Membrane Filtration*), and mix; or constitute, as directed in the labeling, each of 20 containers and transfer a quantity of liquid or suspension, equivalent to about 300 mg of solids, into a sterile 500-mL conical flask, dissolve in about 200 mL of *Fluid A*, and mix. Proceed as directed for *Aqueous Solutions* or *Oils and Oily Solutions*, whichever applies.
Pharmacy Bulk Packages, ≥5 g—From each of 6 containers, aseptically transfer about 1 g of solids into a sterile 500-mL conical flask, dissolve in about 200 mL of *Fluid A*, and mix; or constitute, as directed in the labeling, each of 6 containers and transfer a quantity of liquid, equivalent to about 1 g of solids, into a sterile 500-mL conical flask, dissolve in about 200 mL of *Fluid A*, and mix. Proceed as directed for *Aqueous Solutions*.

ANTIBIOTIC SOLIDS, BULKS, and BLENDS

Aseptically remove a sufficient quantity of solids from the appropriate amount of containers (see *Table 2*), mix to obtain a composite, equivalent to about 6 g of solids, and transfer to a sterile 500-mL conical flask. Dissolve in about 200 mL of *Fluid A*, and mix. Proceed as directed for *Aqueous Solutions*.

STERILE AEROSOL PRODUCTS

For fluid products in pressurized aerosol form, freeze the containers in an alcohol-dry ice mixture at least at –20° for about 1 hour. If feasible, allow the propellant to escape before aseptically opening the container, and transfer the contents to a sterile pooling vessel. Add 100 mL of *Fluid D* to the pooling vessel, and mix gently. Proceed as directed for *Aqueous Solutions* or *Oils and Oily Solutions*, whichever applies.

DEVICES WITH PATHWAYS LABELED STERILE

Aseptically pass not less than 10 pathway volumes of *Fluid D* through each device tested. Collect the fluids in an appropriate sterile vessel, and proceed as directed for *Aqueous Solutions* or *Oils and Oily Solutions*, whichever applies.

In the case of sterile, empty syringes, draw sterile diluent into the barrel through the sterile needle, if attached, or through a sterile needle attached for the purpose of the test, and express the contents into a sterile pooling vessel. Proceed as directed above.♦

Direct Inoculation of the Culture Medium

Transfer the quantity of the preparation to be examined prescribed in *Tables 2* and *3* directly into the culture medium so that the volume of the product is not more than 10% of the volume of the medium, unless otherwise prescribed.

If the product to be examined has antimicrobial activity, carry out the test after neutralizing this with a suitable neutralizing substance or by dilution in a sufficient quantity of culture medium. When it is necessary to use a large volume of the product, it may be preferable to use a concentrated culture medium prepared in such a way that it takes into account the subsequent dilution. Where appropriate, the concentrated medium may be added directly to the product in its container.

OILY LIQUIDS

Use media to which have been added a suitable emulsifying agent at a concentration shown to be appropriate in the *Method Suitability Test,* for example polysorbate 80 at a concentration of 10 g per L.

OINTMENTS and CREAMS

Prepare by diluting to about 1 in 10 by emulsifying with the chosen emulsifying agent in a suitable sterile diluent such as ⧫*Fluid A* (see *Diluting and Rinsing Fluids for Membrane Filtration*).◆ Transfer the diluted product to a medium not containing an emulsifying agent.

Incubate the inoculated media for not less than 14 days. Observe the cultures several times during the incubation period. Shake cultures containing oily products gently each day. However, when *Fluid Thioglycollate Medium* is used for the detection of anaerobic microorganisms, keep shaking or mixing to a minimum in order to maintain anaerobic conditions.

CATGUT and OTHER SURGICAL SUTURES FOR VETERINARIAN USE

Use for each medium not less than the quantities of the product prescribed in *Tables 2* and *3*. Open the sealed package using aseptic precautions, and remove three sections of the strand for each culture medium. Carry out the test on three sections, each 30-cm long, which have been cut off from the beginning, the center, and the end of the strand. Use whole strands from freshly opened cassette packs. Transfer each section of the strand to the selected medium. Use sufficient medium to cover adequately the material to be tested (20 mL to 150 mL).

⧫SOLIDS

Transfer a quantity of the product in the form of a dry solid (or prepare a suspension of the product by adding sterile diluent to the immediate container), corresponding to not less than the quantity indicated in *Tables 2* and *3*. Transfer the material so obtained to 200 mL of *Fluid Thioglycollate Medium,* and mix. Similarly, transfer the same quantity to 200 mL of *Soybean–Casein Digest Medium,* and mix. Proceed as directed above.

PURIFIED COTTON, GAUZE, SURGICAL DRESSINGS, and RELATED ARTICLES

From each package of cotton, rolled gauze bandage, or large surgical dressings being tested, aseptically remove two or more portions of 100- to 500-mg each from the innermost part of the sample. From individually packaged, single-use materials, aseptically remove the entire article. Immerse the portions or article in each medium, and proceed as directed above.

STERILE DEVICES

Articles can be immersed intact or disassembled. To ensure that device pathways are also in contact with the media, immerse the appropriate number of units per medium in a volume of medium sufficient to immerse the device completely, and proceed as directed above. For extremely large devices, immerse those portions of the device that are to come into contact with the patient in a volume of medium sufficient to achieve complete immersion of those portions.

For catheters where the inside lumen and outside are required to be sterile, either cut them into pieces such that the medium is in contact with the entire lumen or fill the lumen with medium, and then immerse the intact unit.◆

OBSERVATION AND INTERPRETATION OF RESULTS

At intervals during the incubation period and at its conclusion, examine the media for macroscopic evidence of microbial growth. If the material being tested renders the medium turbid so that the presence or absence of microbial growth cannot be readily determined by visual examination, 14 days after the beginning of incubation transfer portions (each not less than 1 mL) of the medium to fresh vessels of the same medium, and then incubate the original and transfer vessels for not less than 4 days.

If no evidence of microbial growth is found, the product to be examined complies with the test for sterility. If evidence of microbial growth is found, the product to be examined does not comply with the test for sterility, unless it can be clearly demonstrated that the test was invalid for causes unrelated to the product to be examined. The test may be considered invalid only if one or more of the following conditions are fulfilled:

1. The data of the microbiological monitoring of the sterility testing facility show a fault.
2. A review of the testing procedure used during the test in question reveals a fault.
3. Microbial growth is found in the negative controls.
4. After determination of the identity of the microorganisms isolated from the test, the growth of this species (or these species) may be ascribed unequivocally to faults with respect to the material and or the technique used in conducting the sterility test procedure.

If the test is declared to be invalid, it is repeated with the same number of units as in the original test. If no evidence of microbial growth is found in the repeat test, the product examined complies with the test for sterility. If microbial growth is found in the repeat test, the product examined does not comply with the test for sterility.

APPLICATION OF THE TEST TO PARENTERAL PREPARATIONS, OPHTHALMIC, AND OTHER NONINJECTABLE PREPARATIONS REQUIRED TO COMPLY WITH THE TEST FOR STERILITY

When using the technique of membrane filtration, use, whenever possible, the whole contents of the container, but not less than the quantities indicated in *Table 2,* diluting where necessary to about 100 mL with a suitable sterile solution, such as ⧫*Fluid A* (see *Diluting and Rinsing Fluids for Membrane Filtration*).◆

When using the technique of direct inoculation of media, use the quantities shown in *Table 2,* unless otherwise justified and authorized. The tests for bacterial and fungal sterility are carried out on the same sample of the product to be examined. When the volume or the quantity in a single container is insufficient to carry out the tests, the contents of two or more containers are used to inoculate the different media.

MINIMUM NUMBER OF ITEMS TO BE TESTED

The minimum number of items to be tested in relation to the size of the batch is given in *Table 3*.

⟨197⟩ SPECTROSCOPIC IDENTIFICATION TESTS

Change to read:

INTRODUCTION AND SCOPE

This chapter provides several tests and procedures that are used to confirm the chemical identity of a specific material in its respective monograph. When one of the tests listed in this chapter is referenced in a monograph, it must be used to confirm the identity of the material. Alternative identification procedures may be used, provided they are demonstrated to be equivalent to or better than the specified procedure and if they meet the requirements specified in the *Equivalent/Alternative Tests* section.

Spectroscopic tests contribute meaningfully toward the identification of many official articles. The test procedures that follow are applicable to substances that absorb, transmit, reflect, or scatter electromagnetic radiation in the near-infrared (NIR), infrared (IR), visible or near-infrared (Raman), ultraviolet (UV), visible (Vis), or X-ray ranges (see *Mid-Infrared Spectroscopy* ⟨854⟩, *Ultraviolet-Visible Spectroscopy* ⟨857⟩, *X-Ray Powder Diffraction* ⟨941⟩, *Near-Infrared Spectroscopy—Theory and Practice* ⟨1856⟩, and ▲*Raman Spectroscopy* ⟨858⟩▲ (CN 1-Aug-2020). The NIR, IR, and Raman spectra, or X-ray diffraction pattern of a substance, compared with the spectrum or diffraction pattern obtained with the corresponding USP Reference Standard, provides perhaps the most conclusive evidence of the identity of the substance that can be realized from any single test. The UV or Vis absorption spectrum of a substance, on the other hand, does not exhibit a high degree of specificity in most cases. To provide unambiguous confirmation of the identity of a substance, it may be necessary to execute two (or more) identity tests, as specified in a large proportion of compendial monographs.

IDENTIFICATION METHODOLOGY

Suitable identification methodology must be used for the chemical identification of materials through comparison with the appropriate compendial standards. Where alternative techniques are used, it must be demonstrated that the alternative identification methodology is suitable for the intended application (see *Validation of Compendial Procedures* ⟨1225⟩). Identification procedures should be able to discriminate between materials similar in molecular structure. The lack of specificity of a single technique may be compensated by other supporting analytical procedure(s) or an application of an additional identification technique.

The IR spectrum of a substance, compared with that obtained using equivalent instruments and conditions for the corresponding USP Reference Standard, is the most widely used methodology for chemical identification in compendial monographs. In general practice, the analysis of the sample and the USP Reference Standard are completed at the same time; however, if the USP Reference Standard spectrum was obtained previously using equivalent instruments and conditions, it is appropriate to compare the sample spectrum with the stored USP Reference Standard spectrum.

Under conditions where the IR spectrum lacks specificity for definitive chemical identification, additional spectroscopic information can be used to supplement chemical identification. For example, conformance with both IR and UV test specifications, as specified in a large proportion of compendial monographs, provides complementary information for the definitive identity of the sample under examination. In these instances, the combined spectroscopic information enables discrimination between compounds similar in structure that would not be possible from either IR spectrum or UV spectrum alone.

For preparing the standards used in the applications of this chapter, unless the directions for preparing the USP Reference Standard are explicitly specified in the monograph procedure, the USP Reference Standard must be used in accordance with instructions on its label. For preparation of the test sample, the sample must be prepared in accordance with the directions in the individual monograph procedure. Where no specific instructions are provided for sample preparation, handle the sample in the same manner as described by the USP Reference Standard label. For example, dry the sample as per drying conditions on the label of the corresponding USP Reference Standard.

INFRARED SPECTROSCOPY

Several methods are indicated for the preparation of test samples and USP Reference Standards for analysis by infrared spectroscopy (see *Table 1* and ⟨854⟩). The approaches for the techniques used in IR identity testing are summarized in *Table 1*.

Table 1. Infrared Spectroscopy Sample Preparation Techniques[a]

Method Reference	Sample Preparation
⟨197A⟩	The substance under examination is intimately in contact with an internal reflection element for attenuated total reflection (ATR) analysis.
⟨197D⟩	The substance under examination is mixed intimately with potassium bromide and transferred to a sample container for diffuse reflection (DR) analysis.
⟨197E⟩	The substance under examination is pressed as a thin sample against a suitable plate for IR microscopic analysis.
⟨197F⟩	The substance under examination is a thin film of a neat liquid or semisolid between suitable (e.g., sodium chloride or potassium bromide) plates, or a thin microcrystalline or glassy film deposited from a solution or cooled from a melt.
⟨197K⟩	The substance under examination is mixed intimately with potassium bromide and compressed into a transparent pellet.
⟨197M⟩	The substance under examination (10–20 mg) is finely ground and dispersed in a drop of mulling agent (unless otherwise directed in the monograph, mineral oil is to be used).
⟨197S⟩	A solution of designated concentration is prepared in the solvent specified in the individual monograph. The solution is examined in 0.1-mm cells, unless a different cell path length is specified.

[a] Each of the techniques in the table can be used as alternative methods when any other technique from the table is required in the monograph (see *General Notices, 6.30 Alternative and Harmonized Methods and Procedures*).

In each instance, infrared spectra of both the sample and corresponding USP Reference Standard are obtained using the same sample preparation technique and measurement parameters. Record and compare the spectra of the sample and the corresponding USP Reference Standard over the range from 3800 to 650 cm^{-1}, unless otherwise specified in the individual monograph. The comparison must establish that the IR spectrum of the preparation of the sample exhibits maxima only at the same wavenumbers as that of the appropriately prepared corresponding USP Reference Standard. If there are differences between the spectra, and the sample spectrum was compared with a previously obtained and electronically stored spectrum of the USP Reference Standard, the comparison must be repeated concomitantly with a freshly prepared USP Reference Standard.

Differences between the USP Reference Standard spectrum and sample spectrum that may be observed are sometimes attributable to differences in the solid-state form of the materials, if a solid-state technique is used (e.g., ⟨197A⟩, ⟨197K⟩, or ⟨197M⟩). If a specific crystal form is not specified in the monograph, where spectral differences between the sample and USP Reference Standard are observed, recrystallize both the sample and USP Reference Standard under identical conditions to produce the same solid-state form, unless specific procedures are provided in the individual monographs. Dissolve equal portions of the sample and the USP Reference Standard in equal volumes of a suitable solvent, evaporate the solutions to dryness in similar containers under identical conditions, and repeat the identification test on the residues. Other techniques for recrystallizing the sample and USP Reference Standard based on known scientific principles may be used with appropriate scientific justification.

Change to read:

NEAR-INFRARED AND RAMAN SPECTROSCOPY

The reference ⟨197NIR⟩ in a monograph signifies that a test sample and a standard sample are examined by the NIR spectroscopic technique (see *Near-Infrared Spectroscopy—Theory and Practice* ⟨1856⟩), and the reference ⟨197R⟩ in a monograph signifies that a test sample and a standard sample are examined by the Raman spectroscopic technique (see ▲⟨858⟩▲ (CN 1-Aug-2020)). NIR and Raman spectroscopic procedures may be used to confirm the identity of materials. The approach is similar to that of IR identity testing but is often augmented through the use of spectral libraries and chemometrics. A sample can thus be analyzed by NIR or Raman techniques, with the resulting spectrum compared with stored spectra in the spectral library through the use of multivariate analysis. In general, a visual comparison or simple overlay of the spectra alone may not be sufficient and additional evaluation may be needed.

In both techniques, samples can be directly interrogated with minimal or no sample preparation. Measurement is nondestructive and noninvasive, and data collection can often be made through glass or plastic containers.

A description of these techniques, and the strategy for procedure and chemometrics model development and validation can be found in the appropriate associated chapters when available (see *Chemometrics* ⟨1039⟩, *Near-Infrared Spectroscopy—Theory and Practice* ⟨1856⟩, and ▲*Raman Spectroscopy—Theory and Practice* ⟨1858⟩▲ (CN 1-Aug-2020), and the following chapters to be published at a later date: *Near-Infrared Spectroscopy—Theory and Practice* ⟨1856⟩ and *Raman Spectroscopy—Theory and Practice* ⟨1858⟩).

ULTRAVIOLET-VISIBLE SPECTROSCOPY

The reference ⟨197U⟩ in a monograph signifies that a sample solution and a Standard solution are examined spectroscopically, in 1-cm cells, over the spectral range from 200 to 400 nm, unless otherwise specified in the individual monograph (see ⟨857⟩). Dissolve a portion of the substance under examination in the designated medium to obtain a sample solution having the concentration specified in the monograph. Record and compare the spectra obtained for the sample solution and the Standard solution. Review or calculate the absorptivities and/or absorbance ratios and compare the results, and where appropriate, compare to criteria specified in an individual monograph.

The comparison must establish that the UV spectrum of the preparation of the sample exhibits absorption maxima and minima only at the same wavelengths as those of the appropriately prepared corresponding USP Reference Standard, and that the absorptivities and/or absorbance ratios are within the specified limits. If there are differences in the spectra, and the sample spectrum was compared with a previously obtained and electronically stored spectrum of the USP Reference Standard, the comparison must be concomitantly repeated with a freshly prepared USP Reference Standard.

Unless otherwise specified in the monograph, absorbances indicated for the calculations of the absorptivities and/or absorbance ratios are those measured at the maximum absorbance wavelength (within ±2 nm) specified in the individual monograph. Where the absorbance is to be measured at about the specified wavelength other than that of maximum absorbance, the abbreviations for minimum (min) and shoulder (sh) are used, respectively, in an absorption spectrum.

The reference ⟨197U-LC⟩ in a monograph signifies that, when a diode array detector is used in tandem with an LC procedure test in the monograph, the spectra of the major chromatographic peak(s) of the sample solution and Standard solution are examined spectroscopically.

The requirements are met if the UV spectra of the sample solution and of the Standard solution exhibit maxima and minima at the same wavelengths, and, if applicable, the absorptivities and/or absorbance ratios are within specified limits.

X-RAY POWDER DIFFRACTION

The reference ⟨197XR⟩ in a monograph signifies that a test sample and a standard sample are examined according to ⟨941⟩.

Prepare and mount the specimen as directed in ⟨941⟩. Unless otherwise indicated in the monograph, record the diffraction pattern in a 2θ-range from as near to 0° as possible to at least 32°. Unless otherwise specified in the monograph, the requirements are met if the X-ray diffraction pattern of the test specimen conforms to that of the corresponding USP Reference Standard obtained using equivalent instruments and conditions. Differences in the diffraction line intensities (but not line positions) between the sample and the Standard are acceptable.

The comparison must establish that the diffraction pattern of the preparation of the test specimen conforms to the diffraction pattern of the corresponding USP Reference Standard. If there are differences in the diffraction pattern and the sample diffraction pattern was compared with a previously obtained and electronically stored diffraction pattern of the USP Reference Standard, the comparison must be repeated concomitantly with a freshly prepared USP Reference Standard.

Change to read:

EQUIVALENT/ALTERNATIVE TESTS

In addition to IR, NIR, Raman, X-ray, and UV absorption, several other spectroscopic methodologies can be utilized for the identification of the specimen under examination. The methods cited in this chapter may be used for identification of materials as an alternative method to the method referenced in the monograph, provided that the alternative technique has been determined to be suitable for identification. Suitable identification tests must be able to discriminate between compounds similar in molecular structure that are likely to be present. The choice of such potentially interfering materials must be based on sound scientific judgment, with consideration of interferences that could occur. It is not always possible to demonstrate that an analytical procedure is specific for a particular analyte (complete discrimination).

For information regarding sample preparation and measurement parameters associated with an alternative identification method, refer to the appropriate general chapter (see *Mass Spectrometry* ⟨736⟩, *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩, ⟨854⟩, ⟨857⟩, ⟨941⟩, *Near-Infrared Spectroscopy—Theory and Practice* ⟨1856⟩, and ▲ ⟨858⟩▲ (CN 1-Aug-2020).

⟨221⟩ CHLORIDE AND SULFATE

Change to read:

The following limit tests are provided as general procedures for use where limits for chloride and sulfate are specified in the individual monographs.

Perform the tests and the controls in glass cylinders of the same diameter and matched as closely as practicable in other respects (see ▲*Visual Comparison* ⟨630⟩▲ (CN 1-May-2019)). Use the same quantities of the same reagents for both the solution under test and the control solution containing the specified volume of chloride or sulfate. If, after acidification, the solution is not perfectly clear, pass it through a filter paper that gives negative tests for chloride and sulfate. Add the precipitant, silver nitrate TS or barium chloride TS as required, to both the test solution and the control solution in immediate sequence.

Where the individual monograph calls for applying the test to a specific volume of a solution of the substance, and the limit for chloride or sulfate corresponds to 0.20 mL or less of 0.020 N hydrochloric acid or sulfuric acid, respectively, apply the test to the solution without further dilution. In such cases maintain the same volume relationships for the control solution as specified for the solution under test. In applying the test to the salts of heavy metals, which normally show an acid reaction, omit the acidification and do not neutralize the solution. Dissolve bismuth salts in a few mL of water and 2 mL of nitric acid before treating with the precipitant.

CHLORIDE—Dissolve the specified quantity of the substance under test in 30 to 40 mL of water, or, where the substance is already in solution, add water to make a total volume of 30 to 40 mL, and, if necessary, neutralize the solution with nitric acid to litmus. Add 1 mL each of nitric acid and of silver nitrate TS and sufficient water to make 50 mL. Mix, and allow to stand for 5 minutes protected from direct sunlight. Unless otherwise specified in the monograph, compare the turbidity, if any, with that produced in a solution containing the volume of 0.020 N hydrochloric acid specified in the monograph.

SULFATE—Dissolve the specified quantity of the substance under test in 30 to 40 mL of water, or, where the substance is already in solution, add water to make a total volume of 30 to 40 mL, and, if necessary, neutralize the solution with hydrochloric acid to litmus. Add 1 mL of 3 N hydrochloric acid, 3 mL of barium chloride TS, and sufficient water to make 50 mL. Mix, and allow to stand for 10 minutes. Unless otherwise specified in the monograph, compare the turbidity, if any, with that produced in a solution containing the volume of 0.020 N sulfuric acid specified in the monograph.

⟨281⟩ RESIDUE ON IGNITION

Portions of this general chapter have been harmonized with the corresponding texts of the *European Pharmacopoeia* and the *Japanese Pharmacopoeia*. The portions that are not harmonized are marked with symbols (•,•). The harmonized texts of these pharmacopeias are therefore interchangeable, and the methods of the *European Pharmacopoeia* and/or the *Japanese Pharmacopoeia* may be used for demonstration of compliance instead of the present *United States Pharmacopeia* general chapter. These pharmacopeias have undertaken not to make any unilateral change to this harmonized chapter.

The *Residue on Ignition/Sulfated Ash* test uses a procedure to measure the amount of residual substance not volatilized from a sample when the sample is ignited in the presence of sulfuric acid according to the procedure described below. This test is usually used for determining the content of inorganic impurities in an organic substance.

PROCEDURE

Ignite a suitable crucible (for example, silica, platinum, quartz, or porcelain) at 600 ± 50° for 30 minutes, cool the crucible in a desiccator (silica gel or other suitable desiccant), and weigh it accurately. Weigh accurately •1 to 2 g of the substance, or,• the amount specified in the individual monograph, in the crucible.

Moisten the sample with a small amount (usually 1 mL) of sulfuric acid, then heat gently at a temperature as low as practicable until the sample is thoroughly charred. Cool; then,•unless otherwise directed in the individual monograph,• moisten the residue with a small amount (usually 1 mL) of sulfuric acid; heat gently until white fumes are no longer evolved; and ignite at 600 ± 50°, •unless another temperature is specified in the individual monograph,• until the residue is completely incinerated. Ensure that flames are not produced at any time during the procedure. Cool the crucible in a desiccator (silica gel or other suitable desiccant), weigh accurately, and calculate the percentage of residue.

Unless otherwise specified, if the amount of the residue so obtained exceeds the limit specified in the individual monograph, repeat the moistening with sulfuric acid, heating and igniting as before, using a 30-minute ignition period, until two consecutive weighings of the residue do not differ by more than 0.5 mg or until the percentage of residue complies with the limit in the individual monograph.

•Conduct the ignition in a well-ventilated hood, but protected from air currents, and at as low a temperature as is possible to effect the complete combustion of the carbon. A muffle furnace may be used, if desired, and its use is recommended for the final ignition at 600 ± 50°.

Calibration of the muffle furnace may be carried out using an appropriate digital temperature meter and a working thermocouple probe calibrated against a standard thermocouple traceable to the National Institute of Standards and Technology.

Verify the accuracy of the measuring and controlling circuitry of the muffle furnace by checking the positions in the furnace at the control set point temperature of intended use. Select positions that reflect the eventual method of use with respect to location of the specimen under test. The tolerance is ±25° at each position measured.•

⟨541⟩ TITRIMETRY

DIRECT TITRATIONS

Direct titration is the treatment of a soluble substance, contained in solution in a suitable vessel (the titrate), with an appropriate standardized solution (the titrant), the endpoint being determined instrumentally or visually with the aid of a suitable indicator.

The titrant is added from a suitable buret and is so chosen, with respect to its strength (normality), that the volume added is between 30% and 100% of the rated capacity of the buret.

[NOTE—Where less than 10 mL of titrant is required, a suitable microburet is to be used.]

The endpoint is approached directly but cautiously, and finally the titrant is added dropwise from the buret in order that the final drop added will not overrun the endpoint. The quantity of the substance being titrated may be calculated from the volume and the normality or molarity factor of the titrant and the equivalence factor for the substance given in the individual monograph.

RESIDUAL TITRATIONS

Some Pharmacopeial assays require the addition of a measured volume of a volumetric solution, in excess of the amount actually needed to react with the substance being assayed, the excess of this solution then being titrated with a second volumetric solution. This constitutes a residual titration and is known also as a "back titration." The quantity of the substance

being titrated may be calculated from the difference between the volume of the volumetric solution originally added, corrected by means of a blank titration, and that consumed by the titrant in the back titration, due allowance being made for the respective normality or molarity factors of the two solutions, and the equivalence factor for the substance given in the individual monograph.

COMPLEXOMETRIC TITRATIONS

Successful complexometric titrations depend on several factors. The equilibrium constant for formation of the titrant-analyte complex must be sufficiently large that, at the endpoint, very close to 100% of the analyte has been complexed. The final complex must be formed rapidly enough that the analysis time is practical. When the analytical reaction is not rapid, a residual titration may sometimes be successful.

In general, complexometric indicators are themselves complexing agents. The reaction between metal ion and indicator must be rapid and reversible. The equilibrium constant for formation of the metal-indicator complex should be large enough to produce a sharp color change but must be less than that for the metal-titrant complex. Indicator choice is also restricted by the pH range within which the complexation reaction must be carried out and by interference of other ions arising from the sample or the buffer. Interfering ions may often be masked or "screened" via addition of another complexing agent. (The masking technique is also applicable to redox titrations.)

OXIDATION-REDUCTION (REDOX) TITRATIONS

Determinations may often be carried out conveniently by the use of a reagent that brings about oxidation or reduction of the analyte. Many redox titration curves are not symmetric about the equivalence point, and thus graphical determination of the endpoint is not possible; but indicators are available for many determinations, and a redox reagent can often serve as its own indicator. As in any type of titration, the ideal indicator changes color at an endpoint that is as close as possible to the equivalence point. Accordingly, when the titrant serves as its own indicator, the difference between the endpoint and the equivalence point is determined only by the analyst's ability to detect the color change. A common example is the use of permanganate ion as an oxidizing titrant since a slight excess can easily be detected by its pink color. Other titrants that may serve as their own indicators are iodine, cerium (IV) salts, and potassium dichromate. In most cases, however, the use of an appropriate redox indicator will yield a much sharper endpoint.

It may be necessary to adjust the oxidation state of the analyte prior to titration through use of an appropriate oxidizing or reducing agent; the excess reagent must then be removed, e.g., through precipitation. This is nearly always the practice in the determination of oxidizing agents since most volumetric solutions of reducing agents are slowly oxidized by atmospheric oxygen.

TITRATIONS IN NONAQUEOUS SOLVENTS

Acids and bases have long been defined as substances that furnish, when dissolved in water, hydrogen and hydroxyl ions, respectively. This definition, introduced by Arrhenius, fails to recognize the fact that properties characteristic of acids or bases may be developed also in other solvents. A more generalized definition is that of Brönsted, who defined an acid as a substance that furnishes protons, and a base as a substance that combines with protons. Even broader is the definition of Lewis, who defined an acid as any material that will accept an electron pair, a base as any material that will donate an electron pair, and neutralization as the formation of a coordination bond between an acid and a base.

The apparent strength of an acid or a base is determined by the extent of its reaction with a solvent. In water solution all strong acids appear equally strong because they react with the solvent to undergo almost complete conversion to oxonium ion and the acid anion (leveling effect). In a weakly protophilic solvent such as acetic acid the extent of formation of the acetate acidium ion shows that the order of decreasing strength for acids is perchloric, hydrobromic, sulfuric, hydrochloric, and nitric (differentiating effect).

Acetic acid reacts incompletely with water to form oxonium ion and is, therefore, a weak acid. In contrast, it dissolves in a base such as ethylenediamine, and reacts so completely with the solvent that it behaves as a strong acid. The same holds for perchloric acid.

This leveling effect is observed also for bases. In sulfuric acid almost all bases appear to be of the same strength. As the acid properties of the solvent decrease in the series sulfuric acid, acetic acid, phenol, water, pyridine, and butylamine, the bases become progressively weaker until all but the strongest have lost their basic properties. In order of decreasing strength, the strong bases are sodium 2-aminoethoxide, potassium methoxide, sodium methoxide, and lithium methoxide.

Many water-insoluble compounds acquire enhanced acidic or basic properties when dissolved in organic solvents. Thus the choice of the appropriate solvent permits the determination of a variety of such materials by nonaqueous titration. Furthermore, depending upon which part of a compound is the physiologically active moiety, it is often possible to titrate that part by proper selection of solvent and titrant. Pure compounds can be titrated directly, but it is often necessary to isolate the active ingredient in pharmaceutical preparations from interfering excipients and carriers.

The types of compounds that may be titrated as acids include acid halides, acid anhydrides, carboxylic acids, amino acids, enols such as barbiturates and xanthines, imides, phenols, pyrroles, and sulfonamides. The types of compounds that may be titrated as bases include amines, nitrogen-containing heterocyclic compounds, oxazolines, quaternary ammonium compounds, alkali salts of organic acids, alkali salts of weak inorganic acids, and some salts of amines. Many salts of halogen acids may be titrated in acetic acid or acetic anhydride after the addition of mercuric acetate, which removes halide ion as the unionized mercuric halide complex and introduces the acetate ion.

For the titration of a basic compound, a volumetric solution of perchloric acid in glacial acetic acid is preferred, although perchloric acid in dioxane is used in special cases. The calomel-glass electrode system is useful in this case. In acetic acid solvent, this electrode system functions as predicted by theory.

For the titration of an acidic compound, two classes of titrant are available: the alkali metal alkoxides and the tetraalkylammonium hydroxides. A volumetric solution of sodium methoxide in a mixture of methanol and toluene is used frequently, although lithium methoxide in methanol-benzene solvent is used for those compounds yielding a gelatinous precipitate on titration with sodium methoxide.

The alkali error limits the use of the glass electrode as an indicating electrode in conjunction with alkali metal alkoxide titrants, particularly in basic solvents. Thus, the antimony-indicating electrode, though somewhat erratic, is used in such titrations. The use of quaternary ammonium hydroxide compounds, e.g., tetra-*n*-butylammonium hydroxide and trimethylhexadecylammonium hydroxide (in benzene-methanol or isopropyl alcohol), has two advantages over the other titrants in that (a) the tetraalkylammonium salt of the titrated acid is soluble in the titration medium, and (b) the convenient and well-behaved calomel-glass electrode pair may be used to conduct potentiometric titrations.

Because of interference by carbon dioxide, solvents for acidic compounds need to be protected from excessive exposure to the atmosphere by a suitable cover or by an inert atmosphere during the titration. Absorption of carbon dioxide may be determined by performing a blank titration. The blank should not exceed 0.01 mL of 0.1 N sodium methoxide VS per mL of solvent.

The endpoint may be determined visually by color change, or potentiometrically, as indicated in the individual monograph. If the calomel reference electrode is used, it is advantageous to replace the aqueous potassium chloride salt bridge with 0.1 N lithium perchlorate in glacial acetic acid for titrations in acidic solvents or potassium chloride in methanol for titrations in basic solvents.

Where these or other mixtures are specified in individual monographs, the calomel reference electrode is modified by first removing the aqueous potassium chloride solution and residual potassium chloride, if any, by rinsing with water, then eliminating residual water by rinsing with the required nonaqueous solvent, and finally filling the electrode with the designated nonaqueous mixture.

In nearly all cases, except those where silver ion might interfere, a silver-silver chloride reference electrode may be substituted for the calomel electrode. The silver-silver chloride electrode is more rugged, and its use helps to eliminate toxic mercury salts from the laboratory. Generally, a salt bridge may be used to circumvent interference by silver ion.

The more useful systems for titration in nonaqueous solvents are listed in *Table 1*.

Table 1. Systems for Nonaqueous Titrations

Type of Solvent	Acidic (for titration of bases and their salts)	Relatively Neutral (for differential titration of bases)	Basic (for titration of acids)	Relatively Neutral (for differential titration of acids)
Solvent[1]	Glacial Acetic Acid	Acetonitrile	Dimethylformamide	Acetone
	Acetic Anhydride	Alcohols	*n*-Butylamine	Acetonitrile
	Formic Acid	Chloroform	Pyridine	Methyl Ethyl Ketone
	Propionic Acid	Benzene	Ethylenediamine	Methyl Isobutyl Ketone
	Sulfuryl Chloride	Toluene	Morpholine	*tert*-Butyl Alcohol
		Chlorobenzene		
		Ethyl Acetate		
		Dioxane		
Indicator	Crystal Violet	Methyl Red	Thymol Blue	Azo Violet
	Quinaldine Red	Methyl Orange	Thymolphthalein	Bromothylmol Blue
	p-Naphtholbenzein	*p*-Naphtholbenzein	Azo Violet	*p*-Hydroxyazobenzene
	Alphezurine 2-G		*o*-Nitroaniline	Thymol Blue
	Malachite Green		*p*-Hydroxyazobenzene	
Electrodes	Glass–calomel	Glass–calomel	Antimony–calomel	Antimony–calomel
	Glass–silver–silver chloride	Calomel–silver–silver chloride	Antimony–glass	Glass–calomel
	Mercury–mercuric acetate		Antimony–antimony[2]	Glass–platinum[2]
			Platinum–calomel	
			Glass–calomel	

[1] Relatively neutral solvents of low dielectric constant such as benzene, toluene, chloroform, or dioxane may be used in conjunction with any acidic or basic solvent in order to increase the sensitivity of the titration end-points.
[2] In titrant.

INDICATOR AND POTENTIOMETRIC ENDPOINT DETECTION

The simplest and most convenient method by which the equivalence point, i.e., the point at which the stoichiometric analytical reaction is complete, may be determined is with the use of indicators. These chemical substances, usually colored,

respond to changes in solution conditions before and after the equivalence point by exhibiting color changes that may be taken visually as the endpoint, a reliable estimate of the equivalence point.

A useful method of endpoint determination results from the use of electrochemical measurements. If an indicator electrode, sensitive to the concentration of the species undergoing titrimetric reaction, and a reference electrode, whose potential is insensitive to any dissolved species, are immersed in the titrate to form a galvanic cell, the potential difference between the electrodes may be sensed by a pH meter and used to follow the course of the reaction. Where such a series of measurements is plotted correctly (i.e., for an acid-base titration, pH versus mL of titrant added; for a precipitimetric, complexometric, or oxidation-reduction titration, mV versus mL of titrant added), a sigmoid curve results with a rapidly changing portion (the "break") in the vicinity of the equivalence point. The midpoint of this linear vertical portion or the inflection point may be taken as the endpoint. The equivalence point may also be determined mathematically without plotting a curve. However, it should be noted that in asymmetrical reactions, which are reactions in which the number of anions reacting is not the same as the number of cations reacting, the endpoint as defined by the inflection of the titration curve does not occur exactly at the stoichiometric equivalence point. Thus, potentiometric endpoint detection by this method is not suitable in the case of asymmetric reactions, examples of which are the precipitation reaction,

$$2Ag^{+} + CrO_4^{-2}$$

and the oxidation-reduction reaction,

$$5Fe^{+2} + MnO_4^{-}.$$

All acid-base reactions, however, are symmetrical. Thus, potentiometric endpoint detection may be employed in acid-base titrations and in other titrations involving symmetrical reversible reactions where an indicator is specified, unless otherwise directed in the individual monograph.

Two types of automatic electrometric titrators are available. The first is one that carries out titrant addition automatically and records the electrode potential differences during the course of titration as the expected sigmoid curve. In the second type, titrant addition is performed automatically until a preset potential or pH, representing the endpoint, is reached, at which point the titrant addition ceases.

Several acceptable electrode systems for potentiometric titrations are summarized in *Table 2*.

Table 2. Potentiometric Titration Electrode Systems

Titration	Indicating Electrode	Equation[1]	Reference Electrode	Applicability[2]
Acid-base	Glass	$E = k + 0.0591\ pH$	Calomel or silver–silver chloride	Titration of acids and bases
Precipitimetric (silver)	Silver	$E = E° + 0.0591\ \log [Ag^{+}]$	Calomel (with potassium nitrate salt bridge)	Titration with or of silver involving halides or thiocyanate
Complexometric	Mercury–mercury(II)	$E = E° + 0.0296(\log k' - pM)$	Calomel	Titration of various metals (M), e.g., Mg^{+2}, Ca^{+2} Al^{+3}, Bi^{+3}, with EDTA
Oxidation–reduction	Platinum	$E = E° + (0.0591/n) \times \log [ox]/[red]$	Calomel or silver–silver chloride	Titrations with arsenite, bromine, cerate, dichromate, exacyonoferrate(III), iodate, nitrite, permanganate, thiosulfate

1 Appropriate form of Nernst equation describing the indicating electrode system: k = glass electrode constant; k′ = constant derived from Hg–Hg(II)–EDTA equilibrium; M = any metal undergoing EDTA titration; [ox] and [red] from the equation,

$ox + ne \rightleftarrows red.$

2 Listing is representative but not exhaustive.

BLANK CORRECTIONS

As previously noted, the endpoint determined in a titrimetric assay is an estimate of the reaction equivalence point. The validity of this estimate depends upon, among other factors, the nature of the titrate constituents and the concentration of the titrant. An appropriate *blank correction* is employed in titrimetric assays to enhance the reliability of the endpoint determination. Such a blank correction is usually obtained by means of a *residual blank titration*, wherein the required procedure is repeated in every detail except that the substance being assayed is omitted. In such instances, the actual volume of titrant equivalent to the substance being assayed is the difference between the volume consumed in the residual blank titration and that consumed in the titration with the substance present. The corrected volume so obtained is used in calculating the quantity of the substance being titrated, in the same manner as prescribed under *Residual Titrations*. Where potentiometric endpoint detection is employed, the blank correction is usually negligible.

⟨621⟩ CHROMATOGRAPHY

INTRODUCTION

Chromatographic separation techniques are multistage separation methods in which the components of a sample are distributed between two phases, of which one is stationary and the other is mobile. The stationary phase may be a solid or a liquid supported on a solid or a gel. The stationary phase may be packed in a column, spread as a layer, distributed as a film, or applied by other techniques. The mobile phase may be in a gaseous or liquid form, or a supercritical fluid. The separation may be based on adsorption, mass distribution (partition), or ion exchange; or it may be based on differences among the physicochemical properties of the molecules, such as size, mass, and volume. This chapter contains general procedures, definitions, and calculations of common parameters and describes general requirements for system suitability. The types of chromatography useful in qualitative and quantitative analyses employed in *USP* procedures are column, gas (GC), paper, thin-layer (TLC) [including high-performance thin-layer chromatography (HPTLC)], and pressurized liquid chromatography [commonly called high-pressure or high-performance liquid chromatography (HPLC)].

GENERAL PROCEDURES

This section describes the basic procedures used when a chromatographic method is described in a monograph. These procedures are followed unless otherwise indicated in the individual monograph.

Paper Chromatography

STATIONARY PHASE

The stationary phase is a sheet of paper of suitable texture and thickness. Development may be ascending, in which the solvent is carried up the paper by capillary forces, or descending, in which the solvent flow is also assisted by gravitational force. The orientation of paper grain, with respect to solvent flow, is to be kept constant in a series of chromatograms. The machine direction is usually designated by the manufacturer.

APPARATUS

The essential equipment for paper chromatography consists of a vapor-tight chamber with inlets for the addition of solvent and a rack of corrosion-resistant material about 5 cm shorter than the inside height of the chamber. The rack serves as a support for solvent troughs and antisiphon rods that, in turn, hold up the chromatographic sheets. The bottom of the chamber is covered with the prescribed solvent system or mobile phase. Saturation of the chamber with solvent vapor is facilitated by lining the inside walls with paper wetted with the prescribed solvent system.

SPOTTING

The substance or substances analyzed are dissolved in a suitable solvent. Convenient volumes delivered from suitable micropipettes of the resulting solution, normally containing 1–20 µg of the compound, are placed in 6- to 10-mm spots NLT 3 cm apart.

DESCENDING PAPER CHROMATOGRAPHY PROCEDURE

1. A spotted chromatographic sheet is suspended in the apparatus, using the antisiphon rod to hold the upper end of the sheet in the solvent trough. [NOTE—Ensure that the portion of the sheet hanging below the rods is freely suspended in the chamber without touching the rack, the chamber walls, or the fluid in the chamber.]
2. The chamber is sealed to allow equilibration (saturation) of the chamber and the paper with the solvent vapor. Any excess pressure is released as necessary.
3. After equilibration of the chamber, the prepared mobile phase is introduced into the trough through the inlet.
4. The inlet is closed, and the mobile solvent phase is allowed to travel the desired distance down the paper.
5. The sheet is removed from the chamber.
6. The location of the solvent front is quickly marked, and the sheet is dried.
7. The chromatogram is observed and measured directly or after suitable development to reveal the location of the spots of the isolated drug or drugs.

ASCENDING PAPER CHROMATOGRAPHY PROCEDURE

1. The mobile phase is added to the bottom of the chamber.
2. The chamber is sealed to allow equilibration (saturation) of the chamber and the paper with the solvent vapor. Any excess pressure is released as necessary.
3. The lower edge of the stationary phase is dipped into the mobile phase to permit the mobile phase to rise on the chromatographic sheet by capillary action.

4. When the solvent front has reached the desired height, the chamber is opened, the sheet is removed, the location of the solvent front is quickly marked, and the sheet is dried.
5. The chromatogram is observed and measured directly or after suitable development to reveal the location of the spots of the isolated drug or drugs.

Thin-Layer Chromatography

STATIONARY PHASE

The stationary phase is a relatively thin, uniform layer of dry, finely powdered material applied to a glass, plastic, or metal sheet or plate (typically called the plate). The stationary phase of TLC plates has an average particle size of 10–15 µm, and that of HPTLC plates has an average particle size of 5 µm. Commercial plates with a preadsorbent zone can be used if they are specified in a monograph. The sample applied to the preadsorbent region develops into sharp, narrow bands at the preadsorbent–sorbent interface. The separations achieved may be based on adsorption, partition, or a combination of both effects, depending on the particular type of stationary phase.

APPARATUS

A chromatographic chamber made of inert, transparent material and having the following specifications is used: a flat-bottom or twin trough, a tightly fitted lid, and a size suitable for the plates. The chamber is lined on at least one wall with filter paper. Sufficient mobile phase or developing solvent is added to the chamber so that, after impregnation of the filter paper, a depth appropriate to the dimensions of the plate used is available. The chromatographic chamber is closed and allowed to equilibrate. [NOTE—Unless otherwise indicated, the chromatographic separations are performed in a saturated chamber.]

DETECTION/VISUALIZATION

An ultraviolet (UV) light source suitable for observations under short- (254 nm) and long- (365 nm) wavelength UV light and a variety of other spray reagents, used to make spots visible, is often used.

SPOTTING

Solutions are spotted on the surface of the stationary phase (plate) at the prescribed volume in sufficiently small portions to obtain circular spots of 2–5 mm in diameter (1–2 mm on HPTLC plates) or bands of 10–20 mm × 1–2 mm (5–10 mm × 0.5–1 mm on HPTLC plates) at an appropriate distance from the lower edge and sides of the plate. [NOTE—During development, the application position must be at least 5 mm (TLC) or 3 mm (HPTLC) above the level of the mobile phase.] The solutions are applied on a line parallel to the lower edge of the plate with an interval of at least 10 mm (5 mm on HPTLC plates) between the centers of spots or 4 mm (2 mm on HPTLC plates) between the edges of bands, then allowed to dry.

PROCEDURE

1. Place the plate in the chamber, ensuring that the spots or bands are above the surface of the mobile phase.
2. Close the chamber.
3. Allow the mobile phase to ascend the plate until the solvent front has traveled three-quarters of the length of the plate, or the distance prescribed in the monograph.
4. Remove the plate, mark the solvent front with a pencil, and allow to dry.
5. Visualize the chromatograms as prescribed.
6. Determine the chromatographic *Retardation factor* (R_F) values for the principal spots or zones.
7. Presumptive identification can be made by observation of spots or zones of identical R_F value and about equal magnitude obtained, respectively, with an unknown and a standard chromatographed on the same plate. A visual comparison of the size or intensity of the spots or zones may serve for semiquantitative estimation. Quantitative measurements are possible by means of densitometry (absorbance or fluorescence measurements).

Column Chromatography

SOLID SUPPORT

Purified siliceous earth is used for normal-phase separation. Silanized chromatographic siliceous earth is used for reverse-phase partition chromatography.

STATIONARY PHASE

The solid support is modified by the addition of a stationary phase specified in the individual monograph. If a mixture of liquids is used as the stationary phase, mix the liquids before the introduction of the solid support.

MOBILE PHASE

The mobile phase is specified in the individual monograph. If the stationary phase is an aqueous solution, equilibrate with water. If the stationary phase is a polar organic fluid, equilibrate with that fluid.

APPARATUS

Unless otherwise specified in the individual monograph, the chromatographic tube is about 22 mm in its inside diameter and 200–300 mm long. Attached to it is a delivery tube, without stopcock, about 4 mm in its inside diameter and about 50 mm long.

Apparatus preparation: Pack a pledget of fine glass wool in the base of the tube. Combine the specified volume of stationary phase and the specified amount of solid support to produce a homogeneous, fluffy mixture. Transfer this mixture to the chromatographic tube, and tamp using gentle pressure to obtain a uniform mass. If the specified amount of solid support is >3 g, transfer the mixture to the column in portions of approximately 2 g, and tamp each portion. If the assay or test requires a multisegment column with a different stationary phase specified for each segment, tamp after the addition of each segment, and add each succeeding segment directly to the previous one. Pack a pledget of fine glass wool above the completed column packing. [NOTE—The mobile phase should flow through a properly packed column as a moderate stream or, if reverse-phase chromatography is applied, as a slow trickle.]

If a solution of the analyte is incorporated into the stationary phase, complete the quantitative transfer to the chromatographic tube by scrubbing the beaker used for the preparation of the test mixture with a mixture of about 1 g of solid support and several drops of the solvent used to prepare the sample solution before adding the final portion of glass wool.

PROCEDURE

1. Transfer the mobile phase to the column space above the column packing, and allow it to flow through the column under the influence of gravity.
2. Rinse the tip of the chromatographic column with about 1 mL of mobile phase before each change in composition of mobile phase and after completion of the elution.
3. If the analyte is introduced into the column as a solution in the mobile phase, allow it to pass completely into the column packing, then add the mobile phase in several small portions, allowing each to drain completely, before adding the bulk of the mobile phase.
4. Where the procedure indicates the use of multiple chromatographic columns mounted in series and the addition of mobile phase in divided portions is specified, allow each portion to drain completely through each column, and rinse the tip of each with mobile phase before the addition of each succeeding portion.

Gas Chromatography

LIQUID STATIONARY PHASE

This type of phase is available in packed or capillary columns.

PACKED COLUMN GAS CHROMATOGRAPHY

The liquid stationary phase is deposited on a finely divided, inert solid support, such as diatomaceous earth, porous polymer, or graphitized carbon, which is packed into a column that is typically 2–4 mm in internal diameter and 1–3 m in length.

CAPILLARY COLUMN GAS CHROMATOGRAPHY

In capillary columns, which contain no packed solid support, the liquid stationary phase is deposited on the inner surface of the column and may be chemically bonded to it.

SOLID STATIONARY PHASE

This type of phase is available only in packed columns. In these columns the solid phase is an active adsorbent, such as alumina, silica, or carbon, packed into a column. Polyaromatic porous resins, which are sometimes used in packed columns, are not coated with a liquid phase. [NOTE—Packed and capillary columns must be conditioned before use until the baseline and other characteristics are stable. The column or packing material supplier provides instructions for the recommended conditioning procedure.]

APPARATUS

A gas chromatograph consists of a carrier gas source, injection port, column, detector, and recording device. The injection port, column, and detector are temperature controlled and may be varied as part of the analysis. The typical carrier gas is helium, nitrogen, or hydrogen, depending on the column and detector in use. The type of detector used depends on the nature of the compounds analyzed and is specified in the individual monograph. Detector output is recorded as a function of time, and the instrument response, measured as peak area or peak height, is a function of the amount present.

TEMPERATURE PROGRAM

The length and quality of a GC separation can be controlled by altering the temperature of the chromatographic column. When a temperature program is necessary, the individual monograph indicates the conditions in table format. The table indicates the initial temperature, rate of temperature change (ramp), final temperature, and hold time at the final temperature.

PROCEDURE

1. Equilibrate the column, injector, and detector with flowing carrier gas until a constant signal is received.
2. Inject a sample through the injector septum, or use an autosampler.
3. Begin the temperature program.
4. Record the chromatogram.
5. Analyze as indicated in the monograph.

Liquid Chromatography

LC, as used in the compendia, is synonymous with HPLC (both high-pressure and high-performance). LC is a separation technique based on a solid stationary phase and a liquid mobile phase.

STATIONARY PHASE

Separations are achieved by partition, adsorption, or ion-exchange processes, depending on the type of stationary phase used. The most commonly used stationary phases are modified silica or polymeric beads. The beads are modified by the addition of long-chain hydrocarbons. The specific type of packing needed to complete an analysis is indicated by the "L" designation in the individual monograph (see also *Chromatographic Columns*). The size of the beads is often described in the monograph as well. Changes in the packing type and size are covered in *System Suitability* in this chapter.

CHROMATOGRAPHIC COLUMN

The term "column" includes stainless steel, lined stainless steel, and polymeric columns, packed with a stationary phase. The length and inner diameter of the column affects the separation, and therefore typical column dimensions are included in the individual monograph. Changes to column dimensions are discussed in *System Suitability*. Compendial monographs do not include the name of appropriate columns; this omission avoids the appearance of endorsement of a vendor's product and natural changes in the marketplace. See *Chromatographic Columns* for more information.

In LC procedures, a guard column may be used with the following requirements, unless otherwise indicated in the individual monograph: (a) the length of the guard column must be NMT 15% of the length of the analytical column, (b) the inner diameter must be the same or smaller than that of the analytical column, and (c) the packing material should be the same as the analytical column (e.g., silica) and contain the same bonded phase (e.g., C18). In any case, all system suitability requirements specified in the official procedure must be met with the guard column installed.

MOBILE PHASE

The mobile phase is a solvent or a mixture of solvents, as defined in the individual monograph.

APPARATUS

A liquid chromatograph consists of a reservoir containing the mobile phase, a pump to force the mobile phase through the system at high pressure, an injector to introduce the sample into the mobile phase, a chromatographic column, a detector, and a data collection device.

GRADIENT ELUTION

The technique of continuously changing the solvent composition during the chromatographic run is called gradient elution or solvent programming. The gradient elution profile is presented in the individual monograph as a gradient table, which lists the time and proportional composition of the mobile phase at the stated time.

PROCEDURE

1. Equilibrate the column and detector with mobile phase at the specified flow rate until a constant signal is received.
2. Inject a sample through the injector, or use an autosampler.
3. Begin the gradient program.
4. Record the chromatogram.
5. Analyze as directed in the monograph.

CHROMATOGRAPHIC COLUMNS

A complete list of packings (L), phases (G), and supports (S) used in *USP–NF* tests and assays is located in *USP–NF, Reagents, Indicators, and Solutions—Chromatographic Columns*. This list is intended to be a convenient reference for the chromatographer in identifying the pertinent chromatographic column specified in the individual monograph.

DEFINITIONS AND INTERPRETATION OF CHROMATOGRAMS

Chromatogram: A graphical representation of the detector response, concentration of analyte in the effluent, or other quantity used as a measure of effluent concentration versus effluent volume or time. In planar chromatography, chromatogram may refer to the paper or layer with the separated zones.

Figure 1 represents a typical chromatographic separation of two substances, 1 and 2. t_{R1} and t_{R2} are the respective retention times; h is the height, $h/2$ is the half-height, and $W_{h/2}$ is the width at half-height, for peak 1. W_1 and W_2 are the respective widths of peaks 1 and 2 at the baseline. Air peaks are a feature of gas chromatograms and correspond to the solvent front in LC. The retention time of these air peaks, or unretained components, is designated as t_M.

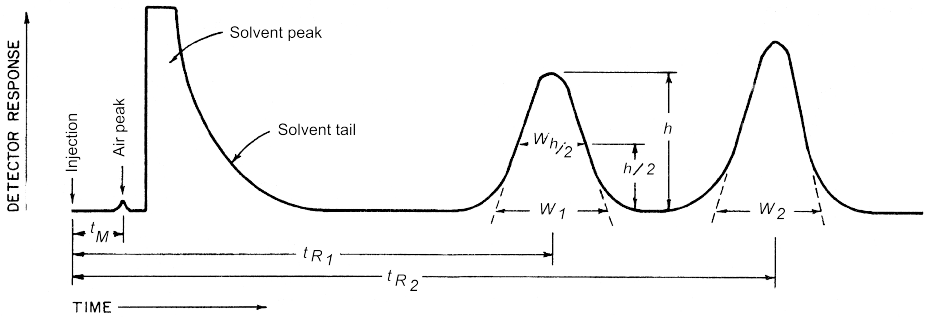


Figure 1. Chromatographic separation of two substances.

Dwell volume (D): Also known as "gradient delay volume", is the volume between the point at which the eluents meet and the top of the column.

Hold-up time (t_M): The time required for elution of an unretained component (see *Figure 1*, shown as an air or unretained solvent peak, with the baseline scale in minutes).

Hold-up volume (V_M): The volume of mobile phase required for elution of an unretained component. It may be calculated from the hold-up time and the flow rate, F, in mL/min:

$$V_M = t_M \times F$$

In size-exclusion chromatography, the symbol V_O is used.

Number of theoretical plates (N):[1] A measure of column efficiency. For Gaussian peaks, it is calculated by:

$$N = 16(t_R/W)^2$$

where t_R is the retention time of the substance, and W is the peak width at its base, obtained by extrapolating the relatively straight sides of the peak to the baseline. The value of N depends upon the substance being chromatographed as well as the operating conditions, such as the flow rate and temperature of the mobile phase or carrier gas, the quality of the packing, the uniformity of the packing within the column, and, for capillary columns, the thickness of the stationary phase film and the internal diameter and length of the column.

Where electronic integrators are used, it may be convenient to determine the number of theoretical plates, by the equation:

$$N = 5.54\left(\frac{t_R}{W_{h/2}}\right)^2$$

where $W_{h/2}$ is the peak width at half-height. However, in the event of dispute, only equations based on peak width at baseline are to be used.

[1] The parameters k, N, r, and r_G were developed for isothermal GC separations and isocratic HPLC separations. Because these terms are thermodynamic parameters, they are only valid for separations made at a constant temperature, mobile phase composition, and flow rate. However, for separations made with a temperature program or solvent gradient, these parameters may be used simply as comparative means to ensure that adequate chromatographic conditions exist to perform the methods as intended in the monographs.

Published August 17, 2020

Peak: The portion of the chromatographic recording of the detector response when a single component is eluted from the column. If separation is incomplete, two or more components may be eluted as one unresolved peak.

Peak-to-valley ratio (p/v): p/v may be employed as a system suitability criterion in a test for related substances when baseline separation between two peaks is not achieved. *Figure 2* represents a partial separation of two substances, where H_p is the height above the extrapolated baseline of the minor peak and H_v is the height above the extrapolated baseline at the lowest point of the curve separating the minor and major peaks:

$$p/v = H_p/H_v$$

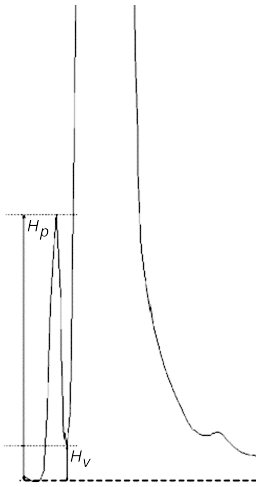


Figure 2. Peak-to-valley ratio determination.

Relative retardation (R_{ret}): The ratio of the distance traveled by the analyte to the distance simultaneously traveled by a reference compound (see *Figure 3*) and is used in planar chromatography.

$$R_{ret} = b/c$$

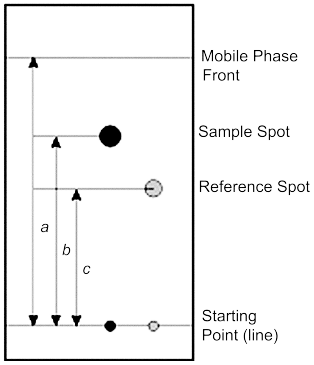


Figure 3. Typical planar chromatography.

Relative retention (r):[1] The ratio of the adjusted retention time of a component relative to that of another used as a reference, obtained under identical conditions:

$$r = (t_{R2} - t_M)/(t_{R1} - t_M)$$

where t_{R2} is the retention time measured from the point of injection of the compound of interest; t_{R1} is the retention time measured from the point of injection of the compound used as reference; and t_M is the retention time of a nonretained marker defined in the procedure, all determined under identical experimental conditions on the same column.

Relative retention time (RRT): Also known as the "unadjusted relative retention". Comparisons in *USP–NF* are normally made in terms of unadjusted relative retention, unless otherwise indicated.

$$\text{RRT} = t_{R2}/t_{R1}$$

The symbol r_G is also used to designate unadjusted relative retention values.

Relative standard deviation in percentage (%RSD):

$$\%\text{RSD} = \frac{100}{\bar{x}}\left(\frac{\sum_{i=1}^{N}\left(x_i - \bar{x}\right)^2}{N-1}\right)^{1/2}$$

Resolution (R_S): The resolution is the separation of two components in a mixture, calculated by:

$$R_S = 2 \times (t_{R2} - t_{R1})/(W_1 + W_2)$$

where t_{R2} and t_{R1} are the retention times of the two components; and W_2 and W_1 are the corresponding widths at the bases of the peaks obtained by extrapolating the relatively straight sides of the peaks to the baseline.

Where electronic integrators are used, it may be convenient to determine the resolution, by the equation:

$$R_S = 1.18 \times (t_{R2} - t_{R1})/(W_{1,h/2} + W_{2,h/2})$$

Retardation factor (R_F): The ratio of the distance traveled by the center of the spot to the distance simultaneously traveled by the mobile phase and is used in planar chromatography. Using the symbols in *Figure 3*:

$$R_F = b/a$$

Retention factor (k):[1] Also known as the "capacity factor (k')". Defined as:

$$k = \frac{\text{amount of substance in stationary phase}}{\text{amount of substance in mobile phase}}$$

or

$$k = \frac{\text{time spent by substance in stationary phase}}{\text{time spent by substance in mobile phase}}$$

The k of a component may be determined from the chromatogram:

$$k = (t_R - t_M)/t_M$$

Retention time (t_R): In LC and GC, the retention time, t_R, is defined as the time elapsed between the injection of the sample and the appearance of the maximum peak response of the eluted sample zone. t_R may be used as a parameter for identification. Chromatographic retention times are characteristic of the compounds they represent but are not unique. The coincidence of retention times of a sample and a reference substance can be used as a partial criterion in construction of an identity profile, but may not be sufficient on its own to establish identity. Absolute retention times of a given compound may vary from one chromatogram to the next.

Retention volume (V_R): The volume of mobile phase required for elution of a component. It may be calculated from the retention time and the flow rate in mL/min:

$$V_R = t_R \times F$$

Separation factor (α): The relative retention calculated for two adjacent peaks (by convention, the value of the separation factor is always >1):

$$\alpha = k_2/k_1$$

Symmetry factor (A_S):[2] Also known as the "tailing factor", of a peak (see *Figure 4*) is calculated by:

$$A_S = W_{0.05}/2f$$

where $W_{0.05}$ is the width of the peak at 5% height and f is the distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from the baseline.

[2] It is also a common practice to measure the "Asymmetry factor" as the ratio of the distance between the vertical line connecting the peak apex with the interpolated baseline and the peak front, and the distance between that line and the peak back measured at 10% of the peak height (see *Figure 4*), which would be $(W_{0.10} - f_{0.10})/f_{0.10}$. However, for the purposes of *USP*, only the formula (A_S) as presented here is valid.

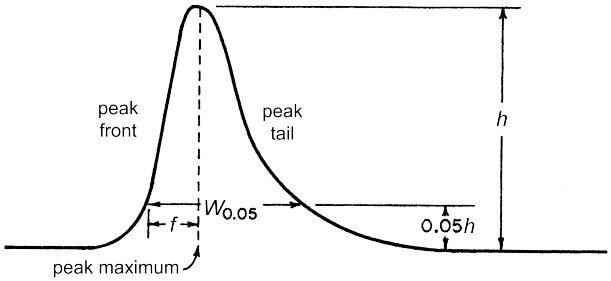


Figure 4. Asymmetrical chromatographic peak.

Tailing factor (*T*): See *Symmetry factor*.

SYSTEM SUITABILITY

System suitability tests are an integral part of GC and LC methods. These tests are used to verify that the chromatographic system is adequate for the intended analysis.

The tests are based on the concept that the equipment, electronics, analytical operations, and samples analyzed constitute an integral system that can be evaluated as such.

Factors that may affect chromatographic behavior include the following:

- Composition, ionic strength, temperature, and apparent pH of the mobile phase
- Flow rate, column dimensions, column temperature, and pressure
- Stationary phase characteristics, including type of chromatographic support (particle-based or monolithic), particle or macropore size, porosity, and specific surface area
- Reverse-phase and other surface modification of the stationary phases, the extent of chemical modification (as expressed by end-capping, carbon loading, and others)

R_S is a function of the number of theoretical plates, *N* (also referred to as efficiency), α, and *k*. [NOTE—All terms and symbols are defined in *Definitions and Interpretation of Chromatograms*.] For a given stationary phase and mobile phase, *N* may be specified to ensure that closely eluting compounds are resolved from each other, to establish the general resolving power of the system, and to ensure that internal standards are resolved from the drug. This is a less reliable means to ensure resolution, as opposed to direct measurement. Column efficiency is, in part, a reflection of peak sharpness, which is important for the detection of trace components.

Replicate injections of a standard preparation or other standard solutions are compared to ascertain whether requirements for precision are met. Unless otherwise specified in the individual monograph, data from five replicate injections of the analyte are used to calculate the relative standard deviation (RSD), if the requirement is ≤2.0%; data from six replicate injections are used if the RSD requirement is >2.0%.

For the assay in a drug substance monograph, where the value is 100% for the pure substance, and no maximum RSD is stated, the maximum permitted %RSD is calculated for a series of injections of the reference solution:

$$\%\text{RSD} = KB\sqrt{n}/t_{90\%,n-1}$$

where *K* is a constant (0.349), obtained from the expression $K = (0.6/\sqrt{2}) \times (t_{90\%,5}/\sqrt{6})$, in which $0.6/\sqrt{2}$ represents the required %RSD after six injections for $B = 1.0$; *B* is the upper limit given in the definition of the individual monograph – 100%; *n* is the number of replicate injections of the reference solution ($3 \le n \le 6$); and $t_{90\%,n-1}$ is the Student's *t* at the 90% probability level (double sided) with $n-1$ degrees of freedom.

Unless otherwise prescribed, the maximum permitted RSD does not exceed the appropriate value given in *Table 1* of repeatability requirements. This requirement does not apply to tests for related substances.

Table 1. RSD Requirements

	Number of Individual Injections			
	3	4	5	6
B (%)	**Maximum Permitted RSD**			
2.0	0.41	0.59	0.73	0.85
2.5	0.52	0.74	0.92	1.06
3.0	0.62	0.89	1.10	1.27

A_S, a measure of peak symmetry, is unity for perfectly symmetrical peaks; its value increases as tailing becomes more pronounced (see *Figure 4*). In some cases, values less than unity may be observed. As peak symmetry moves away from values of 1, integration, and hence precision, become less reliable.

The signal-to-noise (S/N) ratio is a useful system suitability parameter. The S/N ratio is calculated as follows:

$$S/N \text{ ratio} = 2H/h$$

where H is the height of the peak measured from the peak apex to a baseline extrapolated over a distance ≥5 times the peak width at its half-height; and h is the difference between the largest and smallest noise values observed over a distance ≥5 times the width at the half-height of the peak and, if possible, situated equally around the peak of interest (see *Figure 5*).

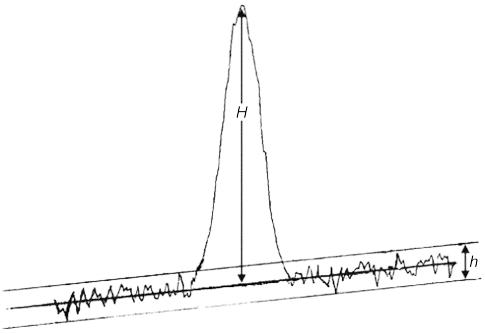


Figure 5. Noise and chromatographic peak, components of the S/N ratio.

These system suitability tests are performed by collecting data from replicate injections of standard or other solutions as specified in the individual monograph.

The specification of definitive parameters in a monograph does not preclude the use of other suitable operating conditions.

Adjustments to the specified chromatographic system may be necessary in order to meet system suitability requirements. Adjustments to chromatographic systems performed in order to comply with system suitability requirements are not to be made in order to compensate for column failure or system malfunction. Adjustments are permitted only when suitable standards (including Reference Standards) are available for all compounds used in the suitability test, and the adjustments or column change yields a chromatogram that meets all the system suitability requirements specified in the official procedure.

If adjustments of operating conditions are necessary in order to meet system suitability requirements, each of the items in the following list is the maximum variation that can be considered, unless otherwise directed in the monograph; these changes may require additional verification data. To verify the suitability of the method under the new conditions, assess the relevant analytical performance characteristics potentially affected by the change. Multiple adjustments can have a cumulative effect on the performance of the system and are to be considered carefully before implementation. In some circumstances, it may be desirable to use an HPLC column with different dimensions to those prescribed in the official procedure (different length, internal diameter, and/or particle size). In either case, changes in the chemical characteristics ("L" designation) of the stationary phase will be considered a modification to the method and will require full validation. Adjustments to the composition of the mobile phase in gradient elution may cause changes in selectivity and are not recommended. If adjustments are necessary, a change in column packing (maintaining the same chemistry), the duration of an initial isocratic hold (when prescribed), and/or the dwell volume are allowed. Additional allowances for gradient adjustment are noted in the text below.

pH of mobile phase (HPLC): The pH of the aqueous buffer used in the preparation of the mobile phase can be adjusted to within ±0.2 units of the value or range specified. Applies to both gradient and isocratic separations.

Concentration of salts in buffer (HPLC): The concentration of the salts used in the preparation of the aqueous buffer employed in the mobile phase can be adjusted to within ±10% if the permitted pH variation (see above) is met. Applies to both gradient and isocratic separations.

Ratio of components in mobile phase (HPLC): The following adjustment limits apply to minor components of the mobile phase (specified as ≤50%). The amounts of these components can be adjusted by ±30% relative. However, the change in any component cannot exceed ±10% absolute (i.e., in relation to the total mobile phase). Adjustment can be made to one minor component in a ternary mixture. Examples of adjustments for binary and ternary mixtures are given below.

Binary mixtures

SPECIFIED RATIO OF 50:50: 30% of 50 is 15% absolute, but this exceeds the maximum permitted change of ±10% absolute in either component. Therefore, the mobile phase ratio may be adjusted only within the range of 40:60–60:40.

SPECIFIED RATIO OF 2:98: 30% of 2 is 0.6% absolute. Therefore, the maximum allowed adjustment is within the range of 1.4:98.6–2.6:97.4.

Ternary mixtures

SPECIFIED RATIO OF 60:35:5: For the second component, 30% of 35 is 10.5% absolute, which exceeds the maximum permitted change of ±10% absolute in any component. Therefore, the second component may be adjusted only within the range of 25%–45% absolute. For the third component, 30% of 5 is 1.5% absolute. In all cases, a sufficient quantity of the first component is used to give a total of 100%. Therefore, mixture ranges of 50:45:5–70:25:5 or 58.5:35:6.5–61.5:35:3.5 would meet the requirement.

Wavelength of UV-visible detector (HPLC): Deviations from the wavelengths specified in the procedure are not permitted. The procedure specified by the detector manufacturer, or another validated procedure, is used to verify that error in the detector wavelength is, at most, ±3 nm.

Stationary phase

COLUMN LENGTH (GC): Can be adjusted by as much as ±70%.

COLUMN LENGTH (HPLC): See *Particle size (HPLC)* below.

COLUMN INNER DIAMETER (HPLC): Can be adjusted if the linear velocity is kept constant. See *Flow rate (HPLC)*.

COLUMN INNER DIAMETER (GC): Can be adjusted by as much as ±50%.

FILM THICKNESS (CAPILLARY GC): Can be adjusted by as much as –50% to 100%.

Particle size (HPLC): For isocratic separations, the particle size and/or the length of the column may be modified provided that the ratio of the column length (*L*) to the particle size (*dp*) remains constant or into the range between –25% and 50% of the prescribed *L*/*dp* ratio. Alternatively (as for the application of particle-size adjustment to superficially porous particles), other combinations of *L* and *dp* can be used provided that the number of theoretical plates (*N*) is within –25% to 50%, relative to the prescribed column. Caution should be used when the adjustment results in a higher number of theoretical plates that generate smaller peak volumes, which may require adjustments to minimize extra-column band broadening by factors such as instrument plumbing, detector cell volume and sampling rate, and injection volume. For gradient separations, changes in length, column inner diameter, and particle size are not allowed.

Particle size (GC): Changing from a larger to a smaller or from a smaller to a larger particle size GC mesh support is acceptable if the chromatography meets the requirements of system suitability and the same particle size range ratio is maintained. The particle size range ratio is defined as the diameter of the largest particle divided by the diameter of the smallest particle.

Flow rate (GC): The flow rate can be adjusted by as much as ±50%. [NOTE—When the monograph specifies a linear velocity parameter, the allowed velocity adjustment is between +50% and –25%, provided the carrier gas system can be maintained under control at the desired set points.]

Flow rate (HPLC): When the particle size is changed, the flow rate may require adjustment, because smaller-particle columns will require higher linear velocities for the same performance (as measured by reduced plate height). Flow rate changes for both a change in column diameter and particle size can be made by:

$$F_2 = F_1 \times [(dc_2^2 \times dp_1)/(dc_1^2 \times dp_2)]$$

where F_1 and F_2 are the flow rates for the original and modified conditions, respectively, dc_1 and dc_2 are the respective column diameters, and dp_1 and dp_2 are the particle sizes.

When a change is made from ≥3-µm to <3-µm particles in isocratic separations, an additional increase in linear velocity (by adjusting flow rate) may be justified, provided that the column efficiency does not drop by >20%. Similarly, a change from <3-µm to ≥3-µm particles may require additional reduction of linear velocity (flow rate) to avoid reduction in column efficiency by >20%. Changes in *F*, *dc*, and *dp* are not allowed for gradient separations.

Additionally, the flow rate can be adjusted by ±50% (isocratic only).

EXAMPLES: Adjustments in column length, internal diameter, particle size, and flow rate can be used in combination to give equivalent conditions (same *N*), but with differences in pressure and run time. *Table 2* lists some of the more popular column configurations to give equivalent efficiency (*N*), by adjusting these variables.

Table 2. Column Configurations

Length (*L*, mm)	Column Diameter (*dc*, mm)	Particle Size (*dp*, µm)	Relative Values				
			L/*dp*	*F*	*N*	Pressure	Run Time
250	4.6	10	25,000	0.5	0.8	0.2	3.3
150	4.6	5	30,000	1.0	1.0	1.0	1.0
150	2.1	5	30,000	0.2	1.0	1.0	1.0
100	4.6	3.5	28,600	1.4	1.0	1.9	0.5
100	2.1	3.5	28,600	0.3	1.0	1.9	0.5
75	4.6	2.5	30,000	2.0	1.0	4.0	0.3
75	2.1	2.5	30,000	0.4	1.0	4.0	0.3
50	4.6	1.7	29,400	2.9	1.0	8.5	0.1
50	2.1	1.7	29,400	0.6	1.0	8.5	0.1

For example, if a monograph specifies a 150-mm × 4.6-mm; 5-µm column operated at 1.5 mL/min, the same separation may be expected with a 75-mm × 2.1-mm; 2.5-µm column operated at 1.5 mL/min × 0.4 = 0.6 mL/min, along with a pressure increase of about four times and a reduction in run time to about 30% of the original.

Injection volume (HPLC): The injection volume can be adjusted as far as it is consistent with accepted precision, linearity, and detection limits. Note that excessive injection volume can lead to unacceptable band broadening, causing a reduction in *N* and resolution, which applies to both gradient and isocratic separations.

Injection volume and split volume (GC): The injection volume and split volume may be adjusted if detection and repeatability are satisfactory.

Column temperature (HPLC): The column temperature can be adjusted by as much as ±10°. Column thermostating is recommended to improve control and reproducibility of retention time, which applies to both gradient and isocratic separations.

Oven temperature (GC): The oven temperature can be adjusted by as much as ±10%.

Oven temperature program (GC): Adjustment of temperatures is permitted as stated above. When the specified temperature must be maintained or when the temperature must be changed from one value to another, an adjustment of up to ±20% is permitted.

Unless otherwise directed in the monograph, system suitability parameters are determined from the analyte peak.

Measured values of R_r, R_F, or t_R for the sample substance do not deviate from the values obtained for the reference compound and mixture by more than the statistically determined reliability estimates from replicate assays of the reference compound. RRT may be provided in monographs for informational purposes only to aid in peak identification. There are no acceptance criteria applied to RRT.

Suitability testing is used to ascertain the effectiveness of the final operating system, which should be subjected to this testing. Make injections of the appropriate preparation(s), as required, in order to demonstrate adequate system suitability (as described in the *Chromatographic system* section of the method in a monograph) throughout the run.

The preparation can be a standard preparation or a solution containing a known amount of analyte and any additional materials (e.g., excipients or impurities) useful in controlling the analytical system. Whenever there is a significant change in the chromatographic system (equipment, mobile phase component, or other components) or in a critical reagent, system suitability is to be reestablished. No sample analysis is acceptable unless the suitability of the system has been demonstrated.

QUANTITATION

During quantitation, disregard peaks caused by solvents and reagents or arising from the mobile phase or the sample matrix.

In the linear range, peak areas and peak heights are usually proportional to the quantity of compound eluting. The peak areas and peak heights are commonly measured by electronic integrators but may be determined by more classical approaches. Peak areas are generally used but may be less accurate if peak interference occurs. The components measured are separated from any interfering components. Peak tailing and fronting is minimized, and the measurement of peaks on tails of other peaks are avoided when possible.

Although comparison of impurity peaks with those in the chromatogram of a standard at a similar concentration is preferred, impurity tests may be based on the measurement of the peak response due to impurities and expressed as a percentage of the area of the drug peak. The standard may be the drug itself at a level corresponding to, for example, 0.5% impurity, assuming similar peak responses. When impurities must be determined with greater certainty, use a standard of the impurity itself or apply a correction factor based on the response of the impurity relative to that of the main component.

External Standard Method

The concentration of the component(s) quantified is determined by comparing the response(s) obtained with the sample solution to the response(s) obtained with a standard solution.

Internal Standard Method

Equal amounts of the internal standard are introduced into the sample solution and a standard solution. The internal standard is chosen so that it does not react with the test material and does not contain impurities with the same retention time as that of the analytes, and is stable and resolved from the component(s) quantified (analytes). The concentrations of the analytes are determined by comparing the ratios of their peak areas or peak heights and the internal standard in the sample solution with the ratios of their peak areas or peak heights and the internal standard in the standard solution.

Normalization Procedure

The percent content of a component of the test material is calculated by determining the area of the corresponding peak as a percentage of the total area of all the peaks, excluding those due to solvents or reagents or arising from the mobile phase or the sample matrix and those at or below the limit at which they can be disregarded.

Calibration Procedure

The relationship between the measured or evaluated signal *y* and the quantity (e.g., concentration or mass) of substance *x* is determined, and the calibration function is calculated. The analytical results are calculated from the measured signal or evaluated signal of the analyte and its position on the calibration curve.

In tests for impurities for both the external standard method, when a dilution of the sample solution is used for comparison, and the normalization procedure, any correction factors indicated in the monograph are applied (e.g., when the relative response factor is outside the range of 0.8–1.2).

When the impurity test prescribes the total of impurities or there is a quantitative determination of an impurity, choice of an appropriate threshold setting and appropriate conditions for the integration of the peak areas is important. In such tests the limit at or below which a peak is disregarded is generally 0.05%. Thus, the threshold setting of the data collection system corresponds to at least half of this limit. Integrate the peak area of any impurity that is not completely separated from the principal peak, preferably by valley-to-valley extrapolation (tangential skim).

Add the following:

▲⟨630⟩ VISUAL COMPARISON

The purpose of this test is to provide the details for the visual comparison of the color and/or turbidance of sample solutions of certain concentration to a standard solution or a series of standard solutions of known concentration. Where a color or turbidity comparison is directed, follow the procedures and conditions outlined below for performing these tests.

Comparison vessels: Color-comparison tubes matched as closely as possible in internal diameter, in depth of sample solution, and in all other respects should be used.

Viewing conditions for turbidity comparison: Tubes should be viewed horizontally against a dark background with the aid of a light source directed from the sides of the tubes.

Viewing conditions for color comparison: Tubes should be viewed downward against a white background. Most of the time, common room lighting is sufficient to perform the assessment. A light source directed from beneath the bottoms of the tubes may be used if needed and if the practice is consistent between the materials under comparison.▲ (USP 1-May-2019)

⟨643⟩ TOTAL ORGANIC CARBON

Total organic carbon (TOC) is an indirect measure of organic molecules present in pharmaceutical waters measured as carbon. Organic molecules are introduced into the water from the source water, from purification and distribution system materials, from biofilm growing in the system, and from the packaging of sterile and nonsterile waters. TOC can also be used as a process control attribute to monitor the performance of unit operations comprising the purification and distribution system. A TOC measurement is not a replacement test for endotoxin or microbiological control. Although there can be a qualitative relationship between a food source (TOC) and microbiological activity, there is no direct numerical correlation.

A number of acceptable methods exist for analyzing TOC. This chapter does not endorse, limit, or prevent any technologies from being used, but this chapter provides guidance on how to qualify these analytical technologies for use as well as guidance on how to interpret instrument results for use as a limit test.

Apparatuses commonly used to determine TOC in water for pharmaceutical use have in common the objective of oxidizing the organic molecules in the water to produce carbon dioxide followed by the measurement of the amount of carbon dioxide produced. Then the amount of CO_2 produced is determined and used to calculate the organic carbon concentration in the water.

All technologies must discriminate between the inorganic carbon, which may be present in the water from sources such as dissolved CO_2 and bicarbonate, and the CO_2 generated from the oxidation of organic molecules in the sample. The discrimination may be accomplished either by determining the inorganic carbon and subtracting it from the total carbon (total carbon is the sum of organic carbon and inorganic carbon), or by purging inorganic carbon from the sample before oxidation. Although purging may entrain organic molecules, such purgeable organic carbon is present in negligible quantities in water for pharmaceutical use.

PROCEDURES

• BULK WATER

The following sections apply to tests for bulk *Purified Water*, *Water for Injection*, *Water for Hemodialysis*, and the condensate of *Pure Steam*.

Apparatus requirements: This test method is performed either as an on-line test or as an off-line laboratory test using a calibrated instrument. The suitability of the apparatus must be periodically demonstrated as described below. In addition, it must have a manufacturer's specified limit of detection of 0.05 mg/L (0.05 ppm) or lower of carbon.

When testing water for quality control purposes, ensure that the instrument and its data are under appropriate control and that the sampling approaches and locations of both on-line and off-line measurements are representative of the quality of the water used. The nature of the water production, distribution, and use should be considered when selecting either on-line or off-line measurement.

Reagent water: Use water having a TOC level of not more than 0.10 mg/L. [NOTE—A conductivity requirement may be necessary in order to ensure method reliability.]

Container preparation: Organic contamination of containers results in higher TOC values. Therefore, use labware and containers that have been scrupulously cleaned of organic residues. Any method that is effective in removing organic matter can be used (see *Cleaning Glass Apparatus* ⟨1051⟩). Use *Reagent water* for the final rinse.

Standard solution: Unless otherwise directed in the individual monograph, dissolve in the *Reagent water* an accurately weighed quantity of USP Sucrose RS to obtain a solution having a concentration of 1.19 mg/L of sucrose (0.50 mg/L of carbon).

System suitability solution: Dissolve in *Reagent water* an accurately weighed quantity of USP 1,4-Benzoquinone RS to obtain a solution having a concentration of 0.75 mg/L (0.50 mg/L of carbon).

Reagent water control: Use a suitable quantity of *Reagent water* obtained at the same time as that used in the preparation of the *Standard Solution* and the *System suitability solution*.

Water sample: Obtain an on-line or off-line sample that suitably reflects the quality of water used.

Other control solutions: Prepare appropriate reagent blank solutions or other specified solutions needed for establishing the apparatus baseline or for calibration adjustments following the manufacturer's instructions, and run the appropriate blanks to zero the instrument, if necessary.

System suitability: Test the *Reagent water control* in the apparatus, and record the response, r_W. Repeat the test using the *Standard solution*, and record the response, r_S. Calculate the corrected *Standard solution* response, which is also the limit response, by subtracting the *Reagent water control* response from the response of the *Standard solution*. The theoretical limit of 0.50 mg/L of carbon is equal to the corrected *Standard solution* response, $r_S - r_W$. Test the *System suitability solution* in the apparatus, and record the response, r_{SS}. Calculate the corrected *System suitability solution* response by subtracting the *Reagent water control* response from the response of the *System suitability solution*, $r_{SS} - r_W$. Calculate the percent response efficiency for the *System suitability solution*:

$$\% \text{ response efficiency} = 100[(r_{SS} - r_W)/(r_S - r_W)]$$

r_{SS} = instrument response to the *System suitability solution*
r_W = instrument response to the *Reagent water control*
r_S = instrument response to the *Standard solution*

The system is suitable if the percent response efficiency is not less than 85% and not more than 115%.

Procedure: Perform the test on the *Water Sample*, and record the response, r_U. The *Water Sample* meets the requirements if r_U is not more than the limit response, $r_S - r_W$. This method can be performed using on-line or off-line instrumentation that meets the *Apparatus requirements*.

• **STERILE WATER**

The following sections apply to tests for *Sterile Water for Injection*, *Sterile Purified Water*, *Sterile Water for Irrigation*, and *Sterile Water for Inhalation*.

Follow the requirements in *Bulk Water*, with the following exceptions.

Apparatus requirements: In addition to the *Apparatus requirements* in *Bulk Water*, the apparatus must have a manufacturer's specified limit of detection of 0.10 mg/L (0.10 ppm) or lower of carbon.

Reagent water: Use water having a TOC level of not more than 0.50 mg/L. [NOTE—A conductivity requirement may be necessary in order to ensure method reliability.]

Standard solution: Unless otherwise directed in the individual monograph, dissolve in the *Reagent water* an accurately weighed quantity of USP Sucrose RS to obtain a solution having a concentration of 19.0 mg/L of sucrose (8.0 mg/L of carbon).

System suitability solution: Dissolve in *Reagent water* an accurately weighed quantity of USP 1,4-Benzoquinone RS to obtain a solution having a concentration of 12.0 mg/L (8.0 mg/L of carbon).

Water sample: Obtain a sample that suitably reflects the quality of water used. Before opening, vigorously agitate the package to homogenize the water sample. Several packages may be required in order to collect sufficient water for analysis.

System suitability: Test the *Reagent water control* in the apparatus, and record the response, r_W. Repeat the test using the *Standard solution*, and record the response, r_S. Calculate the corrected *Standard solution* response, which is also the limit response, by subtracting the *Reagent water control* response from the response of the *Standard solution*. The theoretical limit of 8.0 mg/L of carbon is equal to the corrected *Standard solution* response, $r_S - r_W$. Test the *System suitability solution* in the apparatus, and record the response, r_{SS}. Calculate the corrected *System suitability solution* response by subtracting the *Reagent water control* response from the response of the *System suitability solution*, $r_{SS} - r_W$. Calculate the percent response efficiency for the *System suitability solution*:

$$\% \text{ response efficiency} = 100[(r_{SS} - r_W)/(r_S - r_W)]$$

r_{SS} = instrument response to the *System suitability solution*
r_W = instrument response to the *Reagent water control*
r_S = instrument response to the *Standard solution*

The system is suitable if the percent response efficiency is not less than 85% and not more than 115%.

Procedure: Perform the test on the *Water Sample*, and record the response, r_U. The *Water sample* meets the requirements if r_U is not more than the limit response, $r_S - r_W$, determined in the *System suitability* requirements in *Sterile Water*.

ADDITIONAL REQUIREMENTS

• **USP REFERENCE STANDARDS** ⟨11⟩

USP 1,4-Benzoquinone RS
USP Sucrose RS

⟨645⟩ WATER CONDUCTIVITY

INTRODUCTION

Electrical conductivity in water is a measure of the ion-facilitated electron flow through it. Water molecules dissociate into ions as a function of pH and temperature and result in a very predictable conductivity. Some gases, most notably carbon dioxide, readily dissolve in water and interact to form ions, which predictably affect conductivity also. For the purpose of this discussion, these ions and their resulting conductivity can be considered intrinsic to the water.

Water conductivity is also affected by the presence of extraneous ions. The extraneous ions used in modeling the conductivity specifications described below are the chloride and ammonia ions. The conductivity of the ubiquitous chloride ion (at the theoretical endpoint concentration of 0.47 ppm when chloride was a required attribute test in *USP 22* and earlier revisions) and the ammonium ion (at the limit of 0.3 ppm) represents a major portion of the allowed water ionic impurity level. A balancing quantity of anions (such as chloride, to counter the ammonium ion) and cations (such as sodium, to counter the chloride ion) is included in this allowed impurity level to maintain electroneutrality. Extraneous ions such as these may have a significant effect on the water's chemical purity and suitability for use in pharmaceutical applications.

The procedure in the section *Bulk Water* is specified for measuring the conductivity of waters such as *Purified Water*, *Water for Injection*, *Water for Hemodialysis*, and the condensate of *Pure Steam*. The procedure in the section *Sterile Water* is specified for measuring the conductivity of waters such as *Sterile Purified Water*, *Sterile Water for Injection*, *Sterile Water for Inhalation*, and *Sterile Water for Irrigation*.

The procedures below shall be performed using instrumentation that has been calibrated, has conductivity sensor cell constants that have been accurately determined, and has a temperature compensation function that has been disabled for *Bulk Water* Stage 1 testing. For both online and offline measurements, the suitability of instrumentation for quality control testing is also dependent on the sampling location(s) in the water system. The selected sampling instrument location(s) must reflect the quality of the water used.

INSTRUMENT SPECIFICATIONS AND OPERATING PARAMETERS

Water conductivity must be measured accurately with calibrated instrumentation. An electrical conductivity measurement consists of the determination of the conductance, G (or its inverse, resistance, R), of the fluid between and around the electrodes. The conductance ($1/R$) is directly affected by the geometrical properties of the electrodes; i.e., the conductance is inversely proportional to the distance (d) between the electrodes and proportional to the area (A) of the electrodes. This geometrical ratio (d/A) is known as the cell constant, Θ. Thus the measured conductance is normalized for the cell constant to determine the conductivity, κ, according to the following equation:

$$\text{conductivity, } \kappa \text{ (S/cm)} = \Theta \text{ (cm}^{-1}\text{)}/R \text{ (}\Omega\text{)}$$

It is the cell constant and the resistance measurement that must be verified and adjusted, if necessary.

Cell Constant

The cell constant must be known within ±2%. The cell constant can be verified directly by using a solution of known or traceable conductivity, or indirectly by comparing the instrument reading taken with the conductivity sensor in question to readings from a conductivity sensor of known or traceable cell constant. If necessary, adjust the cell constant following the manufacturer's instrument protocol. The frequency of verification/calibration is a function of the sensor design.

Resistance Measurement

Calibration (or verification) of the resistance measurement is accomplished by replacing the conductivity sensor electrodes with precision resistors having standards traceable to NIST or equivalent national authorities in other countries (accurate to ±0.1% of the stated value) to give a predicted instrument conductivity response. The accuracy of the resistance measurement is acceptable if the measured conductivity with the traceable resistor is within ±0.1 µS/cm of the calculated value according to the equation above. For example, the traceable resistor is 50 kΩ, and the cell constant, Θ, is 0.10 cm^{-1}. The calculated value is 2.0×10^{-6} S/cm or 2.0 µS/cm. The measured value should be 2.0 ± 0.1 µS/cm. The instrument must have a minimum resolution of 0.1 µS/cm on the lowest range.

The target conductivity value(s) should be based on the type of water to be analyzed, and it should be equal to or less than the water conductivity limit for that type of water. Multiple measuring circuits may be embedded in the meter or the sensor, and each circuit may require separate verification or calibration before use. The frequency of recalibration is a function of instrument system design.

System Verification

The cell constant of the user's sensor can be determined with the user's resistance measurement system, or the cell constant can be determined with an independent resistance measurement system. If the cell constant is determined with an independent resistance measurement system, it is recommended that the user verify that the sensor has been properly connected to the resistance measurement system to ensure proper performance. Verification can be made by comparing the conductivity (or

resistivity) values displayed by the measuring equipment with those of an external calibrated conductivity-measuring device. The two non–temperature-compensated conductivity (or resistivity) values should be equivalent to or within ±5% of each other, or should have a difference that is acceptable on the basis of product water criticality and/or the water conductivity ranges in which the measurements are taken. The two conductivity sensors should be positioned close enough together to measure the same water sample at the same temperature and water quality.

Temperature Compensation and Temperature Measurements

Because temperature has a substantial effect on conductivity readings of specimens at high and low temperatures, many instruments automatically correct the actual reading to display the value that theoretically would be observed at the nominal temperature of 25°. This is typically done using a temperature sensor embedded in the conductivity sensor and a software algorithm embedded in the instrument. This temperature compensation algorithm may not be accurate for the various water types and impurities. For this reason, conductivity values used in the *Stage 1* test for *Bulk Water* are non–temperature-compensated measurements. Other conductivity tests that are specified for measurement at 25° can use either temperature-compensated or non–temperature-compensated measurements.

A temperature measurement is required for the *Stage 1* test or for the other tests at 25°. It may be made using the temperature sensor embedded in the conductivity cell sensor. An external temperature sensor positioned near the conductivity sensor is also acceptable. Accuracy of the temperature measurement must be ±2°.

BULK WATER

The procedure and test limits in this section are intended for *Purified Water*, *Water for Injection*, *Water for Hemodialysis*, the condensate of *Pure Steam*, and any other monographs that specify this section.

This is a three-stage test method to accommodate online or offline testing. Online conductivity testing provides real-time measurements and opportunities for real-time process control, decision, and intervention. Precautions should be taken while collecting water samples for offline conductivity measurements. The sample may be affected by the sampling method, the sampling container, and environmental factors such as ambient carbon dioxide concentration and organic vapors. This procedure can be started at *Stage 2* if offline testing is preferred.

Procedure

STAGE 1

Stage 1 is intended for online measurement or may be performed offline in a suitable container.

1. Determine the temperature of the water and the conductivity of the water with a non–temperature-compensated conductivity reading.

2. Using *Table 1*, find the temperature value that is NMT the measured temperature, i.e., the next lower temperature. The corresponding conductivity value on this table is the limit.

[NOTE—Do not interpolate.]

3. If the measured conductivity is NMT the table value determined in step 2, the water meets the requirements of the test for conductivity. If the conductivity is higher than the table value, proceed with *Stage 2*.

Table 1. Stage 1—Temperature and Conductivity Requirements (for non–temperature-compensated conductivity measurements only)

Temperature	Conductivity Requirement (µS/cm)
0	0.6
5	0.8
10	0.9
15	1.0
20	1.1
25	1.3
30	1.4
35	1.5
40	1.7
45	1.8
50	1.9
55	2.1
60	2.2
65	2.4
70	2.5

Table 1. Stage 1—Temperature and Conductivity Requirements (for non–temperature-compensated conductivity measurements only) *(continued)*

Temperature	Conductivity Requirement (µS/cm)
75	2.7
80	2.7
85	2.7
90	2.7
95	2.9
100	3.1

STAGE 2

4. Transfer a sufficient amount of water to a suitable container, and stir the test specimen. Adjust the temperature, if necessary, and, while maintaining it at 25 ± 1°, begin vigorously agitating the test specimen while periodically observing the conductivity. When the change in conductivity (due to uptake of atmospheric carbon dioxide) is less than a net of 0.1 µS/cm per 5 min, note the conductivity.

[NOTE—Conductivity measurements at this stage may be temperature-compensated to 25° or non–temperature-compensated.]

5. If the conductivity is not greater than 2.1 µS/cm, the water meets the requirements of the test for conductivity. If the conductivity is greater than 2.1 µS/cm, proceed with *Stage 3*.

STAGE 3

6. Perform this test within approximately 5 min of the conductivity determination in step 5, while maintaining the sample temperature at 25 ± 1°. Add a saturated potassium chloride solution to the same water sample (0.3 mL per 100 mL of the test specimen), and determine the pH to the nearest 0.1 pH unit, as directed in *pH* ⟨791⟩.

7. Referring to *Table 2*, determine the conductivity limit at the measured pH value. If the measured conductivity in step 4 is NMT the table value determined in step 6, the water meets the requirements of the test for conductivity. If either the measured conductivity is greater than this value or the pH is outside the range of 5.0–7.0, the water does not meet the requirements of the test for conductivity.

Table 2. Stage 3—pH and Conductivity Requirements (for atmosphere- and temperature-equilibrated samples only)

pH	Conductivity Requirement (µS/cm)
5.0	4.7
5.1	4.1
5.2	3.6
5.3	3.3
5.4	3.0
5.5	2.8
5.6	2.6
5.7	2.5
5.8	2.4
5.9	2.4
6.0	2.4
6.1	2.4
6.2	2.5
6.3	2.4
6.4	2.3
6.5	2.2
6.6	2.1
6.7	2.6
6.8	3.1
6.9	3.8
7.0	4.6

STERILE WATER

The procedure and test limits are intended for *Sterile Purified Water*, *Sterile Water for Injection*, *Sterile Water for Inhalation*, and *Sterile Water for Irrigation*, and any other monographs that specify this section. The sterile waters are derived from *Purified Water* or *Water for Injection*, and therefore have been determined to be compliant with the *Bulk Water* requirements before being stored in the container. The specification provided represents the maximum allowable conductivity value, taking into consideration the limitation of the measurement method and reasonable container leaching. Such specification and the sampling volume choices should be defined and validated on the basis of the intended purpose of the water.

Procedure

Obtain a sample that suitably reflects the quality of water used. Before opening, vigorously agitate the package to homogenize the water sample. Several packages may be required to collect sufficient water for analysis.

Transfer a sufficient amount of water to a suitable container, and stir the test specimen. Adjust the temperature, if necessary, and, while maintaining it at 25 ± 1°, begin vigorously agitating the test specimen while periodically observing the conductivity. When the change in conductivity (due to uptake of ambient carbon dioxide) is less than a net of 0.1 µS/cm per 5 min, note the conductivity.

For containers with a nominal volume of 10 mL or less, if the conductivity is NMT 25 µS/cm, the water meets the requirements. For containers with a nominal volume greater than 10 mL, if the conductivity is NMT 5 µS/cm, the water meets the requirements.

⟨831⟩ REFRACTIVE INDEX

The refractive index (n) of a substance is the ratio of the velocity of light in air to the velocity of light in the substance. It is valuable in the identification of substances and the detection of impurities.

Although the standard temperature for Pharmacopeial measurements is 25°, many of the refractive index specifications in the individual monographs call for determining this value at 20°. The temperature should be carefully adjusted and maintained, since the refractive index varies significantly with temperature.

The values for refractive index given in this Pharmacopeia are for the D line of sodium (doublet at 589.0 nm and 589.6 nm). Most instruments available are designed for use with white light but are calibrated to give the refractive index in terms of the D line of sodium light.

The Abbé refractometer measures the range of refractive index for those Pharmacopeial materials for which such values are given. Other refractometers of equal or greater accuracy may be employed.

To achieve the theoretical accuracy of ±0.0001, it is necessary to calibrate the instrument against a standard provided by the manufacturer and to check frequently the temperature control and cleanliness of the instrument by determining the refractive index of distilled water, which is 1.3330 at 20° and 1.3325 at 25°.

⟨841⟩ SPECIFIC GRAVITY

Change to read:

▲Specific gravity is the term for the relative density of a substance when water is used as the reference. The measurement of specific gravity necessarily involves the measurement of the density of the sample at a specified temperature as well as the measurement of the density of water.

The density ρ (g/mL or g/cm^3) means the mass per unit volume, and the relative density means the ratio of the mass of a sample specimen to that of an equal volume of a standard substance. The relative density and specific gravity are unitless values.

The specific gravity ($a_t^{t'}$) means the ratio of the mass of the sample specimen at t' to that of an equal volume of water (H_2O) at t.▲ (USP 1-Aug-2020)Unless otherwise stated in the individual monograph, the specific gravity determination is applicable only to liquids and ▲▲ (USP 1-Aug-2020) is based on the ratio of the weight of a liquid in air at 25° to that of an equal volume of water at the same temperature ▲(i.e., d_{25}^{25}).▲ (USP 1-Aug-2020) Where a temperature is specified in the individual monograph, the specific gravity is the ratio of the weight of the liquid in air at the specified temperature to that of an equal volume of water at the same temperature. ▲If▲ (USP 1-Aug-2020) the substance is a solid at 25°, determine the specific gravity ▲above the melting point▲ (USP 1-Aug-2020) (at the temperature directed in the individual monograph, ▲if specified).▲ (USP 1-Aug-2020)

Unless otherwise stated in the individual monograph, the density is defined as the mass of a unit volume of the substance at 25°, expressed in kilograms per cubic meter or grams per cubic centimeter (1 kg/m^3 = 10^{-3} g/cm^3). Where the density is known, mass can be converted to volume, or volume converted to mass, by ▲using▲ (USP 1-Aug-2020) the formula, volume = mass/density.

▲▲ (USP 1-Aug-2020)

Change to read:

METHOD I

Procedure

Select a ▲ (USP 1-Aug-2020)clean, dry pycnometer that has been previously calibrated by determining its weight and the weight of ▲the deaerated▲ (USP 1-Aug-2020) water contained in it at 25°. Adjust the temperature of the ▲sample▲ (USP 1-Aug-2020) liquid to about 20°, and fill the pycnometer with it. Adjust the temperature of the filled pycnometer to 25°, remove any excess liquid, and weigh. When the monograph specifies a temperature different from 25°, the filled pycnometers must be brought to the temperature of the balance before ▲weighing. The specific gravity ($d_t^{t'}$) can be calculated by use of the following equation:

$$d_t^{t'} = \frac{W_1 - W}{W_2 - W}$$

W_1 = mass of the pycnometer filled with the sample solution at t'
W = mass of the clean, dry pycnometer
W_2 = mass of the pycnometer filled with water at t

The density of water can be used to calculate the density of the sample of the specimen according to the following equation:

$$\rho_{s,t'} = d_t^{t'} \rho_{w,t}$$

$\rho_{s,t'}$ = density of the sample at t'
$\rho_{w,t}$ = density of water at t▲ (USP 1-Aug-2020)

Change to read:

METHOD II

The procedure ▲requires▲ (USP 1-Aug-2020) the use of the oscillating transducer density meter. The apparatus consists of the following:

- A U-shaped tube, usually of borosilicate glass, which contains the liquid to be examined
- A magneto-electrical or piezo-electrical excitation system that causes the tube to oscillate as a cantilever oscillator at a characteristic frequency depending on the density of the liquid to be examined
- A means of measuring the oscillation period (*T*), which may be converted by the apparatus to give a direct reading of density or used to calculate density by using the constants *A* and *B* described below
- A means to measure and/or control the temperature of the oscillating transducer containing the liquid to be tested

The oscillation period is a function of the spring constant (*c*) and the mass of the system:

$$T^2 = \{(M/c) + [(\rho \times V)/c]\} \times 4\pi^2$$

where ρ is the density of the liquid to be tested, *M* is the mass of the tube, and *V* is the volume of the filled tube.

Introduction of two constants $A = c/(4\pi^2 \times V)$ and $B = (M/V)$ leads to the classical equation for the oscillating transducer:

$$\rho = A \times T^2 - B$$

The specific gravity of the liquid is given by the formula:

$$\rho_{(L)}/\rho_{(W)}$$

where $\rho_{(L)}$ and $\rho_{(W)}$ are the densities of the liquid and water, respectively, both determined at 25°, unless otherwise directed in the individual monograph.

▲Samples with high viscosity will dampen the oscillations in the instrument and will introduce an error in the density measurement. Some oscillating transducer density meters are able to provide a correction to the density based on the viscosity of the sample. Samples with viscosities <10 mPa · s can be accurately measured without a density correction. Samples with viscosities >10 mPa · s must be measured with a density correction. If a density correction is not possible for a sample with >10 mPa · s viscosity, use *Method I*.▲ (USP 1-Aug-2020)

Calibration

The constants *A* and *B* are determined by operating the instrument with the U-shaped tube filled with two different samples of known density (e.g., degassed water and air). Perform the control measurements daily, using degassed water. The results displayed for the control measurement using degassed water ▲should▲ (USP 1-Aug-2020) not deviate from the reference value (ρ_{25} = 0.997043 g/cm³) by more than its specified error. Precision is a function of the repeatability and stability of the oscillator

frequency. Density meters are able to achieve measurements with an error on the order of 1×10^{-3} g/cm³ to 1×10^{-5} g/cm³ and a repeatability of 1×10^{-4} g/cm³ to 1×10^{-6} g/cm³. For example, an instrument specified to $\pm 1 \times 10^{-4}$ g/cm³ must display 0.9970 ± 0.0001 g/cm³ in order to be suitable for further measurement, otherwise a readjustment is necessary. Calibration with certified reference materials should be carried out regularly.

Procedure

▲Verify the viscosity of the sample to be analyzed. If the viscosity is >10 mPa · s and the oscillating transducer density meter is unable to provide a density correction based on the viscosity, use *Method I*.▲ (USP 1-Aug-2020)

Using the manufacturer's instructions, perform the measurements using the same procedure as for *Calibration*. If necessary, equilibrate the liquid▲ ▲ (USP 1-Aug-2020) at 25° before introduction into the tube to avoid the formation of bubbles and to reduce the time required for measurement. Factors affecting accuracy include the following:

- Temperature uniformity throughout the tube
- Nonlinearity over a range of density
- Parasitic resonant effects
- Viscosity, if the oscillating transducer density meters used do not provide automatic compensation of sample viscosity influence

▲The oscillating transducer density meter provides the reportable value of the sample density at the selected temperature. The specific gravity of the sample is calculated from this measured density according to the following equation:

$$d_t^{t'} = \frac{\rho_{s,t'}}{\rho_{w,t}}$$

$\rho_{s,t'}$ = density of the sample at t'
$\rho_{w,t}$ = density of water at t

[NOTE—The density of water used in the calculation of the specific gravity should be the accepted value of the density of water at t (e.g., at 25° use 0.9970 g/cm³) rather than the value reported by the instrument.▲ (USP 1-Aug-2020)]

⟨921⟩ WATER DETERMINATION

Many Pharmacopeial articles either are hydrates or contain water in adsorbed form. As a result, the determination of the water content is important in demonstrating compliance with the Pharmacopeial standards. Generally one of the methods given below is called for in the individual monograph, depending upon the nature of the article. In rare cases, a choice is allowed between two methods. When the article contains water of hydration, *Method I (Titrimetric)*, *Method II (Azeotropic)*, or *Method III (Gravimetric)* is employed, as directed in the individual monograph, and the requirement is given under the heading *Water.*

The heading *Loss on Drying* (see *Loss on Drying* ⟨731⟩) is used in those cases where the loss sustained on heating may be not entirely water.

METHOD I (TITRIMETRIC)

Determine the water by *Method Ia,* unless otherwise specified in the individual monograph.

Method Ia (Direct Titration)

PRINCIPLE

The titrimetric determination of water is based upon the quantitative reaction of water with an anhydrous solution of sulfur dioxide and iodine in the presence of a buffer that reacts with hydrogen ions.

In the original titrimetric solution, known as Karl Fischer Reagent, the sulfur dioxide and iodine are dissolved in pyridine and methanol. The test specimen may be titrated with the *Reagent* directly, or the analysis may be carried out by a residual titration procedure. The stoichiometry of the reaction is not exact, and the reproducibility of a determination depends upon such factors as the relative concentrations of the *Reagent* ingredients, the nature of the inert solvent used to dissolve the test specimen, and the technique used in the particular determination. Therefore, an empirically standardized technique is used in order to achieve the desired accuracy. Precision in the method is governed largely by the extent to which atmospheric moisture is excluded from the system. The titration of water is usually carried out with the use of anhydrous methanol as the solvent for the test specimen. In some cases, other suitable solvents may be used for special or unusual test specimens. In these cases, the addition of at least 20% of methanol or other primary alcohol is recommended.

APPARATUS

Any apparatus may be used that provides for adequate exclusion of atmospheric moisture and determination of the endpoint. In the case of a colorless solution that is titrated directly, the endpoint may be observed visually as a change in color from canary

yellow to amber. The reverse is observed in the case of a test specimen that is titrated residually. More commonly, however, the endpoint is determined electrometrically with an apparatus employing a simple electrical circuit that serves to impress about 200 mV of applied potential between a pair of platinum electrodes immersed in the solution to be titrated. At the endpoint of the titration a slight excess of the reagent increases the flow of current to between 50 and 150 microamperes for 30 s to 30 min, depending upon the solution being titrated. The time is shortest for substances that dissolve in the reagent. With some automatic titrators, the abrupt change in current or potential at the endpoint serves to close a solenoid-operated valve that controls the buret delivering the titrant. Commercially available apparatus generally comprises a closed system consisting of one or two automatic burets and a tightly covered titration vessel fitted with the necessary electrodes and a magnetic stirrer. The air in the system is kept dry with a suitable desiccant, and the titration vessel may be purged by means of a stream of dry nitrogen or current of dry air.

REAGENT

Prepare the Karl Fischer Reagent as follows. Add 125 g of iodine to a solution containing 670 mL of methanol and 170 mL of pyridine, and cool. Place 100 mL of pyridine in a 250-mL graduated cylinder, and, keeping the pyridine cold in an ice bath, pass in dry sulfur dioxide until the volume reaches 200 mL. Slowly add this solution, with shaking, to the cooled iodine mixture. Shake to dissolve the iodine, transfer the solution to the apparatus, and allow the solution to stand overnight before standardizing. One mL of this solution when freshly prepared is equivalent to approximately 5 mg of water, but it deteriorates gradually; therefore, standardize it within 1 h before use, or daily if in continuous use. Protect from light while in use. Store any bulk stock of the reagent in a suitably sealed, glass-stoppered container, fully protected from light, and under refrigeration. For determination of trace amounts of water (less than 1%), it is preferable to use a *Reagent* with a water equivalency factor of not more than 2.0, which will lead to the consumption of a more significant volume of titrant.

A commercially available, stabilized solution of Karl Fischer type reagent may be used. Commercially available reagents containing solvents or bases other than pyridine or alcohols other than methanol may be used also. These may be single solutions or reagents formed in situ by combining the components of the reagents present in two discrete solutions. The diluted *Reagent* called for in some monographs should be diluted as directed by the manufacturer. Either methanol or other suitable solvent, such as ethylene glycol monomethyl ether, may be used as the diluent.

TEST PREPARATION

Unless otherwise specified in the individual monograph, use an accurately weighed or measured amount of the specimen under test estimated to contain 2–250 mg of water. The amount of water depends on the water equivalency factor of the *Reagent* and on the method of endpoint determination. In most cases, the minimum amount of specimen, in mg, can be estimated using the formula:

$$FCV/KF$$

in which F is the water equivalency factor of the *Reagent,* in mg per mL; C is the used volume, in percent, of the capacity of the buret; V is the buret volume, in mL; and KF is the limit or reasonable expected water content in the sample, in percent. C is generally between 30% and 100% for manual titration, and between 10% and 100% for the instrumental method endpoint determination.

[NOTE—It is recommended that the product of FCV be greater than or equal to 200 for the calculation to ensure that the minimum amount of water titrated is greater than or equal to 2 mg.]

Where the specimen under test is an aerosol with propellant, store it in a freezer for not less than 2 h, open the container, and test 10.0 mL of the well-mixed specimen. In titrating the specimen, determine the endpoint at a temperature of 10° or higher.

Where the specimen under test is capsules, use a portion of the mixed contents of not fewer than four capsules.

Where the specimen under test is tablets, use powder from not fewer than four tablets ground to a fine powder in an atmosphere of temperature and relative humidity known not to influence the results.

Where the monograph specifies that the specimen under test is hygroscopic, take an accurately weighed portion of the solid into the titration vessel, proceeding as soon as possible and taking care to avoid moisture uptake from the atmosphere. If the sample is constituted by a finite amount of solid as a lyophilized product or a powder inside a vial, use a dry syringe to inject an appropriate volume of methanol, or other suitable solvent, accurately measured, into a tared container, and shake to dissolve the specimen. Using the same syringe, remove the solution from the container and transfer it to a titration vessel prepared as directed for *Procedure.* Repeat the procedure with a second portion of methanol, or other suitable solvent, accurately measured, add this washing to the titration vessel, and immediately titrate. Determine the water content, in mg, of a portion of solvent of the same total volume as that used to dissolve the specimen and to wash the container and syringe, as directed for *Standardization of Water Solution for Residual Titration,* and subtract this value from the water content, in mg, obtained in the titration of the specimen under test. Dry the container and its closure at 100° for 3 h, allow to cool in a desiccator, and weigh. Determine the weight of specimen tested from the difference in weight from the initial weight of the container.

When appropriate, the water may be desorbed or released from the sample by heat in an external oven connected with the vessel, to where it is transferred with the aid of an inert and dried gas such as pure nitrogen. Any drift due to the transport gas should be considered and corrected. Care should be taken in the selection of the heating conditions to avoid the formation of water coming from dehydration due to decomposition of the sample constituents, which may invalidate this approach.

STANDARDIZATION OF THE REAGENT

Place enough methanol or other suitable solvent in the titration vessel to cover the electrodes, and add sufficient *Reagent* to give the characteristic endpoint color, or 100 ± 50 microamperes of direct current at about 200 mV of applied potential.

Purified Water, sodium tartrate dihydrate, a USP Reference Standard, or commercial standards with a certificate of analysis traceable to a national standard may be used to standardize the *Reagent*. The reagent equivalency factor, the recommended titration volume, buret size, and amount of standard to measure are factors to consider when deciding which standard and how much to use.[1] For *Purified Water* or water standards, quickly add the equivalent of between 2 and 250 mg of water. Calculate the water equivalency factor, *F,* in mg of water per mL of reagent:

$$W/V$$

in which *W* is the weight, in mg, of the water contained in the aliquot of standard used; and *V* is the volume, in mL, of the *Reagent* used in the titration. For sodium tartrate dihydrate, quickly add 20–125 mg of sodium tartrate dihydrate ($C_4H_4Na_2O_6 \cdot 2H_2O$), accurately weighed by difference, and titrate to the endpoint. The water equivalence factor *F,* in mg of water per mL of reagent, is given by the formula:

$$W/V\ (36.04/230.08)$$

in which 36.04 is two times the molecular weight of water and 230.08 is the molecular weight of sodium tartrate dihydrate; *W* is the weight, in mg, of sodium tartrate dihydrate; and *V* is the volume, in mL, of the *Reagent* consumed in the second titration. Note that the solubility of sodium tartrate dihydrate in methanol is such that fresh methanol may be needed for additional titrations of the sodium tartrate dihydrate standard.

PROCEDURE

Unless otherwise specified, transfer enough methanol or other suitable solvent to the titration vessel, ensuring that the volume is sufficient to cover the electrodes (approximately 30–40 mL), and titrate with the *Reagent* to the electrometric or visual endpoint to consume any moisture that may be present. (Disregard the volume consumed, because it does not enter into the calculations.) Quickly add the *Test Preparation,* mix, and again titrate with the *Reagent* to the electrometric or visual endpoint. Calculate the water content of the specimen taken, in mg:

$$SF$$

in which *S* is the volume, in mL, of the *Reagent* consumed in the second titration; and *F* is the water equivalence factor of the *Reagent.*

Method Ib (Residual Titration)

PRINCIPLE

See the information given in the section *Principle* under *Method Ia.* In the residual titration, excess *Reagent* is added to the test specimen, sufficient time is allowed for the reaction to reach completion, and the unconsumed *Reagent* is titrated with a standard solution of water in a solvent such as methanol. The residual titration procedure is applicable generally and avoids the difficulties that may be encountered in the direct titration of substances from which the bound water is released slowly.

APPARATUS, REAGENT, AND TEST PREPARATION

Use *Method Ia.*

STANDARDIZATION OF WATER SOLUTION FOR RESIDUAL TITRATION

Prepare a *Water Solution* by diluting 2 mL of water with methanol or other suitable solvent to 1000 mL. Standardize this solution by titrating 25.0 mL with the *Reagent,* previously standardized as directed under *Standardization of the Reagent.* Calculate the water content, in mg per mL, of the *Water Solution* taken:

$$V'F/25$$

in which *V'* is the volume of the *Reagent* consumed, and *F* is the water equivalence factor of the *Reagent.* Determine the water content of the *Water Solution* weekly, and standardize the *Reagent* against it periodically as needed.

PROCEDURE

Where the individual monograph specifies that the water content is to be determined by *Method Ib,* transfer enough methanol or other suitable solvent to the titration vessel, ensuring that the volume is sufficient to cover the electrodes (approximately 30–40 mL), and titrate with the *Reagent* to the electrometric or visual endpoint. Quickly add the *Test Preparation,* mix, and add an accurately measured excess of the *Reagent.* Allow sufficient time for the reaction to reach completion, and titrate the unconsumed *Reagent* with standardized *Water Solution* to the electrometric or visual endpoint. Calculate the water content of the specimen, in mg, taken:

[1] Consider a setup in which the reagent equivalency factor is 5 mg/mL, and the buret volume is 5 mL and an instrumental endpoint. Standard amounts equivalent to between 2.5 mg and 22.5 mg of water (10%–90% of buret capacity) could be used based on the buret and the reagent equivalency factor. The upper end of this range would involve an excessive amount of sodium tartrate dihydrate. If *Purified Water* or a standard is weighed, an analytical balance appropriate to the amount weighed is required.

$$F(X' - XR)$$

in which *F* is the water equivalence factor of the *Reagent*; *X′* is the volume, in mL, of the *Reagent* added after introduction of the specimen; *X* is the volume, in mL, of standardized *Water Solution* required to neutralize the unconsumed *Reagent;* and *R* is the ratio, *V′*/25 (mL *Reagent*/mL *Water Solution*), determined from the *Standardization of Water Solution for Residual Titration.*

Method Ic (Coulometric Titration)

PRINCIPLE

The Karl Fischer reaction is used in the coulometric determination of water. Iodine, however, is not added in the form of a volumetric solution but is produced in an iodide-containing solution by anodic oxidation. The reaction cell usually consists of a large anode compartment and a small cathode compartment that are separated by a diaphragm. Other suitable types of reaction cells (e.g., without diaphragms) may also be used. Each compartment has a platinum electrode that conducts current through the cell. Iodine, which is produced at the anode electrode, immediately reacts with water present in the compartment. When all the water has been consumed, an excess of iodine occurs, which usually is detected electrometrically, thus indicating the endpoint. Moisture is eliminated from the system by pre-electrolysis. Changing the Karl Fischer solution after each determination is not necessary because individual determinations can be carried out in succession in the same reagent solution. A requirement for this method is that each component of the test specimen is compatible with the other components, and no side reactions take place. Samples are usually transferred into the vessel as solutions by means of injection through a septum. Gases can be introduced into the cell by means of a suitable gas inlet tube. Precision in the method is predominantly governed by the extent to which atmospheric moisture is excluded from the system; thus, the introduction of solids into the cell may require precautions, such as working in a glove-box in an atmosphere of dry inert gas. Control of the system may be monitored by measuring the amount of baseline drift, which does not preclude the need of any blank correction when used as a vehicle for sample introduction. This method is particularly suited to chemically inert substances like hydrocarbons, alcohols, and ethers. In comparison with the volumetric Karl Fischer titration, coulometry is a micro-method.

When appropriate, the water may be desorbed or released from the sample by heat in an external oven connected with the vessel, to where it is transferred with the aid of an inert and dried gas such as pure nitrogen. Any drift due to the transport gas should be considered and corrected. Care should be taken in the selection of the heating conditions to avoid the formation of water coming from dehydration due to decomposition of the sample constituents, which may invalidate this approach.

APPARATUS

Any commercially available apparatus consisting of an absolutely tight system fitted with the necessary electrodes and a magnetic stirrer is appropriate. The instrument's microprocessor controls the analytical procedure and displays the results. Calibration of the instrument is not necessary, as the current consumed can be measured absolutely.

REAGENT

See the manufacturer's recommendations.

TEST PREPARATION

Where the specimen is a soluble solid, an appropriate quantity, accurately weighed, may be dissolved in anhydrous methanol or other suitable solvents.

Where the specimen is an insoluble solid, an appropriate quantity, accurately weighed, may be extracted using a suitable anhydrous solvent, and may be injected into the anolyte solution. Alternatively, an evaporation technique may be used in which water is released and evaporated by heating the specimen in a tube in a stream of dry inert gas. The gas is then passed into the cell.

Where the specimen is to be used directly without dissolving in a suitable anhydrous solvent, an appropriate quantity, accurately weighed, may be introduced into the chamber directly.

Where the specimen is a liquid, and is miscible with anhydrous methanol or other suitable solvents, an appropriate quantity, accurately weighed, may be added to anhydrous methanol or other suitable solvents.

PROCEDURE

Using a dry device, inject or add directly an accurately measured amount of the sample or sample preparation estimated to contain between 0.5 and 5 mg of water, or an amount recommended by the instrument manufacturer into the anolyte, mix, and perform the coulometric titration to the electrometric endpoint. Read the water content of the liquid *Test Preparation* directly from the instrument's display, and calculate the percentage that is present in the substance. Perform a blank determination, as needed, and make any necessary corrections.

METHOD II (AZEOTROPIC—TOLUENE DISTILLATION)

Apparatus

Use a 500-mL glass flask A connected by means of a trap B to a reflux condenser C by ground glass joints (see *Figure 1*).

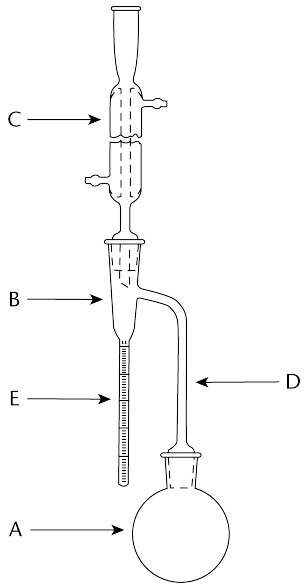


Figure 1. Toluene moisture apparatus.

The critical dimensions of the parts of the apparatus are as follows. The connecting tube D is 9–11 mm in internal diameter. The trap is 235–240 mm in length. The condenser, if of the straight-tube type, is approximately 400 mm in length and not less than 8 mm in bore diameter. The receiving tube E has a 5-mL capacity, and its cylindrical portion, 146–156 mm in length, is graduated in 0.1-mL subdivisions, so that the error of reading is not greater than 0.05 mL for any indicated volume. The source of heat is preferably an electric heater with rheostat control or an oil bath. The upper portion of the flask and the connecting tube may be insulated.

Clean the receiving tube and the condenser with a suitable cleanser, thoroughly rinse with water, and dry in an oven. Prepare the toluene to be used by first shaking with a small quantity of water, separating the excess water, and distilling the toluene.

Procedure

Place in the dry flask a quantity of the substance, weighed accurately to the nearest centigram, which is expected to yield 2–4 mL of water. If the substance is of a pasty character, weigh it in a boat of metal foil of a size that will just pass through the neck of the flask. If the substance is likely to cause bumping, add enough dry, washed sand to cover the bottom of the flask, or a number of capillary melting-point tubes, about 100 mm in length, sealed at the upper end. Place about 200 mL of toluene in the flask, connect the apparatus, and fill the receiving tube E with toluene poured through the top of the condenser. Heat the flask gently for 15 min and, when the toluene begins to boil, distill at the rate of about two drops per s until most of the water has passed over, then increase the rate of distillation to about four drops per s. When the water has apparently all distilled over, rinse the inside of the condenser tube with toluene while brushing down the tube with a tube brush attached to a copper wire and saturated with toluene. Continue the distillation for five min, then remove the heat, and allow the receiving tube to cool to room temperature. If any droplets of water adhere to the walls of the receiving tube, scrub them down with a brush consisting of a rubber band wrapped around a copper wire and wetted with toluene. When the water and toluene have separated completely, read the volume of water, and calculate the percentage that was present in the substance.

METHOD III (GRAVIMETRIC)

Procedure for Chemicals

Proceed as directed in the individual monograph preparing the chemical as directed under *Loss on Drying* ⟨731⟩.

Procedure for Biologics

Proceed as directed in the individual monograph.

Procedure for Articles of Botanical Origin

Place about 10 g of the drug, prepared as directed (see *Methods of Analysis* under *Articles of Botanical Origin* ⟨561⟩) and accurately weighed, in a tared evaporating dish. Dry at 105° for 5 h, and weigh. Continue the drying and weighing at 1-h intervals until the difference between two successive weighings corresponds to not more than 0.25%.

General Information Chapters

⟨1231⟩ WATER FOR PHARMACEUTICAL PURPOSES

TABLE OF CONTENTS

Published August 17, 2020

1. INTRODUCTION

Water is widely used as a raw material, inactive ingredient, medicinal vehicle, and solvent in the processing, formulation, and manufacture of pharmaceutical products (dosage forms), active pharmaceutical ingredients (APIs), API intermediates, compendial articles, and analytical reagents as well as in cleaning applications.

This is an informational chapter on pharmaceutical water topics and includes some of the chemical and microbiological concerns unique to water and its preparation and uses. The chapter provides information about water quality attributes (that may or may not be included within a water monograph) and processing techniques that can be used to improve water quality. It also discusses water system validation and gives a description of minimum water quality standards that should be considered when selecting a water source including sampling and system controls. It is equally important for water systems to be operated and maintained in a state of control to provide assurance of operational stability and therefore the capability to provide water that meets established water quality standards.

This informational chapter is intended to be educational, and the user should also refer to existing regulations or guidelines that cover U.S. and international [International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) or World Health Organization (WHO)] good manufacturing practice (GMP) issues, as well as operational and engineering guides and/or other regulatory guidance for water [e.g., from the Food and Drug Administration (FDA), Environmental Protection Agency (EPA), or WHO]. This chapter is not, and should not be considered, an all-inclusive document on pharmaceutical waters. It contains basic information and points to be considered for the processing, holding, monitoring, and use of water. It is the user's responsibility to ensure that:

1. The selection of the type and specifications of water is appropriate for its intended use.
2. Water production and quality meet applicable governmental regulations and guidance.
3. The pharmacopeial specifications for the types of water used in monographed articles are met.
4. Water used in the preparation of reagents for analysis or the performance of required tests meets USP requirements.

Control and monitoring of the chemical and endotoxin purity of waters are important for complying with the requirements of the monographs in this compendium. Attributes listed in *USP* monographs should be considered the "minimum" requirements. More stringent requirements may be needed for some applications to ensure suitability for particular uses. Basic guidance on the appropriate applications of waters can be found in the monographs and is also discussed further in this chapter.

Control of the microbiological quality of water is also important for many of its uses. This attribute is intentionally not specified in most water monographs. Microbiological control is discussed throughout this chapter, but especially in sections *4. Validation and Qualification of Water Purification, Storage, and Distribution Systems*, *5. Design and Operation of Purified Water and Water for Injection Systems*, *6. Sampling*, *8. Microbial Evaluations*, and *9. Alert and Action Levels and Specifications*.

This chapter contains various chemical, microbiological, processing, and engineering concepts of importance to users of water. Water system validation, process control levels, and specifications are also presented later in this chapter.

2. SOURCE WATER CONSIDERATIONS

Source water is the water that enters the facility. The origin of this source water can be from natural surface waters like rivers and reservoirs, deep-bed well waters, sea waters, or some combination of these, potentially including multiple locations of each type of source water. Thus, source water can be supplied from these various origins (public or private), from municipalities' on-site water sourcing, or by external delivery such as a truck. It is possible that source water may not be potable and safe to drink. Such water may require pretreatment to ensure that it meets drinking water standards. It is the responsibility of the users of any source water to ensure that the water used in the production of drug substances (API), as well as water for indirect drug product contact or for purification system feed water purposes meets, at a minimum, drinking (potable) water standards as defined by the requirements of the National Primary Drinking Water Regulations (NPDWR) (40 CFR 141) issued by the U.S. EPA or the drinking water regulations of the European Union (EU) or Japan, or the WHO drinking water guidelines (see *3.3.1*

Drinking Water). These regulations establish limits on the types and quantities of certain chemical and microbiological contaminants and ensure that the water will contain safe quantities of chemical and microbial species.

Where water supplies are from regulated water utility companies, less stringent monitoring may be possible because the attributes may be tested regularly and ensured by the supplier (see *9.4.5 Source Water Control*). Water being withdrawn from a nonregulated supply should be sampled and tested appropriately at a suitable frequency that takes into account local environmental and seasonal changes and other quality fluctuations. Testing should ensure conformance with one of the drinking water standards discussed above.

The use of water complying with one of these designated drinking waters as a source water allows water pretreatment systems to only be challenged to remove small quantities of potentially difficult-to-remove chemicals. Control of objectionable chemical contaminants at the source water stage eliminates the need to specifically test for some of them [e.g., trihalomethanes and elemental impurities (see *Elemental Impurities—Limits* ⟨232⟩)] after the water has been further purified, assuming there is no opportunity for recontamination.

Source waters can be used for nonproduct contact purposes such as for non-contact cooling systems. Such water may not normally be required to meet drinking water standards. Under such circumstances, the quality standards for this water when used in a pharmaceutical facility should be subject to quality standards established by the user and defensible to regulatory agencies.

Change to read:

3. WATERS USED FOR PHARMACEUTICAL MANUFACTURING AND TESTING PURPOSES

There are many different grades of water used for pharmaceutical purposes. Several are described in *USP* monographs that specify uses, acceptable methods of preparation, and quality attributes. These waters can be divided into two general types: bulk waters, which are typically produced on-site where they are used; and sterile waters, which are produced, packaged, and sterilized to preserve microbial quality throughout their packaged shelf life. There are several specialized types of sterile waters that differ in their designated applications, packaging limitations, and other quality attributes. Monographed waters must meet the quality attributes as specified in the related monographs, and any *Notes* appearing in those monographs should be considered and addressed.

With the exception of Bacteriostatic Water for Injection, the monographed bulk and sterile waters have a statement indicating that there are no added substances, or no added antimicrobial agents. In the case of antimicrobial agents, the purpose is to ensure that the sterile water product is rendered sterile based solely on its preparation, packaging, and storage. In the case of the more general statement, "no added substances", this requirement is intended to mean "no added substances that aren't sufficiently removed". Two specific examples support this intention, but there are many examples. First, the use of softeners is commonplace. A softener replaces calcium and magnesium ions ▲(also known as hardness ions)▲ 1S (USP41) with sodium, so technically you are adding two sodium ions for each hard ion. The purpose of sodium displacement is to protect downstream equipment from the hard water. The sodium ions are eventually removed sufficiently, and this is proven when the water sample passes the test in *Water Conductivity* ⟨645⟩. Another specific example is the use of ozone as a sanitant that is added to the storage tank for microbial control. This could be considered an added substance, unless the ozone is destroyed before use, as is normally the case. Other notable examples include the addition of chlorine to kill bacteria in the pretreatment system, use of bisulfite to chemically reduce chlorine to chloride and protect downstream equipment, and use of a nitrogen blanket for protection from atmospheric contamination.

There are also other types of water for which there are no monographs. These are waters with names given for descriptive purposes only. Many of these waters are used in specific analytical methods. The descriptive titles may imply certain quality attributes or modes of preparation, but these nonmonographed waters may not necessarily adhere strictly to the stated or implied modes of preparation or specified attributes. Waters produced by other means or controlled by other test attributes, or even a monographed water, may equally satisfy the intended uses for these waters. It is the user's responsibility to ensure that such waters, even if produced and controlled exactly as stated, are suitable for their intended use. Wherever the term "water" is used within this compendium without other descriptive adjectives or clauses, the intent is that water of no less purity than USP *Purified Water* be used (see *3.1.1 Purified Water*). A brief description of the various types of waters commonly associated with pharmaceutical applications and their significant uses or attributes follows.

Figure 1 may be helpful in understanding some of the various types of waters, their preparation, and uses.

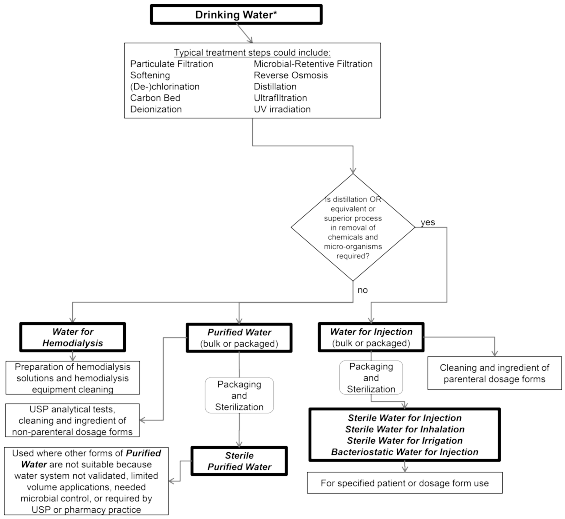


Figure 1. Water for pharmaceutical purposes. [a]Complying with U.S. EPA NPDWR or the drinking water regulations of EU or Japan or WHO.

3.1 Bulk Monographed Waters and Steam

The following waters are generally produced in large volumes using a multiple-unit operation water system. These waters are typically distributed in a piping system for use at the same site.

3.1.1 PURIFIED WATER

Purified Water (see the *USP* monograph) is used as an excipient in the production of nonparenteral preparations and in other pharmaceutical applications, such as the cleaning of nonparenteral product-contact components and equipment. Unless otherwise specified, *Purified Water* is also to be used as the minimum water quality for all tests and assays in which "water" is indicated (see *General Notices, 8.230.30 Water in a Compendial Procedure*). This applies regardless of the font and letter case used in its spelling.

The minimal quality of source water for the production of Purified Water is Drinking Water whose attributes are prescribed by the U.S. EPA, EU, Japan, or WHO. This source water may be purified using unit operations that include deionization, distillation, ion exchange, reverse osmosis, filtration, or other suitable purification procedures. Purified Water must meet the requirements for ionic and organic chemical purity and must be protected from microbial contamination. Purified Water systems must be validated to reliably and consistently produce and distribute water of acceptable chemical and microbiological quality. Purified Water systems that function under ambient conditions are particularly susceptible to the establishment of biofilms of microorganisms, which can be the source of undesirable levels of viable microorganisms or endotoxins in the water. These ambient Purified Water systems require frequent sanitization and microbiological monitoring to ensure that the water reaching the points of use has appropriate microbiological quality.

The *Purified Water* monograph also allows bulk packaging for commercial use elsewhere. In contrast to Sterile Purified Water, packaged Purified Water is not required to be sterile. Because there is potential for microbial contamination and other quality changes in this packaged nonsterile water, this form of Purified Water should be prepared and stored in a manner that limits microbial growth, and/or should be used in a timely fashion before microbial proliferation renders it unsuitable for its intended use. Also, depending on the material used for packaging, extractable compounds could be leaching into the water from the packaging. Although this article is required to meet the same chemical purity standards as the bulk water, extractables from the packaging will likely render the packaged water less chemically pure than the bulk water. The nature of these impurities may even render the water an inappropriate choice for some applications. It is the user's responsibility to ensure fitness for use of this packaged article when it is used in manufacturing, clinical, or analytical applications where the purer bulk form of the water is indicated.

3.1.2 WATER FOR INJECTION

Water for Injection (see the *USP* monograph) is used as an excipient in the production of parenteral and other preparations where product endotoxin content must be controlled, and in other pharmaceutical applications, such as the cleaning of certain equipment and parenteral product-contact components.

The minimal quality of source water for the production of Water for Injection is Drinking Water whose attributes are prescribed by the U.S. EPA, EU, Japan, or WHO. This source water may be treated to render it suitable for subsequent final purification steps, such as distillation (or whatever other validated process is used, according to the monograph). The finished water must meet all of the chemical requirements specified in the monograph, as well as an additional bacterial endotoxin specification. Because endotoxins are produced by the kinds of microorganisms that are prone to inhabit water systems, the equipment and procedures used by the system to purify, store, and distribute Water for Injection should be designed to control microbial contamination and must be designed to remove incoming endotoxins from the source water. Water for Injection systems must be validated to reliably and consistently produce and distribute this quality of water.

The *Water for Injection* monograph also allows bulk packaging for commercial use. In contrast to Sterile Water for Injection, packaged Water for Injection is not required to be sterile. However, to preclude significant changes in its microbial and endotoxins content during storage, this form of Water for Injection should be prepared and stored in a manner that limits microbial introduction and growth and/or should be used in a timely fashion before microbial proliferation renders it unsuitable for its intended use. Also, depending on the material used for packaging, extractable compounds could be leaching into the water from the packaging. Although this article is required to meet the same chemical purity standards as the bulk water, extractables from the packaging will likely render the packaged water less chemically pure than the bulk water. The nature of these impurities may even render the water an inappropriate choice for some applications. It is the user's responsibility to ensure fitness for use of this packaged article when it is used in manufacturing, clinical, or analytical applications where the purer bulk form of the water is indicated.

3.1.3 WATER FOR HEMODIALYSIS

Water for Hemodialysis (see the *USP* monograph) is used for hemodialysis applications, primarily the dilution of hemodialysis concentrate solutions. The minimal quality of source water for the production of Water for Hemodialysis is Drinking Water whose attributes are prescribed by the U.S. EPA, EU, Japan, or WHO. Water for Hemodialysis has been further purified to reduce chemical and microbiological components, and it is produced and used on site. This water contains no added antimicrobial agents, and it is not intended for injection. Water for Hemodialysis must meet all of the chemical requirements specified in the monograph as well as an additional bacterial endotoxin specification. The microbial limits attribute for this water is unique among the "bulk" water monographs, but is justified on the basis of this water's specific application, which has microbial content requirements related to its safe use. The bacterial endotoxins attribute is likewise established at a level related to its safe use.

3.1.4 PURE STEAM

Pure Steam (see the *USP* monograph) is also sometimes referred to as "clean steam". It is used where the steam or its condensate would directly contact official articles or article-contact surfaces, such as during their preparation, sterilization, or cleaning where no subsequent processing step is used to remove any impurity residues. These Pure Steam applications include, but are not limited to, porous load sterilization processes, product or cleaning solutions heated by direct steam injection, or humidification of processes where steam injection is used to control the humidity inside processing vessels where the official articles or their in-process forms are exposed. The primary intent of using this quality of steam is to ensure that official articles or article-contact surfaces exposed to it are not contaminated by residues within the steam.

The minimal quality of source water for the production of Pure Steam is Drinking Water whose attributes are prescribed by the U.S. EPA, EU, Japan, or WHO, and which has been suitably treated. The water is then vaporized with suitable mist elimination, and distributed under pressure. The sources of undesirable contaminants within Pure Steam could arise from entrained source water droplets, anticorrosion steam additives, or residues from the steam production and distribution system itself. The chemical tests in the *Pure Steam* monograph should detect most of the contaminants that could arise from these sources. If an official article is exposed to Pure Steam and it is intended for parenteral use or other applications where the pyrogenic content must be controlled, the Pure Steam must additionally meet the specification for *Bacterial Endotoxins Test* ⟨85⟩.

These purity attributes are measured in the condensate of the article, rather than the article itself. This, of course, imparts great importance to the cleanliness of the process for Pure Steam condensate generation and collection, because it must not adversely impact the quality of the resulting condensed fluid.

Other steam attributes not detailed in the monograph, particularly the presence of even small quantities of noncondensable gases or the existence of a superheated or dry state, may also be important for applications such as sterilization. The large release of energy (latent heat of condensation) as water changes from the gaseous to the liquid state is the key to steam's sterilization efficacy and its efficiency, in general, as a heat transfer agent. If this phase change (condensation) is not allowed to happen because the steam is extremely hot and is in a persistent superheated, dry state, then its usefulness could be seriously compromised. Noncondensable gases in steam tend to stratify or collect in certain areas of a steam sterilization chamber or its load. These surfaces would thereby be at least partially insulated from the steam condensation phenomenon, preventing them from experiencing the full energy of the sterilizing conditions. Therefore, control of these kinds of steam attributes, in addition to its chemical purity, may also be important for certain Pure Steam applications. However, because these additional attributes are use-specific, they are not mentioned in the *Pure Steam* monograph.

Note that lower-purity "plant steam" may be used in the following applications: 1) for steam sterilization of nonproduct-contact nonporous loads, 2) for general cleaning of nonproduct-contact equipment, 3) as a nonproduct-contact heat-exchange medium, and 4) in all compatible applications involved in bulk pharmaceutical chemical and API manufacture.

Finally, because Pure Steam is lethal to microbes, monitoring of microbial control within a steam system is unnecessary, as is microbial analysis of the steam condensate.

3.2 Sterile Monographed Waters

The following monographed waters are packaged forms of either Purified Water or Water for Injection that have been sterilized to preserve their microbiological properties. These waters may have specific intended uses as indicated by their names, and may also have restrictions on the packaging configurations related to those uses. In general, these sterile waters may be used in a variety of applications in lieu of the bulk forms of water from which they were derived. However, there is a substantial difference between the acceptance criteria for the chemical purities of these bulk waters versus sterile waters. The specifications for sterile waters differ from those of bulk waters to accommodate a wide variety of packaging types, properties, volumes, and uses. As a result, the inorganic and organic impurity specifications are not equivalent for bulk and packaged waters. The packaging materials and elastomeric closures are the primary sources of these impurities, which tend to increase over the shelf life of these packaged articles. Therefore, due consideration must be given to the chemical purity suitability at the time of use of the sterile forms of water when used in manufacturing, analytical, and cleaning applications in lieu of the bulk waters from

which these waters were derived. It is the user's responsibility to ensure fitness for use of these sterile packaged waters in these applications. Nevertheless, for the applications discussed below for each sterile water, their respective purities and packaging restrictions generally render them suitable by definition.

3.2.1 STERILE PURIFIED WATER

Sterile Purified Water (see the *USP* monograph) is Purified Water, packaged and rendered sterile. It can be used in the preparation of nonparenteral compendial dosage forms or in analytical applications requiring Purified Water where 1) access to a validated Purified Water system is not practical, 2) only a relatively small quantity is needed, 3) Sterile Purified Water is required by specific monograph or pharmacy practice, or 4) bulk packaged Purified Water is not suitably controlled for the microbiological quality for its intended use.

3.2.2 STERILE WATER FOR INJECTION

Sterile Water for Injection (see the *USP* monograph) is Water for Injection packaged and rendered sterile. It is used for extemporaneous prescription compounding and as a sterile diluent for parenteral products. It may also be used for other applications where bulk Water for Injection or Purified Water is indicated but access to a validated water system is not practical, or where only a relatively small quantity is needed. Sterile Water for Injection is packaged in single-dose containers not larger than 1 L.

3.2.3 BACTERIOSTATIC WATER FOR INJECTION

Bacteriostatic Water for Injection (see the *USP* monograph) is Water for Injection, packaged and rendered sterile, to which has been added one or more suitable antimicrobial preservatives. It is intended to be used as a diluent in the preparation of parenteral products, most typically for multi-dose products that require repeated content withdrawals. It may be packaged in single-dose or multiple-dose containers not larger than 30 mL.

3.2.4 STERILE WATER FOR IRRIGATION

Sterile Water for Irrigation (see the *USP* monograph) is Water for Injection packaged and sterilized in single-dose containers that may be larger than 1 L and allow rapid delivery of their contents. Due to its usage, Sterile Water for Irrigation is not required to meet *Particulate Matter in Injections* ⟨788⟩. It may also be used in other applications that do not have particulate matter specifications, where bulk Water for Injection or Purified Water is indicated but where access to a validated water system is not practical, or where somewhat larger quantities are needed than are provided as Sterile Water for Injection.

3.2.5 STERILE WATER FOR INHALATION

Sterile Water for Inhalation (see the *USP* monograph) is Water for Injection that is packaged and rendered sterile and is intended for use in inhalators and in the preparation of inhalation solutions. This monograph has no requirement to meet ⟨788⟩; it carries a less stringent specification for bacterial endotoxins than Sterile Water for Injection, and therefore is not suitable for parenteral applications.

3.3 Nonmonographed Waters

In addition to the bulk monographed waters described above, nonmonographed waters can also be used in pharmaceutical processing steps such as cleaning and synthetic steps, and also as a starting material for further purification or testing purposes. Unless otherwise specified in the compendium, the minimum quality of water is *Purified Water*. [NOTE—The information in this chapter is not an all-inclusive discussion of all nonmonographed waters identified in the *USP–NF*.]

3.3.1 DRINKING WATER

Drinking Water can be referred to as Potable Water (meaning drinkable or fit to drink), National Primary Drinking Water, Primary Drinking Water, or EPA Drinking Water. Except where a singular drinking water specification is stated (such as the U.S. EPA's NPDWR, as cited in 40 CFR Part 141), this water must comply with the quality attributes of either the NPDWR or the drinking water regulations of the EU or Japan, or the *WHO Guidelines for Drinking-Water Quality*. Drinking Water may originate from a variety of sources including a public water supply, a private water supply (e.g., a well), or a combination of these sources (see *2. Source Water Considerations*).

Drinking Water may be used in the early stages of cleaning pharmaceutical manufacturing equipment and product-contact components. Drinking Water is also the minimum quality of water that should be used for the preparation of official substances and other bulk pharmaceutical ingredients. Where compatible with the processes, the contaminant levels allowed in Drinking Water are generally considered safe for use in preparing official substances and other drug substances. However, where required by the processing of the materials to achieve their required final purity, higher qualities of water may be needed for these manufacturing steps, perhaps even water as pure as Water for Injection or Purified Water. Such higher-purity waters, however, might require only selected attributes to be of higher purity than Drinking Water (see *Figure 2a* and *Figure 2b*). Drinking Water is the prescribed source or feed water for the production of bulk monographed pharmaceutical waters. The use of Drinking Water specifications establishes a reasonable set of maximum allowable levels of chemical and microbiological contaminants with which a water purification system will be challenged. Because seasonal variations in the quality attributes of the Drinking Water supply can occur, it is important to give due consideration to its uses. The processing steps in the production of pharmaceutical waters must be designed to accommodate this variability.

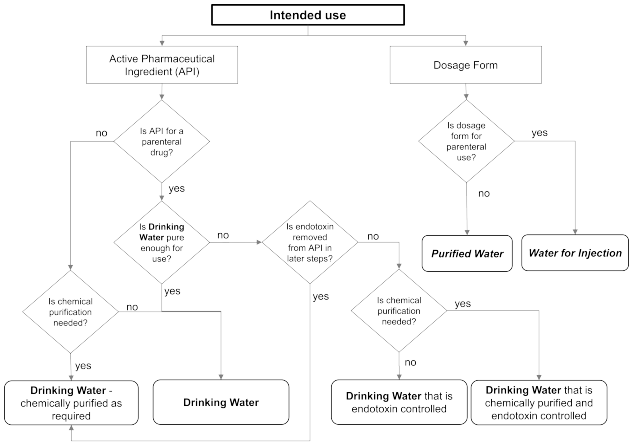


Figure 2a. Selection of water for pharmaceutical purposes: APIs and dosage forms.

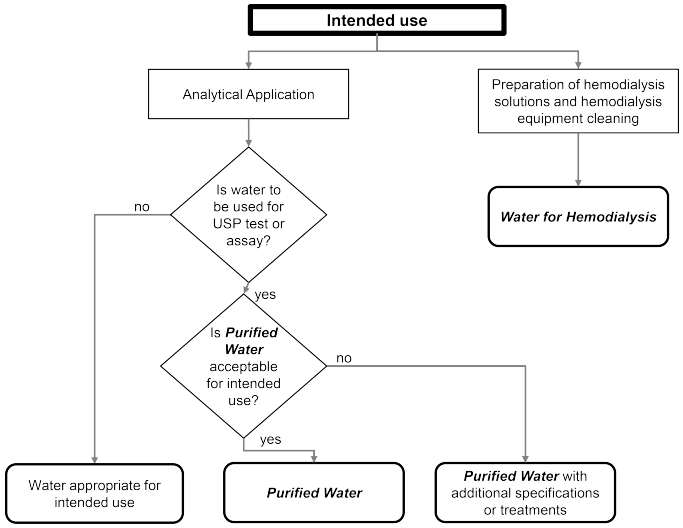


Figure 2b. Selection of water for pharmaceutical purposes: Analytical reagents.

3.3.2 OTHER NONMONOGRAPHED WATERS

In addition to Drinking Water, this compendium discusses waters with various other designations. These include waters of various quality levels for special uses such as, but not limited to, cleaning and testing purposes.

Both *General Notices and Requirements* (see *General Notices, 8.230.30 Water in a Compendial Procedure*) and *Reagents, Indicators, and Solutions* clearly state that where the term "water" is indicated for use in analyses without grammatical qualification or other specification, the quality of the water must be *Purified Water*. However, numerous such qualifications do exist. Some of these qualifications involve adjectives describing methods of preparation, ranging from specifying the primary purification step to specifying additional purification. Other qualifications call for specific attribute "absences" to be met that might otherwise interfere with analytical processes. In most of these cases, the required attribute absences are not specifically tested. Sometimes, a further "purification process" is specified that ostensibly allows the water to adequately meet this required "absence attribute".

However, preparation instructions for many reagents were carried forward from the innovator's laboratories to the originally introduced monograph for a particular *USP–NF* article or general test chapter. The quality of the reagent water described in these tests may reflect the water quality designation of the innovator's laboratory. These specific water designations may have originated without the innovator's awareness of the requirement for *Purified Water* in *USP–NF* tests. Regardless of the original reason for the creation of these numerous special analytical waters, it is possible that the attributes of these special waters could now be met by the basic preparation steps and current specifications of *Purified Water*. In some cases, however, some of the cited post-processing steps are still necessary to reliably achieve the required attributes.

Users are not obligated to utilize specific and perhaps archaically generated forms of analytical water where alternatives with equal or better quality, availability, or analytical performance may exist. The consistency and reliability of operations for producing these alternative analytical waters should be verified so that the desired attributes are produced. In addition, any alternative analytical water must be evaluated on an application-by-application basis by the user to ensure its suitability. The following is a summary of the various types of nonmonographed analytical waters that are cited in the *USP–NF*. This is not an exhaustive listing. Those listed below are used in multiple locations. Several nonmonographed analytical waters are not included below because they are only found in one or perhaps two locations within this compendium.

Note that the names of many of the waters below imply a very low chemical impurity level. For example, "deionized water" implies that all the ions have been removed. However, in most cases discussed below, exposure of the water to air will result in the ingress of carbon dioxide (CO_2), leading to the formation of bicarbonate and hydrogen ions. Therefore, the removal of ions cannot be completely maintained for most analytical applications.

3.3.3 AMMONIA-FREE WATER

From a functional standpoint, Ammonia-Free Water must have a negligible ammonia concentration to avoid interference in tests sensitive for or to ammonia. Due to the nature of the uses of this water, *Purified Water* could be a reasonable alternative for these applications.

3.3.4 CARBON DIOXIDE-FREE WATER

Carbon dioxide-free water is defined in the *Reagents, Indicators, and Solutions* section of *USP–NF* as Purified Water that has been vigorously boiled for NLT 5 min, then cooled and protected from absorption of atmospheric carbon dioxide. Alternatively, this could be *Purified Water* that has a resistivity of NLT 18 megohm-cm at 25°.

Because the absorption of atmospheric carbon dioxide lowers the pH of high-purity waters, most of the uses of Carbon Dioxide-Free Water are either associated as a solvent in pH-related or pH-sensitive determinations or as a solvent in bicarbonate-sensitive reagents or determinations.

The term "Carbon Dioxide-Free Water" is sometimes used improperly. Besides its use for pH or acidity/alkalinity tests, the purpose for using this water is not always clear. The intention could be to use water that was deaerated (free of dissolved air) or deionized (free of extraneous ions), or even Purified Water with an additional boiling step. Although boiling is highly effective for removing carbon dioxide as well as all other dissolved gasses, these gases are readily re-absorbed unless the water is protected. Even with protection, such as use of a stoppered container, re-absorption will occur over time as air will readily transmit through seals and diffuse through most materials. Deionization is also an efficient process for removing dissolved carbon dioxide. Carbon dioxide forms ionic bicarbonate in water, and will be subsequently removed by ion-exchange resins. However, the same problem of carbon dioxide re-absorption will occur after the deionized water is exposed to air. Also, the deionization approach for creating Carbon Dioxide-Free Water does not deaerate the water or remove other dissolved gases such as oxygen (O_2); it only removes carbon dioxide and other ions.

Depending on the application, *Purified Water* may meet the requirements where Carbon Dioxide-Free Water is called for. This could also include pH or acidity or alkalinity tests. The pH of a sample of pure Deionized Water is, by definition, 7.0. When that same sample is exposed to typical environmental atmospheric conditions, the water sample will absorb carbon dioxide and result in a pH range of approximately 5.4–6.2 ($[H^+]$ is in the range of 4.0×10^{-6} M to 6.3×10^{-7} M). The added acidity caused by carbon dioxide absorption may be insignificant compared to the material being analyzed.

3.3.5 DISTILLED WATER

Distilled Water is produced by vaporizing Drinking Water or a higher quality of water and condensing it into a purer state. It is used primarily as a solvent for reagent preparation, and it is also specified in the execution of other aspects of tests, such as for rinsing an analyte, transferring a test material as a slurry, as a calibration standard or analytical blank, and for test apparatus cleaning. Distilled Water is also cited as the starting water to be used for making High-Purity Water (see *3.3.10 High-Purity Water*). Because none of the cited uses of this water imply a need for a particular purity attribute that can only be derived by distillation, water meeting the requirements for *Purified Water* derived by other means of purification or *Water for Injection* could be equally suitable where Distilled Water is specified. It is the user's responsibility to verify the suitability of Purified Water or Water for Injection.

3.3.6 FRESHLY DISTILLED WATER

Freshly Distilled Water or "recently distilled water" is produced in the same manner as Distilled Water and should be used soon after its generation. This implies the need to avoid endotoxin contamination, as well as any other forms of contamination from the air or containers, that could arise with prolonged storage. Freshly Distilled Water is used for preparing solutions for subcutaneous test-animal injections and for a reagent solvent in tests for which there appears to be no particularly high water purity needed that could be ascribable to being "freshly distilled". In the test-animal application, the term "freshly distilled" and its testing use imply a chemical, endotoxin, and microbiological purity that could be equally satisfied by *Water for Injection* (although no reference is made to these chemical, endotoxin, or microbial attributes or specific protection from recontamination). For non-animal uses, water meeting the requirements for *Purified Water* derived by other means of purification and/or storage periods could be equally suitable where "recently distilled water" or Freshly Distilled Water is specified. It is the user's responsibility to verify the suitability of Purified Water or Water for Injection.

3.3.7 DEIONIZED WATER

Deionized Water can be produced by starting with either Drinking Water or Purified Water, depending upon monograph or testing procedures defined in the compendia. Deionized Water is produced by an ion-exchange process in which the cations and anions are replaced with H+ and OH– ions by use of ion-exchange resins. Similar to Distilled Water, Deionized Water is used primarily as a solvent for reagent preparation, but it is also specified in the execution of other aspects of tests, such as for transferring an analyte within a test procedure, as a calibration standard or analytical blank, and for test apparatus cleaning. Also, none of the cited uses of this water imply any needed purity attribute that can only be achieved by deionization. Therefore, water meeting the requirements for *Purified Water* that is derived by other means of purification could be equally suitable where Deionized Water is specified. It is the user's responsibility to verify the suitability of Purified Water.

3.3.8 DEIONIZED DISTILLED WATER

Deionized Distilled Water is produced by deionizing (see *3.3.7 Deionized Water*) Distilled Water. This water is used as a reagent in a liquid chromatography test that requires a low ionic or organic impurity level. Because of the importance of this high purity, water that meets the requirements for *Purified Water* may not be acceptable. High-Purity Water (see *3.3.10 High-Purity Water*) could be a reasonable alternative to this water. It is the user's responsibility to verify the suitability of the alternative water used.

3.3.9 FILTERED WATER

Filtered Water is Purified Water that has been filtered to remove particles that could interfere with the analysis where this water is specified. It is sometimes used synonymously with Particle-Free Water and Ultra-Filtered Water and is cited in some monographs and general chapters as well as in *Reagents, Indicators, and Solutions*. Depending on its referenced location in *USP–NF*, it is variously defined as water that has been passed through filters rated as 1.2, 0.2, or 0.22 µm, or unspecified porosity rating. Even though the water names and the filter ratings used to produce these waters are defined inconsistently, the use of 0.2-µm or 0.22-µm filtered *Purified Water* should be universally acceptable for all applications where Particle-Free Water, Filtered Water, or Ultra-Filtered Water are specified.

3.3.10 HIGH-PURITY WATER

High-Purity Water may be prepared by deionizing previously distilled water and then filtering it through a 0.45-µm rated membrane. This water must have an in-line conductivity of NMT 0.15 µS/cm (NLT 6.67 megohm-cm) at 25°. If the water of this purity contacts the atmosphere even briefly as it is being used or drawn from its purification system, its conductivity will immediately increase by as much as about 1.0 µS/cm ▲at 25°▲ 1S (USP41) as atmospheric carbon dioxide dissolves in the water and equilibrates to hydrogen and bicarbonate ions. Therefore, if the analytical use requires that water conductivity remains as low as possible or the bicarbonate/carbon dioxide levels be as low as possible, the water should be protected from atmospheric exposure. High-Purity Water is used as a reagent, as a solvent for reagent preparation, and for test apparatus cleaning where less stringent water specifications would not be considered acceptable. However, if a user's routinely available *Purified Water* is filtered and meets or exceeds the conductivity specifications of High-Purity Water, it could be used in lieu of High-Purity Water.

3.3.11 DEAERATED WATER

Deaerated Water or "degassed water" is Purified Water that has been treated to reduce the content of dissolved air by "suitable means" such as boiling, sonication, and/or stirring during the application of a partial vacuum, followed by immediate use or protection from air reabsorption.

3.3.12 OXYGEN-FREE WATER

Oxygen-Free Water is Purified Water that has been treated to remove or reduce dissolved oxygen. Such treatment could involve deaerating by boiling or sparging with an inert gas such as nitrogen or helium, followed by inert gas blanketing to prevent oxygen reabsorption. Any procedure used for removing oxygen should be verified as reliably producing water that is fit for use.

3.3.13 WATER FOR BACTERIAL ENDOTOXINS TEST

Water for Bacterial Endotoxins Test (BET) is also referred to as Limulus Amebocyte Lysate (LAL) Reagent Water. This type of water is often Water for Injection, which may have been sterilized. It is free from a level of endotoxin that would yield any detectable reaction or interference with the LAL reagent used in the BET (see ⟨85⟩).

Change to read:

4. VALIDATION AND QUALIFICATION OF WATER PURIFICATION, STORAGE, AND DISTRIBUTION SYSTEMS

4.1 Validation Requirement

Establishing the reliability of pharmaceutical water purification, storage, and distribution systems requires demonstrating control of the process through an appropriate period of monitoring and observation. Finished water is typically continuously produced and used, while product and process attributes may only be periodically assessed. The quality of bulk finished water cannot be established by only testing monograph attributes. The unit operations in the pharmaceutical water system need to demonstrate that they are in control through monitoring of the process parameters and water quality. The advent of using conductivity and total organic carbon (TOC) to define chemical purity allows the user to more quantitatively assess the water's chemical purity and its variability as a function of routine treatment system maintenance and regeneration. Treatment processes must also demonstrate control of microbial attributes within the overall system. Some unit operations that are needed for chemical treatment may significantly increase microbial and bacterial endotoxin levels. These are later controlled by downstream unit operations. Knowledge of the treatment system processes and the effectiveness of control measures is needed to ensure that the pharmaceutical waters are acceptable for use.

Efficacy of the design, operation, sanitization, and control of the pharmaceutical water system is demonstrated through the monitoring of chemical and microbial attributes. A typical water system validation program involves an initial increased

frequency of monitoring of the treatment system process parameters and sampling and testing of major process points to demonstrate the ability to produce the acceptable water and to characterize the operation of the system. This is followed by a life cycle approach of validation maintenance and monitoring.

4.2 Validation Approach

Validation is the program of documenting, to a high level of assurance, that a specific process is capable of consistently delivering product conforming to an established set of quality attributes. A validation program qualifies and documents the design, installation, operation, and performance of the system. A graphical representation of a typical water system validation life cycle is shown in *Figure 3*.

The validation protocol should be based on the boundaries of the water system and the critical water quality and process attributes needed to maintain consistent performance. The system boundary may stop at the point of use or may include the water transfer process. If the transfer process from the distribution system outlets to the water use locations (typically either with hoses or hard-piped equipment connections) is defined as outside the water system boundary, then this transfer process still needs to be validated to not adversely affect the quality of the water as it is delivered for use. Because routine quality control (QC) microbial monitoring is performed for the same transfer process and components (e.g., hoses and heat exchangers) as that of routine water use (see *6.1.2 QC Sampling*), there is some logic to include this water transfer process within the distribution system validation.

4.2.1 VALIDATION ELEMENTS

Validation is accomplished through the use of a structured, documented process. The phases of this process include Design Qualification (DQ), Installation Qualification (IQ), Operational Qualification (OQ), Performance Qualification (PQ), and Validation Maintenance. The process is documented in a validation protocol. The elements may be in individual protocols for each phase, or integrated into variations of a DQ/IQ/OQ/PQ combined document format. The protocols are formally approved quality documents. Factory Acceptance Testing (FAT), Site Acceptance Testing (SAT), and commissioning testing of the system may supplement qualification tests for IQ or OQ provided that they are properly documented and reviewed; and if it can be shown that the system functionality is not affected by the transport and installation.

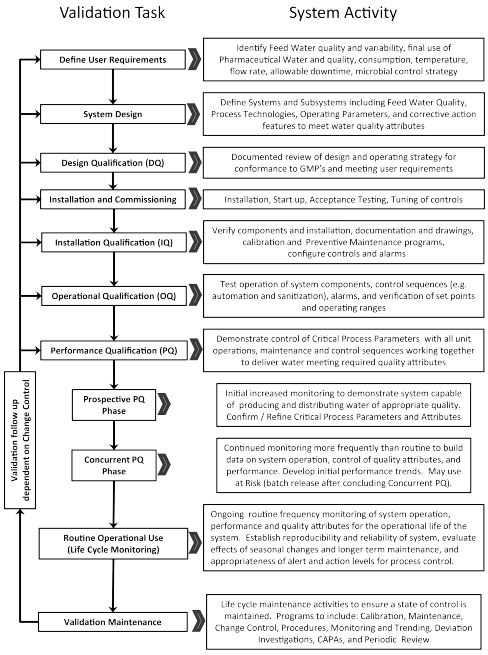


Figure 3. Water system validation life cycle.

4.2.2 USER REQUIREMENTS SPECIFICATION AND DESIGN QUALIFICATION

The user requirements for the water system should identify the design, operation, maintenance, and quality elements needed to produce the desired water type from the available source water, including its anticipated attribute variability. The essential elements of quality need to be built in at this stage and any GMP risks mitigated to an acceptable level.

The review of the specifications, system design, components, functions, and operation should be performed to demonstrate that the system complies with GMPs and verify that the design meets the user requirements. This documented review may be performed as part of the overall design process or as a separate DQ.

4.2.3 IQ

An IQ protocol for a water system confirms that the system has been properly installed and documented. This may include verification of components, piping, installation, and weld quality; documentation of the specifications for all system components present; inspections to verify that the drawings accurately depict the final configuration of the water system and, where necessary, special tests to verify that the installation meets the design requirements. Additionally, the water system is readied for operational testing, including calibration of instruments, configuration of alarm levels and adjustment of operating parameters (e.g., flow rate, pressure).

4.2.4 OQ

The OQ phase consisting of tests and inspections to verify that the equipment, system alerts, and controls are operating reliably and that appropriate Alert and Action Levels are established (this phase of qualification may overlap with aspects of IQ and PQ). During this phase of validation specific testing is performed for alarms, verifying control sequences, equipment functional checks, and verification of operating ranges. SOPs for all aspects of water system operation, maintenance, water use, water sampling, and testing, etc. should be in place and operator training completed. At the completion of the OQ, the water system has demonstrated that the components are operational and the system is producing suitable water.

4.2.5 PQ

The prospective PQ stage considers two aspects of the water system: critical process parameters and critical water attribute parameters. These are evaluated in parallel by monitoring the water quality and demonstrating acceptable quality attributes while demonstrating control of the process parameters (see *6.3 Validation Sampling Plans*). The initial PQ stage may result in refinement of process parameters to yield appropriate water quality. This PQ stage includes an increased frequency of monitoring for approximately 2–4 weeks, or sufficient time to generate adequate data to demonstrate that water meeting the appropriate quality attributes is produced and distributed. One of the reasons for this duration is that biofilm, the source of planktonic organisms in water samples, takes time to develop and to determine if the sanitization unit operations and processes are adequate to control microbial proliferation. The chemical control program adequacy is typically apparent in less time than it takes to see microbial control adequacy. However, chemical purification can be compromised by poor microbial control and, to a lesser degree, vice versa.

Once a level of control of microbial and chemical attributes has been demonstrated, the next phase of PQ is to continue the frequency of monitoring for approximately 2–4 weeks at a somewhat reduced level that will still give adequate data on system performance while using the pharmaceutical water. The water may be used for manufacturing at risk, and the associated products may be released only after water quality attributes have been determined to be acceptable and this validation phase has been completed. At the completion of the second phase, the data should be formally reviewed and the system approved for operational use.

4.3 Operational Use

When the water system has been placed into operational use, monitoring of the water quality attributes and the system process parameters is performed at a routine frequency (see *6.4 Routine Sampling Plans*) to ensure that they remain with a state of control during long-term variability from seasonal variations in source water quality, unit operation maintenance, system sanitization processes, and earlier-established Alert and Action Levels.

The water system should continue to be monitored and evaluated on an on-going basis following a life cycle approach using online instruments or samples for laboratory-based testing. The use of online instruments and process automation technology, such as conductivity, TOC, temperature, flow rate, and pressure can facilitate improved operational control of the attributes and parameters and for process release. Manual observation of operating parameters and laboratory-based testing is also appropriate and acceptable for monitoring and trend evaluation.

4.3.1 MONITORING

The frequency of routine monitoring should be based on the criticality of the finished water, capabilities of the process, and ability to maintain product water quality trends. Monitoring may be adjusted from the initial validation monitoring program when there is sufficient data to support a change (see *6.4 Routine Sampling Plans*).

4.3.2 VALIDATION MAINTENANCE

Maintaining the validated state of control requires a life cycle approach. After the completion of the PQ and release of the water system for use, ongoing activities and programs have to be in place to maintain the validated state of control after the system has been validated and placed into service (see *5.4 Operation, Maintenance, and Control*). This includes unit operation, calibration, corrective maintenance, preventive maintenance, procedures, manuals and drawings, standardization of instruments, process parameter and quality attribute trending, change control, deviations, corrective and preventive actions (CAPA), training, records retention, logbooks, etc.

4.3.3 CHANGE CONTROL

Identification and control of changes made to unit operations and other system components, operation parameters, system sanitization, and laboratory processes or procedures need to be established. Not all changes will require validation follow up, but even minor ones, such as gasket elastomer changes could have an impact on quality attributes. The impact of the change

on process parameters and quality attributes must be identified, evaluated and remediated. This may result in a selective validation activity to demonstrate the ongoing state of control for the system and ability to maintain water quality attributes.

Certain calibration and preventive maintenance activities may be considered routine tasks if they do not impact on system operation or water quality. Replacement of components needs to be carefully evaluated. Replacement of components using exact parts generally does not affect system operation or control. Replacement of components with ones that ▲are not exact parts but have▲ 1S (USP41) similar functional specifications can be performed at risk with the critical specifications (e.g., material of construction, dimensions, flow rate, response factors) having been evaluated and the differences determined to be acceptable and documented within the change control system.

4.3.4 PERIODIC REVIEW

The water system qualification, maintenance history, calibration records, quality and process data, issues with the unit operations and any process variability, change control, and other validation maintenance data should be assessed periodically to determine impact on the state of control.

The review may result in adjustments to operating or sanitization processes, calibration or maintenance plans, or monitoring plans. This may also result in additional testing or repeating certain qualification tasks (re-qualification).

Change to read:

5. DESIGN AND OPERATION OF PURIFIED WATER AND WATER FOR INJECTION SYSTEMS

The design, installation, and operation of systems to produce Purified Water and Water for Injection include similar components, control techniques, and procedures. The quality attributes of the two waters differ in their bioburden expectation, the presence of a bacterial endotoxin requirement for Water for Injection, and in their methods of preparation. The similarities in the quality attributes provide considerable common ground in the design of water systems to meet either requirement. The critical difference is the degree of control of the system and the final purification steps needed to ensure removal of bacteria and bacterial endotoxins and reductions in opportunities for biofilm re-development within those purification steps that could become in situ sources of bacteria and endotoxin in the finished water.

Many aspects of system design and operation relate to control and elimination of biofilm. Unit operations can cause the deterioration of water microbial attributes and the formation of biofilm on unit operation surfaces, even when properly maintained (see *8.2 Biofilm Formation in Water Systems*).

Production of pharmaceutical water involves sequential unit operations (processing steps) that address specific water quality attributes and protect the operation of subsequent treatment steps. A typical evaluation process for selecting an appropriate water quality for a particular pharmaceutical purpose is shown in the decision trees in *Figure 2a* and *Figure 2b*. This diagram may be used to assist in defining requirements for specific water uses and in the selection of unit operations. The final unit operation used to produce Water for Injection is limited to distillation or other processes equivalent or superior to distillation in the removal of chemical impurities as well as microorganisms and their components, such as bacterial endotoxins. Distillation coupled with suitable pretreatment technologies has a long history of generally reliable performance (though not completely infallible) and can be validated as a unit operation for the production of Water for Injection. Other combinations of purification technologies may also be suitable in the production of Water for Injection if they can be shown through validation to be as effective and reliable as distillation in the removal of chemicals and microorganisms. The development of new designs and materials of construction for other technologies (such as reverse osmosis, electrodeionization, and ultrafiltration) that allow intermittent or continuous operation at hot bactericidal conditions show promise for a valid use in producing Water for Injection.

5.1 Unit Operations Considerations

To achieve the quality attributes for pharmaceutical waters, multiple-unit operations are required. The design of the water purification system needs to take into consideration different aspects, including the source water quality, sanitization, pharmaceutical water quality attributes, uses of the water, and maintenance programs. Each unit operation contributes specific purification attributes associated with chemical and microbiological parameters.

The following is a brief description of selected unit operations and the design, installation, operation, maintenance, and monitoring parameter considerations associated with them. Not all unit operations are discussed, nor are all potential shortcomings addressed.

5.1.1 PREFILTRATION

The purpose of prefiltration—also referred to as initial, coarse, particulate, or depth filtration—is to remove solid contaminants from the incoming source water supply and protect downstream system components from particulates that can inhibit equipment performance and shorten their effective life. This coarse filtration technology primarily uses sieving effects for particle capture and a depth of filtration medium that has a high “dirt load” capacity. Such filtration units are available in a wide range of designs and for various applications. Removal efficiencies and capacities differ significantly, from granular bed filters such as multimedia or sand for larger water systems, to depth cartridges for smaller water systems. Unit and system configurations vary widely in the type of filtering media and the location in the process. Granular or cartridge prefilters are often situated at the beginning of the water purification system prior to unit operations designed to remove the source water disinfectants. Cartridge-type coarse filters may also be used to capture fines released from granular beds such as activated carbon and deionization beds. These locations, however, do not preclude the need for periodic microbial evaluation.

Design and operational issues that may impact the performance of depth filters include channeling of the filtering media, blockage from silt, microbial growth, and filtering-media loss during improper backwashing. Control methods involve pressure

and flow monitoring during use and backwashing, sanitizing, and replacing filtering media. An important design concern is sizing of the filter to prevent channeling or media loss resulting from inappropriate water flow rates as well as proper sizing to minimize excessively frequent or infrequent backwashing or cartridge filter replacement.

5.1.2 ACTIVATED CARBON

Activated carbon beds, depending on the type and placement, are used to adsorb low-molecular-weight organic material, bacterial endotoxins, and oxidizing additives such as chlorine and chloramine compounds, removing them from the water. They are used to achieve certain quality attributes and to protect against reactions with downstream unit operations, stainless steel surfaces, resins, and membranes.

The chief operating concerns regarding activated carbon beds include the propensity to support bacterial growth, the potential for hydraulic channeling, the organic adsorption capacity, and insufficient contact time. Operation deficiencies may result in the release of bacteria, endotoxins, organic chemicals, and fine carbon particles.

Control measures may involve monitoring water flow rates and differential pressures, sanitizing with hot water or steam, backwashing, testing for adsorption capacity, and frequent replacement of the carbon bed. Monitoring of carbon bed unit operation may also include microbial loading, disinfectant chemical reduction, and TOC if used for TOC reduction. The use of hot water or steam for carbon bed sanitization is ineffective if there is channeling rather than even permeation through the bed. Channeling can be mitigated through design and proper flow rates during sanitization.

Microbial biofilm development on the surface of the granular carbon particles can cause adjacent bed granules to agglomerate. This may result in ineffective removal of trapped debris and fragile biofilm during backwashing, and ineffective sanitization.

Alternative technologies to activated carbon beds can be used to avoid their microbial challenges. These include disinfectant-neutralizing chemical additives and intense ultraviolet (UV) light for removal of chlorine, and regenerable organic scavenging deionizing resins for removal of organics.

5.1.3 ADDITIVES

Chemical additives are used in water systems 1) to control microorganisms by use of sanitizing agents, such as chlorine compounds and ozone; 2) to enhance the removal of suspended solids by use of flocculating agents; 3) to remove chlorine compounds; 4) to avoid scaling on reverse osmosis membranes; and 5) to adjust pH for more effective removal of carbonate and ammonia compounds by reverse osmosis. These additives do not constitute “added substances” as long as they are either removed by subsequent processing steps or are otherwise absent from the finished water. Control of additives to ensure a continuously effective concentration and subsequent monitoring to ensure their removal should be designed into the system and included in the monitoring program.

5.1.4 ORGANIC SCAVENGERS

Organic scavenging devices use macroreticular, weakly basic anion-exchange resins capable of removing negatively charged organic material and endotoxins from the water. Organic scavenger resins can be regenerated with appropriate biocidal caustic brine solutions. Operating concerns are associated with organic scavenging capacity; particulate, chemical, and microbiological fouling of the reactive resin surface; flow rate; regeneration frequency; and shedding of fines from the fragile resins. Control measures include TOC testing of influent and effluent, backwashing, monitoring hydraulic performance, and using downstream filters to remove resin fines.

5.1.5 SOFTENERS

Water softeners may be located either upstream or downstream of disinfectant removal units. They utilize sodium-based cation-exchange resins to remove water-hardness ions, such as calcium and magnesium, that could foul or interfere with the performance of downstream processing equipment such as reverse osmosis membranes, deionization devices, and distillation units. Water softeners can also be used to remove other lower affinity cations, such as the ammonium ion, that may be released from chloramine disinfectants commonly used in drinking water. If ammonium removal is one of its purposes, the softener must be located downstream of the disinfectant removal operation. Water softener resin beds are regenerated with concentrated sodium chloride solution (brine).

Concerns include microorganism proliferation, channeling, appropriate water flow rates and contact time, ion-exchange capacity, organic and particulate resin fouling, organic leaching from new resins, fracture of the resin beads, resin degradation by excessively chlorinated water, and contamination from the brine solution used for regeneration.

Control measures involve recirculation of water during periods of low water use; periodic sanitization of the resin and brine system; use of microbial control devices (e.g., UV light and chlorine); locating the unit upstream of the disinfectant removal step (if used only for softening); appropriate regeneration frequency; effluent chemical monitoring (e.g., hardness ions and possibly ammonium); and downstream filtration to remove resin fines. If a softener is used for ammonium removal from chloramine-containing source water, then the capacity, contact time, resin surface fouling, pH, and regeneration frequency are very important.

5.1.6 DEIONIZATION

Deionization (DI) and continuous electrodeionization (CEDI) are effective methods of improving the chemical quality attributes of water by removing cations and anions. DI systems have charged resins that require periodic regeneration with an acid and base. Typically, cation resins are regenerated with either hydrochloric or sulfuric acid, which replace the captured positive ions with hydrogen ions. Anion resins are regenerated with sodium hydroxide or potassium hydroxide, which replace captured negative ions with hydroxide ions. Because free endotoxin is negatively charged, some removal of endotoxin is

achieved by the anion resin. The system can be designed so that the cation and anion resins are in separate or "twin" beds, or they can be blended together to form a "mixed" bed.

The CEDI system uses a combination of ion-exchange materials such as resins or grafted material, selectively permeable membranes, and an electric charge, providing continuous flow (of product and waste concentrate) and continuous regeneration. Water enters both the resin section and the waste (concentrate) section. The resin acts as a conductor, enabling the electrical potential to drive the captured cations and anions through the resin and appropriate membranes for concentration and removal in the waste water stream. As the water passes through the resin, it is deionized to become product water. The electrical potential also separates the water in the resin (product) section into hydrogen and hydroxide ions. This permits continuous regeneration of the resin without the need for regenerant additives. However, unlike conventional deionization, CEDI units must start with water that is already partially purified because they generally cannot achieve the conductivity attribute of Purified Water when starting with the heavier ion load of source water.

Concerns for all forms of DI units include microbial and endotoxin control; chemical additive impact on resins and membranes; and loss, degradation, and fouling of resin. Issues of concern specific to DI units include regeneration frequency and completeness; channeling caused by biofilm agglomeration of resin particles; organic leaching from new resins; complete resin separation for mixed bed regeneration; and bed fluidization air contamination (mixed beds).

Control measures may include continuous recirculation loops, effluent microbial control by UV light, conductivity monitoring, resin testing, microporous filtration of bed fluidization air, microbial monitoring, frequent regeneration to minimize and control microorganism growth, sizing the equipment for suitable water flow and contact time, and use of elevated temperatures. Internal distributor and regeneration piping for DI bed units should be configured to ensure that regeneration chemicals contact all internal bed and piping surfaces and resins.

Rechargeable canisters can be the source of contamination and should be carefully monitored. Full knowledge of previous resin use, minimum storage time between regeneration and use, and appropriate sanitizing procedures are critical factors for ensuring proper performance.

5.1.7 REVERSE OSMOSIS

Reverse osmosis (RO) units use semipermeable membranes. The "pores" of RO membranes are intersegmental spaces among the polymer molecules. They are big enough for permeation of water molecules, but they limit the passage of hydrated chemical ions, organic compounds, and microorganisms. RO membranes can achieve chemical, microbial, and endotoxin quality improvement. Many factors, including pH, temperature, source water hardness, permeate and reject flow rate, and differential pressure across the membrane, affect the selectivity and effectiveness of this permeation. The process streams consist of supply water, product water (permeate), and waste water (reject). Depending on the source water, pretreatment and system configuration variations and chemical additives may be necessary to achieve the desired performance and reliability. For most source waters, a single stage of RO filtration is usually not enough to meet Purified Water conductivity specifications. A second pass of this permeate water through another RO stage usually achieves the necessary permeate purity if other factors such as pH and temperature have been appropriately adjusted and the ammonia from source water that has been previously treated with chloramines is removed.

Concerns associated with the design and operation of RO units include membrane materials that are sensitive to sanitizing agents and to particulate, chemical, and microbial membrane fouling; membrane and seal integrity; and the passage of dissolved gases, such as carbon dioxide and ammonia. Failure of membrane or seal integrity will result in product water contamination. Methods of control involve suitable pretreatment of the influent water stream; appropriate membrane material selection; membrane design and heat tolerance; periodic sanitization; and monitoring of differential pressures, conductivity, microbial levels, and TOC.

The development of RO units that can tolerate sanitizing water temperatures and also operate efficiently and continuously at elevated temperatures has added greatly to their microbial control ability and to the avoidance of biofouling. RO units can be used alone or in combination with DI and CEDI units, as well as ultrafiltration, for operational and quality enhancements.

5.1.8 ULTRAFILTRATION

Ultrafiltration is a technology that is often used near the end of a pharmaceutical water purification system for removing endotoxins from a water stream though upstream uses are possible. Ultrafiltration can use semipermeable membranes, but unlike RO, these typically use polysulfone membranes with intersegmental "pores" that have been purposefully enlarged. Membranes with differing molecular weight "cutoffs" can be created to preferentially reject molecules with molecular weights above these ratings.

Ceramic ultrafilters are another molecular sieving technology. Ceramic ultrafilters are self-supporting and extremely durable; they can be backwashed, chemically cleaned, and steam sterilized. However, they may require higher operating pressures than do membrane-type ultrafilters.

All ultrafiltration devices work primarily by a molecular sieving principle. Ultrafilters with molecular weight cutoff ratings in the range of 10,000–20,000 Da are typically used in water systems for removing endotoxins. This technology may be appropriate as an intermediate or final purification step. As with RO, successful performance is dependent upon pretreatment of the water by upstream unit operations.

Issues of concern for ultrafilters include compatibility of membrane material with heat and sanitizing agents, membrane integrity, fouling by particles and microorganisms, and seal integrity. Control measures involve filter membrane composition, sanitization, flow design (dead end vs. tangential), cartridge replacement, elevated feed water temperature, and monitoring TOC and differential pressure.

5.1.9 MICROBIAL-RETENTIVE FILTRATION

Microbial-retentive membrane filters have a larger effective "pore size" than ultrafilters and are intended to prevent the passage of microorganisms and similarly sized particles without unduly restricting flow. This type of filtration is widely employed within water systems for filtering the bacteria out of both water and compressed gases as well as for vent filters on tanks and stills and other unit operations.

In water systems, a filter's microbial retention characteristics exhibit different phenomena than in other aseptic filtration applications.

The following factors interact to create the retention phenomena for water system microorganisms: the variability in the range and average pore sizes created by the various membrane fabrication processes; the variability of the surface chemistry and three-dimensional structure related to the different polymers used in these filter matrices; and the size and surface properties of the microorganism intended to be retained by the filters. ▲▲ 1S (USP41) In some situations, the appearance of water system microorganisms on the downstream sides of some 0.2- to 0.22-µm rated filters after a ▲▲ 1S (USP41) period of use (days to weeks) seems to support the idea that ▲water-borne microorganisms can penetrate the 0.2- to 0.22-µm rated filters.▲ 1S (USP41) It is not known whether this downstream appearance is caused by ▲exceeding the retentive capabilities of the filters due to high prefiltration bioburden levels of water-borne microorganisms and extended filtration times. These conditions can lead to a "pass-through" phenomenon resulting from tiny cells or less cell "stickiness", or perhaps by a "grow-through" phenomenon in which cells hypothetically replicate their way through the pores to the downstream side.▲ 1S (USP41) Whatever the penetration mechanism, 0.2- to 0.22-µm rated membranes may not be the best choice for some water system uses (see ▲*Sterility Assurance* ⟨1211⟩).▲ (CN 1-May-2018)

Nevertheless, microbial retention success in water systems has been reported with the use of filters rated as 0.2 or 0.1 µm. There is general agreement that, for a given manufacturer, their 0.1-µm rated filters are tighter than their 0.2- to 0.22-µm rated filters. However, comparably rated filters from different manufacturers may not have equivalent performance in water filtration applications because of the different filter materials, different fabrication processes, and nonstandardized microbial retention challenge processes currently used for defining the 0.1-µm filter rating. It should be noted that filters with a 0.1-µm rating may result in a lower flow rate compared to 0.2- to 0.22-µm filters, so whatever filters are chosen for a water system application, the user must verify that they are suitable for their intended application, use period, and use process, including flow rate.

For microbial retentive gas filtrations, the same sieving and adsorptive retention phenomena are at work as in liquid filtration, but the adsorptive phenomenon is enhanced by additional electrostatic interactions between the particles and filter matrix. These electrostatic interactions are so strong, particle retention for a given filter rating is significantly more efficient in gas filtration than in water or product-solution filtrations. These additional adsorptive interactions render filters rated at 0.2–0.22 µm unquestionably suitable for microbial retentive gas filtrations. When microbial retentive filters are used in these applications, the membrane surface is typically hydrophobic (non-wettable by water). A significant area of concern for gas filtration is blockage of tank vents by condensed water vapor, which can cause mechanical damage to the tank. Control measures include electrical or steam tracing and a self-draining orientation of vent filter housings to prevent accumulation of vapor condensate. However, a continuously high filter temperature will take an oxidative toll on polypropylene components of the filter, so sterilization of the unit prior to initial use, and periodically thereafter, as well as regular visual inspections, integrity tests, and filter cartridge changes are recommended control methods.

In water applications, microbial retentive filters may be used downstream of unit operations that tend to release microorganisms or upstream of unit operations that are sensitive to microorganisms. Microbial retentive filters may also be used to filter water feeding the distribution system. It should be noted that regulatory authorities allow the use of microbial retentive filters within distribution systems or even at use points if they have been properly validated and are appropriately maintained. A point-of-use filter should only be intended to "polish" the microbial quality of an otherwise well-maintained system and not to serve as the primary microbial control device. The efficacy of system microbial control measures can only be assessed by sampling the water upstream of the filters. As an added measure of protection, in-line UV lamps, appropriately sized for the flow rate (see *5.3 Sanitization*), may be used just upstream of microbial retentive filters to inactivate microorganisms prior to their capture by the filter. This tandem approach tends to greatly delay potential microbial penetration phenomena and can substantially extend filter service life.

5.1.10 ULTRAVIOLET LIGHT

The use of low-pressure UV lights that emit a 254-nm wavelength for microbial control is discussed in *5.3 Sanitization*, but the application of UV light in chemical purification is also emerging. This 254-nm wavelength is also useful in the destruction of ozone. At wavelengths around 185 nm (as well as at 254 nm), medium-pressure UV lights have demonstrated utility in the destruction of the chlorine-containing disinfectants used in source water as well as for interim stages of water pretreatment. High intensities of ▲185 nm alone or 254 nm▲ 1S (USP41) in combination with other oxidizing sanitants, such as hydrogen peroxide, have been used to lower TOC levels in recirculating distribution systems. The organics are typically converted to carbon dioxide, which equilibrates to bicarbonate, and incompletely oxidized carboxylic acids, both of which can easily be removed by polishing ion-exchange resins.

Areas of concern include inadequate UV intensity and residence time, gradual loss of UV emissivity with bulb age, gradual formation of a UV-absorbing film at the water contact surface, incomplete photodegradation during unforeseen source water hyperchlorination, release of ammonia from chloramine photodegradation, unapparent UV bulb failure, and conductivity degradation in distribution systems using 185-nm UV lights.

Control measures include regular inspection or emissivity alarms to detect bulb failures or film occlusions, regular UV bulb sleeve cleaning and wiping, downstream chlorine detectors (when used for dechlorination), downstream polishing deionizers (when used for TOC reduction), and regular (approximately yearly) bulb replacement. UV lamps generate heat during operation, which can cause failure of the lamps or increase the temperature of the water. Precautions should be in place to ensure that water flow is present to control excessive temperature increase.

5.1.11 DISTILLATION

Distillation units provide chemical and microbial purification via thermal vaporization, mist elimination, and water vapor condensation. A variety of designs is available, including single effect, multiple effect, and vapor compression. The latter two configurations are normally used in larger systems because of their generating capacity and efficiency. Source water controls must provide for the removal of hardness and silica impurities that may foul or corrode the heat transfer surfaces, as well as the removal of those impurities that could volatize and condense along with the water vapor. In spite of general perceptions, even the best distillation process does not ensure absolute removal of contaminating ions, organics, and endotoxins. Most stills are recognized as being able to accomplish at least a 3–4 log reduction in these impurity concentrations. They are highly effective in sterilizing the feed water.

Areas of concern include carryover of volatile organic impurities such as trihalomethanes (see *2. Source Water Considerations*) and gaseous impurities such as ammonia and carbon dioxide, faulty mist elimination, evaporator flooding, inadequate blow down, stagnant water in condensers and evaporators, pump and compressor seal design, pinhole evaporator and condenser leaks, and conductivity (quality) variations during start-up and operation.

Methods of control may involve the following: preliminary steps to remove both dissolved carbon dioxide and other volatile or noncondensable impurities; reliable mist elimination to minimize feed water droplet entrainment; visual or automated high-water-level indication to detect boiler flooding and boil over; use of sanitary pumps and compressors to minimize microbial and lubricant contamination of feed water and condensate; proper drainage during inactive periods to minimize microbial growth and accumulation of associated endotoxin in boiler water; blow down control to limit the impurity concentration effect in the boiler to manageable levels; on-line conductivity sensing with automated diversion to waste to prevent unacceptable water upon still start-up or still malfunction from getting into the finished water distribution system; and periodic testing for pinhole leaks to routinely ensure that condensate is not compromised by nonvolatized source water contaminants.

5.1.12 STORAGE TANKS

Storage tanks are included in water distribution systems to optimize processing equipment capacity. Storage also allows for routine maintenance within the purification system while maintaining continuous supply to meet manufacturing needs. Design and operation considerations are needed to prevent or minimize the development of biofilm, to minimize corrosion, to aid in the use of chemical sanitization of the tanks, and to safeguard mechanical integrity.

Areas of concern include microbial growth or corrosion due to irregular or incomplete sanitization and microbial contamination from unalarmed rupture disk failures caused by condensate-occluded vent filters.

Control considerations may include using closed tanks with smooth interiors, the ability to spray the tank headspace using spray balls on recirculating loop returns, and the use of heated, jacketed/insulated tanks. This minimizes corrosion and biofilm development and aids in thermal or chemical sanitization. Storage tanks require venting to compensate for the dynamics of changing water levels. This can be accomplished with a properly oriented and heat-traced filter housing fitted with a hydrophobic microbial retentive membrane filter affixed to an atmospheric vent. Alternatively, an automatic membrane-filtered compressed gas blanketing system may be used. In both cases, rupture disks equipped with a rupture alarm device should be used as a further safeguard for the mechanical integrity of the tank.

5.1.13 DISTRIBUTION SYSTEMS

Distribution system configuration should allow for the continuous flow of water in the piping by means of recirculation. Use of no recirculating, dead-end, or one-way systems or system segments should be avoided whenever possible. If not possible, these systems should be flushed periodically and monitored more closely. Experience has shown that continuously recirculated systems are easier to maintain. Pumps should be designed to deliver fully turbulent flow conditions to facilitate thorough heat distribution (for hot-water sanitized systems) as well as thorough chemical sanitant distribution. Turbulent flow also appears to either retard the development of biofilms or reduce the tendency of those biofilms to shed bacteria into the water. If redundant components, such as pumps or filters, are used, they should be configured and used to avoid microbial contamination of the system.

Components and distribution lines should be sloped and fitted with drain points so that the system can be completely drained. In distribution systems, dead legs and low-flow conditions should be avoided, and valved tie-in points should have length-to-diameter ratios of six or less. In systems that operate at self-sanitizing temperatures, precautions should be taken to avoid cool points where biofilm development could occur. If drainage of components or distribution lines is intended as a microbial control strategy, they should also be configured to be dried completely using dry compressed gas because drained but still moist surfaces will still support microbial proliferation. Water exiting from the distribution system should not be returned to the system without first passing through all or a portion of the purification system.

The distribution design should include the placement of sampling valves in the storage tank and at other locations, such as in the return line of the recirculating water system. Direct connections to processes or auxiliary equipment should be designed to prevent reverse flow into the controlled water system. Hoses and heat exchangers that are attached to points of use to deliver water must not chemically or microbiologically degrade the water quality. The distribution system should permit sanitization for microorganism control. The system may be continuously operated at sanitizing conditions or sanitized periodically.

5.1.14 NOVEL/EMERGING TECHNOLOGIES

New water treatment technologies are being developed continuously. Before these technologies are utilized in pharmaceutical water systems, they should be evaluated for acceptable use in a GMP environment. Other considerations should include the treatment process, reliability and robustness, use of added substances, materials of construction, and ability to validate. Consideration should be given to recognize the areas of concern during the evaluation and to identify control measures for the technology. This should include impact on chemical and microbial attributes.

5.2 Installation, Materials of Construction, and Component Selection

Installation techniques are important because they can affect the mechanical, corrosive, and sanitary integrity of the system. Valve installation should promote gravity drainage. Pipe supports should provide appropriate slopes for drainage and should be designed to support the piping adequately under worst-case thermal and flow conditions. The methods of connecting system components—including units of operation, tanks, and distribution piping—require careful attention to preclude potential operational and microbial problems.

Stainless steel welds should provide reliable joints that are internally smooth and corrosion-free. Low-carbon stainless steel, compatible wire filler where necessary, inert gas, automatic welding machines, and regular inspection and documentation help to ensure acceptable weld quality. Follow-up cleaning and passivation of metal surfaces after installation are important for removing contamination and corrosion products and to re-establish the passive corrosion-resistant surface.

Plastic materials can be fused (welded) in some cases, and also require smooth, uniform internal surfaces. Adhesive glues and solvents should be avoided due to the potential for voids and organic extractables. Mechanical methods of joining, such as flange fittings, require care to avoid the creation of offsets, gaps, penetrations, and voids. Use of plastic materials may contribute to TOC levels.

Control measures include good alignment, properly sized gaskets, appropriate spacing, uniform sealing force, and the avoidance of threaded fittings.

Materials of construction should be selected to be compatible with control measures such as sanitizing, cleaning, or passivation. Temperature rating is a critical factor in choosing appropriate materials because surfaces may be required to handle elevated operating and sanitization temperatures. If chemicals or additives will be used to clean, passivate, or sanitize the system, materials resistant to these chemicals or additives must be utilized. Materials should be capable of handling turbulent flow and elevated velocities without erosion of the corrosion-resistant film (such as the passive chromium oxide surface of stainless steel) or reduction in wall thickness for plastics. The finish on metallic materials such as stainless steel, whether it is a refined mill finish, polished to a specific grit, or an electropolished treatment, should complement the system design and provide satisfactory corrosion and microbial activity resistance. The finish should also be a material that can be chemically sanitized. Auxiliary equipment and fittings that require seals, gaskets, diaphragms, filter media, and membranes should exclude materials that permit the possibility of extractables, shedding, and microbial activity. Insulating materials exposed to stainless steel surfaces should be free of chlorides to avoid the phenomenon of stress corrosion cracking that can lead to system contamination and the destruction of tanks and critical system components.

Specifications are important to ensure proper selection of materials and to serve as a reference for system qualification and maintenance. Information such as the manufacturer's metallurgical reports for stainless steel and reports of composition, ratings, and material-handling capabilities for nonmetallic substances should be reviewed for suitability and retained for reference. Component (auxiliary equipment) selection should be made with assurance that it does not create a source of contamination intrusion. Heat exchangers should be constructed to prevent leakage of heat transfer medium into the pharmaceutical water and, for heat exchanger designs where prevention may fail, there should be a means to detect leakage. Pumps should be of sanitary design with seals that prevent contamination of the water. Valves should have smooth internal surfaces with the seat and closing device exposed to the flushing action of water, such as occurs in diaphragm valves. Valves with pocket areas or closing devices (e.g., ball, plug, gate, globe) that move into and out of the flow area should be avoided.

5.3 Sanitization

Microbial control in water systems is achieved primarily through sanitization practices. Systems can be sanitized using either thermal or (photo-)chemical means.

5.3.1 THERMAL SANITIZATION

Thermal approaches to system sanitization include periodic or continuously circulating hot water and the use of steam. Temperatures of 65°–80° are most commonly used for thermal sanitization. Continuously recirculating water of at least 65° at the coldest location in the distribution system has also been used effectively in stainless steel distribution systems when attention is paid to uniformity and distribution of such self-sanitizing temperatures. These techniques are limited to systems that are compatible with the higher temperatures needed to achieve sanitization. Frequent use of thermal sanitization at appropriate temperatures should eliminate the need for other sanitization methods.

The use of thermal methods at temperatures above 80° is contraindicated because it does not add to microbial control of the system or reduction of biofilm. Some methods (e.g., steam sanitizing, hot water circulation at temperatures ≥100°) can be less effective or even destructive because of the need to eliminate condensate or manipulate system components, stress materials of construction, deform filters, and its adverse impact on instrumentation.

Although thermal methods control biofilm development by either continuously inhibiting its growth or, in intermittent applications, by killing the microorganisms within developing biofilms, they are not effective in removing established biofilms. Killed but intact biofilms can become a nutrient source for rapid biofilm regrowth after the sanitizing conditions are removed or halted. In cases of infrequent thermal sanitizations that allow biofilm development between treatments, a combination of routine thermal treatment and periodic supplementation with chemical sanitization may be more effective. The more frequent the thermal sanitization, the more likely it is that biofilm re-development can be eliminated.

5.3.2 CHEMICAL SANITIZATION

Chemical methods, where compatible, can be used on a wider variety of construction materials. These methods typically use oxidizing agents such as ozone, hydrogen peroxide, peracetic acid, or combinations thereof. Halogenated compounds can be effective sanitizers but are less aggressive oxidizing agents and may be difficult to flush from the system. Chemical agents may not penetrate the full biofilm matrix or extend into all biofilm locations (such as crevices at gasketed fittings) and may

leave biofilms incompletely inactivated. Compounds such as ozone, hydrogen peroxide, and peracetic acid oxidize bacteria and biofilms with reactive peroxides and by forming very reactive free radicals (notably hydroxyl radicals). The short half-life of ozone in particular, and its limitation on achievable concentrations, require that it be added continuously during the sanitization process. Hydrogen peroxide and ozone rapidly degrade to water and/or oxygen, and peracetic acid degrades to oxygen and acetic acid. The ease of degradation of ozone to oxygen using 254-nm UV lights in circulating loops allows it to be used effectively on a continuously sanitizing basis in holding tanks and on an intermittent basis (e.g., daily or weekly) in the distribution loops. The highly reactive nature of ozone requires the use of system materials and components that are even more oxidation resistant than those typically used with the other oxidizing agents.

It is important to note that microorganisms in a well-developed biofilm can be extremely difficult to kill, even by using aggressive oxidizing chemicals. The less developed and therefore thinner the biofilm, the more effective the biofilm inactivation. Therefore, optimal microbial control is achieved by using oxidizing chemicals at a frequency that does not permit significant biofilm development between treatments.

Validation of chemical sanitization requires demonstration of adequate chemical concentrations throughout the system, exposure to all wetted surfaces including the body of use point valves, and complete removal of the sanitant from the system at the completion of treatment. Methods validation for the detection and quantification of residues of the sanitant or its objectionable degradants is an essential part of the validation program.

5.3.3 UV SANITIZATION

In-line UV light at a wavelength of 254 nm can also be used to continuously "sanitize" only the water circulating in the system, but these devices must be properly sized for the water flow. Such devices inactivate a high percentage (but not 100%) of microorganisms that flow through the device but cannot be used to directly control existing biofilm upstream or downstream of the device. However, when coupled with conventional thermal or chemical sanitization technologies or located immediately upstream of a microbially retentive filter, UV light is most effective and can prolong the interval between needed system re-sanitizations.

5.3.4 SANITIZATION PROCEDURES

Sanitization steps require validation to demonstrate the ability to reduce and hold microbial contamination at acceptable levels. Validation of thermal methods should include a heat distribution study to demonstrate that sanitization temperatures are achieved throughout the system, including the body of use point valves; sampling ports; instrument side branches; and fittings, couplings, and adapters, relying on water convection and thermal conduction through system materials for heat transfer to wetted surfaces.

The routine frequency of sanitization should be supported by the results of system microbial monitoring. Conclusions derived from trend analysis of the microbiological data should be used as the alert mechanism for the need for extraordinary maintenance. The routine frequency of sanitization should be established in such a way that the system operates in a state of microbiological control and does not regularly exceed Alert and Action Levels (see *9.4 Defining Alert and Action Levels and Specifications*).

5.4 Operation, Maintenance, and Control

A preventive maintenance program should be established to ensure that the water system remains in a state of control. The program should include 1) procedures for operating the system, 2) monitoring programs for critical quality attributes and operating conditions including calibration of critical instruments, 3) schedule for periodic sanitization, 4) preventive maintenance of components, and 5) control of changes to the mechanical system and to operating conditions.

5.4.1 OPERATING PROCEDURES

Operating procedures for the water system and for performing routine maintenance and corrective action should be written, and they should also define the point when action is required. The procedures should be well documented, and should detail the function of each job, assign who is responsible for performing the work, describe how the job is to be done, and identify acceptable operating parameters. The effectiveness of these procedures should be assessed during water system validation.

5.4.2 PROCESS MONITORING PROGRAM

A process-monitoring program should establish the critical quality attributes and operating parameters that are documented and monitored. The program may include a combination of in-line sensors and/or automated instruments (e.g., for temperature, TOC conductivity, hardness, and chlorine), automated or manual documentation of operational parameters (e.g., flow rates or pressure drop across a carbon bed, filter, or RO unit), and laboratory tests (e.g., total microbial counts). The frequency of sampling, the requirement for evaluating test results, and the necessity of initiating corrective action should be included.

5.4.3 ROUTINE MICROBIAL CONTROL

Sanitization may be integral to operation and maintenance, and necessary on a routine basis, depending on system design and the selected units of operation, to maintain the system in a state of microbial control. Technologies for sanitization are described above in more detail in *5.3 Sanitization*.

5.4.4 PREVENTIVE MAINTENANCE

A preventive maintenance program should be in effect. The program should establish what preventive maintenance is to be performed, the frequency of maintenance work, and how the work should be documented.

5.4.5 CHANGE CONTROL

The mechanical configuration, operating conditions, and maintenance activities of the water system must be controlled. Proposed changes should be evaluated for their impact on the whole system. The need to requalify the system after changes are made should be determined. After a decision is made to modify a water system, the affected drawings, manuals, and procedures should be revised. Portions or operations of the water system that are affected by the modification should be tested to demonstrate a continued state of control. The extent and duration of testing should be related to the risk impact of the change to the system.

Change to read:

6. SAMPLING

The testing of water samples from a water system is critical to the ongoing control of the system and assessment of the quality of the water being used. If improperly collected, a sample could yield a test result that is unrepresentative of the sample's purpose. This could lead to inaction when remediation is needed or to unnecessary remediation when none is necessary. It could also lead to misinterpretations of product impact. Therefore, properly collecting water samples, understanding their purpose, and establishing appropriate water system sampling plans are essential to water quality control and system control.

6.1 Purposes and Procedures

To assess a particular water ▲▲ 1S (USP41) attribute, a sample of the water usually must be removed from a water system for specific ▲▲ 1S (USP41) attribute testing. ▲The sample needs to be obtained from specific locations that are representative for the purpose being monitored.▲ 1S (USP41) This sample may be analyzed by in-line/on-line instruments or it may be completely removed from the system as a "grab sample" in a container for off-line testing. In-line/on-line testing avoids the exogenous contamination potential of grab samples that could lead to artifactually variable data trends and incorrect decisions on system performance, maintenance, and utilized ▲water,▲ 1S (USP41) as well as initiating fruitless causative investigations. ▲Grab samples may be appropriate where the water in the system is not homogeneous for certain attributes.▲ 1S (USP41)

The data from water testing are generally used for one of two purposes: for process control (PC) of the water purification and distribution system or for ▲release or▲ 1S (USP41) QC of the water being drawn from the system for some application or use. In many cases, depending on the sampling location and sampling process, the resulting data can be used for both PC and QC purposes.

6.1.1 PC SAMPLING

Because PC sampling is intended to reflect the quality of the water behind the valve and within the distribution system, coming from the purification system, or between its purification steps, efforts should be made to avoid contaminating the water as it ▲is▲ 1S (USP41) drawn from the system so that its test results accurately reflect the water quality within the system at that location. This may require the use of strategically located sampling ports, in addition to points of use.

If microbial testing is needed for PC purposes, the sampling valve should have a properly installed, sanitary design that uses vigorous pre-sampling flushing. This flushing shears off fragile biofilm structures growing on surfaces within the valve and water path before the sample is collected. This avoids biasing the microbial count of perhaps pristine water in the system behind that valve. A fully open valve flush (at >8 ft/s velocity within the valve and connector) for at least 30 s typically provides sufficient shear forces to adequately remove any fragile biofilm structures. Additional control measures for preventing sample contamination could also include stringent pre- and post-sampling outlet sanitation, the use of sterile hoses and gaskets or other connectors to direct the water flow, and other measures.

The data from PC sampling indicate how well the system is maintaining the water quality at that sampling location. These data are subsequently used to signal when some extraordinary intervention might be needed, in addition to normal maintenance and system sanitization operations, to restore the system to the expected level of purity.

PC sampling can only be used to indicate the quality of the water being delivered to the points of use (for QC purposes) if it has been shown to be representative of that point-of-use quality. This may be possible with chemical attributes that are typically not affected by the fluid path of the water delivery process, but is generally not possible with microbial attributes, which can be greatly affected by localized biofilms along that fluid path. If this fluid path is not utilized for PC sampling, then the resulting data typically cannot be used for QC purposes.

6.1.2 QC SAMPLING

QC sampling is intended to reflect the quality of water that is being used. These samples should be collected at the true point of use; that is, where the water is delivered for use, not where it leaves the water system. QC sampling must utilize that same delivery path and components utilized for a water transfer during actual water use. This includes the same valves, hoses, heat exchangers, flow totalizers, hard-piped connections, and other components utilized during water use.

In addition to the water transfer components, QC sampling must also use the same water transfer process employed during water use, including the same pre-use outlet and delivery path flushing procedure and the same outlet, fitting, and hose

sanitization practices employed during actual water use. The water delivery process and components used for QC sampling must be identical to manufacturing practices at every system outlet for the QC sample to mimic the quality of water being used by accumulating the same chemical and microbial contaminant levels it would during actual use from that outlet location.

Where permanent connections from the water system to equipment are present, accommodation should be made in the design to collect samples from locations as close to the equipment as possible. For example, samples can be collected from special sample ports or other valves near the equipment connection that allow the collected water sample to accurately reflect the water quality that is used. Where the water transfer conduit is designed and/or definitively treated to eliminate all contaminating influences prior to water transfer through that conduit, PC sampling locations within the distribution system can reflect the quality of the water that is actually used for QC purposes at those permanent connections. However, the success of the design and treatments intended to eliminate these contaminating influences must be verified. This is typically done during water system validation.

Where routine water use practices involve contamination-prone activities, such as no pre-use flushing or poor hose storage/sanitization/replacement practices, these water use practices should be improved to reduce the potential for delivering contaminated water from the water system and for unacceptable QC sample testing results that reflect that same contamination.

6.2 Attributes and Sampling Locations

The tests being performed on the samples are relevant to the sampling location and purpose of the sample. In-process monitoring of nonmonograph attributes may be indicated for specific unit operations. For instance, before and after a softener, it may be important to determine water hardness to verify softener efficacy. Before and after an activated carbon bed/filter, it may be important to verify chlorine or TOC removal and/or reduction or test for an increase in microbial count. Before a distillation unit, it may be important to quantitate the incoming bacterial endotoxin level to ensure that the still is not being over-challenged beyond its typical 3–4 log purification capability. However, once the water is in the distribution system, the compendial attributes of importance typically include at least conductivity, TOC, and microbial count. In Water for Injection systems and other systems or system locations where bacterial endotoxin control is important, endotoxin is also assayed. Other tests may be necessary depending on the intended uses of the water.

6.2.1 CHEMICAL ATTRIBUTES

Dissolved chemical contaminants detected by conductivity or TOC testing tend to be uniformly distributed in the water throughout the water system. However, there are exceptions where localized chemical contamination sources can occur, such as from a coolant-leaking heat exchanger in a sub-loop, or at a point of use, or within a dead leg. These chemical contaminants may only be seen at the associated outlets and not systemically. However, in the absence of localized contamination influences, chemical attributes are candidates for on-line testing at fixed strategic locations within the distribution system, such as near a circulating loop return, and are generally reflective of the same chemical quality at all locations and points of use within the distribution system. Nevertheless, the suitability of the on-line locations of these instruments for QC release purposes must be verified as being representative of the use-point water quality. This is usually done during water system validation.

6.2.2 MICROBIAL ATTRIBUTES

The same uniformity scenario cannot be assumed for microbial attributes. Planktonic organisms in a water sample could have originated from biofilms in the purification or distribution systems releasing more or less uniform levels of planktonic organisms into the circulating water, as detectable in samples from all outlets. However, a local biofilm developing within a water delivery conduit (such as a use-point outlet valve and transfer hose) in an otherwise pristine biofilm-free water system could release planktonic organisms detectable only in water delivered through that conduit. Therefore, QC release samples for assessing the quality of water that is delivered by the system during water use must be collected after the water has traversed the same fluid conduit (including the same preparatory activities such as outlet sanitization and pre-flushing) from the water distribution system to the specific locations where the water is used.

On-line microbial water sampling/testing has value in pharmaceutical water systems only for PC purposes unless the water is taken from the point of use in the same manner as routine water usage, in which case the data can also have a QC release purpose. Microbial counts detected from strategic sampling ports continue to have PC and investigational value, but generally cannot be substituted for QC release testing except in certain scenarios, as described in *6.1.2 QC Sampling*.

6.3 Validation Sampling Plans

The initial sampling plan for a pharmaceutical water system is usually developed for a validation program (see *4. Validation and Qualification of Water Purification, Storage, and Distribution Systems*). This strategy is for characterization of the system's ability to purify, distribute, and deliver pharmaceutical water. Typically, the initial validation sampling is for a short duration (e.g., at least 2–4 weeks) at a high sampling frequency to generate a significant body of data that will allow detection of short-term or localized chemical or microbial quality deviations from all outlets. These data provide an initial assessment of system performance to guide decisions about using the water for operational purposes.

The initial validation sampling plan is re-evaluated when the pharmaceutical water is placed into operation, typically to reduce the amount of data being generated while not compromising the ability to identify anomalous operations/events, especially during the early life cycle of the water system. In the absence of such quality deviations during the initial sampling period, the sampling frequency can be lessened for a period of time (e.g., at least 2–4 additional weeks) to ensure that somewhat longer-term adverse quality trends are not apparent. During this second period of time, the water may be considered for at-risk routine use, pending the acceptable completion of the second validation sampling period. After successful completion, monitoring can eventually be lessened again to what will become the routine sampling plan.

Periodic review of the water system operation and monitoring needs to be performed to assess seasonal source water variability, effectiveness of sanitization, and routine maintenance events. Periodic review should be performed during the complete life cycle of the water system, typically annually, for evidence of longer-term data trends and quality deviations.

The routine sampling plan should be re-evaluated periodically based on the available data to determine the appropriate frequency and sample locations. This review offers an opportunity to improve data evaluation and reduce workloads based on what that data indicate relative to process and quality control. The routine sampling plan should have a rationale for the frequency and locations that are selected to justify how the resulting data will be used to characterize the overall operation of the system and the release of the water for use.

6.4 Routine Sampling Plans

6.4.1 SOURCE WATER SAMPLING

As mentioned in earlier sections, the source water for pharmaceutical water systems must comply with the standards for one of the Drinking Waters listed in the associated compendial water monograph or in *General Notices*. When a municipality or other water authority is providing this Drinking Water, they are required to comply with the local Drinking Water Regulations for the water supplied to a drinking or potable water distribution piping grid for that region. The quality of that water by the time it reaches the pharmaceutical user is dependent on a number of factors including distance from the input source, duration of travel within the piping, and condition of the piping in that potable water distribution grid, any of which could have adversely affected some of its initial chemical and/or microbial attributes. Based on a risk assessment, it may be prudent to verify full compliance with regulations using water collected from sample ports prior to the pretreatment system, or other equivalent Drinking Water outlets within the facility. If the water complies, then continued assurance of compliance could be verified using Drinking Water Regulation test results provided by the water authority or by periodic retesting of selected or all the Drinking Water attributes by the user or by both the user and the water authority. If private sourced water is utilized, it is the user's responsibility to demonstrate full Drinking Water regulation compliance, using water samples from such sampling ports on a periodic basis as determined by a risk analysis.

These pre-pretreatment sampling ports could, at the user's discretion, be used to periodically monitor other source water attributes that could affect specific pretreatment or purification unit operations. Depending on the user's source water quality consistency and a risk assessment of its potential impact on the purification process, the periodically monitored attributes could include microbial count, absence of coliforms, bacterial endotoxin levels, conductivity, TOC, pH, hardness, chlorine, silica, turbidity or silt density index, and others. These data could be useful in investigations and for operational adjustments to critical unit operation parameters and maintenance procedures, or for feedback to the potable water provider if unusual trends are observed.

6.4.2 PRETREATMENT AND PURIFICATION SYSTEM SAMPLING

The location and frequency of sampling from ports within the pretreatment and purification systems may be selected based on a risk analysis of unit operation purpose. The purpose of this sampling is primarily for PC, for example, to ensure maintenance of acceptable unit operation performance, to assess maintenance procedure efficacy, and to investigate the need for remedial action. Quality deviations in the early portions of the purification process can affect unit operation efficiency but usually do not impact the finished water quality or acceptable use.

6.4.3 PURIFIED WATER DISTRIBUTION SYSTEM SAMPLING

Purified Water distribution system sampling is intended to provide continuing assurance of ongoing PC and compliance with the user's finished water chemical and microbiological requirements. Generally, the locations for that sampling and the frequency of testing the specific attributes are a matter of process and quality control consistency, as well as risk tolerance in the event of a deviation.

Depending on the water system design, the chemical attributes of a water system tend to be relatively constant and more uniformly distributed than the microbiological attributes. Therefore, less frequent sampling at only selected locations could be justified for chemical testing based on familiarity with system design and the existence of historically consistent operational data. However, with some purification system designs, the chemical quality could change dramatically in a short period of time (such as from the exhaustion of deionization beds), so frequent or even continuous in-line/on-line monitoring of the chemical attributes would be advisable to be able to recognize and correct the cause of the problem before non-compliant water is produced and used.

For microbial testing, all use points and critical sample ports in a distribution system are typically sampled routinely, including those that are infrequently used by manufacturing. There is no prescribed sampling frequency for Purified Water system outlets, so typical outlet sampling frequencies vary from daily to monthly, with sampling occurring somewhere in the system at least at weekly intervals.

A risk analysis is suggested for determining the sampling plan for a Purified Water system. Factors in this analysis could include (but are not limited to) the test result history for the entire water system as well as specific outlets, the criticality of specific outlets to manufacturing, the usefulness of selected sample ports as indicators of ongoing system control, and the scope of impact on products and activities should an unfavorable test result occur. For the scope of impact, the less frequent the sampling, the more products and processes will be impacted by an unfavorable test result.

6.4.4 WATER FOR INJECTION DISTRIBUTION SYSTEM SAMPLING

The sampling plans for Water for Injection distribution systems (as well as any water system where some level of bacterial endotoxin control is needed) utilize the same general sampling approaches as do Purified Water systems. However, the

regulatory expectations for Water for Injection distribution system sampling plans are more prescriptive because microbial control must be much more stringent as it is related to the bacterial endotoxin attribute. In general, water sampling for microbial and bacterial endotoxin testing is expected to occur daily somewhere in the system, with each outlet being sampled periodically, based on a risk assessment, to characterize the quality of the water.

6.5 Non-Routine Sampling

Non-routine sampling can also be performed on the water system for episodic events or reasons for which the routine sampling plans are insufficient to capture the needed information. Examples include change control purposes such as evaluating potential changes to sampling, testing, maintenance procedures, or system design; data or event excursion investigation purposes; or simply for long-term informational purposes and establishing baselines for future investigational value. The purpose of the non-routine sampling dictates the sampling procedures to be used, the attributes to be tested, and the location and repeating occurrence (if any) of that testing. It should also be noted that such non-routine sampling may be done from sampling ports that may or may not be routinely tested. Sampling ports can be positioned in a water system purely for investigational, non-routine sampling, and as such, they do not need to be part of a routine sampling plan.

Change to read:

7. CHEMICAL EVALUATIONS

7.1 Chemical Tests for Bulk Waters

The chemical attributes of *Purified Water* and *Water for Injection* that were in effect prior to *USP 23* were specified by a series of chemistry tests for various specific and nonspecific attributes with the intent of detecting chemical species indicative of incomplete or inadequate purification. Although these methods could have been considered barely adequate to control the quality of these waters, they nevertheless stood the test of time. This was partly because the operation of water systems was, and still is, based on on-line conductivity measurements and specifications generally thought to preclude the failure of these archaic chemistry attribute tests.

In 1996, USP moved away from these chemical attribute tests, switching to contemporary analytical technologies for the bulk waters Purified Water and Water for Injection. The intent was to upgrade the analytical technologies without tightening the quality requirements. The two contemporary analytical technologies employed were TOC and conductivity. The TOC test replaced the test for *Oxidizable Substances* that primarily targeted organic contaminants. A multi-staged conductivity test that detects ionic (mostly inorganic) contaminants replaced, with the exception of the test for *Heavy Metals*, all of the inorganic chemical tests (i.e., *Ammonia*, *Calcium*, *Carbon Dioxide*, *Chloride*, *Sulfate*).

Replacing the heavy metals attribute was considered unnecessary because 1) the source water specifications (found in the U.S. EPA's NPDWR) for individual heavy metals were tighter than the approximate limit of detection of the *Heavy Metals* test for *USP XXII* Water for Injection and Purified Water (approximately 0.1 ppm), 2) contemporary water system construction materials do not leach heavy metal contaminants, and 3) test results for this attribute have uniformly been negative; there has not been a confirmed occurrence of a singular test failure (failure of only the *Heavy Metals* test with all other attributes passing) since the current heavy metal drinking water standards have been in place.

Total Solids and *pH* were the only tests not covered by conductivity testing. The test for *Total Solids* was considered redundant because the nonselective tests of conductivity and TOC could detect most chemical species other than silica, which could remain undetected in its colloidal form. Colloidal silica in Purified Water and Water for Injection is easily removed by most water pretreatment steps, and even if present in the water, it constitutes no medical or functional hazard except in extreme and rare situations. In such extreme situations, other attribute extremes are also likely to be detected. It is, however, the user's responsibility to ensure fitness for use. If silica is a significant component in the source water, and the purification unit operations could fail and selectively allow silica to be released into the finished water (in the absence of co-contaminants detectable by conductivity), then either silica-specific testing or a total-solids type testing should be utilized to monitor for and control this rare problem.

The *pH* attribute was eventually recognized to be redundant to the conductivity test (which included *pH* as an aspect of the test and specification); therefore, *pH* was discontinued as a separate attribute test.

The rationale used by USP to establish its Purified Water and Water for Injection conductivity specifications took into consideration the conductivity contributed by the two least-conductive former attributes of *Chloride* and *Ammonia*, thereby precluding their failure had those wet chemistry tests been performed. In essence, the *Stage 3* conductivity specifications (see *Water Conductivity* ⟨645⟩*, Bulk Water, Procedure, Stage 3*) were established from the sum of the conductivities of the limit concentrations of chloride ions (from pH 5.0 to 6.2) and ammonia ions (from pH 6.3 to 7.0), plus the unavoidable contribution of other conductivity-contributing ions from water (H^+ and OH^-), dissolved atmospheric carbon dioxide (as HCO_3^-), and an electro-balancing quantity of either sodium (Na^+) or chlorine (Cl^-), depending on the pH-induced ionic imbalance (see *Table 1*). The *Stage 2* conductivity specification is the lowest value in this table, 2.1 µS/cm. The *Stage 1* specifications, designed primarily for on-line measurements, were derived by essentially summing the lowest values in ▲individual (H^+, OH^-, HCO_3^-) and group (Cl^-, Na^+, NH_4^+) of▲ 1S (USP41) contributing ion columns for each of a series of tables similar to *Table 1*, created for each 5° increment between 0° and 100°. For example purposes, the italicized values in *Table 1*, the conductivity data table for 25°, were summed to yield a conservative value of 1.3 µS/cm, the *Stage 1* specification for a nontemperature-compensated, nonatmosphere-equilibrated water sample that actually had a measured temperature of 25°–29°. Each 5° increment in the table was similarly treated to yield the individual values listed in the table of *Stage 1* specifications (see *Water Conductivity* ⟨645⟩*, Bulk Water*).

Table 1. Contributing Ion Conductivities of the Chloride-Ammonia Model as a Function of pH (in atmosphere-equilibrated water at 25°)

Conductivity (µS/cm)								
pH	H^+	OH^-	HCO_3^-	Cl^-	Na^+	NH_4^+	Combined Conductivities	Stage 3 Limit
5.0	3.49	*0*	*0.02*	*1.01*	*0.19*	*0*	4.71	4.7
5.1	2.77	0	0.02	1.01	0.29	0	4.09	4.1
5.2	2.20	0	0.03	1.01	0.38	0	3.62	3.6
5.3	1.75	0	0.04	1.01	0.46	0	3.26	3.3
5.4	1.39	0	0.05	1.01	0.52	0	2.97	3.0
5.5	1.10	0	0.06	1.01	0.58	0	2.75	2.8
5.6	0.88	0	0.08	1.01	0.63	0	2.60	2.6
5.7	0.70	0	0.10	1.01	0.68	0	2.49	2.5
5.8	0.55	0	0.12	1.01	0.73	0	2.41	2.4
5.9	0.44	0	0.16	1.01	0.78	0	2.39	2.4
6.0	0.35	0	0.20	1.01	0.84	0	2.40	2.4
6.1	0.28	0	0.25	1.01	0.90	0	2.44	2.4
6.2	0.22	0	0.31	1.01	0.99	0	2.53	2.5
6.3	0.18	0	0.39	0.63	0	1.22	2.42	2.4
6.4	0.14	0.01	0.49	0.45	0	1.22	2.31	2.3
6.5	0.11	0.01	0.62	0.22	0	1.22	2.18	2.2
6.6	0.09	0.01	0.78	0	0.04	1.22	2.14	2.1
6.7	0.07	0.01	0.99	0	0.27	1.22	2.56	2.6
6.8	0.06	0.01	1.24	0	0.56	1.22	3.09	3.1
6.9	0.04	0.02	1.56	0	0.93	1.22	3.77	3.8
7.0	*0.03*	0.02	1.97	0	1.39	1.22	4.63	4.6

As stated above, this rather radical change to utilizing a conductivity attribute as well as the inclusion of a TOC attribute allowed for on-line measurements. This was a major philosophical change and allowed industry to realize substantial savings. The TOC and conductivity tests can also be performed off-line in the laboratories using collected samples, although sample collection tends to introduce opportunities for adventitious contamination that can cause false high readings. The collection of on-line data is not, however, without challenges. The continuous readings tend to create voluminous amounts of data, where previously only a single data point was available. As stated in *6. Sampling*, continuous in-process data are excellent for understanding how a water system performs during all of its various usage and maintenance events in real time, but this is too much data for QC purposes. Therefore, for example, one can use a justifiable portion of the data (at a designated daily time or at the time of batch manufacturing) or the highest value in a given period as a worst case representation of the overall water quality for that period. Data averaging is generally discouraged because of its ability to obscure short-lived extreme quality events.

7.2 Chemical Tests for Sterile Waters

Packaged/sterile waters present a particular dilemma relative to the attributes of conductivity and TOC. The package itself is the major source of chemicals (inorganics and organics) that leach over time into the packaged water and can easily be detected by the conductivity and TOC tests. The irony of organic leaching from plastic packaging is that before the advent of bulk water TOC testing, when the *Oxidizable Substances* test was the only "organic purity" test for both bulk and packaged/sterile water monographs in *USP*, the insensitivity of that test to many of the organic leachables from plastic and elastomeric packaging materials was largely unrecognized, allowing organic levels in packaged/sterile water to be quite high (possibly many times the TOC specification for bulk water).

Similarly, glass containers can also leach inorganics, such as sodium, which are easily detected by conductivity but poorly detected by the former wet chemistry attribute tests. Most of these leachables are considered harmless based on current perceptions and standards at the rather significant concentrations present. Nevertheless, they effectively degrade the quality of the high-purity waters placed into these packaging systems. Some packaging materials contain more leachables than others and may not be as suitable for holding water and maintaining its purity.

The attributes of conductivity and TOC tend to reveal more about the packaging leachables than they do about the water's original purity. These currently "allowed" leachables could render the sterile packaged versions of originally equivalent bulk water essentially unsuitable for many uses where the bulk waters are perfectly adequate.

Therefore, to better control the ionic packaging leachables, ⟨645⟩ is divided into two sections. The first, *Water Conductivity* ⟨645⟩*, Bulk Water*, applies to *Purified Water*, *Water for Injection*, *Water for Hemodialysis*, and *Pure Steam*, and includes the three-stage conductivity testing instructions and specifications. The second, *Water Conductivity* ⟨645⟩*, Sterile Water*, applies to *Sterile Purified Water*, *Sterile Water for Injection*, *Sterile Water for Inhalation*, and *Sterile Water for Irrigation*. The *Sterile Water* section includes conductivity specifications similar to the *Water Conductivity* ⟨645⟩*, Bulk Water, Procedure, Stage 2* testing approach because it is intended as a laboratory test, and these sterile waters were made from bulk water that already complied with the three-stage conductivity test. In essence, packaging leachables are the primary target analytes of the conductivity specifications in *Water Conductivity* ⟨645⟩*, Sterile Water*. The effect on potential leachables from different container sizes is the rationale for having two different specifications, one for small packages containing nominal volumes of 10 mL or less and another for larger packages. These conductivity specifications are harmonized with the *European Pharmacopoeia* conductivity specifications for Sterile Water for Injection. All monographed waters, except *Bacteriostatic Water for Injection*, have a conductivity specification that directs the user to either the *Bulk Water* or the *Sterile Water* section. For the sterile packaged water monographs, this water conductivity specification replaces the redundant wet chemistry limit tests intended for inorganic contaminants that had previously been specified in these monographs.

Controlling the organic purity of these sterile packaged waters, particularly those in plastic packaging, is more challenging. Although the TOC test can better detect these impurities and therefore can be better used to monitor and control these impurities than the current *Oxidizable Substances* test, the latter has a history of use for many decades and has the flexibility to test a variety of packaging types and volumes that are applicable to these sterile packaged waters. Nevertheless, TOC testing of these currently allowed sterile, plastic-packaged waters reveals substantial levels of plastic-derived organic leachables that render the water perhaps orders of magnitude less organically pure than is typically achieved with bulk waters. Therefore, usage of these packaged waters for analytical, manufacturing, and cleaning applications should only be exercised after the purity of the water for the application has been confirmed as suitable.

▲7.3 Storage and Hold Times for Chemical Tests

Due to the homogeneous nature of chemical impurities in water, unlike the challenges of microbial impurities, the storage requirements and impact of holding times are very practically determined. In general, the chemical purity of high-purity water samples can only degrade over time, possibly generating a failed result of the sample that would have passed if it were tested immediately or on-line. The general fact is that the longer samples are stored, the greater the potential to be adversely impacted by containers or conditions.

For off-line chemical tests of waters, there are no compendial requirements for storage time and conditions. However, the general recommendation is to perform testing as soon as practical to avoid false adverse results. Where possible, store cool and measure as quickly as practical. This reduces the chances that a water sample gets contaminated over time, and this would reduce unwarranted and unnecessary investigations of false positives.

7.3.1 CONTAINERS

When sampling water for off-line analysis, the selection and cleanliness of the container play a significant part in obtaining accurate data. For samples to be tested for chemical impurities according to ⟨645⟩ and *Total Organic Carbon* ⟨643⟩, the proper container should be one that does not contaminate the sample during the storage/hold time. For example, the use and preparation of glass containers could be very acceptable for storing samples for TOC testing, but some glass containers do leach ions over time (hours and days), and they can adversely impact a conductivity test by creating a false positive result—if the storage time is too long. Likewise, there are some polymer materials that can adversely impact the TOC chemical impurity in water. However, many polymer materials are very inert.

In any case, cleanliness of the container is crucial because trace quantities of soaps and fingerprints will adversely impact the chemical purity of the water. Properly cleaned containers are acceptable because chemical impurities are easily rinsed away. Extensive chemical cleaning methods such as acid or caustic rinsing should never be needed. If they are needed, consider replacing the containers.

7.3.2 STORAGE TIME AND CONDITIONS

There are no specific recommendations for storage of samples for water analyses. If there is some trace interaction of the container and water, then generally colder and shorter storage times are better than warmer and longer storage times. Chemical dissolution and reactivity are usually enhanced by increased temperature. Furthermore, time is always an element because the water sample can only get worse in a container, and it never gets better with time.

7.4 Elemental Impurities in Pharmaceutical Waters

Elemental impurities (EI) have the most restrictive limits for Water for Injection used in manufacturing parenterals, in particular large-volume injections (see *Injections and Implanted Drug Products* ⟨1⟩ for a definition of large-volume injections) because of the large dose. The most restrictive permissible daily exposure (PDE) of EI resides with lead, mercury, cadmium, and arsenic. Other EI listed in ⟨232⟩ permit a substantially higher PDE, and are therefore less restrictive.

Water that meets U.S. EPA National Primary Drinking Water Regulations or WHO Drinking Water Guidelines that has been purified by conventional technologies used to produce Water for Injection can comply with ⟨232⟩ for parenterals.

Table 2 shows that source water that meets US EPA NPDWR or WHO Drinking Water Guidelines has maximum contaminant levels (concentration) for lead, mercury, cadmium, and arsenic that are NMT 10 times (1-log) higher than the EI limits for parenterals, based on a daily dose of 2000 mL. For a smaller volume injection, the allowed parenteral daily dose of EI is correspondingly higher. The purification technologies needed to produce Water for Injection that reduce the impurities by a factor of 100 to 1000 will assure compliance with ⟨232⟩, provided there are no elemental impurities added during processing, packaging, delivery, or storage.

Table 2. Elemental Impurity Limits for Drug Products and their Water Components per ⟨232⟩

Element	Parenteral PDE (µg/day)	Parenteral Daily Dose (µg/mL[a])	U.S. EPA National Primary Drinking Water Regulations (µg/mL[b])	WHO Drinking Water Guidelines (µg/mL[b])	Result of 2-Log Reduction of EI Concentration for WFI (µg/mL[c])
Cadmium	2	0.001	0.005	0.003	0.00005
Lead	5	0.0025	0.015	0.01	0.00015
Inorganic arsenic	15	0.0075	0.01	0.01	0.0001
Inorganic mercury	3	0.0015	0.002	0.006	0.00006

[a] Concentration based on a daily dose of 2000 mL, and all drug product elemental impurities coming from the water component.
[b] Drinking Water Regulations state these Maximum Contaminant Levels (MCLs) as mg/L, which equals µg/mL or ppm.
[c] Determined from the greater of the US EPA Regulations column and WHO Guidelines column for each element, then divided by 100 (2–log).

Chemical purification technologies for Purified Water are similarly efficient in removing EI as those for Water for Injection production. Because all sterile waters are prepared from Purified Water or Water for Injection, the assurance of compliance to ⟨232⟩ extends to sterile waters, provided there are no elemental impurities added during processing, packaging, delivery, or storage.

Further discussion can be found in *Pharmacopeial Forum* [see Bevilacqua A, Soli TC, USP Chemical Analysis Expert Committee. Elemental impurities in pharmaceutical waters. *Pharm Forum.* 2013;39(1)].▲ 1S (USP41)

Change to read:

8. MICROBIAL EVALUATIONS

This section of the chapter presents a discussion about the types and sources of microorganisms and whether certain microbes are prone to colonize pharmaceutical water systems. This section also addresses microbiological examination of water samples, including a discussion on recovery methods.

8.1 Microorganism Types

Microorganisms are ubiquitous and their natural habitats are extremely diverse. Based on comparative ribosomal RNA sequencing, the phylogenetic tree of life consists of three domains: Bacteria and Archaea (both prokaryotes), and Eukarya (eukaryotes). Most microorganisms that contaminate pharmaceutical products are prokaryotic bacteria and eukaryotic fungi (yeasts and molds). These microbes are typical isolates from pharmaceutical environments, including the associated personnel, and a few are frank or opportunistic pathogens. Contamination with viruses is a concern in bioprocessing that uses animal cells.

8.1.1 ARCHAEANS

Microbes from the domain Archaea are phylogenetically related to prokaryotes but are distinct from bacteria. Many are extremophiles, with some species capable of growing at very high temperatures (hyperthermophiles) or in other extreme environments beyond the tolerance of any other life form. In general, most extremophiles are anaerobic or microaerophilic chemolithoautotrophs. Because of their unique habitats, metabolism, and nutritional requirements, Archaeans are not known to be frank or opportunistic pathogens, and they are not capable of colonizing a pharmaceutical water system.

8.1.2 BACTERIA

Bacteria are of immense importance because of their rapid growth, mutation rates, and ability to exist under diverse and adverse conditions; ▲some of them are human pathogens.▲ 1S (USP41) Some are very small and can pass through 0.2-µm rated filters. Others form spores, which are not part of their reproductive cycle. Bacterial spore formation is a complex developmental process that allows the organisms to produce a dormant and highly resistant cell in times of extreme stress. Bacterial endospores can survive high temperatures, strong UV irradiation, desiccation, chemical damage, and enzymatic destruction, which would normally kill vegetative bacteria.

Using a traditional cellular staining technique based on cell wall compositional differences, bacteria are categorized into Gram positive and Gram negative, although many sub-groups exist within each category based on genomic similarities and differences.

8.1.2.1 Gram-positive bacteria: Gram-positive bacteria are common in a pharmaceutical manufacturing environment but not in water systems. This is because they are generally not suited to surviving in a liquid environment that has the chemical purity of a pharmaceutical-grade water system. Gram-positive bacteria include the spore-forming bacteria from the genus *Bacillus*,

which are common soil and dust ▲microorganisms,▲ 1S (USP41) and the non-sporulating bacteria from the genera *Staphylococcus*, *Streptococcus*, and *Micrococcus*, which normally colonize human skin and mucous membranes. Other types of Gram-positive bacterial ▲microorganisms▲ 1S (USP41) include organisms from the genera *Corynebacterium*, *Mycobacterium*, *Arthrobacter*, *Propionibacterium*, *Streptomyces*, and *Actinomyces.* This latter group of microbes can be found in various natural habitats including the human skin and soil.

Although Gram-positive bacteria can be detected in pharmaceutical water samples, their recovery is often associated with faulty aseptic technique during sampling or testing, or associated with exogenous contamination sources. Although these non-aquatic microorganisms could be present in source water and could, in rare circumstances, make their way into the early stages of a water purification unit operation, Gram-positive bacteria are not known to colonize water systems. In addition, these microbes will likely be removed by one or more of the purification unit operations prior to the ultimate creation of the pharmaceutical-grade water.

8.1.2.2 Gram-negative bacteria: These types of bacteria are found in soil, water, plants, and animals. Gram-negative bacteria are ▲relevant▲ 1S (USP41) to pharmaceutical manufacturers, primarily due to their production of endotoxins ▲as well as their ability to populate water systems,▲ 1S (USP41) a topic discussed in *8.4 Endotoxin*. Some Gram-negative bacteria prefer aquatic habitats and tend to colonize water systems and other wet environments as biofilms, a topic discussed in *8.2 Biofilm Formation in Water Systems*.

8.1.2.3 Mycoplasma: Organisms from the genus Mycoplasma are the smallest of the bacteria. Unlike other bacteria, these organisms do not have a cell wall and many exist as intracellular or animal▲/plant▲ 1S (USP41) parasites. Mycoplasmas also ▲may▲ 1S (USP41) require specific nutrients for survival, including ▲sterols,▲ 1S (USP41) and they cannot survive in a hypotonic environment such as pure water. Based on these facts, this type of bacteria is not a concern for pharmaceutical-grade water systems.

8.1.3 FUNGI

Fungi are mainly aerobic mesophilic microbes. They exist as unicellular (yeast) and multicellular filamentous (mold) organisms. Molds are often found in wet/moist but usually non-aquatic environments, such as soil and decaying vegetation. ▲Yeasts are often associated with humans and vegetation, and both yeasts and molds also can be found in pharmaceutical environment.▲ 1S (USP41) As mold matures it develops spores, which, unlike bacterial spores, are part of its reproductive cycle and are less resistant to adverse conditions. Mold spores are easily spread through air and materials, and could contaminate water samples.

▲▲ 1S (USP41)

Neither yeasts nor molds are suited for colonization or survival in pharmaceutical water systems. Their recovery is often associated with faulty aseptic technique during sampling or testing, or associated with exogenous contamination sources. These non-aquatic microorganisms, if present in source water, could make their way into the early stages of a water purification system; however, they will likely be removed by one or more of the purification unit operations.

8.1.4 VIRUSES

A virus is a small infectious agent unlike eukaryotes and prokaryotes. This is because viruses have no metabolic abilities of their own. Viruses are genetic elements containing either DNA or RNA that replicate within host cells. Human pathogenic viruses, especially those of fecal origin, could be present in source water. However, they are easily neutralized by typical water purification treatments, such as chlorination. Therefore, it is unlikely that ▲human pathogenic▲ 1S (USP41) viruses will be present or will proliferate (due to the absence of host cells) in pharmaceutical-grade waters.

8.1.5 THERMOPHILES

Thermophiles are heat-loving organisms and can be either bacteria or molds. Thermophilic and hyperthermophilic aquatic microorganisms (see *8.1.1 Archaeans*) require unique environmental and nutritional conditions to survive ▲(e.g., presence of specific inorganic or organic nutrients and their concentrations, extreme pH, presence or absence of oxygen).▲ 1S (USP41) These conditions do not exist in the high-purity water of pharmaceutical water systems, whether ambient or hot, to support their growth. Bacteria that are able to inhabit hot pharmaceutical water systems are invariably found in much cooler locations within these hot systems; for example, within infrequently used outlets, ambient subloops off of hot loops, use-point and sub-loop cooling heat exchangers, transfer hoses and connecting pipes, or dead legs. These bacterial contaminants are the same mesophilic (moderate temperature-loving) types found in ambient water systems and are not thermophiles. Based on these facts, thermophilic bacteria are not a concern for hot pharmaceutical-grade water systems.

8.2 Biofilm Formation in Water Systems

A biofilm is a three-dimensional structured community of sessile microbial cells embedded in a matrix of extracellular polymeric substances (EPS). Biofilms form when bacteria attach to surfaces in moist environments and produce a slimy, glue-like substance, the EPS matrix, while proliferating at that location. This slimy matrix facilitates biofilm adhesion to surfaces as well as the attachment of additional planktonic cells to form a microbial community.

The EPS matrix of biofilms that colonize water systems also facilitates adsorption and concentration of nutrients from the water and retains the metabolites and waste products produced by the embedded biofilm cells, which can serve as nutrients for other biofilm community members.

This EPS matrix is also largely responsible for biofilm's resistance to chemical sanitizers, which must penetrate completely through the matrix to contact and kill the biofilm cells within the matrix. Heat sanitization approaches do not generally have

these EPS matrix penetration difficulties, so they are usually considered superior to chemicals in killing biofilms where materials of construction allow.

The three-dimensional structure of a well-developed biofilm, as well as the biofilm's creation and release of small, motile "pioneer cells" for further colonization, are facilitated through gene expression modulating "quorum sensing" chemicals released in tiny amounts by individual biofilm cells and concentrated to a functional level within this same EPS matrix. So, the EPS matrix of biofilms is primarily responsible for the biofilm's success in colonizing and proliferating in very low nutrient-containing high-purity water systems. The EPS matrix also explains the difficulty in killing and/or removing biofilms from water purification and distribution system surfaces.

8.2.1 BIOFILM-FORMING BACTERIA IN WATER SYSTEMS

Common microorganisms recovered from water system samples include Gram-negative bacteria from the genera *Pseudomonas*, *Ralstonia*, *Burkholderia*, *Stenotrophomonas*, *Comamonas*, *Methylobacterium*, and many other types of *Pseudomonas*-like organisms known collectively as pseudomonads ▲(members of the family *Pseudomonadaceae*).▲ 1S (USP41) These types of microbes, found in soil and source water, tend to colonize all water system distribution and purification system surfaces including activated carbon beds, deionizing resin beds, RO systems, membrane filtration modules, connecting piping, hoses, and valves. If not controlled, they can compromise the functionality of purification steps in the system and spread downstream, possibly forming biofilms on the distribution system surfaces such as tanks, piping, valves, hoses, and other surfaces, from where they can be sheared or otherwise released into the finished water used in processes and products.

Some of the biofilm pseudomonads are opportunistic human pathogens and may possess resistance to commonly used pharmaceutical product preservatives, particularly when embedded in EPS matrix flocs sheared from water system biofilms. Several pseudomonads are also capable of utilizing a wide variety of carbon sources as nutrients, allowing them to colonize austere, adventitious nutrient environments such as water systems. This nutritional diversity also makes them capable of growing to very high numbers in some pharmaceutical products and raw materials, thus leading to product adulteration and potential risk to patient health. Given that these bacteria are commonly found in aqueous environments, endotoxin control for Water for Injection systems (and some Purified Water systems) through biofilm control becomes critical.

8.2.2 NON-BIOFILM-FORMING BACTERIA IN WATER SYSTEMS

Other types of non-pseudomonad Gram-negative bacteria, ▲such as▲ 1S (USP41) the genera *Escherichia*, *Salmonella*, *Shigella*, *Serratia*, *Proteus*, *Enterobacter*, and *Klebsiella*, are used as indicators of fecal contamination. Although some of these bacteria ▲are also plant pathogens, others▲ 1S (USP41) can be human enteric pathogens, ▲and can contaminate potable water supplies.▲ 1S (USP41) These non-pseudomonads are not suited to colonizing or surviving in pharmaceutical water systems owing to the water's chemical purity. In fact, non-pseudomonad enteric bacteria are extremely unlikely contaminants of pharmaceutical water systems unless local sewage and source water controls are not in place. Such controls are required in order to comply with the source water requirements for making USP-grade waters as described in their respective monographs.

8.3 Microorganism Sources

8.3.1 EXOGENOUS CONTAMINATION

Exogenous microbial contamination of bulk pharmaceutical water comes from numerous possible sources, including source water. At a minimum, source water should meet the microbial quality attributes of Drinking Water, which is the absence of fecal coliforms (*E. coli*). A wide variety of other types of microorganisms, chiefly Gram-negative bacteria, may be present in the incoming water. If appropriate steps are not taken to reduce their numbers or eliminate them, these microorganisms may compromise subsequent water purification steps.

Exogenous microbial contamination can also arise from maintenance operations, equipment design, and the process of monitoring, including:

- Unprotected, faulty, or absent vent filters or rupture disks
- Backflow from interconnected equipment
- Non-sanitized distribution system openings for component replacements, inspections, repairs, and expansions
- Inadequate drain air-breaks
- Innate bioburden of activated carbon, ion-exchange resins, regenerant chemicals, and chlorine-neutralizing chemicals
- Inappropriate rinsing water quality after regeneration or sanitization
- Poor sanitization of use points, hard-piped equipment connectors, and other water transfer devices such as hoses
- Deficient techniques for use, sampling, and operation

The exogenous contaminants may not be normal aquatic bacteria but rather microorganisms of soil, air, or even human origin. The detection of non-aquatic microorganisms may be an indication of sampling or testing contamination or a system component failure, which should trigger investigation and remediation. Sufficient care should be given to sampling, testing, system design, and maintenance to minimize microbial contamination from exogenous sources.

8.3.2 ENDOGENOUS CONTAMINATION

Endogenous sources of microbial contamination can arise from unit operations in a water purification system that is not properly maintained and operated. Microorganisms present in source water may adsorb to carbon bed media, ion-exchange resins, filter membranes, and other equipment surfaces, and initiate the formation of biofilms.

Downstream colonization can occur when microorganisms are shed from existing biofilm-colonized surfaces and carried to other areas of the water system. Microorganisms may also attach to suspended particles such as carbon bed fines or fractured resin particles. When the microorganisms become planktonic, they serve as a source of contamination to subsequent purification equipment and to distribution systems.

Another source of endogenous microbial contamination is the distribution system itself. Microorganisms can colonize pipe surfaces, rough welds, misaligned flanges, valves, and dead legs, where they proliferate and form biofilms. Once formed, biofilms can become a continuous source of microbial contamination, which is very difficult to eradicate. Therefore, biofilm development must be managed by methods such as frequent cleaning and sanitization, as well as process and equipment design.

8.4 Endotoxin

▲Bacterial endotoxin is a lipopolysaccharide (LPS) that is a component of the outer cell membrane of Gram-negative bacteria. Endotoxins may occur as collections of LPS molecules associated with living microorganisms, fragments of dead microorganisms, the EPS matrix surrounding biofilm bacteria, or free molecular clusters or micelles containing many lipopolysaccharide molecules. The monomeric form of the endotoxin molecule does not exist in high-purity water because of the molecule's amphipathic nature.▲ 1S (USP41) Some grades of pharmaceutical waters, such as those used in parenteral applications (e.g., Water for Injection, Water for Hemodialysis, and the sterilized packaged waters made from Water for Injection) strictly limit the amount of endotoxins that may be present because these compounds are pyrogenic.

8.4.1 SOURCES

▲▲ 1S (USP41) Endotoxins may be introduced into the system from the source water or may be released from cell surfaces of bacteria ▲in water system biofilms.▲ 1S (USP41) For example, a spike in endotoxin may occur following sanitization as a result of endotoxin release from killed cells. Endotoxin quantitation in water samples is not a good indicator of the level of biofilm development in a water system because of the multiplicity of endotoxin sources.

8.4.2 REMOVAL AND CONTROL

To control endotoxin levels in water systems, it is important to control all potential sources of contamination with Gram-negative bacteria as well as free endotoxin in the water. Contamination control includes the use of upstream unit operations to reduce bioburden from incoming water, as well as engineering controls (e.g., heat sanitization, equipment design, UV sanitizers, filters, material surface, and flow velocity) to minimize biofilm development on piping surfaces and to reduce re-inoculation of the system with free-floating bacteria.

Endotoxin remediation may be accomplished through the normal exclusion or removal action afforded by various unit operations within the treatment system. Examples of endotoxin removal steps in a water purification train include RO, deionization, ultrafilters, ▲distillation,▲ 1S (USP41) and endotoxin-adsorptive filters.

8.5 Test Methods

Microbes in water systems can be detected as exampled in this section or by methods adapted from *Microbial Enumeration Tests* ⟨61⟩, *Tests for Specified Microorganisms* ⟨62⟩, or the current edition of *Standard Methods for the Examination of Water and Wastewater* by the American Public Health Association. This section describes classical culture approaches to bioburden testing, with a brief discussion on rapid microbiological methods.

Every water system has a unique microbiome. It is the user's responsibility to perform method validation studies to demonstrate the suitability of the chosen test media and incubation conditions for bioburden recovery. In general, users should select the method that recovers the highest planktonic microbial counts in the shortest time, thus allowing for timely investigations and remediation. Such studies are usually performed before or during system validation. ▲▲ 1S (USP41)

The steady state condition can take months or even years to be achieved, and can be affected by a change in source water quality, changes in finished water purity by using modified or increasingly inefficient purification processes, changes in finished water use patterns and volumes, changes in routine and preventative maintenance or sanitization procedures and frequencies, or any type of system intrusion (e.g., component replacement, removal, or addition).

8.5.1 MICROBIAL ENUMERATION CONSIDERATIONS

Most microbial contaminants in water systems are found primarily as biofilms on surfaces, with only a very small percentage of the microbiome suspended in the water, or planktonic, at any given time. Although it would seem logical to directly monitor biofilm development on surfaces, current technology for surface evaluations in an operating water system makes this impractical in a GMP environment. Therefore, an indirect approach must be used: the detection and enumeration of planktonic microorganisms that have been released from biofilms. This planktonic microbiome will impact the processes or products where the water is used.

The detection and enumeration of the planktonic microbiome can be accomplished by collecting samples from water system outlets. Planktonic organisms are associated with the presence of biofilms as well as free-floating bacteria introduced into the system (pioneer cells), which may eventually form new biofilms. Therefore, by enumerating the microorganisms in water ▲▲ 1S (USP41) samples, the overall state of control over biofilm development can be assessed. This assessment has historically been accomplished with classical cultural techniques, which are viewed as the traditional method. However, nutritional limitations of the growth media may not satisfy growth requirements of organisms present in the water system that originated from a

biofilm. As a result, traditional cultural methods may only detect a fraction of the biofilm bacteria present in the water sample. Other options are available, such as rapid microbiological methods.

There is no ideal cultural enumeration method that will detect all microorganisms in a water sample, although some media or incubation temperatures may be better than others. However, from a PC perspective, this limitation is acceptable because it is the relative changes in the trends for water sample microbial counts that indicate the state of PC.

▲Consideration should also be given to the timeliness of microbial testing after sample collection. The number of detectable organisms in a sample collected in a sterile, scrupulously clean sample container will usually decrease as time passes. The organisms within the sample may die or adhere to the container walls, reducing the number that can be withdrawn from the sample for testing. The opposite effect can also occur if the sample container is not scrupulously clean and contains a low concentration of nutrients that could promote microbial growth. Because the number of organisms in the water can change over time after sample collection, it is best to test the samples as soon as possible. If it is not possible to test the sample within 2 h of collection, the sample should be held at refrigerated temperatures (2°–8°) and tested within 24 h. In situations where even 24 h is not possible (such as when using off-site contract laboratories), it is particularly important to qualify the microbiological sample hold times and storage conditions to avoid significant changes in the microbial population during sample storage.▲ 1S (USP41)

8.5.2 THE CLASSICAL CULTURAL APPROACH

Classical cultural approaches for microbial testing of water include but are not limited to pour plates, spread plates, membrane filtration, and most probable number (MPN) tests. These methods are generally easy to perform, and provide excellent sample processing throughput. Method sensitivity can be increased via the use of larger sample sizes. This strategy is used in the membrane filtration method. Cultural approaches are further defined by the type of medium used in combination with the incubation temperature and duration. This combination should be selected according to the monitoring needs of a specific water system and its ability to recover the microorganisms of interest, i.e., those that could have a detrimental effect on the products manufactured or process uses, as well as those that reflect the microbial control status of the system.

8.5.2.1 Growth media: The traditional categorization is that there are two basic forms of media available: "high nutrient" and "low nutrient". Those media traditionally categorized as high-nutrient include Plate Count Agar (TGYA), Soybean Casein Digest Agar (SCDA or TSA), and m-HPC Agar (formerly m-SPC Agar). These media are intended for the general isolation and enumeration of heterotrophic or copiotrophic bacteria. Low-nutrient media, such as R2A Agar and NWRI Agar (HPCA), have a larger variety of nutrients than the high-nutrient media. These low-nutrient media were developed for use with potable water due to their ability to recover a more nutritionally diverse population of microorganisms found in these environments. The use of R2A may not be the best choice for high-purity water systems. Even though high-purity water creates an oligotrophic environment, it has been shown empirically that in many high-purity compendial waters, the microbial count disparity between low- and high-nutrient media is dramatically less to nil, compared to potable water. Nevertheless, using the medium that has been demonstrated ▲as acceptable through comparative media analysis is recommended.▲ 1S (USP41)

8.5.2.2 Incubation conditions: Duration and temperature of incubation are also critical aspects of microbiological testing. Classical compendial methods (e.g., ⟨61⟩) specify the use of high-nutrient media, typically incubated at 30°–35° for NLT 48 h. Given the types of microbes found in many water systems, incubation at lower temperatures (e.g., ranges of 20°–25° or 25°–30°) for longer periods (at least 4 days) could recover higher microbial counts than classical compendial methods. Low-nutrient media typically require longer incubation conditions (at least 5 days) because the lower nutrient concentrations promote slower growth. Even high-nutrient media can sometimes yield higher microbial recovery with longer and cooler incubation conditions.

▲8.5.2.3 Selection of method conditions: The decision to test a particular system using high- or low-nutrient media, higher or lower incubation temperatures, and longer or shorter incubation times should be based on comparative cultivation studies using the native microbiome of the water system. The decision to use media requiring longer incubation periods to recover higher counts also should be balanced with the timeliness of results. Detection of marginally higher counts at the expense of a significantly longer incubation period may not be the best approach for monitoring water systems, particularly when the slow growers are not new species but the same as those recovered within shorter incubation times. Some cultural conditions using low-nutrient media lead to the development of microbial colonies that are much less differentiated in colonial appearance, an attribute that microbiologists rely on when selecting representative microbial types for further characterization. The nature of some of the slow growers and the extended incubation times needed for their development into visible colonies also may lead to those colonies becoming dysgonic and difficult to subculture. That could limit their further characterization, depending on the microbial identification technology used. The selection of method parameters should provide conditions that adequately recover microorganisms from the water system, including those that are objectionable for the intended water use.▲ 1S (USP41)

8.5.3 SUGGESTED CLASSICAL CULTURAL METHODS

▲Example methods are presented in *Table 3*.▲ 1S (USP41)

Table ▲3.▲ 1S (USP41) Example Culture Methods

Drinking Water	Pour plate method or membrane filtration method[a]
	Suggested sample volume: 1.0 mL[b]
	Growth medium: ▲Plate Count Agar▲ 1S (USP41)[c]
	Incubation time: 48–72 h[d]
	Incubation temperature: 30°–35°[e]

Table ▲3.▲ 1S (USP41) Example Culture Methods *(continued)*

Purified Water	Pour plate method or membrane filtration method[b]
	Suggested sample volume: 1.0 mL for pour plate or ▲up to▲ 1S (USP41) 100 mL for membrane filtration[b]
	Growth medium: ▲Plate Count Agar▲ 1S (USP41)[c]
	Incubation time: 48–72 h[d]
	Incubation temperature: 30°–35°[e]
Water for Injection	Membrane filtration method[a]
	Suggested sample volume: 200 mL[b]
	Growth medium: ▲Plate Count Agar▲ 1S (USP41)[c]
	Incubation time: 48–72 h[d]
	Incubation temperature: 30°–35°[e]

[a] A membrane filter with a rating of 0.45 µm is generally considered preferable to smaller porosity membranes.
[b] Sample size must be appropriate for the expected microbial count of the water in order to derive statistically valid colony counts.
[c] For optimum recovery, an alternative medium may be more appropriate (e.g., m-HPC, TSA/SCDA, R2A).
[d] For optimum recovery, alternative incubation times may be needed.
[e] For optimum recovery, alternative incubation temperatures may be needed.

For media growth promotion, use at a minimum *Pseudomonas aeruginosa* ATCC 9027 and *Bacillus subtilis* ATCC 6633. Additional organisms should be used to represent those that are considered objectionable and/or typically isolated from the water system (house isolates).

8.5.4 MICROBIAL IDENTIFICATION

In addition to the enumeration of the bioburden in the water, there is a need to identify and/or select certain microbial species that could be detrimental to products or processes. Some bacteria may also be resistant to preservatives and other antimicrobial chemicals used in ▲nonsterile▲ 1S (USP41) liquid and semi-solid products, thus leading to potential product spoilage. For example, *Pseudomonas aeruginosa* and *Burkholderia cepacia*, as well as some other pseudomonads, are known opportunistic pathogens ▲under certain conditions.▲ 1S (USP41) As such, it may be appropriate to consider these species as objectionable microorganisms for the type of water used to manufacture ▲nonsterile▲ 1S (USP41) liquid and semi-solid products. There is a higher risk of infection if these organisms are found in products targeted for susceptible patient populations (e.g., the very young, the very old, and the immunocompromised) or products contacting highly susceptible tissues (e.g., inhaled products or some topical products). However, if the product where the water is used carries an absence specification for a particular pathogenic species that is not capable of living in a high-purity water system ▲(e.g., *Staphylococcus aureus* or *Escherichia coli*),▲ 1S (USP41) then these non-aquatic species should not be candidates for routine ▲recovery testing from water samples.▲ 1S (USP41) For more information, see *Microbiological Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use* ⟨1111⟩, *Microbial Characterization, Identification, and Strain Typing* ⟨1113⟩, and *Microbiological Best Laboratory Practices* ⟨1117⟩.

For PC and QC, it is valuable to know the microbial species present in the normal microbiome of a water system, even if they are not specifically objectionable. If a new species is detected, it may be an indication of a subtle process change or an exogenous intrusion. The identity of the microorganism may be a clue as to its origin and can help with implementation of corrective or preventive action. Therefore, it is industry practice to identify the microorganisms in samples that yield results exceeding established Alert and Action Levels. It is also of value to periodically identify the normal microbiome in a water system, even if counts are below established Alert Levels. This information can provide perspective on the species recoveries from Alert and Action Level excursion samples, indicating whether they are new species or just higher levels of the normal microbiome. Water system isolates may be incorporated into a company culture collection for use in tests such as antimicrobial effectiveness tests, microbial method validation/suitability testing, and media growth promotion. The decision to use ▲water isolates in these studies should be risk-based because many such isolates may not grow well on the high-nutrient media required. And because once adapted to laboratory media, they may not perform like their wild type progenitors.▲ 1S (USP41)

8.5.5 RAPID MICROBIOLOGICAL METHODS

In recent years, new technologies that enhance microbial detection and the timeliness of test results have been adopted by pharmaceutical QC testing labs. Rapid Microbiological Methods (RMM) are divided into four categories: Growth-Based, Viability-Based, ▲Metabolite-Based,▲ 1S (USP41) and Nucleic Acid-Based. Examples of RMM used for the evaluation of microbial quality of water systems include:

- Microscopic visual epifluorescence membrane counting techniques
- Automated laser scanning membrane counting approaches
- Early colony detection methods based on autofluorescence, adenosine triphosphate (ATP) bioluminescence, or vital staining
- Genetic-based detection/quantitation

See *Validation of Alternative Microbiological Methods* ⟨1223⟩ for further information on rapid microbiological methods.

Change to read:

9. ALERT AND ACTION LEVELS AND SPECIFICATIONS

9.1 Introduction

Establishment of Alert and Action Levels for any manufacturing process facilitates appropriate and timely control. In the case of a pharmaceutical water system, the key PC parameters can be specific chemical, physical, and microbiological attributes of the water produced. Typically, most chemical attributes can be determined in real time or in the lab within a few minutes after sample collection. Physical attributes such as the pressure drop across a filter, temperature, and flow rate—which are sometimes considered critical for operation or sanitization of the water system—must be measured in situ during operation. Obtaining timely microbial data is more challenging compared to chemical and physical attributes, often taking several days. This limits the ability to control microbial attributes in a timely manner, and therefore requires a more challenging evaluation of the test results and conservative implementation of PC levels. This section provides guidance on the establishment and use of Alert and Action Levels, as well as Specifications to assess the suitability of the water and the water system for use in production.

9.2 Examples of Critical Parameter Measurements

Examples of measurements and parameters that are important to water system processes and products are described below. The list, which is not intended to be exhaustive or required, contains some examples of parameters that could be measured to demonstrate that the system is in a state of control.

Examples of measurements that could be critical to the purification or sanitization process include:

- Temperature, for thermally sanitized systems
- Percent rejection of an RO system
- Endotoxin levels of feed water to a distillation system
- Chlorine presence immediately prior to an RO system

Examples of measurements that could be critical to the water distribution process include:

- Return/end-of-loop line pressure, to forewarn of ▲the potential to aspirate air or fluids because of▲ 1S (USP41) simultaneous use of too many outlets
- ▲Temperature to assure the self-sanitizing conditions are maintained for a hot water system▲ 1S (USP41)
- Flow rate, to ensure that sufficient water is available for operations

Examples of measurements that could be critical to final water quality include:

- Conductivity
- TOC
- Endotoxin—for Water for Injection systems
- ▲▲ 1S (USP41)
- Bioburden
- Ozone or other chemicals—for chemically sanitized systems

9.3 Purpose of the Measurements

Although the purpose of each measurement varies, the results can be used to provide system performance feedback, often immediately, serving as ongoing PC and product quality indicators. At the same time, the results provide information necessary for making decisions regarding the immediate processing and usability of the water (see *6.1 Purposes and Procedures*). However, some attributes may not be monitored continuously or may have a long delay in data availability (e.g., microbial data). Regardless, both real-time data and data with longer cycle times can be used to properly establish Alert and Action Levels, which can serve as an early warning or indication of a potentially approaching quality shift.

As PC indicators, Alert and Action Levels are trigger points for the potential need for investigation and/or remedial action, to prevent a system from deviating from normal conditions and producing water unsuitable for its intended use. This "intended use" minimum quality is sometimes referred to as a "Specification" or "Limit", and may include limits for conductivity and TOC listed in water monographs, or other specifications required for these waters that have been defined by the user internally.

In all cases, the validity of the data should be verified to ensure that the data are accurate and consistently representative of the water quality in the system, regardless of whether the sample was collected from a sampling port or use point. The resulting data must not be unduly biased, positively or negatively, due to the sampling method, the environment in the vicinity of the sampling location, the test procedure, instrumentation, or other artifacts that could obscure or misrepresent the true quality of the water intended by the purpose of the sampling, i.e., for PC or for QC.

9.4 Defining Alert and Action Levels and Specifications

Data generated from routine water system monitoring should be trended to ensure that the system operates in a state of chemical and microbiological control. To assist with the evaluation of system performance, companies should establish in-process control levels based on historical data or a fraction of the water Specifications (as long as this latter approach yields values with relevance to process performance).

When establishing Alert and Action Levels and Specifications, a two- or three-tier approach is typically used. In a three-tier approach, the typical structure is to establish in-process controls using "Alert Level", "Action Level", and "Specifications". Alert

and Action Levels are used as proactive approaches to system management prior to exceeding Specifications. The criteria for defining and reacting to adverse trends should be set by the user. These levels should be set at values that allow companies to take action to prevent the system from producing water that is unfit for use. Water Specifications or Limits represent the suitability for use of the water.

In a two-tier approach, a combination of the above terminology is used, depending on the parameter to be monitored. For example, if the attribute does have a monograph specification, the two tiers are Alert Level (or Action Level) and Specification. If the attribute does not have a limit/specification, the two tiers are usually Alert Level and Action Level.

A single-tier approach is possible, but this is risky and difficult to manage. With this approach, where the water/system is either acceptable or not acceptable, the single-tier method does not allow for any adjustment, correction, or investigation prior to stopping production.

However, certain sampling locations, such as sampling ports that are not used for manufacturing products or processes, do not represent the finished water quality where a Specification could be applied. In these locations, a two-tier approach (Alert and Action Levels only) could be applied. In some sampling locations, a single PC level might possibly be appropriate, depending on the attribute.

9.4.1 ALERT LEVEL

An Alert Level for a measurement or parameter should be derived from the normal operating range of the water system. Specifically, Alert Levels are based on the historical operating performance under production conditions, and then are established at levels that are just beyond the majority of the normal historical data. The Alert Level for a parameter is often a single value or a range of values, such as:

- Higher than typical conductivity or TOC
- Higher than typical microbial count
- Higher than typical endotoxin level
- Low temperature during thermal sanitization
- pH range control prior to an RO
- Ozone concentration in a storage tank

Various methods, tools, and statistical approaches are available for establishing Alert Levels, and the user needs to determine the approaches that work for their application. Some numerical examples are two or three standard deviations ▲▲ 1S (USP41) (or more) in excess of the mean value, or some percentage above the mean value but below a Specification. An event-based example could be the appearance of a new microorganism or a non-zero microbial count where zero is the norm.

When an Alert Level is exceeded, this indicates that a process or product may have drifted from its normal operating condition or range. Alert Level excursions represent a warning and do not necessarily require a corrective action. However, Alert Level excursions may warrant notification of personnel involved in water system operation, as well as the quality assurance (QA) personnel. Alert Level excursions may also lead to additional monitoring, with more intense scrutiny of the resulting and neighboring data as well as other process indicators.

9.4.2 ACTION LEVEL

An Action Level is also based on the same historical data, but the levels are established at values (or ranges) that exceed the Alert Levels. The values/ranges are determined using the same types of numerical or event-based tools as the Alert Levels, but at different values▲/ranges.▲ 1S (USP41)

In a three-tier approach, it is good practice to select an Action Level that is more than the Alert Level, but less than the Specification to allow the user to make corrective actions before the water would go out of compliance.

Exceeding a quantitative Action Level indicates that the process has allowed the product quality or other critical parameter to drift outside of its normal operating range. An Action Level can also be event-based. In addition to exceeding quantitative Action Levels, some examples of event-based Action Level excursions include, but are not limited to:

- Exceeding an Alert Level repeatedly
- Exceeding an Alert Level in multiple locations simultaneously
- The recovery of specific objectionable microorganisms
- A repeating non-zero microbial count where zero is the norm

If an Action Level is exceeded, this should prompt immediate notification of both QA staff and the personnel involved in water system operations and use, so that corrective actions can be taken to restore the system back to its normal operating range. Such remedial actions should also include investigative efforts to understand what happened and eliminate or reduce the probability of recurrence. Depending on the nature of the Action Level excursion, it may be necessary to evaluate its impact on the water uses during the period between the previous acceptable test result and the next acceptable test result.

9.4.3 SPECIAL ALERT AND ACTION LEVEL SITUATIONS

In new or significantly altered water systems, where there is limited or no historical data from which to derive trends, it is common to establish initial Alert and Action Levels based on equipment design capabilities. These initial levels should be within the process and product Specifications where water is used. It is also common for new water systems, especially ambient water systems, to undergo changes, both chemically and microbiologically, over time as various unit operations (such as RO membranes) exhibit the effects of aging. This type of system aging effect is most common during the first year of use. As the system ages, a steady state ▲microbiome▲ 1S (USP41) (microorganism types and levels) may develop due to the collective effects of system design, source water, maintenance, and operation, including the frequency of re-bedding, backwashing, regeneration, and sanitization. This established or mature ▲microbiome▲ 1S (USP41) may be higher than the one detected when

the water system was new. Therefore, there is cause for the impurity levels to increase over this maturation period and eventually stabilize.

Some water systems are so well controlled microbially—such as continuously or intermittently hot Water for Injection distribution systems—that microbial counts and endotoxin levels are essentially nil or below the limit of reasonable detectability. This common scenario often coincides with a very low Specification that is poorly quantifiable due to imprecision (as much as two-fold variability) of the test methods that may be near their limits of detection. In such systems, quantitative data trending has little value, and therefore, quantitative PC levels also have little value. The non-zero values in such systems could be due to sporadic sampling issues and not indicative of a water system PC deviation; however, if these non-zero values occur repeatedly, they could be indicative of process problems. So, an alternative approach for establishing Alert and Action Levels with these data could be the use of the incident rate of non-zero values, with the occasional single non-zero "hit" perhaps being an Alert Level (regardless of its quantitative value), and multiple or sequential "hits" being an Action Level. Depending on the attribute, perhaps single hits may not even warrant being considered an Alert Level, so only a multiple-hit situation would be considered actionable. It is up to the user to decide on their approach for system control, i.e., whether to use one, two, or three levels of controls for a given water system and sampling location, and whether to establish Alert and Action Levels as quantitative or qualitative hit-frequency values.

9.4.4 SPECIFICATIONS

Water Specifications or Limits are set based on direct potential product and/or process impact and they represent the suitability for use of the water. The various bulk water monographs contain tests for *Conductivity*, *TOC*, and *Bacterial Endotoxins* (for *Water for Injection*). Aside from the monographs for *Water for Hemodialysis* and multiple sterile waters, microbial specifications for the bulk waters are intentionally not included in their monograph tests.

The need for microbial specifications for bulk waters (*Purified Water* and *Water for Injection*) depends on the water use(s), some of which may require strict control (e.g., very low bioburden, absence of objectionable organisms, or low ionic strength) while others may require no specification due to the lack of impact. For example, microbial specifications are appropriate and typically expected for water that is used in product formulations and final equipment rinses. ▲Where the water is used for analytical reagent preparations and the analytical method is not affected by microbial contaminants,▲ 1S (USP41) or for cleaning processes that conclude with a final antimicrobial heat drying or solvent rinsing step, the microbial quality of the water is likely less of a concern. The decision to establish microbial Specifications for bulk pharmaceutical waters should be based on a formal risk assessment of its uses and justified by scientific rationale.

It is very important to understand the chemical and microbial quality of the water in its final form as it is delivered from a water system to the locations where it is used in manufacturing activities and other points of use. The quality of the water within the water system could be compromised if it picks up chemical or microbial contaminants during its delivery from the system to the points of use. These points of use, where cumulative contamination could be present, are the locations where compliance with all the water Specifications is mandated.

As discussed above, compliance with chemical Specifications can be confirmed periodically between uses, immediately prior to use, or even while the water is being utilized in product manufacturing. While the use of RMM may provide for timely microbial data, the use of conventional cultivative microbiological testing usually delays confirmation of microbial compliance until after the water has been used. However, for some applications, this logistical limitation should not eliminate the need for establishing microbial Specifications for this very important raw material.

The manufacturing risk imposed by these logistics accentuates the value of validated microbial control for a water system. It also emphasizes the value of ▲unbiased sampling for microbial monitoring (e.g., influences from technique, hoses, flushing)▲ 1S (USP41) of samples collected from pertinent locations, with evaluation of the resulting data against well-chosen, preferably trend-derived Alert and Action Levels, which can facilitate remedial PC to preclude Specification excursions.

Users should establish their own quantitative microbial Specifications suited to their water uses. But these values should not be greater than 100 cfu/mL for *Purified Water* or 10 cfu/100 mL for *Water for Injection* unless specifically justified, because these values generally represent the highest microbial levels for pharmaceutical water that are still suitable for manufacturing use.

A Specification excursion should prompt an out-of-specification (OOS) investigation. The investigation is performed to determine 1) the root cause of the excursion so that CAPA may be taken for remediation purposes, and 2) assess the impact on affected processes and finished products where the water was used. Product disposition decisions must be made and are dependent on factors that could include:

- Role of water in the product or in-process material
- Chemical or microbial nature of the attribute whose Specification value was exceeded
- Level of product contamination by the water
- Presence of objectionable microorganisms
- Any downstream processing of affected in-process materials that could mitigate the OOS attribute
- Physical and chemical properties of the finished product where the water was used that could mitigate the OOS attribute
- Product administration routes and potentially sensitive/susceptible users

9.4.5 SOURCE WATER CONTROL

The chemical and microbial attributes of the starting source water are important to the ability of the water system to remove or reduce these impurities to meet the finished water Specifications (see *2. Source Water Considerations*). Using the example microbial enumeration methods in *Table* ▲*3*▲ 1S (USP41) a reasonable maximum bacterial Action Level for source water is 500 cfu/mL. This number is derived from U.S. EPA NPDWR where it is used as an Action Level for the water authority indicating the need for improving disinfection and water filtration to avoid the penetration of viral, bacterial, and protozoal pathogens into

the finished Drinking Water. It is not, however, a U.S. EPA heterotrophic plate count Specification or Maximum Contaminant Level (MCL) for Drinking Water.

Nevertheless, of particular importance could be the microbial and chemical quality of this starting water because the water is often delivered to the facility at a great distance from its source and in a condition over which the user has little or no control. High microbial and chemical levels in source water may indicate a municipal potable water system upset, a change in the supply or original water source, a broken water main, or inadequate disinfection, and therefore, potentially contaminated water with objectionable or new microorganisms or coincidental chemical contaminants.

Considering the potential concern about objectionable microorganisms and chemical contaminants in the source water, contacting the water provider about the problem should be an immediate first step. In-house remedial actions could also be needed, including performance of additional testing on the incoming water (as well as the finished water in some cases) or pretreating the water with additional microbial and chemical purification operations (see *5.1 Unit Operations Considerations*).

General Provisions and Requirements Applying to Specifications, Tests, and Assays of the *Food Chemicals Codex*

The General Provisions provide, in summary form, guidelines for the interpretation and application of the standards, tests and assays, and other specifications of the *Food Chemicals Codex* and make it unnecessary to repeat throughout the book those requirements that are pertinent in numerous instances. Where exceptions to the General Provisions are made, the wording in the individual monograph or general test chapter takes precedence and specifically indicates the directions or the intent.

TITLE OF BOOK

The title of this book, including its supplements, is the *Food Chemicals Codex*, Twelfth Edition. It may be abbreviated to *FCC 12*. Where the term *FCC* is used without further qualification in the text of this book, it applies to the *Food Chemicals Codex*, Twelfth Edition.

APPROPRIATE USE OF THE *FOOD CHEMICALS CODEX*

As a compendium that addresses known food ingredients used in food products either in the United States or internationally, the *FCC* has many practical applications in industry, research, and academia. The *FCC* does not, however, provide information on the regulatory status or safety of food chemicals, nor does the presence or absence of standards for a particular food ingredient indicate in any way USP's endorsement (or lack thereof) of that item for use in foods or food processing. It is the responsibility of the user to determine the safety and regulatory status of a particular food ingredient for any specific application.

FCC standards have been developed in cooperation with regulatory authorities and industry in the United States and elsewhere both under the stewardship of the Institute of Medicine and, more recently, USP. While USP makes great efforts to dialog with the U.S. Food and Drug Administration (FDA) regarding creating or revising monograph standards in the *FCC*, USP has no official legislative authority to establish legal requirements for food ingredients in the United States.[1] The *FCC* serves as a resource for companies that manufacture, process, purchase, or use food ingredients and seek to determine appropriate minimum standards for components of their food products.

The structure and format of the *FCC* monographs and informational chapters allow users to quickly access the following types of information:

- General information about food ingredients
- Chemical information specific to food ingredients
- Information regarding laboratory method validation components
- Guidance for establishing and using Good Manufacturing Practices
- Validated testing methods (including enzyme assays and methods that use highly-characterized reference standards)
- Minimum standards for identity, purity, and quality of food ingredients

Food ingredient manufacturers, processors, and purchasers often use the *FCC*'s standards as the basis for establishing minimum requirements for identity, purity, and quality of their ingredients. *FCC* standards are also used to define these parameters within commercial purchase agreements between buyers and sellers of ingredients and food and, thus, help to promote food quality and food safety programs in industry. The validated test methods included in the *FCC* can be used to demonstrate the identity, quality, and purity of food ingredients, or they can be a starting point in developing new test methods. Manufacturers, processors, and purchasers of food ingredients will find these validated test methods useful, as will regulatory agency labs, contract labs, and students of chemistry or food science. In addition to being a resource for purchasing and quality control operations, portions of the *FCC* are useful to quality assurance groups and can serve as references for internal Standard Operating Procedures (SOPs) and quality manuals used by the food industry. The *FCC* is an excellent resource that may be used to provide important information in order

[1] For further information about the legal status of *FCC*, see *Legal Recognition of FCC Standards*, in the *Preface*.

Published August 17, 2020

to ascertain identity, quality, and purity of ingredients. In addition, the *FCC* can be an important part of a food manufacturer or purchaser's comprehensive food quality program and it provides a common basis for evaluations of food ingredients in all aspects of food research and the food industry.

FCC SPECIFICATIONS

FCC specifications are presented in monograph form for each substance or group of related substances. They are designed to ensure that food ingredients have the specified identity and a sufficiently high level of quality to be safe under usual conditions of intended use in foods or in food processing. Thus, *FCC* specifications generally represent acceptable levels of quality and purity of food-grade ingredients available in the United States (or in other countries or instances in which *FCC* specifications are recognized).

Manufacturers, vendors, and users of *FCC* substances are expected to exercise good manufacturing practices (GMPs) (see *General Information*). They are also expected to establish food safety assurance systems such as Hazard Analysis and Critical Control Points (HACCP) to ensure that *FCC* substances are safe and otherwise suitable for their intended use. *FCC* substances must meet applicable regulatory requirements, including microbiological criteria, for safety and quality.

The name of the substance on a container label, plus the designation "Food Chemicals Codex Grade," "FCC Grade," or simply "FCC," is a representation by the manufacturer, vendor, or user of the substance that at the time of shipment, the substance conforms to the specifications in the effective edition of *FCC*, including any *Supplement* that is effective at the time. When an *FCC* substance is available commercially in solution form as a component of a mixture and there is no provision in the *FCC* for such solution or mixture, the manufacturer, vendor, or user may indicate on the label that the product contains substances meeting *FCC* specifications by use of the initials "FCC" after the name of those components that meet the *FCC* specifications. For the labeling of *FCC* substances in which added substances are permitted, see *Added Substances*.

Added Substances *FCC* specifications are intended for application to individual substances and not to proprietary blends or other mixtures. Some specifications, however, allow "added substances" (i.e., functional secondary ingredients such as anti-caking agents, antioxidants, diluents, emulsifiers, and preservatives) intentionally added when necessary to ensure the integrity, stability, utility, or functionality of the primary substance in commercial use.

If an *FCC* monograph allows such additions, each added substance must meet the following requirements: (1) it is permitted for use in foods by the FDA or by the responsible government agency in other countries; (2) it is of appropriate food-grade quality and meets the requirements of the *FCC*, if listed therein; (3) it is used in an amount not to exceed the minimum required to impart its intended technical effect or function in the primary substance; (4) its use will not result in concentrations of contaminants exceeding permitted levels in any food as a consequence of the affected *FCC* primary substance's being used in food; and (5) it does not interfere with the tests and assays prescribed for determining compliance with the *FCC* requirements for the primary substance, unless the monograph for the primary substance has provided for such interferences. Where added substances are specifically permitted in an *FCC* substance, the label shall state the name(s) of any added substance(s).

Adding substances not specifically provided for and mentioned by name or function in the monograph of an *FCC* substance will cause the substance to no longer be designated as an *FCC* substance. Such a combination is a mixture to be described by disclosure of its ingredients, including any that are not *FCC* substances.

Title of Monograph The titles of *FCC* monographs are in most instances the common or usual names. *FCC* specifications apply equally to substances bearing the main titles, synonyms listed under the main titles, and names derived by transposition of definitive words in main titles. The nomenclature used for flavoring agents may not be consistent with other authoritative sources.

Molecular Structures and Chemical Formulas Molecular structures, chemical formulas, and formula weights immediately following titles are included for the purpose of information and are not to be considered an indication of the purity of the substance. Molecular formulas given in specifications, tests, and assays, however, denote the pure chemical entity.

CAS Number If available, Chemical Abstracts Service (CAS) registry numbers are included for informational purposes. Additional *CAS numbers* may be relevant.

INS Numbers If available, numbers adopted by the Codex Alimentarius Commission under the International Numbering System for Food Additives are included for informational purposes.

FEMA Numbers If available, numbers assigned by the Flavor and Extract Manufacturers Association of the United States (FEMA) are included for informational purposes.

UNII The Unique Ingredient Identifier (UNII) is a nonproprietary, free, unique, unambiguous, nonsemantic, alphanumeric identifier based on a substance's molecular structure and/or descriptive information issued through the joint FDA/USP Substance Registration System (SRS) to support health information technology initiatives for substances in drugs, biologics, foods, and devices.

Alternative Analytical Procedures Although the tests and assays described constitute procedures upon which the specifications of the *FCC* depend, analysts are not prevented from applying alternative procedures if supporting data shows that the procedures used will produce results of equal or greater accuracy. In the event of the doubt or disagreement concerning a substance purported to comply with the specifications of the *FCC*, only the methods described herein are applicable and authoritative.

Labeling For purpose of compliance with *FCC* monographs, "labeling" means all labels and other written, printed, or graphic matter (1) on any article of any of its containers or wrappers or (2) accompanying such article, or otherwise provided by vendors to purchasers for purposes of product identification.

Sulfiting agents If an *FCC* substance contains 10 mg/kg or more of any sulfiting agent, the presence of such sulfiting agent shall be indicated on the labeling.

Requirements for Listing Substances in the *FCC*

The *FCC* is intended to be an international compendium of food ingredient standards. The requirements for listing substances in the *FCC* are as follows: (1) the substance is permitted for use in food or in food processing in the United States or in other countries, (2) it is commercially available, and (3) suitable specifications and analytical test procedures are available to determine its identity and purity.

GENERAL SPECIFICATIONS AND STATEMENTS

Certain specifications and statements in the monographs of the *FCC* are not amenable to precise description and accurate determination within narrow limiting ranges. Because of the subjective or general nature of these specifications, good judgment, based on experience, must be used in interpreting and attaching significance to them.

Description Characteristics described and statements made in the *Description* section of a monograph are not requirements, but are provided as information that may assist with the overall evaluation of a food ingredient. The section includes a description of physical characteristics such as color and form and information on stability under certain conditions of exposure to air and light. It may also include odor terms that are general descriptors and do not necessarily indicate the source of the material. Statements in this section may also cover approximate indications of properties such as solubility (see below) in various solvents, pH, melting point, and boiling point, with numerical values modified by "about," "approximately," "usually," "~," and other comparable nonspecific terms.

Function A statement of function is provided to indicate the technical effect(s) of the substance in foods or in food processing or a principle application such as "Nutrient". The statement is not intended to limit in any way the choice or use of the substance or to indicate that it has no other utility. The term "Source of..." is used to describe the function of materials that may, following ingestion, exhibit a functional effect on the human body, in a manner similar to that of some nutrients. These substances are products of an emerging science, and a comprehensive understanding of their beneficial effects has yet to be developed. The inclusion of monographs for these materials should not be interpreted as implying an endorsement of the claimed potential health or other benefits.

Odorless This term, when used in describing a flavoring material, applies to the examination, after exposure to air for 15 min, of about 25 g of the material that has been transferred from the original container to an open evaporating dish of about 100-mL capacity. If the package contains 25 g or less, the entire contents should be examined.

Packaging and Storage Statements in monographs relating to packaging and storage are advisory in character and are intended only as general information to emphasize instances where deterioration may be accelerated under adverse packaging and storage conditions, such as exposure to air, light, or temperature extremes, or where safety hazards are involved. Additionally, to reduce the risk of intentional or accidental introduction of undesirable materials into food substances, containers should be equipped with tamper-resistant closures.

Cool Place A cool place is one where the temperature is between 8° and 15° (46° and 59°F). Alternatively, it may be a refrigerator, unless otherwise specified in the monograph.

Excessive Heat Any temperature above 40° (104°F).

Storage under Nonspecific Conditions Where no specific storage directions or limitations are provided in the individual monograph, the conditions of storage and distribution include protection from moisture, freezing, and excessive heat. Containers should be stored in secure areas when not in use to reduce the possibility of tampering.

Containers The container is the device that holds the substance and that is or may be in direct contact with it. The immediate container is in direct contact with the substance at all times. The closure is a part of the container. Closures should be tamper-resistant and tamper-evident. The container should not interact physically or chemically with the material that it holds so as to alter its strength, quality, or purity. The food ingredient contact surface of the container should comply with relevant regulations promulgated under the Federal Food, Drug, and Cosmetic Act (or with applicable laws and regulations in other countries). Polyunsaturated fats and oils are particularly susceptible to oxidation when stored in metal containers, at elevated temperatures, and/or in open containers. Oxidation can be minimized by storing them in closed, nonmetal containers with minimal headspace or flushed with nitrogen gas.

Light-Resistant Container A light-resistant container is designed to prevent deterioration of the contents beyond the prescribed limits of strength, quality, or purity under the ordinary or customary conditions of handling, shipments, storage, and sale. A colorless container may be made light resistant by enclosing it in an opaque carton or wrapper (see also *Apparatus*, below).

Well-Closed Container A well-closed container protects the contents from extraneous solids and from loss of the chemical under the ordinary or customary conditions of handling, shipment, storage, and sale.

Tight Container A tight container protects the contents from contamination of extraneous liquids, solids, or vapors; from loss of the chemical; and from efflorescence, deliquescence, or evaporation under the ordinary or customary conditions of handling, shipment, storage, and sale, and is capable of tight reclosure.

Product Security Tamper-evident packaging closures and security tags should be used. Containers that appear to have been opened or otherwise altered by unauthorized persons should not be used until the purity of the substance has been confirmed.

Solubility Statements included in a monograph under a heading such as *Solubility in Alcohol* express exact requirements and constitute quality specifications. Statements relating to solubility given in the *Description*, however, are intended as information regarding approximate solubilities only and are not to be considered as exact *FCC*-quality specifications. Such statements are considered to be of minor significance as a means of identification or determination of purity. For those purposes, dependence must be placed upon other *FCC* specifications.

Approximate solubilities given in the *Description* are indicated by the following descriptive terms:

Descriptive Term	Parts of Solvent Required for 1 part of Solute
Very Soluble	less than 1
Freely Soluble	from 1 to 10
Soluble	from 10 to 30
Sparingly Soluble	from 30 to 100
Slightly Soluble	from 100 to 1000
Very Slightly Soluble	from 1000 to 10,000
Practically Insoluble or Insoluble	more than 10,000

Soluble substances, when brought into solution, may show slight physical impurities, such as fragments of filter paper, fibers, and dust particles unless excluded by definite tests or other requirements. Significant amounts of black specks, metallic chips, glass fragments, or other insoluble matter are not permitted.

TESTS AND ASSAYS

Every substance in commerce that claims or purports to conform to *FCC*, when tested in accordance with its tests and assays, meets all of the requirements in the *FCC* monograph defining it.

The methods and analytical procedures described in the *FCC* are designed for use by properly trained personnel in a suitably equipped laboratory. In common with many laboratory procedures, test methods in the *FCC* frequently involve hazardous materials. In performing the test procedures and assays in the *FCC*, safe laboratory practices must be followed. This includes the use of precautionary measures, protective equipment, and work practices consistent with the chemicals and procedures used. Before undertaking any assay or procedures described in the *FCC*, the individual should be aware of the hazards associated with the chemicals and of the procedures and means of protecting against them. Material Safety Data Sheets, which contain precautionary information related to safety and health concerns, are available from manufacturers and distributors of chemicals such as USP and should provide helpful information about the safe use of such chemicals. Certain chemical reagents specified in *FCC* test procedures may be considered to be hazardous or toxic by the Occupational Safety and Health Administration, by the Environmental Protection Agency (under provisions of the Toxic Substances Control Act), or by health authorities in other countries. Where such reagents are specified, the analyst is encouraged to investigate the use of suitable substitute reagents, as appropriate, and to inform the *USP FCC* Liaison (*fcc@usp.org*) of the results so obtained.

Analytical Samples In the description of tests and assays, the approximate quantity of the analytical sample to be used is usually indicated. The quantity actually used, however, should not deviate by more than 10% from the stated amount. Tests or assays sometimes call for a sample taken to be "previously dried." Where a test for *Loss on Drying* or *Loss on Ignition* is included in a monograph, the conditions specified for these procedures are to be used to dry the sample prior to performing the test procedure or assay, unless otherwise specified. Often, the results of tests or assays that do not call for use of a "previously dried" sample are expressed as calculated on the dried, anhydrous, or ignited basis. In such cases, a test for *Loss on Drying, Water*, or *Loss on Ignition* is included in the monograph and the result of such a test is used for the calculation on the dried, anhydrous, or ignited basis, provided that any moisture or other volatile matter in the undried sample does not interfere with the specified test procedures and assays.

In editions of the *FCC* prior to the *Seventh* edition, the terms "exactly," "accurately weighed," and "accurately measured" are used in connection with gravimetric or volumetric measurements and linked directly to a sample weight or volume. These terms indicate that an operation should be carried out within the limits of error prescribed under *Volumetric Apparatus* or *Weights and Balances*, Appendix I. In the *Seventh* edition and each subsequent edition, these terms have been removed from most monographs, to be more concise. Nonetheless, it shall be understood that all quantitative measurements are to be performed "accurately" and in conformance with the provisions in *Volumetric Apparatus* or *Weights and Balances*, Appendix I, unless otherwise indicated by qualifiers such as "about" or by the particular nature of the test procedure.

The word "transfer," when used in describing tests and assays, means that the procedure should be carried out quantitatively.

Apparatus With the exception of volumetric flasks and other exact measuring or weighing devices, directions to use a definite size or type of container or other laboratory apparatus are intended only as recommendations, unless otherwise specified. Where an instrument for physical measurement, such as a thermometer, spectrophotometer, or gas chromatograph, is designated by its distinctive name or trade name in a test or assay, a similar instrument of equivalent or greater sensitivity of accuracy may be employed. An instrument may be substituted for the specified instrument if the substitute uses the same fundamental principles of operation and is of equivalent or greater sensitivity and accuracy. These characteristics must be validated as appropriate. Where low-actinic or light-resistant containers are specified, clear glass containers that have been rendered opaque by application of a suitable coating or wrapping may be used. Where a particular brand or source of a material, instrument, or piece of equipment, or the name and address of the manufacturer, or distributor, is mentioned (ordinarily in a footnote), this identification is furnished solely for informational purposes as a matter of convenience, without implication of approval, endorsement, or certification.

Atomic Weights The atomic weights used in computing formula weights and volumetric and gravimetric factors stated in tests and assays are those recommended in 1991 by the IUPAC Commission on Isotopic Abundances and Atomic Weights.

Blank Tests Where a blank determination is specified in a test or assay, it is to be conducted using the same quantities of the same reagents and by the same procedure repeated in every detail except that the substance being tested is omitted.

A residual blank titration may be stipulated in tests and assays involving a back titration in which a volume of a volumetric solution larger than is required to react with the sample is added, and the excess of this solution is then titrated with a second volumetric solution. Where a residual blank titration is specified or where the procedure involves such a titration, a blank is run as directed in the preceding paragraph. The volume of the titrant consumed in the back titration is then subtracted from the volume required for the blank. The difference between the two, equivalent to the actual volume consumed by the sample, is the corrected volume of the volumetric solution to be used in calculating the quantity of the substance being determined.

Centrifuge Where the use of a centrifuge is indicated, unless otherwise specified, the directions are predicated on the use of the apparatus having an effective radius of about 20 cm (8 in) and driven at a speed sufficient to clarify the supernatant layer within 15 min. If necessary, determine the gravity by using the equation $g = \{[(rpm \times 2 \times \pi)/60] \times r_m\}/980$, in which rpm is the rotor speed and r_m is the mean radius, in cm, of the tube holding the sample in the rotor.

Desiccators and Desiccants The expression "in a desiccator" means using a tightly closed container of appropriate design in which a low moisture content can be maintained by means of a suitable desiccant. Preferred desiccants include anhydrous calcium sulfate, magnesium perchlorate, phosphorus pentoxide, and silica gel.

Filtration Where it is directed to "filter," without further qualification, the intent is that the liquid be filtered through suitable filter paper or an equivalent device until the filtrate is clear.

Identification The tests described under this heading in monographs are designed for application to substances

taken from labeled containers and are provided only as an aid to substantiate identification. These tests, regardless of their specificity, are not necessarily sufficient to establish proof of identity, but failure of a substance taken from a labeled container to meet the requirements of a prescribed identification test means that it does not conform to the requirements of the monograph.

Indicators The quantity of an indicator solution used should be 0.2 mL (approximately 3 drops) unless otherwise directed in a test or assay.

mg/kg and Percent The term "mg/kg" is used in expressing the concentrations of trace amounts of substances, such as impurities, up to 10 mg/kg. Above 10 mg/kg, percent (by weight) is used. For example, a monograph requirement equivalent to 20 mg/kg is expressed as 0.002%, or 0.0020%, depending on the number of significant figures justified by the test specified for use in conjunction with the requirement.

Microbial Limit Tests The *FCC* directly references the procedures in the *FDA Bacteriological Analytical Manual* (BAM) (http://www.fda.gov/Food/default.htm) for its microbial limit tests. Where the sample size is not defined in the limit, the results are based on the sampling procedures described in BAM.

Negligible The term "negligible," as used in some *Residue on Ignition* specifications, indicates a quantity not exceeding 0.5 mg.

Pressure Measurements The term "mm Hg" used with respect to pressure within an apparatus, or atmospheric pressure, refers to the use of a suitable manometer or barometer calibrated in terms of the pressure exerted by a column of mercury of the stated height.

Reagents Specifications for reagents are not included in the *FCC*. Unless otherwise specified, reagents required in tests and assays should conform to the specifications of the current editions of *Reagent Chemicals: American Chemical Society Specifications* or in the section on *Reagent Specifications* in the *United States Pharmacopeia*. Reagents not covered by any of these specifications should be of a grade suitable to the proper performance of the method of test or assay involved.

Acids and Ammonium Hydroxide When ammonium hydroxide, glacial acetic acid, hydrochloric acid, hydrofluoric acid, nitric acid, phosphoric acid, or sulfuric acid is called for in tests and assays, reagents of ACS grade and strengths are to be used. (These reagents sometimes are called "concentrated," but this term is not used in the *FCC*.)

Alcohol, Ethyl Alcohol, Ethanol When one of these substances is called for in tests and assays, use ACS-grade *Ethyl Alcohol* (95%) or USP-grade *Alcohol*.

Alcohol Absolute, Anhydrous Alcohol, Dehydrated Alcohol When one of these substances is called for in tests and assays, use ACS-grade *Ethyl Alcohol, Absolute* or USP-grade *Dehydrated Alcohol*.

Water When water is called for in tests and assays or in the preparation of solutions, it shall have been prepared by distillation, ion-exchange treatment, or reverse osmosis.

Water, Carbon Dioxide-Free When this type of water is called for, it shall have been boiled vigorously for 5 min or more, and allowed to cool while protected from absorption of carbon dioxide from the atmosphere.

"Deaerated water" or "degassed water" is water that has been treated to reduce the content of dissolved air by suitable means, such as by boiling vigorously for 5 min and cooling while protected from air or by the application of ultrasonic vibration.

Reference Standards Test and assay results are determined on the basis of comparison of the test sample with a reference standard that has been freed from or corrected for volatile residues or water content, as instructed on the reference standard label. The requirements for any new *FCC* standards, tests, or assays for which a new USP or *FCC* Reference Standard or Authentic Substance is specified are not in effect until the specified Reference Standard or Authentic Substance is available. If a reference standard is required to be dried before use, transfer a sufficient amount to a clean, dry vessel. Do not use the original container as the drying vessel, and do not dry a reference standard repeatedly at temperatures above 25°. Where the titrimetric determination of water is required at the time a reference standard is to be used, proceed as directed in the *Karl Fischer Titrimetric Method* under *Water Determination*, Appendix IIB. Unless a reference standard bears a specific potency or content, assume that the reference standard is 100% pure in the compendial application. [Directions for use printed on the label text of USP and *FCC* reference standards are lot-specific, and they take precedence over any other indication listed in the *FCC*.]

Significant Figures When tolerance limits are expressed numerically, the values are significant to the number of digits indicated. Record the observed or calculated analytical result with only one digit included in the decimal place to the right of the last place in the limit expression. If this digit is smaller than 5, eliminate it and leave the preceding digit unchanged. If this digit is greater than 5, eliminate it and increase the preceding digit by one. If this digit equals 5, eliminate it and increase the preceding digit by one. For example, a requirement of not less than 96.0% would not be met by a result of 95.94%, but would be met by results of 95.96% or 95.95%, both of which would be rounded to 96.0%. When a range is stated, the upper and lower limits are inclusive so that the range consists of the two values themselves, properly rounded, and all values between them.

Solutions Prepare all solutions, unless otherwise specified, with water prepared by distillation, ion-exchange treatment, reverse osmosis, or as otherwise indicated in the monograph. Expressions such as "1:10" or "10%" mean that *1 part by volume* of a liquid or *1 part by weight* of a solid is to be dissolved in a volume of the diluent or solvent sufficient to make the finished solution 10 parts by volume. Directions for the preparation of colorimetric solutions (CS), test solutions (TS), and volumetric solutions (VS), are provided in the section on *Solutions and Indicators*. Prepare a volumetric solution to have a normality (molarity) within 10% of the stated value and to be standardized to four significant figures. When volumetric equivalence factors are provided in tests and assays, the term "0.X N(M)" is understood to mean a VS having a normality (molarity) of exactly 0.X000 N(M). If the normality (molarity) of the VS employed in a particular procedure differs from 0.X000, apply an appropriate correction factor.

Specific Gravity Numerical values for specific gravity, unless otherwise noted, refer to the ratio of the weight of a substance in air at 25° to that of an equal volume of water at the same temperature. Determine specific gravity by any reliable method, unless otherwise specified.

Temperatures Unless otherwise specified, temperatures are expressed in Celsius (centigrade) degrees, and all measurements are to be made at 25°, unless otherwise directed.

Time Limits Unless otherwise specified, allow 5 min for a reaction to take place when conducting limit tests for trace

impurities such as chloride or iron. Expressions such as "exactly 5 min" mean that the stated period should be accurately timed.

Tolerances Minimum purity tolerance limits presented in monographs neither bar the use of lots of articles that more nearly approach 100% purity nor constitute a basis for a claim that such lots exceed the quality prescribed by the *FCC*. When no maximum assay tolerance is given, the assay should show the equivalent of not more than 100.5%.

Trace Impurities Tests for inherent trace impurities are provided to limit such substances to levels that are consistent with good manufacturing practice and that are safe and otherwise unobjectionable under conditions in which the food additive or ingredient is customarily employed. It is impossible for *FCC* to provide limits and tests in each monograph for the detection of all possible unusual or unexpected impurities, the presence of which would be inconsistent with good manufacturing practice. The limits and tests provided in *FCC* are those considered to be necessary according to currently recognized methods of manufacture and are based on information available to or provided to the Food Ingredients Expert Committee. If other methods of manufacture or other than the usual raw materials are used, or if other possible impurities may be present, additional tests may be required and should be applied, as necessary, by the manufacturer, vendor, or user to demonstrate that the substance is suitable for its intended application. Such tests should be submitted to the USP *FCC* Liaison (*fcc@usp.org*) for consideration for inclusion in the *FCC*.

Vacuum The unqualified use of the term "in vacuum" means a pressure at least as low as that obtainable by an efficient aspirating water pump (not higher than 20 mm Hg).

Water and Loss on Drying In general, for compounds containing water of crystallization or adsorbed water, a limit test, to be determined by the *Karl Fischer Titrimetric Method*, is provided under the heading *Water*. For compounds in which the *Loss on Drying* may not necessarily be attributable to water, a limit test, to be determined by other methods, is provided under the heading *Loss on Drying*.

Weighing Practices

Constant Weight A direction that a substance is to be "dried to constant weight" means that the drying should continue until two consecutive weighings differ by not more than 0.5 mg/g of the sample taken, the second weighing to follow an additional hour of drying. The direction "ignite to constant weight" means that the ignition should be continued at 800 ± 25°, unless otherwise specified, until two consecutive weighings do not differ by more than 0.5 mg/g of the sample taken, the second weighing to follow an additional 15 min of ignition.

Tared Container When a tared container, such as a glass filtering crucible, a porcelain crucible, or a platinum dish, is called for in an analytical procedure, it shall be treated as is specified in the procedure, e.g., dried or ignited for a specified time or to constant weight, cooled in a desiccator as necessary, and weighed accurately.

Weights and Measures, Symbols and Abbreviations: The International System of Units (SI), to the extent possible, is used in most specifications, tests, and assays in this edition of *FCC*. The SI metric units, and other units and abbreviations commonly employed, are as follows:

° = degrees Celsius
kg = kilogram
g = gram
mg = milligram
µg = microgram
ng = nanogram
pg = picogram
L = liter
mL = milliliter
µL = microliter
m = meter
cm = centimeter
dm = decimeter
mm = millimeter
µm = micrometer (0.001 mm)
nm = nanometer
~ = approximately
C = coulomb
A = ampere
V = volt
mV = millivolt
W = watt
dc = direct current
ft = foot
in = inch
in^3 = cubic inch
gal = gallon
lb = pound
oz = ounce
mEq = milliequivalents
mg/kg = parts per million (by weight)
µg/kg = parts per billion (by weight)
ng/kg = parts per trillion (by weight)
psi = pounds per square inch
psia = pounds per square inch absolute
kPa = kilopascal
sp. gr. = specific gravity
b.p. = boiling point
m.p. = melting point
id = inside diameter
od = outside diameter
h = hour
min = minute
s = second
N = normality
M = molarity
mM = millimolar
mmol = millimole
µM = micromolar
µmol = micromole
CFU = colony-forming unit(s)
ACS = American Chemical Society
AOAC = AOAC International
AOCS = American Oil Chemists Society
ASTM = ASTM (American Society for Testing and Materials) International
CAS = Chemical Abstracts Service
CFR = Code of Federal Regulations (U.S.)
FDA = United States Food and Drug Administration
FEMA = Flavor and Extract Manufacturers Association of the United States

INS = International Numbering System of the Codex Alimentarius
IUPAC = International Union of Pure and Applied Chemistry
NIST = National Institute of Standards and Technology
UNII = Unique Ingredient Identifier (as defined by US FDA)

Published August 17, 2020

FCC Monographs

Ethyl Alcohol

Alcohol

Ethanol

C_2H_6O Formula wt 46.07
CAS: [64-17-5]

DESCRIPTION

Ethyl Alcohol occurs as a clear, colorless, mobile liquid. It is miscible with water, with ether, and with chloroform. It boils at about 78° and is flammable. Its refractive index at 20° is about 1.364.

[NOTE—This monograph applies only to undenatured ethyl alcohol.]

Function: Extraction solvent; carrier solvent

Packaging and Storage: Store in tight containers, remote from fire.

ASSAY

• **SPECIFIC GRAVITY:** Determine by any reliable method (see *General Provisions*).

Acceptance criteria: NMT 0.8096 at 25°/25° (equivalent to 0.8161 at 15.56°/15.56°), and equivalent to NLT 94.9% by volume (92.3% by weight) of C_2H_6O

IMPURITIES

Inorganic Impurities

• **LEAD,** *Lead Limit Test, Atomic Absorption Spectrophotometric Graphite Furnace Method, Method I, Appendix IIIB*

Sample: 10 g

Acceptance criteria: NMT 0.5 mg/kg

Organic Impurities

• **FUSEL OIL**

Sample: 10 mL

Analysis: Mix the *Sample* with 1 mL of glycerin and 1 mL of water, and allow to evaporate from a piece of clean, odorless, absorbent paper.

Acceptance criteria: No foreign odor is perceptible when the last traces of alcohol leave the paper.

• **KETONES, ISOPROPYL ALCOHOL**

Sample: 1 mL

Analysis: Transfer the *Sample*, 3 mL of water, and 10 mL of *mercuric sulfate TS* to a test tube; mix; and heat in a boiling water bath.

Acceptance criteria: No precipitate forms within 3 min.

• **METHANOL**

Analysis: To 1 drop of sample in a test tube, add 1 drop of 1:20 phosphoric acid and 1 drop of 50 mg/mL potassium permanganate solution, mix, and allow to stand for 1 min. Add, dropwise, 100 mg/mL sodium bisulfite solution until the permanganate color disappears. If a brown color remains, add 1 drop of the phosphoric acid solution. Add 5 mL of freshly prepared chromotropic acid TS to the colorless solution, and heat it in a water bath at 60° for 10 min.

Acceptance criteria: No violet color appears.

• **SUBSTANCES DARKENED BY SULFURIC ACID**

Sample: 10 mL

Analysis: Transfer 10 mL of sulfuric acid into a small Erlenmeyer flask, cool to 10° and, with constant agitation, add the *Sample*, dropwise.

Acceptance criteria: The mixture is colorless or has no more color than either the acid or the sample before mixing.

• **SUBSTANCES REDUCING PERMANGANATE**

Sample: 20 mL

Analysis: Transfer the *Sample*, previously cooled to 15°, to a glass-stoppered cylinder, add 0.1 mL of 0.1 N potassium permanganate, mix, and allow to stand for 5 min.

Acceptance criteria: The pink color does not entirely disappear.

SPECIFIC TESTS

• **ACIDITY (AS ACETIC ACID)**

Analysis: Transfer 10 mL of sample to a glass-stoppered flask containing 25 mL of water, add 0.5 mL of phenolphthalein TS, and then add 0.02 N sodium hydroxide to the first appearance of a pink color that persists after shaking for 30 s. Add an additional 25 mL of sample, mix, and titrate with 0.02 N sodium hydroxide until the pink color is restored.

Acceptance criteria: NMT 0.5 mL of 0.02 N sodium hydroxide is required to restore the pink color. (NMT 0.003%)

• **ALKALINITY (AS NH_3)**

Sample: 25 mL

Analysis: Add 2 drops of methyl red TS to 25 mL of water, add 0.02 N sulfuric acid until a red color just appears, then add the *Sample*, and mix.

Acceptance criteria: NMT 0.2 mL of 0.02 N sulfuric acid is required to restore the red color. (NMT 3 mg/kg)

• **NONVOLATILE RESIDUE**

Sample: 125 mL (about 100 g)

Analysis: Evaporate the *Sample* to dryness in a tared dish on a steam bath, dry the residue at 105° for 30 min, cool, and weigh.

Acceptance criteria: NMT 0.003%

• **SOLUBILITY IN WATER**

Analysis: Transfer 50 mL of sample to a 100-mL glass-stoppered graduated cylinder, dilute to 100 mL with water, and mix. Place the graduated cylinder, in a water bath maintained at 10°, and allow it to stand for 30 min.

Acceptance criteria: No haze or turbidity develops.

Isopropyl Alcohol

2-Propanol

Isopropanol

C_3H_8O Formula wt 60.10
CAS: [67-63-0]

DESCRIPTION

Isopropyl Alcohol occurs as a clear, colorless, flammable liquid. It is miscible with water, with ethyl alcohol, with ether, and with many other organic solvents.

Function: Extraction solvent

Packaging and Storage: Store in tight containers, remote from fire.

IDENTIFICATION

• **Refractive Index,** *Appendix IIB* [Note—Use an Abbé or other refractometer of equal or greater accuracy.]
Acceptance criteria: 1.377–1.380 at 20°

ASSAY

• **Procedure**
System suitability solution: USP 2-Propanol System Suitability RS
Chromatographic system, *Appendix IIA*
Mode: Gas chromatography
Detector: Flame ionization
Column: 60-m × 0.25-mm fused silica column with 1.4-µm film thickness of 6% cyanopropylphenyl/94% dimethylpolysiloxane stationary phase[1] with a 4-mm straight liner
Temperature
Injector: 150°
Detector: 200°
Column: Hold at 35° for 5 min; ramp to 45° at 1°/min; ramp to 100° at 10°/min; hold at 100° for 1 min
Carrier gas: Helium
Linear velocity: 35 cm/s
Injection size: 1 µL
Split ratio: 50:1
System suitability
Sample: *System suitability solution*
[Note—Approximate relative retention times for ethyl ether, acetone, isopropyl alcohol, diisopropyl ether, 1-propanol, and 2-butanol are 0.7, 0.9, 1.0, 1.4, 1.5, and 2.0, respectively.]
Suitability requirement 1: The relative standard deviation for the main isopropyl alcohol peak is NMT 2.0% for replicate injections.
Suitability requirement 2: The resolution for the acetone and isopropyl alcohol peaks is NLT 2.0.
Suitability requirement 3: The signal-to-noise ratio is NLT 10 for any of the following peaks: ethyl ether, acetone, diisopropyl ether, 1-propanol, and 2-butanol.
Analysis: Inject the sample into the chromatograph, and record the resulting chromatogram. Determine the percentage of C_3H_8O present in the sample through peak area normalization:

$$\text{Result} = (R_i/R_T) \times 100$$

R_i = peak area for isopropyl alcohol

R_T = sum of all of the peak areas

Acceptance criteria: NLT 99.7% of C_3H_8O

IMPURITIES

Inorganic Impurities

• **Lead,** *Lead Limit Test, Atomic Absorption Spectrophotometric Graphite Furnace Method, Method I, Appendix IIIB*
Acceptance criteria: NMT 1 mg/kg

SPECIFIC TESTS

• **Acidity (as Acetic Acid)**
Sample: 50 mL (about 39 g)
Analysis: Add 2 drops of phenolphthalein TS to 100 mL of water, then add 0.01 N sodium hydroxide to the first pink color that persists for at least 30 s. Add the *Sample* to this solution, and mix. Continue the addition of 0.01 N sodium hydroxide until the pink color is restored.
Acceptance criteria: NMT 0.7 mL of sodium hydroxide is required to restore the pink color (NMT 10 mg/kg).
• **Distillation Range,** *Appendix IIB*
Acceptance criteria: Within a range of 1°, including 82.3°
• **Nonvolatile Residue**
Sample: 125 mL (about 100 g)
Analysis: Evaporate the *Sample* to dryness in a tared dish on a steam bath, dry the residue at 105° for 30 min, cool, and weigh.
Acceptance criteria: NMT 10 mg/kg
• **Solubility in Water**
Sample: 10 mL
Analysis: Mix the *Sample* with 40 mL of water.
Acceptance criteria: After 1 h, the solution is as clear as an equal volume of water.
• **Specific Gravity:** Determine by any reliable method (see *General Provisions*).
Acceptance criteria: NMT 0.7840 at 25°/25° (equivalent to 0.7870 at 20°/20°)
• **Substances Reducing Permanganate**
Sample: 50 mL
Analysis: Transfer the *Sample* into a 50-mL glass-stoppered cylinder, add 0.25 mL of 0.1 N potassium permanganate, mix, and allow to stand for 10 min.
Acceptance criteria: The pink color is not entirely discharged.
• **Water,** *Water Determination, Appendix IIB*
Acceptance criteria: NMT 0.2%

Hydrogen Peroxide

H_2O_2 Formula wt 34.01
CAS: [7722-84-1]

DESCRIPTION

Hydrogen Peroxide occurs as a clear, colorless liquid. The grades of Hydrogen Peroxide suitable for food use usually have a concentration between 30% and 50%. It is miscible with water.

[Note—Although Hydrogen Peroxide undergoes exothermic decomposition in the presence of dirt and other foreign materials, it is safe and stable under recommended conditions of handling and storage. Information on safe handling and use may be obtained from the supplier.]

Function: Bleaching, oxidizing agent; starch modifier; antimicrobial agent

Packaging and Storage: Store in a cool place in containers with a vent in the stopper.

IDENTIFICATION

• **Procedure**
Sample: 1 mL
Analysis: Shake the *Sample* with 10 mL of water containing 1 drop of 2 N sulfuric acid, and add 2 mL of ether. Add one drop of potassium dichromate TS.
Acceptance criteria: An evanescent blue color is produced in the water layer that, upon agitation and standing, passes into the ether layer.

[1] Restek Rtx®-1301, or equivalent. Available at www.restek.com.

ASSAY

• **PROCEDURE**

Sample solution: Dilute an amount of sample equivalent to 300 mg of H_2O_2 to 100 mL with water.

Analysis: Add 25 mL of 2 N sulfuric acid to 20.0 mL of *Sample solution*, and titrate with 0.1 N potassium permanganate. Each mL of 0.1 N potassium permanganate is equivalent to 1.701 mg of H_2O_2.

Acceptance criteria: NLT the labeled concentration or within the range stated on the label

IMPURITIES

Inorganic Impurities

• **IRON**

Sample: 18 mL (20 g)

Analysis: Evaporate the *Sample* to dryness with 10 mg of sodium chloride on a steam bath. Dissolve the residue in 2 mL of hydrochloric acid and dilute to 50 mL with water. Add 40 mg of ammonium persulfate crystals and 10 mL of ammonium thiocyanate TS, and mix.

Acceptance criteria: Any red or pink color produced by the *Sample* does not exceed that produced by 1.0 mL of *Iron Standard Solution* (10 μg Fe) in an equal volume of solution containing the quantities of the reagents used in the test. (NMT 0.5 mg/kg)

• **LEAD,** *Lead Limit Test, Flame Atomic Absorption Spectrophotometric Method, Appendix IIIB*

Analysis: Determine as directed with the following modifications: (1) Prepare only one *Diluted Standard Lead Solution* by transferring 40 mL of *Lead Nitrate Stock Solution* into a 1000-mL volumetric flask and diluting to volume with water to obtain a solution containing 4 μg/mL of lead (Pb) ion; (2) Replace the first paragraph under *Sample Preparation* with the following: Transfer 10 g of sample, into an evaporation dish; (3) Under *Procedure*, determine the absorbances of the *Sample Preparation* and *Diluted Standard Lead Solution* only.

Acceptance criteria: The absorbance of the *Sample Preparation* is NMT that of the *Diluted Standard Lead Solution*. (NMT 4 mg/kg)

• **PHOSPHATE**

Sample: 400 mg

Analysis: Evaporate the *Sample* to dryness on a steam bath. Dissolve the residue in 25 mL of 0.5 N sulfuric acid, add 1 mL of a 50 mg/mL ammonium molybdate tetrahydrate solution and 1 mL of *p*-methylaminophenol sulfate TS, and allow it to stand for 2 h. Prepare a *Control* using 2.0 mL of *Phosphate Standard Solution* (20 μg PO_4) (see *Solutions and Indicators*) in an equal volume of solution containing the quantities of the reagents used for the *Sample*.

Acceptance criteria: Any blue color produced by the *Sample* does not exceed that produced by the *Control*. (NMT 0.005%)

• **TIN**

Aluminum chloride solution: 8.93 mg/mL of aluminum chloride ($AlCl_3 \cdot 6H_2O$)

Gelatin solution: 2 mg/mL of gelatin in boiled water that has been cooled to between 50° and 60°. [NOTE—Prepare on the day of use.]

Standard stock solution: Dissolve 250.0 mg of lead-free tin foil in 10 to 15 mL of hydrochloric acid, and dilute to 250.0 mL with 1:2 hydrochloric acid.

Standard solution: Transfer 5.0 mL of *Standard stock solution* into a 100-mL volumetric flask, dilute to volume with water, and mix. Transfer 2.0 mL of this solution (100 μg Sn) into a 250-mL Erlenmeyer flask, and add 15 mL of water, 5 mL of nitric acid, and 2 mL of sulfuric acid. Place a small, stemless funnel in the mouth of the flask, and heat until strong fumes of sulfuric acid evolve. Cool, add 5 mL of water, evaporate again to strong fumes, and cool. Repeat the addition of water and heating to strong fumes, then add 15 mL of water, heat to boiling, and cool. Dilute to about 35 mL with water, add 1 drop of methyl red TS and 2.0 mL of the *Aluminum chloride solution*, and mix. Make the solution just alkaline by adding, dropwise, ammonium hydroxide and stirring gently, then add 0.1 mL in excess. [**CAUTION**—To avoid dissolving the aluminum hydroxide precipitate, do not add more ammonium hydroxide than 0.1 mL in excess.] Centrifuge for about 15 min at 4000 rpm, and then decant the supernatant liquid as completely as possible without disturbing the precipitate. Dissolve the precipitate in 5 mL of 1:2 hydrochloric acid, add 1.0 mL of the *Gelatin solution*, and dilute to 20.0 mL with a saturated solution of aluminum chloride. [NOTE—Prepare on the day of use.]

Sample solution: Transfer 9 mL (10 g) of sample into a 250-mL Erlenmeyer flask, and add 15 mL of water, 5 mL of nitric acid, and 2 mL of sulfuric acid. Mix, and heat gently on a hot plate to initiate and maintain a vigorous decomposition. When decomposition is complete, place a small, stemless funnel in the mouth of the flask, and continue as directed for the *Standard solution*, beginning with "and heat until strong fumes of sulfuric acid evolve."

Analysis: Rinse a polarographic cell or other vessel with a portion of the *Standard solution*, then add a suitable volume to the cell, immerse it in a constant-temperature bath maintained at 35° ± 0.2°, and deaerate by bubbling oxygen-free nitrogen or hydrogen through the solution for at least 10 min. Insert the dropping mercury electrode of a suitable polarograph, and record the polarogram from –0.2 to –0.7 V at a sensitivity of 0.0003 μA/mm, using a saturated calomel reference electrode. In the same manner, record a polarogram of a portion of the *Sample solution* at the same current sensitivity.

Acceptance criteria: The height of the wave produced by the *Sample solution* is not greater than that produced by the *Standard solution* at the same half-wave potential. (NMT 10 mg/kg)

SPECIFIC TESTS

• **ACIDITY (AS H_2SO_4)**

Sample: 9 mL (10 g)

Analysis: Dilute the *Sample* in 90 mL of carbon dioxide-free water, add methyl red TS and titrate with 0.02 N sodium hydroxide. Perform a blank determination by repeating the preceding, omitting the addition of the *Sample*.

Acceptance criteria: The volume of sodium hydroxide solution required for titration of the *Sample* should not be more than 3 mL greater than the volume required for the blank titration. (NMT 0.03%)

• **RESIDUE ON EVAPORATION**

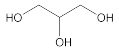
Sample: 25 g

Analysis: Evaporate the *Sample* to dryness in a tared porcelain or silica dish on a steam bath, and continue drying to constant weight at 105°.

Acceptance criteria: The weight of the residue does not exceed 1.5 mg. (NMT 0.006%)

Glycerin

Glycerol



$C_3H_8O_3$ **Formula wt:** 92.09
INS: 422 CAS: [56-81-5]

DESCRIPTION

Glycerin occurs as a clear, colorless, viscous liquid. It is hygroscopic, and its solutions are neutral. Glycerin is miscible with water and with alcohol. It is insoluble in chloroform, in ether, and in fixed and volatile oils. [NOTE—An informational GC method (not a monograph requirement) for the identification and quantification of diethylene glycol and ethylene glycol in glycerin is available for *FCC* users interested in testing food-grade materials for these potential adulterants. See *Diethylene Glycol and Ethylene Glycol in Glycerin, Appendix XIII*.]

Function: Humectant; solvent; bodying agent; plasticizer
Packaging and Storage: Store in tight containers.

IDENTIFICATION

• **A. INFRARED ABSORPTION,** *Spectrophotometric Identification Tests, Appendix IIIC*
Reference standard: USP Glycerin RS
Sample and standard preparation: *F*
Acceptance criteria: The spectrum of the sample exhibits maxima at the same wavelengths as those in the spectrum of the *Reference standard*. [NOTE—A very strong absorption band in the glycerin spectrum at about 10.1 µm can be useful for differentiating glycerin from diethylene glycol and ethylene glycol, which both lack this band.]

• **B. PROCEDURE**
Standard solution: 2.0 mg/mL of USP Glycerin RS and 0.050 mg/mL of USP Diethylene Glycol RS in methanol
Sample solution: 50 mg/mL in methanol
Chromatographic system, *Appendix IIA*
Mode: GC
Detector: Flame-ionization
Column: 0.53-mm × 30-m fused-silica analytical; coated with 3.0-µm 6% cyanopropylphenyl–94% dimethylpolysiloxane stationary phase[1]
Temperatures
Injector: 220°
Detector: 250°
Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
100	—	100	4
100	50	120	10
120	50	220	6

Carrier gas: Helium
Injection volume: 1.0 µL
Flow rate: 4.5 mL/min
Injection type: Split flow ratio is about 10:1
System suitability
Sample: *Standard solution*
Suitability requirements
Resolution: NLT 1.5 between diethylene glycol and glycerin
Analysis: Separately inject equal volumes of the *Standard solution* and the *Sample solution*. [NOTE—The relative retention times for diethylene glycol and glycerin are about 0.8 and 1.0, respectively.]
Acceptance criteria: The retention time of the glycerin peak of the *Sample solution* corresponds to that of the glycerin peak of the *Standard solution*.

[1] DB-624 (J & W Scientific), or equivalent.

ASSAY

• **PROCEDURE**
Sodium periodate solution: Dissolve 60 g of sodium metaperiodate ($NaIO_4$) in sufficient water containing 120 mL of 0.1 N sulfuric acid to make 1000 mL. Do not heat to dissolve the periodate. If the solution is not clear, pass through a sintered-glass filter. Store the solution in a glass-stoppered, light-resistant container. Test the suitability of this solution as follows: Pipet 10 mL into a 250-mL volumetric flask, dilute to volume, and mix. Dissolve about 550 mg of sample in 50 mL of water, and add 50 mL of the diluted periodate solution by pipet. For a blank, pipet 50 mL of the diluted periodate solution into a flask containing 50 mL of water. Allow the solutions to stand for 30 min, then add 5 mL of hydrochloric acid and 10 mL of potassium iodide TS to each, and rotate to mix. Allow to stand for 5 min, add 100 mL of water, and titrate with 0.1 N sodium thiosulfate, shaking continuously and adding starch TS near the endpoint. The ratio of the volume of 0.1 N sodium thiosulfate required for the sample:periodate mixture to that required for the blank should be between 0.750 and 0.765.
Sample: 400 mg
Analysis: Transfer the *Sample* into a 600-mL beaker, dilute with 50 mL of water, add bromothymol blue TS, and acidify with 0.2 N sulfuric acid to a definite green or green-yellow color. Neutralize with 0.05 N sodium hydroxide to a definite blue endpoint free of green color. Prepare a blank containing 50 mL of water, and neutralize in the same manner. Pipet 50 mL of the *Sodium periodate solution* into each beaker, mix by swirling gently, cover with a watch glass, and allow to stand for 30 min at room temperature (not above 35°) in the dark or in subdued light. Add 10 mL of a mixture consisting of equal volumes of ethylene glycol and water to each beaker, and allow to stand for 20 min. Dilute each solution to about 300 mL with water, and titrate with 0.1 N sodium hydroxide to a pH of 8.1 ± 0.1 for the *Sample* and 6.5 ± 0.1 for the blank, using a pH meter previously calibrated with pH 4.0 *Acid Phthalate Standard Buffer Solution* (see *Solutions and Indicators*). Each mL of 0.1 N sodium hydroxide, after correction for the blank, is equivalent to 9.210 mg of glycerin ($C_3H_8O_3$).
Acceptance criteria: 99.0%– 101.0% of glycerin ($C_3H_8O_3$) on the as-is basis

IMPURITIES

Inorganic Impurities

• **LEAD,** *Lead Limit Test, Atomic Absorption Spectrophotometric Graphite Furnace Method, Method I, Appendix IIIB*
Acceptance criteria: NMT 1 mg/kg

Organic Impurities

• **FATTY ACIDS AND ESTERS**
Sample: 40.0 mL (50 g)
Analysis: Mix the *Sample* with 50 mL of recently boiled water and 5.0 mL of 0.5 N sodium hydroxide. Boil the mixture for 5 min, cool, add phenolphthalein TS, and titrate the excess alkali with 0.5 N hydrochloric acid.

Acceptance criteria: NMT 1 mL of 0.5 N sodium hydroxide is consumed.

SPECIFIC TESTS

- **CHLORINATED COMPOUNDS (AS CL)**
  Sample: 5.0 g
  Analysis: Transfer the *Sample* into a dry, 100-mL round-bottom, ground-joint flask, and add 15 mL of morpholine to it. Connect the flask with a ground joint reflux condenser, and reflux the mixture gently for 3 h. Rinse the condenser with 10 mL of water, receiving the washing into the flask, and cautiously acidify with nitric acid. Transfer the solution to a suitable comparison tube, add 0.5 mL of silver nitrate TS, dilute to 50.0 mL, and mix thoroughly.
  Control: 150 µg of chloride in an equal volume of solution containing the quantities of reagents used in the *Analysis*, but omitting the refluxing
  Acceptance criteria: Any turbidity produced by the *Sample* does not exceed that produced by the *Control*. (NMT 0.003% as Cl)
- **COLOR**
  Sample: 50 mL
  Control: 0.40 mL of ferric chloride CS diluted with water to 50 mL
  Analysis: Transfer the *Sample* and the *Control* to separate 50-mL Nessler tubes of the same diameter and color and view the tubes downward against a white surface.
  Acceptance criteria: The color of the *Sample* is not darker than that of the *Control*.
- **READILY CARBONIZABLE SUBSTANCES,** *Appendix IIB*
  Sample: 5 mL
  Analysis: Rinse a glass-stoppered 25-mL cylinder with 95% sulfuric acid, and allow it to drain for 10 min. Add the *Sample* and 5 mL of 95% sulfuric acid, gently mix for 1 min at 18°–20°, and allow to stand for 1 h.
  Acceptance criteria: The resulting mixture has no more color than *Matching Fluid H*.
- **RESIDUE ON IGNITION**
  Sample: 50 g
  Analysis: Heat the *Sample* in a tared, open dish, and ignite the vapors, allowing them to burn until the sample has been completely consumed. After cooling, moisten the residue with 0.5 mL of sulfuric acid, and complete the ignition by heating for 15-min periods at 800 ± 25° to constant weight.
  Acceptance criteria: NMT 0.01%
- **SPECIFIC GRAVITY:** Determine by any reliable method (see *General Provisions*).
  Acceptance criteria: NLT 1.259
- **WATER,** *Water Determination, Method I, Appendix IIB*
  Acceptance criteria: NMT 1.0%

Published August 17, 2020

FCC Solutions

STANDARD BUFFER SOLUTIONS

Reagent Solutions Before mixing, dry the crystalline reagents, except the boric acid, at 110° to 120°, and use water that has been previously boiled and cooled in preparing the solutions. Store the prepared reagent solutions in chemically resistant glass or polyethylene bottles, and use within 3 months. Discard if molding is evident.

Potassium Chloride, 0.2 M Dissolve 14.91 g of potassium chloride (KCl) in sufficient water to make 1000.0 mL.

Potassium Biphthalate, 0.2 M Dissolve 40.84 g of potassium biphthalate [$KHC_6H_4(COO)_2$] in sufficient water to make 1000.0 mL.

Potassium Phosphate, Monobasic, 0.2 M Dissolve 27.22 g of monobasic potassium phosphate (KH_2PO_4) in sufficient water to make 1000.0 mL.

Boric Acid–Potassium Chloride, 0.2 M Dissolve 12.37 g of boric acid (H_3BO_3) and 14.91 g of potassium chloride (KCl) in sufficient water to make 1000.0 mL.

Hydrochloric Acid, 0.2 M, and Sodium Hydroxide, 0.2 M Prepare and standardize as directed under *Volumetric Solutions* in this section.

Procedure To prepare 200 mL of a standard buffer solution having a pH within the range 1.2 to 10.0, place 50.0 mL of the appropriate 0.2 M salt solution, prepared as above, in a 200-mL volumetric flask, add the volume of 0.2 M hydrochloric acid or of sodium hydroxide specified for the desired pH in the *accompanying table*, dilute with water to volume, and mix.

STANDARD SOLUTIONS FOR THE PREPARATION OF CONTROLS AND STANDARDS

The following solutions are used in tests for impurities that require the comparison of the color or turbidity produced in a solution of the test substance with that produced by a known amount of the impurity in a control. Directions for the preparation of other standard solutions are given in the monographs or under the general tests in which they are required (see also *Index*).

Ammonium Standard Solution (10 µg NH_4 in 1 mL) Dissolve 296.0 mg of ammonium chloride (NH_4Cl) in sufficient water to make 100.0 mL, and mix. Transfer 10.0 mL of this solution into a 1000-mL volumetric flask, dilute with water to volume, and mix.

Barium Standard Solution (100 µg Ba in 1 mL) Dissolve 177.9 mg of barium chloride ($BaCl_2 \cdot 2H_2O$) in water in a 1000-mL volumetric flask, dilute with water to volume, and mix.

Iron Standard Solution (10 µg Fe in 1 mL) Dissolve 702.2 mg of ferrous ammonium sulfate [$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$] in 10 mL of 2 N sulfuric acid in a 100-mL volumetric

Composition of Standard Buffer Solutions

Hydrochloric Acid Buffer		Acid Phthalate Buffer		Neutralized Phthalate Buffer		Phosphate Buffer		Alkaline Borate Buffer	
To 50.0 mL of 0.2 M KCl add the mL of HCl specified		To 50.0 mL of 0.2 M $KHC_6H_4(COO)_2$ add the mL of HCl specified		To 50.0 mL of 0.2 M $KHC_6H_4(COO)_2$ add the mL of NaOH specified		To 50.0 mL of 0.2 M KH_2PO_4 add the mL of NaOH specified		To 50.0 mL of 0.2 M H_3BO_3-KCl add the mL of NaOH specified	
pH	0.2 M HCl (mL)	pH	0.2 M HCl (mL)	pH	0.2 M NaOH (mL)	pH	0.2 M NaOH (mL)	pH	0.2 M NaOH (mL)
1.2	85.0	2.2	49.5	4.2	3.0	5.8	3.6	8.0	3.9
1.3	67.2	2.4	42.2	4.4	6.6	6.0	5.6	8.2	6.0
1.4	53.2	2.6	35.4	4.6	11.1	6.2	8.1	8.4	8.6
1.5	41.4	2.8	28.9	4.8	16.5	6.4	11.6	8.6	11.8
1.6	32.4	3.0	22.3	5.0	22.6	6.6	16.4	8.8	15.8
1.7	26.0	3.2	15.7	5.2	28.8	6.8	22.4	9.0	20.8
1.8	20.4	3.4	10.4	5.4	34.1	7.0	29.1	9.2	26.4
1.9	16.2	3.6	6.3	5.6	38.8	7.2	34.7	9.4	32.1
2.0	13.0	3.8	2.9	5.8	42.3	7.4	39.1	9.6	36.9
2.1	10.2	4.0	0.1	—	—	7.6	42.4	9.8	40.6
2.2	7.8	—	—	—	—	7.8	44.5	10.0	43.7
						8.0	46.1	—	—

Dilute all final solutions to 200.0 mL (see *Procedure*). The standard pH values given in this table are considered to be reproducible to within ±0.02 of the pH unit specified at 25°.

Published August 17, 2020

flask, dilute with water to volume, and mix. Transfer 10.0 mL of this solution into a 1000-mL volumetric flask, add 10 mL of 2 N sulfuric acid, dilute with water to volume, and mix.

Magnesium Standard Solution (50 µg Mg in 1 mL) Dissolve 50.0 mg of magnesium metal (Mg) in 1 mL of hydrochloric acid in a 1000-mL volumetric flask, dilute with water to volume, and mix.

Phosphate Standard Solution (10 µg PO_4 in 1 mL) Dissolve 143.3 mg of monobasic potassium phosphate (KH_2PO_4) in water in a 100-mL volumetric flask, dilute with water to volume, and mix. Transfer 10.0 mL of this solution into a 1000-mL volumetric flask, dilute with water to volume, and mix.

TEST SOLUTIONS (TS) AND OTHER REAGENTS

Certain of the following test solutions are intended for use as acid-base indicators in volumetric analyses. Such solutions should be adjusted so that when 0.15 mL of the indicator solution is added to 25 mL of carbon dioxide-free water, 0.25 mL of 0.02 N acid or alkali, respectively, will produce the characteristic color change.

In general, the directive to prepare a solution "fresh" indicates that the solution is of limited stability and must be prepared on the day of use.

Acetic Acid Use ACS reagent-grade *Acetic Acid, Glacial* (99.7% of CH_3COOH;approximately 17.5 N).

Acetic Acid TS, Diluted (1 N) A solution containing about 6% (w/v) of CH_3COOH. Prepare by diluting 60.0 mL of glacial acetic acid, or 166.6 mL of 36% acetic acid (6 N), with sufficient water to make 1000 mL.

Acetic Acid TS, Strong (5 N) A solution containing 30% (v/v) of CH_3COOH. Prepare by diluting 300.0 mL of glacial acetic acid with sufficient water to make 1000 mL.

Acetic Periodic Acid TS Dissolve 2.7 g of periodic acid (H_5IO_6) in 50 mL of water, add 950 mL of glacial acetic acid, and mix thoroughly. [**CAUTION**—This solution is an oxidizing agent and is dangerous in contact with organic materials. Do not use cork or rubber stoppers on storage bottles.] [NOTE—Store this solution protected from light.]

Alcohol (*Ethanol; Ethyl Alcohol;* C_2H_5OH) Use ACS reagent-grade *Ethyl Alcohol* (NLT 95.0%, by volume, of C_2H_5OH).

[NOTE—For use in assays and tests involving ultraviolet spectrophotometry, use ACS reagent-grade *Ethyl Alcohol Suitable for Use in Ultraviolet Spectrophotometry*.]

Alcohol, Absolute (*Anhydrous Alcohol*; *Dehydrated Alcohol*) Use ACS reagent-grade *Ethyl Alcohol, Absolute* (NLT 99.5%, by volume, of C_2H_5OH).

Alcohol, Diluted A solution containing 41.0%–42.0%, by weight, corresponding to 48.4%–49.5%, by volume, at 15.56°, of C_2H_5OH.

Alcohol, 70% (at 15.56°) A 38.6:15 mixture (v/v) of 95% alcohol and water, having a specific gravity of 0.884 at 25°. To prepare 100 mL, dilute 73.7 mL of alcohol to 100 mL with water at 25°.

Alcohol, 80% (at 15.56°) A 45.5:9.5 mixture (v/v) of 95% alcohol and water, having a specific gravity of 0.857 at 25°. To prepare 100 mL, dilute 84.3 mL of alcohol to 100 mL with water at 25°.

Alcohol, 90% (at 15.56°) A 51:3 mixture (v/v) of 95% alcohol and water, having a specific gravity of 0.827 at 25°. To prepare 100 mL, dilute 94.8 mL of alcohol to 100 mL with water at 25°.

Alcohol, Aldehyde-Free Dissolve 2.5 g of lead acetate in 5 mL of water, add the solution to 1000 mL of alcohol contained in a glass-stoppered bottle, and mix. Dissolve 5 g of potassium hydroxide in 25 mL of warm alcohol, cool, and add slowly, without stirring, to the alcoholic solution of lead acetate. Allow to stand for 1 h, then shake the mixture vigorously, allow to stand overnight, decant the clear liquid, and recover the alcohol by distillation. *Ethyl Alcohol FCC*, *Alcohol USP*, or *USSD #3A* or *#30* may be used. If the titration of a 250-mL sample of the alcohol by *Hydroxylamine Hydrochloride TS* does not exceed 0.25 mL of 0.5 N alcoholic potassium hydroxide, the above treatment may be omitted.

Alcoholic Potassium Hydroxide TS See *Potassium Hydroxide TS, Alcoholic*.

Alkaline Cupric Tartrate TS (*Fehling's Solution*) See *Cupric Tartrate TS, Alkaline*.

Alkaline Mercuric Potassium Iodide TS (*Nessler's Reagent*) See *Mercuric Potassium Iodide TS, Alkaline.*

Ammonia–Ammonium Chloride Buffer TS (approximately pH 10) Dissolve 67.5 g of ammonium chloride (NH_4Cl) in water, add 570 mL of ammonium hydroxide (28%), and dilute with water to 1000 mL.

Ammonia TS (6 N in NH_3) A solution containing 9.5%–10.5% of NH_3. Prepare by diluting 400 mL of ammonium hydroxide (28%) with sufficient water to make 1000 mL.

Ammonia TS, Stronger (15.2 N in NH_3) (*Ammonium Hydroxide*; *Stronger Ammonia Water*) Use ACS reagent-grade *Ammonium Hydroxide*, which is a practically saturated solution of ammonia in water, containing 28%–30% of NH_3.

Ammoniacal Silver Nitrate TS Add 6 N ammonium hydroxide, dropwise, to a 1:20 solution of silver nitrate until the precipitate that first forms is almost, but not entirely, dissolved. Filter the solution, and place in a dark bottle.

[**CAUTION**—*Ammoniacal Silver Nitrate TS* forms explosive compounds on standing. Do not store this solution, but prepare a fresh quantity for each series of determinations. Neutralize the excess reagent and rinse all glassware with hydrochloric acid immediately after completing a test.]

Ammonium Acetate TS Dissolve 10 g of ammonium acetate ($NH_4C_2H_3O_2$) in sufficient water to make 100 mL.

Ammonium Carbonate TS Dissolve 20 g of ammonium carbonate and 20 mL of *Ammonia TS* in sufficient water to make 100 mL.

Ammonium Chloride TS Dissolve 10.5 g of ammonium chloride (NH_4Cl) in sufficient water to make 100 mL.

Ammonium Molybdate TS Dissolve 6.5 g of finely powdered molybdic acid (85%) in a mixture of 14 mL of water and 14.5 mL of ammonium hydroxide. Cool the solution, and add it slowly, with stirring, to a well-cooled mixture of 32 mL of nitric acid and 40 mL of water. Allow to stand for 48 h, and pass through a fine-porosity, sintered-glass crucible lined at the bottom with a layer of glass wool. This solution deteriorates upon standing and is unsuitable for use if, upon the addition of 2 mL of *Sodium Phosphate TS* to 5 mL of the solution, an abundant yellow precipitate does not form at once or after slight warming. Store it in the dark. If a precipitate forms during storage, use only the clear, supernatant solution.

Ammonium Oxalate TS Dissolve 3.5 g of ammonium oxalate [$(NH_4)_2C_2O_4 \cdot H_2O$] in sufficient water to make 100 mL.

Ammonium Sulfanilate TS To 2.5 g of sulfanilic acid add 15 mL of water and 3 mL of 6 N ammonium hydroxide, and mix. Add, with stirring, more 6 N ammonium hydroxide, if necessary, until the acid dissolves, adjust the pH of the solution to about 4.5 with 2.7 N hydrochloric acid, using *Bromocresol Green TS* as an outside indicator, and dilute to 25 mL.

Ammonium Sulfide TS Saturate 6 N ammonium hydroxide with hydrogen sulfide (H_2S), and add two-thirds of its volume of 6 N ammonium hydroxide. Residue upon ignition: NMT 0.05%. The solution is not rendered turbid either by *Magnesium Sulfate TS* or by *Calcium Chloride TS* (*carbonate*). This solution is unsuitable for use if an abundant precipitate of sulfur is present. Store it in small, well-filled, dark amber-colored bottles in a cold, dark place.

Ammonium Thiocyanate TS (1 N) Dissolve 8 g of ammonium thiocyanate (NH_4SCN) in sufficient water to make 100 mL.

Anthrone TS Carefully dissolve about 0.1 g of anthrone in 100 g of sulfuric acid. Use a freshly prepared solution.

Antimony Trichloride TS Dissolve 20 g of antimony trichloride ($SbCl_3$) in chloroform to make 100 mL. Filter if necessary.

Barium Chloride TS Dissolve 12 g of barium chloride ($BaCl_2 \cdot 2H_2O$) in sufficient water to make 100 mL.

Barium Diphenylamine Sulfonate TS Dissolve 300 mg of *p*-diphenylamine sulfonic acid barium salt in 100 mL of water.

Barium Hydroxide TS Use a saturated solution of barium hydroxide in recently boiled water. Use a freshly prepared solution.

Benedict's Qualitative Reagent See *Cupric Citrate TS, Alkaline*.

Benzidine TS Dissolve 50 mg of benzidine in 10 mL of glacial acetic acid, dilute with water to 100 mL, and mix.

Bismuth Nitrate TS Reflux 5 g of bismuth nitrate [$Bi(NO_3)_3 \cdot 5H_2O$] with 7.5 mL of nitric acid and 10 mL of water until dissolved, cool, filter, and dilute with water to 250 mL.

Bromine TS (*Bromine Water*) Prepare a saturated solution of bromine by agitating 2–3 mL of bromine (Br_2) with 100 mL of cold water in a glass-stoppered bottle, the stopper of which should be lubricated with petrolatum. Store it in a cold place protected from light.

Bromocresol Blue TS Use *Bromocresol Green TS*.

Bromocresol Green TS Dissolve 50 mg of bromocresol green in 100 mL of alcohol, and filter if necessary.

Bromocresol Purple TS Dissolve 250 mg of bromocresol purple in 20 mL of 0.05 N sodium hydroxide, and dilute with water to 250 mL.

Bromophenol Blue TS Dissolve 100 mg of bromophenol blue in 100 mL of 1:2 alcohol, and filter if necessary.

Bromothymol Blue TS Dissolve 100 mg of bromothymol blue in 100 mL of 1:2 alcohol, and filter if necessary.

Calcium Chloride TS Dissolve 7.5 g of calcium chloride ($CaCl_2 \cdot 2H_2O$) in sufficient water to make 100 mL.

Calcium Hydroxide TS A solution containing approximately 140 mg of $Ca(OH)_2$ in each 100 mL. To prepare, add 3 g of calcium hydroxide [$Ca(OH)_2$] to 1000 mL of water, and agitate the mixture vigorously and repeatedly for 1 h. Allow the excess calcium hydroxide to settle, and decant or draw off the clear, supernatant liquid.

Calcium Sulfate TS A saturated solution of calcium sulfate in water.

Carr-Price Reagent See *Antimony Trichloride TS*.

Ceric Ammonium Nitrate TS Dissolve 6.25 g of ceric ammonium nitrate [$(NH_4)_2Ce(NO_3)_6$] in 100 mL of 0.25 N nitric acid. Prepare the solution fresh every third day.

Chlorine TS (*Chlorine Water*) A saturated solution of chlorine in water. Place the solution in small, completely filled, light-resistant containers. *Chlorine TS*, even when kept from light and air, is apt to deteriorate. Store it in a cold, dark place. For full strength, prepare this solution fresh.

Chromotropic Acid TS Dissolve 50 mg of chromotropic acid or its sodium salt in 100 mL of 75% sulfuric acid (made by cautiously adding 75 mL of 95%–98% sulfuric acid to 33.3 mL of water).

Cobaltous Chloride TS Dissolve 2 g of cobaltous chloride ($CoCl_2 \cdot 6H_2O$) in 1 mL of hydrochloric acid and sufficient water to make 100 mL.

Cobalt–Uranyl Acetate TS Dissolve, with warming, 40 g of uranyl acetate [$UO_2(C_2H_3O_2)_2 \cdot 2H_2O$] in a mixture of 30 g of glacial acetic acid and sufficient water to make 500 mL. Similarly, prepare a solution containing 200 g of cobaltous acetate [$Co(C_2H_3O_2)_2 \cdot 4H_2O$] in a mixture of 30 g of glacial acetic acid and sufficient water to make 500 mL. Mix the two solutions while still warm, and cool to 20°. Maintain the temperature at 20° for about 2 h to separate the excess salts from solution, and then pass through a dry filter.

Congo Red TS Dissolve 500 mg of congo red in a mixture of 10 mL of alcohol and 90 mL of water.

Copper Sulfate TS Dissolve 12.5 g of cupric sulfate in sufficient water to make 100 mL.

Cresol Red TS Triturate 100 mg of cresol red in a mortar with 26.2 mL of 0.01 N sodium hydroxide until solution is complete, then dilute the solution with water to 250 mL.

Cresol Red–Thymol Blue TS Add 15 mL of *Thymol Blue TS* to 5 mL of *Cresol Red TS*, and mix.

Crystal Violet TS Dissolve 100 mg of crystal violet in 10 mL of glacial acetic acid.

Cupric Citrate TS, Alkaline (*Benedict's Qualitative Reagent*) With the aid of heat, dissolve 173 g of sodium citrate ($C_6H_5Na_3O_7 \cdot 2H_2O$) and 117 g of sodium carbonate ($Na_2CO_3 \cdot H_2O$) in about 700 mL of water, and filter through paper, if necessary. In a separate container, dissolve 17.3 g of cupric sulfate ($CuSO_4 \cdot 5H_2O$) in about 100 mL of water, and slowly add this solution, with constant stirring, to the first solution. Cool the mixture, dilute to 1000 mL, and mix.

Cupric Nitrate TS Dissolve 2.4 g of cupric nitrate [$Cu(NO_3)_2 \cdot 3H_2O$] in sufficient water to make 100 mL.

Cupric Sulfate TS Dissolve 12.5 g of cupric sulfate ($CuSO_4 \cdot 5H_2O$) in sufficient water to make 100 mL, and mix.

Cupric Tartrate TS, Alkaline (*Fehling's Solution*) *The Copper Solution* (*A*): Dissolve 34.66 g of carefully selected, small crystals of cupric sulfate ($CuSO_4 \cdot 5H_2O$) showing no trace of efflorescence or of adhering moisture, in sufficient water to make 500 mL. Store this solution in small, tight containers. *The Alkaline Tartrate Solution* (*B*): Dissolve 173 g of crystallized potassium sodium tartrate ($KNaC_4H_4O_6 \cdot 4H_2O$) and 50 g of sodium hydroxide (NaOH) in sufficient water to make 500 mL. Store this solution in small, alkali-resistant containers. For use, mix exactly equal volumes of solutions *A* and *B* at the time required.

Cyanogen Bromide TS Dissolve 5 g of cyanogen bromide in water to make 50 mL.

[**CAUTION**—Prepare this solution in a hood, as cyanogen bromide volatilizes at room temperature, and the vapor is highly irritating and poisonous.]

Denigès' Reagent See *Mercuric Sulfate TS*.

Dichlorophenol–Indophenol TS Warm 100 mg of 2,6-dichlorophenol–indophenol sodium with 100 mL of water. Filter and use within 3 days.

2,7-Dihydroxynaphthalene TS Dissolve 100 mg of 2,7-dihydroxynaphthalene in 1000 mL of sulfuric acid, and allow the solution to stand until the initial color disappears. If the solution is very dark, discard it and prepare a new solution from a different supply of sulfuric acid. This solution is stable for approximately 1 month if stored in a dark bottle.

Diphenylamine TS Dissolve 1 g of diphenylamine in 100 mL of sulfuric acid. The solution should be colorless.

Diphenylcarbazone TS Dissolve about 1 g of diphenylcarbazone ($C_{13}H_{12}N_4O$) in sufficient alcohol to make 100 mL. Store this solution in a brown bottle.

α,α-Dipyridyl TS Dissolve 100 mg of α,α-dipyridyl ($C_{10}H_8N_2$) in 50 mL of absolute alcohol.

Dithizone TS Dissolve 25.6 mg of dithizone in 100 mL of alcohol.

Eosin Y TS (adsorption indicator) Dissolve 50 mg of eosin Y in 10 mL of water.

Eriochrome Black TS Dissolve 200 mg of eriochrome black T and 2 g of hydroxylamine hydrochloride ($NH_2OH \cdot HCl$) in sufficient methanol to make 50 mL, and filter. Store the solution in a light-resistant container and use within 2 weeks.

***p*-Ethoxychrysoidin TS** Dissolve 50 mg of *p*-ethoxychrysoidin monohydrochloride in a mixture of 25 mL of water and 25 mL of alcohol, add 3 drops of hydrochloric acid, stir vigorously, and filter if necessary to obtain a clear solution.

Fehling's Solution See *Cupric Tartrate TS, Alkaline*.

Ferric Ammonium Sulfate TS Dissolve 8 g of ferric ammonium sulfate [$FeNH_4(SO_4)_2 \cdot 12H_2O$] in sufficient water to make 100 mL.

Ferric Chloride TS Dissolve 9 g of ferric chloride ($FeCl_3 \cdot 6H_2O$) in sufficient water to make 100 mL.

Ferric Chloride TS, Alcoholic Dissolve 100 mg of ferric chloride ($FeCl_3 \cdot 6H_2O$) in 50 mL of absolute alcohol. Prepare this solution fresh.

Ferric Sulfate TS, Acid Add 7.5 mL of sulfuric acid to 100 mL of water, and dissolve 80 g of ferrous sulfate in the mixture with the aid of heat. Mix 7.5 mL of nitric acid and 20 mL of water, warm, and add to this the ferrous sulfate solution. Concentrate the mixture until, upon the sudden disengagement of ruddy vapors, the black color of the liquid changes to red. Test for the absence of ferrous iron, and, if necessary, add a few drops of nitric acid and heat again. When the solution is cold, add sufficient water to make 110 mL.

Ferroin TS Dissolve 0.7 g of ferrous sulfate and 1.76 g of phenanthroline hydrochloride in 70 mL of water. Transfer the solution to a 100-mL volumetric flask, and dilute with water to volume. Test the sensitivity of *Ferroin TS* by adding 0.1 mL of *Ferroin TS* and 0.15 mL of osmium tetroxide solution (2.5 g/L of osmium tetroxide in 0.05 M sulfuric acid) to 50 mL of 1 M sulfuric acid. Add 0.1 mL of a 0.1 M ammonium cerium (IV) nitrate solution; a color change from red to light blue should be observed.

Ferrous Sulfate TS Dissolve 8 g of clear crystals of ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in about 100 mL of recently boiled and thoroughly cooled water. Prepare this solution fresh.

Formaldehyde TS A solution containing approximately 37.0% (w/v) of HCHO. It may contain methanol to prevent polymerization.

Fuchsin–Sulfurous Acid TS Dissolve 200 mg of basic fuchsin in 120 mL of hot water, and allow the solution to cool. Add a solution of 2 g of anhydrous sodium sulfite in 20 mL of water, and then add 2 mL of hydrochloric acid. Dilute the solution with water to 200 mL, and allow to stand for at least 1 h. Prepare this solution fresh.

Hydrochloric Acid Use ACS reagent-grade *Hydrochloric Acid* (36.5%–38.0% of HCl;approximately 12 N).

Hydrochloric Acid TS, Diluted (2.7 N) A solution containing 10% (w/v) of HCl. Prepare by diluting 226 mL of hydrochloric acid (36%) with sufficient water to make 1000 mL.

Hydrogen Peroxide TS A solution containing 2.5–3.5 g of H_2O_2 in each 100 mL. It may contain suitable preservatives, totaling not more than 0.05%.

Hydrogen Sulfide TS A saturated solution of hydrogen sulfide made by passing H_2S into cold water. Store it in small, dark, amber-colored bottles, filled nearly to the top. It is unsuitable unless it possesses a strong odor of H_2S, and unless it produces at once a copious precipitate of sulfur when added to an equal volume of *Ferric Chloride TS*. Store in a cold, dark place.

Hydroxylamine Hydrochloride TS Dissolve 3.5 g of hydroxylamine hydrochloride ($NH_2OH \cdot HCl$) in 95 mL of 60% alcohol, and add 0.5 mL of a 1:1000 solution of bromophenol blue and 0.5 N alcoholic potassium hydroxide until a green tint develops in the solution. Then add sufficient 60% alcohol to make 100 mL.

8-Hydroxyquinoline TS Dissolve 5 g of 8-hydroxyquinoline (oxine) in sufficient alcohol to make 100 mL.

Indigo Carmine TS (*Sodium Indigotindisulfonate TS*) Dissolve a quantity of sodium indigotindisulfonate, equivalent to 180 mg of $C_{16}H_8N_2O_2(SO_3Na)_2$, in sufficient water to make 100 mL. Use within 60 days.

Iodine TS Dissolve 14 g of iodine (I_2) in a solution of 36 g of potassium iodide (KI) in 100 mL of water, add 3 drops of hydrochloric acid, dilute with water to 1000 mL, and mix.

Iodinated Zinc Chloride Dissolve 10 g of potassium iodide (KI) and 0.15 g of iodine (I) in 10 mL of water. Add this solution to 100 mL of a 60% solution of zinc chloride, $ZnCl_2$, in water (sp. gr. 1.8). Keep a few crystals of iodine in the solution.

Isopropanol [*Isopropyl Alcohol*; *2-Propanol*; $(CH_3)_2CHOH$] Use ACS reagent-grade *Isopropyl Alcohol*.

[NOTE—For use in assays and tests involving ultraviolet spectrophotometry, use ACS reagent-grade *Isopropyl Alcohol Suitable for Use in Ultraviolet Spectrophotometry*.]

Isopropanol, Anhydrous (*Dehydrated Isopropanol*) Use isopropanol that has been previously dried by shaking with anhydrous calcium chloride, followed by filtering.

Lead Acetate TS Dissolve 9.5 g of clear, transparent crystals of lead acetate [$Pb(C_2H_3O_2)_2 \cdot 3H_2O$] in sufficient recently boiled water to make 100 mL. Store in well-stoppered bottles.

Lead Subacetate TS Triturate 14 g of lead monoxide (PbO) to a smooth paste with 10 mL of water, and transfer

the mixture to a bottle, using an additional 10 mL of water for rinsing. Dissolve 22 g of lead acetate [$Pb(C_2H_3O_2)_2 \cdot 3H_2O$] in 70 mL of water, and add the solution to the lead oxide mixture. Shake it vigorously for 5 min, then set it aside, shaking it frequently during 7 days. Finally, filter, and add enough recently boiled water through the filter to make 100 mL.

Lead Subacetate TS, Diluted Dilute 3.25 mL of *Lead Subacetate TS* with sufficient water, recently boiled and cooled, to make 100 mL. Store in small, well-fitted, tight containers.

Litmus TS Digest 25 g of powdered litmus with three successive 100-mL portions of boiling alcohol, continuing each extraction for about 1 h. Filter, wash with alcohol, and discard the alcohol filtrate. Macerate the residue with about 25 mL of cold water for 4 h, filter, and discard the filtrate. Finally, digest the residue with 125 mL of boiling water for 1 h, cool, and filter.

Magnesia Mixture TS Dissolve 5.5 g of magnesium chloride ($MgCl_2 \cdot 6H_2O$) and 7 g of ammonium chloride (NH_4Cl) in 65 mL of water, add 35 mL of 6 *N* ammonium hydroxide, set the mixture aside for a few days in a well-stoppered bottle, and filter. If the solution is not perfectly clear, filter it before using.

Magnesium Sulfate TS Dissolve 12 g of crystals of magnesium sulfate ($MgSO_4 \cdot 7H_2O$), selected for freedom from efflorescence, in water to make 100 mL.

Malachite Green TS Dissolve 1 g of malachite green oxalate in 100 mL of glacial acetic acid.

Mayer's Reagent See *Mercuric–Potassium Iodide TS*.

Mercuric Acetate TS Dissolve 6 g of mercuric acetate [$Hg(C_2H_3O_2)_2$] in sufficient glacial acetic acid to make 100 mL. Store in tight containers protected from direct sunlight.

Mercuric Chloride TS Dissolve 6.5 g of mercuric chloride ($HgCl_2$) in water to make 100 mL.

Mercuric–Potassium Iodide TS (*Mayer's Reagent*) Dissolve 1.358 g of mercuric chloride ($HgCl_2$) in 60 mL of water. Dissolve 5 g of potassium iodide (KI) in 10 mL of water. Mix the two solutions, and add water to make 100 mL.

Mercuric–Potassium Iodide TS, Alkaline (*Nessler's Reagent*) Dissolve 10 g of potassium iodide (KI) in 10 mL of water, and add slowly, with stirring, a saturated solution of mercuric chloride until a slight red precipitate remains undissolved. To this mixture add an ice-cold solution of 30 g of potassium hydroxide (KOH) in 60 mL of water, then add 1 mL more of the saturated solution of mercuric chloride. Dilute with water to 200 mL. Allow the precipitate to settle, and draw off the clear liquid. A 2-mL portion of this reagent, when added to 100 mL of a 1:300,000 solution of ammonium chloride in ammonia-free water, instantly produces a yellow-brown color.

Mercuric Sulfate TS (*Denigès' Reagent*) Mix 5 g of yellow mercuric oxide (HgO) with 40 mL of water, and while stirring, slowly add 20 mL of sulfuric acid, then add another 40 mL of water, and stir until completely dissolved.

Mercurous Nitrate TS Dissolve 15 g of mercurous nitrate in a mixture of 90 mL of water and 10 mL of 2 N nitric acid. Store in dark, amber-colored bottles in which a small globule of mercury has been placed.

Methanol (*Methyl Alcohol*) Use ACS reagent-grade *Methanol*.

Methanol, Anhydrous (*Dehydrated Methanol*) Use *Methanol*.

***p*-Methylaminophenol Sulfate TS** Dissolve 2 g of *p*-methylaminophenol sulfate [$(HOC_6H_4NHCH_3)_2 \cdot H_2SO_4$] in 100 mL of water. To 10 mL of this solution add 90 mL of water and 20 g of sodium bisulfite. Confirm the suitability of this solution by the following test: Add 1 mL of the solution to each of four tubes containing 25 mL of 0.5 N sulfuric acid and 1 mL of *Ammonium Molybdate TS*. Add 5 µg of phosphate (PO_4) to one tube, 10 µg to a second, and 20 µg to a third, using 0.5 mL, 1.0 mL, and 2.0 mL, respectively, of *Phosphate Standard Solution*, and allow to stand for 2 h. The solutions in the three tubes should show readily perceptible differences in blue color corresponding to the relative amounts of phosphate added, and the one to which 5 µg of phosphate was added should be perceptibly bluer than the blank.

Methylene Blue TS Dissolve 125 mg of methylene blue in 100 mL of alcohol, and dilute with alcohol to 250 mL.

Methyl Orange TS Dissolve 100 mg of methyl orange in 100 mL of water, and filter if necessary.

Methyl Red TS Dissolve 100 mg of methyl red in 100 mL of alcohol, and filter if necessary.

Methyl Red–Methylene Blue TS Add 10 mL of *Methyl Red TS* to 10 mL of *Methylene Blue TS*, and mix.

Methylrosaniline Chloride TS See *Crystal Violet TS*.

Methyl Violet TS See *Crystal Violet TS*.

Millon's Reagent To 2 mL of mercury in an Erlenmeyer flask add 20 mL of nitric acid. Shake the flask in a hood to break the mercury into small globules. After about 10 min add 35 mL of water, and if a precipitate or crystals appear, add sufficient 1:5 nitric acid (prepared from nitric acid from which the oxides have been removed by blowing air through it until it is colorless) to dissolve the separated solid. Add a 1:10 solution of sodium hydroxide, dropwise, with thorough mixing, until the curdy precipitate that forms after the addition of each drop no longer redissolves but is dispersed to form a suspension. Add 5 mL more of the dilute nitric acid, and mix well. Prepare this solution fresh.

α-Naphtholbenzein TS Dissolve 0.2 g of α-naphtholbenzein in glacial acetic acid to make 100 mL. *Sensitivity*: Add 100 mL of freshly boiled and cooled water to 0.2 mL of a 1:1000 solution of α-naphtholbenzein in ethanol, and add 0.1 mL of 0.1 N sodium hydroxide: a green color develops. Add subsequently 0.2 mL of 0.1 N hydrochloric acid: the color of the solution changes to yellow-red.

Naphthol Green TS Dissolve 500 mg of naphthol green B in water to make 1000 mL.

Nessler's Reagent See *Alkaline Mercuric–Potassium Iodide TS*.

Neutral Red TS Dissolve 100 mg of neutral red in 100 mL of 50% alcohol.

Nickel Standard Solution TS (10 mg/kg) Prepare a 0.40% (w/v) solution of analytical reagent-grade nickel chloride ($NiCl_2 \cdot 6H_2O$) with water. Pipet 1.0 mL of the solution into a 100-mL volumetric flask, and dilute with water to volume.

Ninhydrin TS See *Triketohydrindene Hydrate TS*.

Nitric Acid Use ACS reagent-grade *Nitric Acid* (approximately 15.7 N).

Nitric Acid TS, Diluted (1.7 N) A solution containing about 10% (w/v) of HNO_3. Prepare by diluting 105 mL of nitric acid (70%) with water to make 1000 mL.

Orthophenanthroline TS Dissolve 150 mg of orthophenanthroline ($C_{12}H_8N_2 \cdot H_2O$) in 10 mL of a solution of ferrous sulfate, prepared by dissolving 700 mg of clear

crystals of ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in 100 mL of water. The ferrous sulfate solution must be prepared immediately before dissolving the orthophenanthroline. Store the solution in well-closed containers.

Oxalic Acid TS Dissolve 6.3 g of oxalic acid ($H_2C_2O_4 \cdot 2H_2O$) in water to make 100 mL.

Phenol Red TS (*Phenolsulfonphthalein TS*) Dissolve 100 mg of phenolsulfonphthalein in 100 mL of alcohol, and filter if necessary.

Phenolphthalein TS Dissolve 1 g of phenolphthalein in 100 mL of alcohol.

Phenolsulfonphthalein TS See *Phenol Red TS*.

***p*-Phenylphenol TS** On the day of use, dissolve 750 mg of *p*-phenylphenol in 50 mL of *Sodium Hydroxide TS*.

Phosphoric Acid Use ACS reagent-grade *Phosphoric Acid* (NLT 85.0% of H_3PO_4).

Phosphotungstic Acid TS Dissolve 1 g of phosphotungstic acid (approximately $24WO_3 \cdot 2H_3PO_4 \cdot 48H_2O$) in water to make 100 mL.

Picric Acid TS See *Trinitrophenol TS*.

Potassium Acetate TS Dissolve 10 g of potassium acetate ($KC_2H_3O_2$) in water to make 100 mL.

Potassium Chromate TS Dissolve 10 g of potassium chromate (K_2CrO_4) in water to make 100 mL.

Potassium Dichromate TS Dissolve 7.5 g of potassium dichromate ($K_2Cr_2O_7$) in water to make 100 mL.

Potassium Ferricyanide TS (10%) Dissolve 1 g of potassium ferricyanide [$K_3Fe(CN)_6$] in 10 mL of water. Prepare this solution fresh.

Potassium Ferrocyanide TS Dissolve 1 g of potassium ferrocyanide [$K_4Fe(CN)_6 \cdot 3H_2O$] in 10 mL of water. Prepare this solution fresh.

Potassium Hydroxide TS (1 N) Dissolve 6.5 g of potassium hydroxide (KOH) in water to make 100 mL.

Potassium Hydroxide TS, Alcoholic Use *0.5 N Alcoholic Potassium Hydroxide* (see *Volumetric Solutions* in this section).

Potassium Iodide TS Dissolve 16.5 g of potassium iodide (KI) in water to make 100 mL. Store in light-resistant containers.

Potassium Permanganate TS Use *0.1 N Potassium Permanganate* (see *Volumetric Solutions* in this section).

Potassium Pyroantimonate TS Dissolve 2 g of potassium pyroantimonate in 95 mL of hot water. Cool quickly, and add a solution containing 2.5 g of potassium hydroxide in 50 mL of water and 1 mL of an 8.5:100 solution of sodium hydroxide. Allow to stand for 24 h, filter, and dilute with water to 150 mL.

Potassium Sulfate TS Dissolve 1 g of potassium sulfate (K_2SO_4) in sufficient water to make 100 mL.

Quimociac TS Dissolve 70 g of sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$) in 150 mL of water (*Solution A*). Dissolve 60 g of citric acid in a mixture of 85 mL of nitric acid and 150 mL of water, and cool (*Solution B*). Gradually add *Solution A* to *Solution B*, with stirring, to produce *Solution C*. Dissolve 5.0 mL of natural or synthetic quinoline in a mixture of 35 mL of nitric acid and 100 mL of water (*Solution D*). Gradually add *Solution D* to *Solution C*, mix well, and allow to stand overnight. Filter the mixture, add 280 mL of acetone to the filtrate, dilute with water to 1000 mL, and mix. Store in a polyethylene bottle.

[**CAUTION**—This reagent contains acetone. Do not use it near an open flame. Operations involving heating or boiling should be conducted in a well-ventilated hood.]

Quinaldine Red TS Dissolve 100 mg of quinaldine red in 100 mL of glacial acetic acid.

Schiff's Reagent, Modified Dissolve 200 mg of rosaniline hydrochloride ($C_{20}H_{20}ClN_3$) in 120 mL of hot water. Cool, add 2 g of sodium bisulfite ($NaHSO_3$) followed by 2 mL of hydrochloric acid, and dilute with water to 200 mL. Store in a brown bottle at 15° or lower.

Silver Nitrate TS Use *0.1 N Silver Nitrate* (see *Volumetric Solutions* in this section).

Sodium Bisulfite TS Dissolve 10 g of sodium bisulfite ($NaHSO_3$) in water to make 30 mL. Prepare this solution fresh.

Sodium Bitartrate TS Dissolve 1 g of sodium bitartrate ($NaHC_4H_4O_6 \cdot H_2O$) in water to make 10 mL. Prepare this solution fresh.

Sodium Borate TS Dissolve 2 g of sodium borate ($Na_2B_4O_7 \cdot 10H_2O$) in water to make 100 mL.

Sodium Carbonate TS Dissolve 10.6 g of anhydrous sodium carbonate (Na_2CO_3) in water to make 100 mL.

Sodium Cobaltinitrite TS Dissolve 10 g of sodium cobaltinitrite [$Na_3Co(NO_2)_6$] in water to make 50 mL, and filter if necessary.

Sodium Fluoride TS Dry about 500 mg of sodium fluoride (NaF) at 200° for 4 h. Weigh accurately 222 mg of the dried sodium fluoride, and dissolve it in sufficient water to make exactly 100 mL. Transfer 10.0 mL of this solution into a 1000-mL volumetric flask, dilute with water to volume, and mix. Each mL of this final solution corresponds to 10 µg of fluorine (F).

Sodium Hydroxide TS (1 N) Dissolve 4.3 g of sodium hydroxide (NaOH) in water to make 100 mL.

Sodium Indigotindisulfonate TS See *Indigo Carmine TS*.

Sodium Nitroferricyanide TS Dissolve 1 g of sodium nitroferricyanide [$Na_2Fe(NO)(CN)_5 \cdot 2H_2O$] in water to make 20 mL. Prepare this solution fresh.

Sodium Phosphate TS Dissolve 12 g of clear crystals of dibasic sodium phosphate ($Na_2HPO_4 \cdot 7H_2O$) in water to make 100 mL.

Sodium Sulfide TS Dissolve 1 g of sodium sulfide ($Na_2S \cdot 9H_2O$) in water to make 10 mL. Prepare this solution fresh.

Sodium Tetraphenylborate TS Dissolve 1.2 g of sodium tetraphenylborate in water to make 200 mL. If necessary, stir for 5 min with 1 g of freshly prepared hydrous aluminum oxide, and filter to clarify.

Sodium Thiosulfate TS Use *0.1 N Sodium Thiosulfate* (see *Volumetric Solutions* in this section).

Stannous Chloride TS Dissolve 40 g of reagent-grade stannous chloride dihydrate ($SnCl_2 \cdot 2H_2O$) in 100 mL of hydrochloric acid.

Starch TS Mix 1 g of a suitable starch and sufficient cold water to make a thin paste. Add 20 mL of boiling water, boil for 1 min with continuous stirring, and cool. Use only the clear solution. Test the sensitivity of the *Starch TS* as follows: Prepare a solution of 50 mg/kg chlorine by diluting 1 mL of a commercial 5% sodium hypochlorite (NaOCl) solution in 1000 mL of water. Combine 5 mL of *Starch TS* with 100 mL of water and add 0.5 mL of 0.1 N potassium iodide. Addition of one drop of the 50 mg/kg chlorine solution should give a swirl of color where the drop hits. Addition of 1 mL of 50 mg/kg chlorine solution should give a deep blue color throughout the solution. The deep blue color produced is discharged by addition of 0.05 mL of 0.1 N

sodium thiosulfate. Prepare fresh solution when *Starch TS* no longer passes the sensitivity test.

Starch Iodide Paste TS Heat 100 mL of water in a 250-mL beaker to boiling, add a solution of 750 mg of potassium iodide (KI) in 5 mL of water, then add 2 g of zinc chloride ($ZnCl_2$) dissolved in 10 mL of water, and while the solution is boiling, add with stirring a smooth suspension of 5 g of potato starch in 30 mL of cold water. Continue to boil for 2 min, then cool. Store in well-closed containers in a cool place. This mixture must show a definite blue streak when a glass rod dipped in a mixture of 1 mL of 0.1 M sodium nitrite, 500 mL of water, and 10 mL of hydrochloric acid is streaked on a smear of the paste.

Sulfanilic Acid TS Dissolve 800 mg of sulfanilic acid (*p*-$NH_2C_6H_4SO_3H \cdot H_2O$) in 100 mL of acetic acid. Store in tight containers.

Sulfuric Acid Use ACS reagent-grade *Sulfuric Acid* (95.0%–98.0% of H_2SO_4;approximately 36 N).

Sulfuric Acid TS (95%) Add a quantity of sulfuric acid of known concentration to sufficient water to adjust the final concentration to 94.5%–95.5% of H_2SO_4. Because the acid concentration may change upon standing or upon intermittent use, the concentration should be checked frequently and solutions assaying more than 95.5% or less than 94.5% discarded or adjusted by adding either diluted or fuming sulfuric acid, as required.

Sulfuric Acid TS, Diluted (2 N) A solution containing 10% (w/v) of H_2SO_4. Prepare by cautiously adding 57 mL of sulfuric acid (95%–98%) or *Sulfuric Acid TS* to about 100 mL of water, then cool to room temperature, and dilute with water to 1000 mL.

Tannic Acid TS Dissolve 1 g of tannic acid (tannin) in 1 mL of alcohol, and add water to make 10 mL. Prepare this solution fresh.

Thymol Blue TS Dissolve 100 mg of thymol blue in 100 mL of alcohol, and filter if necessary.

Thymolphthalein TS Dissolve 100 mg of thymolphthalein in 100 mL of alcohol, and filter if necessary.

Triketohydrindene Hydrate TS (*Ninhydrin TS*) Dissolve 200 mg of triketohydrindene hydrate ($C_9H_4O_3 \cdot H_2O$) in water to make 100 mL. Prepare this solution fresh.

Trinitrophenol TS (*Picric Acid TS*) Dissolve the equivalent of 1 g of anhydrous trinitrophenol in 100 mL of hot water. Cool the solution, and filter if necessary.

Xylenol Orange TS Dissolve 100 mg of xylenol orange in 100 mL of alcohol.

Published August 17, 2020

FCC Appendices

APPENDIX II: PHYSICAL TESTS AND DETERMINATIONS

A. CHROMATOGRAPHY

[NOTE—Chromatographic separations may also be characterized according to the type of instrumentations or apparatus used. The types of chromatography that may be used in the *Food Chemicals Codex* (*FCC*) are column, thin-layer, gas, and high-pressure or high-performance liquid chromatography.

The Committee on *Food Chemicals Codex* recognizes that the field of chromatography continues to advance. Accordingly, the use of equivalent or improved systems is acceptable with appropriate validation.]

For the purposes of the *FCC*, chromatography is defined as an analytical technique whereby a mixture of chemicals may be separated by virtue of their differential affinities for two immiscible phases. One of these, the stationary phase, consists of a fixed bed of small particles with a large surface area, while the other, the mobile phase, is a gas or liquid that moves constantly through, or over the surface of, the fixed phase. Chromatographic systems achieve their ability to separate mixtures by selectively retarding the passage of some compounds through the stationary phase while permitting others to move more freely. Therefore, the chromatogram may be evaluated qualitatively by determining the R_F, or retardation factor, for each of the eluted substances. The R_F is a measure of that fraction of its total elution time that any compound spends in the mobile phase. Because this fraction is directly related to the fraction of the total amount of the solute that is in the mobile phase, the R_F can be expressed as:

$$R_F = V_m C_m/(V_m C_m + V_s C_s)$$

in which V_m and V_s are the volumes of the mobile and stationary phase, respectively, and C_m and C_s are the concentrations of the solute in either phase at any time. This can be simplified to:

$$R_F = V_m/(V_m + KV_s)$$

in which $K = C_s/C_m$ and is an equilibrium constant that indicates this differential affinity of the solute for the phases. Alternatively, a new constant, k, the capacity factor, may be introduced, giving another form of the expression:

$$R_F = 1/(1 + k)$$

in which $k = KV_s/V_m$. The capacity factor, k, which is normally constant for small samples, is a parameter that expresses the ability of a particular chromatographic system to interact with a solute. The larger the k value, the more the sample is retarded.

Both the retardation factor and the capacity factor may be used for qualitative identification of a solute or for developing strategies for improving separation. In terms of parameters easily obtainable from the chromatogram, the R_F is defined as the ratio of the distance traveled by the solute band to the distance traveled by the mobile solvent in a particular time. The capacity factor, k, can be evaluated by the expression:

$$k = (t_r - t_o)/t_o$$

in which t_r, the retention time, is the elapsed time from the start of the chromatogram to the elution maximum of the solute, and t_o is the retention time of a solute that is not retained by the chromatographic system.

Retardation of the solutes by the stationary phase may be achieved by one or a combination of mechanisms. Certain substances, such as alumina or silica gel, interact with the solutes primarily by *adsorption*, either *physical adsorption*, in which the binding forces are weak and easily reversible, or *chemisorption*, in which strong bonding to the surface can occur. Another important mechanism of retardation is *partition*, which occurs when the solute dissolves in the stationary phase, usually a liquid coated as a thin layer on the surface of an inert particle or chemically bonded to it. If the liquid phase is a polar substance (e.g., polyethylene glycol) and the mobile phase is nonpolar, the process is termed *normal-phase chromatography*. When the stationary phase is nonpolar (e.g., octadecylsilane) and the mobile phase is polar, the process is *reversed-phase chromatography*. For the separation of mixtures of ionic species, insoluble polymers called *ion exchangers* are used as the stationary phase. Ions of the solutes contained in the mobile phase are adsorbed onto the surface of the ion exchanger while at the same time displacing an electrically equivalent amount of less strongly bound ions to maintain the

electroneutrality of both phases. The chromatographic separation of mixtures of large molecules such as proteins may be accomplished by a mechanism called *size exclusion chromatography*. The stationary phases used are highly cross-linked polymers that have imbibed a sufficient amount of solvent to form a gel. The separation is based on the physical size of the solvated solutes; those that are too large to fit within the interstices of the gel are eluted rapidly, while the smaller molecules permeate into the pores of the gel and are eluted later. In any chromatographic separation, more than one of the above mechanisms may be occurring simultaneously.

Chromatographic separations may also be characterized according to the type of instrumentation or apparatus used. The types of chromatography that may be used in the *FCC* are column, thin-layer, gas, and high-performance liquid chromatography.

Column Chromatography

APPARATUS

The equipment needed for column chromatography is not elaborate, consisting only of a cylindrical glass or Teflon tube that has a restricted outflow orifice. The dimensions of the tube are not critical and may vary from 10 to 40 mm in inside diameter and from 100 to 600 mm in length. For a given separation, greater efficiency may be obtained with a long narrow column, but the resultant flow rate will be lower. A fritted-glass disk may be seated in the end of the tube to act as a support for the packing material. The column is fitted at the end with a stopcock or other flow-restriction device to control the rate of delivery of the eluant.

PROCEDURE

The stationary phase is introduced into the column either as a dry powder or as a slurry in the mobile phase. Because a homogeneous bed free of void spaces is necessary to achieve maximum separation efficiency, the packing material is introduced in small portions and allowed to settle before further additions are made. Settling may be accomplished by allowing the mobile phase to flow through the bed, by tapping or vibrating the column if a dry powder is used, or by compressing each added portion using a tamping rod. The rod can be a solid glass, plastic, or metal cylinder whose diameter is slightly smaller than that of the column, or it can be a thinner rod onto the end of which has been attached a disk of suitable diameter. Ion-exchange resins and exclusion polymers are never packed as dry powders because after introduction of the mobile phase, they will swell and create sufficient pressure to shatter the column. When the packing has been completed, the sample is introduced onto the top of the column. If the sample is soluble, it is dissolved in a minimum amount of the mobile phase, pipetted onto the column, and allowed to percolate into the top of the bed. If it is not soluble or if the volume of solution is too large, it may be mixed with a small amount of the column packing. This material is then transferred to the chromatographic tube to form the top of the bed.

The chromatogram is then developed by adding the mobile phase to the column in small portions and allowing it to percolate through the packed bed either by gravity or under the influence of pressure or vacuum. Development of the chromatogram takes place by selective retardation of the components of the mixture as a result of their interaction with the stationary phase. In column chromatography, the stationary phase may act by adsorption, partition, ion exchange, exclusion of the solutes, or a combination of these effects.

When the development is complete, the components of the sample mixture may be detected and isolated by either of two procedures. The entire column may be extruded carefully from the tube, and if the compounds are colored or fluorescent under ultraviolet light, the appropriate segments may be cut from the column using a razor blade. If the components are colorless, they may be visualized by painting or spraying a thin longitudinal section of the surface of the chromatogram with color-developing reagents. The chemical may then be separated from the stationary phase by extraction with a strong solvent such as methanol and subsequently quantitated by suitable methods.

In the second procedure, the mobile phase may be allowed to flow through the column until the components of the mixture successively appear in the effluent. This eluate may be collected in fractions and the mobile phase evaporated if desired. The chemicals present in each fraction may then be determined by suitable analytical techniques.

Paper Chromatography

In this type of chromatography, the stationary phase ordinarily consists of a sheet of paper of suitable texture and thickness. The paper used is made from highly purified cellulose, which has a great affinity for water and other polar solvents since it has many hydroxyl functional groups. The tightly bound water acts as the stationary phase, and therefore the mechanism that predominates is liquid–liquid or partition chromatography. Adsorption of solutes to the cellulose surface may also occur, but this is of lesser importance. Papers especially impregnated to permit ion-exchange or reverse-phase chromatography are also available.

APPARATUS

The essential equipment for paper chromatography consists of the following:

Vapor-tight chamber: The chamber is constructed preferably of glass, stainless steel, or porcelain. It is provided with inlets for the addition of solvent or for releasing internal pressure, and it is designed to permit observation of the progress of the chromatographic run without being opened. Tall glass cylinders are convenient if they are made vapor-tight with suitable covers and a sealing compound.

Supporting rack: The rack serves as a support for the solvent troughs and antisiphoning rods. It is constructed of a corrosion-resistant material about 5 cm shorter than the inside height of the chamber.
Solvent troughs: The troughs, made of glass, are designed to be longer than the width of the chromatographic sheets and to contain a volume of solvent greater than that required for one chromatographic run.
Antisiphoning rods: Constructed of heavy glass, the rods are placed on the rack and arranged to run outside of, parallel to, and slightly above the edge of the glass trough.
Chromatographic sheets: Special chromatographic filter paper is cut to length approximately equal to the height of the chamber. The sheet is a least 2.5 cm wide but not wider than the length of the trough. A fine pencil line is drawn horizontally across the filter paper at a distance from one end such that when the sheet is suspended from the antisiphoning rods with the upper end of the paper resting in the trough and the lower portion hanging free into the chamber, the line is located a few cm below the rods. Care is necessary to avoid contaminating the paper by excessive handling or by contact with dirty surfaces.

PROCEDURE FOR DESCENDING CHROMATOGRAPHY

Separation of substances by descending chromatography is accomplished by allowing the mobile phase to flow downward on the chromatographic sheet.

The substance or substances to be analyzed are dissolved in a suitable solvent. Convenient volumes of the resulting solution, normally containing 1–20 µg of the compound, are placed in 6–10-mm spots along the pencil line not less than 3 cm apart. If the total volume to be applied would produce spots of a diameter greater than 6–10 mm, it is applied in separate portions to the same spot, each portion being allowed to dry before the next is added.

The spotted chromatographic sheet is suspended in the chamber by use of the antisiphoning rod and an additional heavy glass rod that holds the upper end of the sheet in the solvent trough. The bottom of the chamber is covered with a mixture containing both phases of the prescribed solvent system. It is important to ensure that the portion of the sheet hanging below the rods is freely suspended in the chamber without touching the rack or the chamber walls. The chamber is sealed to allow equilibration (saturation) of the chamber and the paper with solvent vapor. Any excess pressure is released as necessary. For large chambers, equilibration overnight may be necessary.

A volume of the mobile phase in excess of the volume required for complete development of the chromatogram is saturated with the immobile phase. After equilibration of the chamber, the prepared mobile solvent is introduced into the trough through the inlet. The inlet is closed, and the mobile phase is allowed to travel down the paper the desired distance. Precautions must be taken against allowing the solvent to run down the sheet when opening the chamber and removing the chromatogram. The location of the solvent front is quickly marked, and the sheets are dried.

The chromatogram is observed and measured directly or after suitable development to reveal the location of the spots of the isolated components of the mixture.

PROCEDURE FOR ASCENDING CHROMATOGRAPHY

In ascending chromatography, the lower edge of the sheet (or strip) is dipped into the mobile phase to permit the mobile phase to rise on the chromatographic sheet.

The test materials are applied to the chromatographic sheet as directed under *Procedure for Descending Chromatography*. Enough of both phases of the solvent mixture to cover the bottom of the chamber is added. Empty solvent troughs are placed on the bottom of the chamber, and the chromatographic sheet is suspended so that the end near which the spots have been added hangs free inside the empty trough.

The chamber is sealed, and equilibration is allowed to proceed as described under *Procedure for Descending Chromatography*. Then the solvent is added through the inlet to the trough in excess of the quantity of solvent required for complete moistening of the chromatographic sheet. The chamber is resealed. When the solvent front has reached the desired height, the chamber is opened and the sheet is removed, the location of the solvent front is quickly marked, and the sheet is dried.

Small cylinders may be used without troughs so that only the mobile phase is placed on the bottom. The chromatographic sheet is suspended during equilibration with the lower end just above the solvent, and chromatography is started by lowering the sheet so that it touches the solvent.

DETECTION OF CHROMATOGRAPHIC BANDS

After the chromatogram has been fully developed, the bands corresponding to the various solutes may be detected by means similar to those described in *Column Chromatography*. If the compounds are colored or fluorescent under ultraviolet light, they may be visualized directly. Colorless compounds may be detected by spraying the paper with color-developing reagents. The bands corresponding to the individual components can be cut from the paper, and the chemical substances eluted from the cellulose by the use of a strong solvent such as methanol.

IDENTIFICATION OF SOLUTES

Since the chromatographic mobilities of the solutes may change from run to run due to varying experimental conditions, presumptive identification of a substance should be based on comparison with a reference standard. The R_F values of the unknown substance and the standard on the same chromatogram must be identical. Alternatively, the ratio between the distances traveled by a given compound and a reference substance, the R_r value, must be 1.0. Identification may also be made by mixing a small amount of the reference substance with the unknown and chromatographing. The resulting

chromatogram should contain only one spot. Definitive identification of solutes may be achieved by eluting them from the paper and subjecting them to IR, NMR, or mass spectrometry.

Thin-Layer Chromatography

In thin-layer chromatography (TLC), the stationary phase is a uniform layer of a finely divided powder that has been coated on the surface of a glass or plastic sheet and that is held in place by a binder. The capacity of the system is dependent on the thickness of the layer, which may range from 0.1–2.0 mm. The thinner layers are used primarily for analytical separations, while the thicker layers, because of their greater sample-handling ability, are useful for preparative work.

Substances that are used as coatings in TLC include silica gel, alumina, cellulose, and reversed-phase packings. Separations occur because of adsorption of the solutes from the mobile phase onto the surface of the thin layer. However, adsorption of water from the air or solvent components from the mobile phase can give rise to partition or liquid–liquid chromatography. The stationary phase of TLC plates has an average particle size of 10–15 µm, and that of high-performance TLC (HPTLC) plates has an average particle size of 5 µm. Commercial plates with a preadsorbent zone can be used if they are specified in a monograph. Sample applied to the preadsorbent region develops into sharp, narrow bands at the preadsorbent-sorbent interface. The separations achieved may be based on adsorption, partition, or a combination of both effects, depending on the particular type of stationary phase. Specially coated plates are available that permit ion-exchange or reversed-phase separations.

APPARATUS

Acceptable apparatus and materials for thin-layer chromatography consist of the following:

TLC plates: Flat plates of uniform thickness throughout their areas. Common sizes are 20 cm, 10 cm, and 5 cm × 20 cm. (Plates are typically glass, plastic, or metal.)

Aligning tray: An aligning tray or other suitable flat surface is used to align and hold plates during application of the adsorbent.

Adsorbent: The adsorbent may consist of finely divided adsorbent materials for chromatography. It can be applied directly to the glass plate, or it can be bonded to the plate by means of plaster of Paris or with starch paste. Pretreated chromatographic plates are available commercially.

Spreader: A suitable spreading device that, when moved over the glass plate, applies a uniform layer of adsorbent of the desired thickness over the entire surface of the plate.

Storage rack: A rack of convenient size to hold the prepared plates during drying and transportation.

Developing chamber: A glass chamber that can accommodate one or more plates and can be properly closed and sealed. It is fitted with a plate-support rack that can support the plates when the lid of the chamber is in place.

[NOTE—Preformed TLC plates available commercially may also be used.]

PROCEDURE

A general method for preparation of TLC plates is included below. In the case of both TLC and HPTLC, commercially-prepared plates are readily available from multiple suppliers and are commonly used.

Clean the plates scrupulously, as by immersion in a chromic acid cleansing mixture, rinse them with copious quantities of water until the water runs off the plates without leaving any visible water or oily spots, and dry.

Arrange the plate or plates on the aligning tray, and secure them so that they will not slip during the application of the adsorbent. Mix an appropriate quantity of adsorbent and liquid, usually water, which when shaken for 30 s gives a smooth slurry that will spread evenly with the aid of a spreader. Transfer the slurry to the spreader, and apply the coating at once before the binder begins to harden. Move the spreader smoothly over the plates from one end of the tray to the other. Remove the spreader, and wipe away excess slurry. Allow the plates to set for 10 min, and then place them in the storage rack, and dry at 105° for 30 min or as directed in the individual monograph. Store the finished plates in a desiccator.

Equilibrate the atmosphere in the *Developing Chamber* by placing in it a volume of the mobile phase in excess of that required for complete development of the chromatogram, cover the chamber with its lid, and allow it to stand for at least 30 min.

The *Sample Solution* and the *Standard Solution* are spotted on the surface of the stationary phase (plate) at the prescribed volume in sufficiently small portions to obtain circular spots of 2–5 mm in diameter (1–2 mm on HPTLC plates) or bands of 10–20 mm × 1–2 mm (5–10 mm × 0.5–1 mm on HPTLC plates) at an appropriate distance from the lower edge and sides of the plate. [NOTE—During development, the application position must be at least 5 mm (TLC) or 3 mm (HPTLC) above the level of the mobile phase.] The solutions are applied on a line parallel to the lower edge of the plate with an interval of at least 10 mm (5 mm on HPTLC plates) between the centers of spots, or 4 mm (2 mm on HPTLC plates) between the edges of bands, then allowed to dry.

Arrange the plate on the supporting rack (sample spots on the bottom), and introduce the rack into the developing chamber. The solvent in the chamber must be deep enough to reach the lower edge of the adsorbent, but must not touch the spot points. Seal the cover in place, and maintain the system until the solvent ascends to a point 10–15 cm above the initial spots. Remove the plates, and dry them in air. Measure and record the distance of each spot from the point of origin. If so directed, spray the spots with the reagent specified, observe, and compare the sample with the standard chromatogram.

DETECTION AND IDENTIFICATION

Detection and identification of solute bands is done by methods essentially the same as those described in *Column Chromatography*. However, in TLC an additional method called *fluorescence quenching* is also used. In this procedure, an inorganic phosphor is mixed with the adsorbent before it is coated on the plate. When the developed chromatogram is irradiated with ultraviolet light, the surface of the plate fluoresces with a characteristic color, except in those places where ultraviolet-absorbing solutes are situated. These quench the fluorescence and are detectable as dark spots.

Detection with an ultraviolet light source suitable for observations with short (254-nm) and long (360-nm) ultraviolet wavelengths may be called for in some cases.

QUANTITATIVE ANALYSIS

Two methods are available if quantitation of the solute is necessary. In the first, the bands are detected and their positions marked. Those areas of adsorbent containing the compounds of interest are scraped from the surface of the plate into a centrifuge tube. The chemicals are extracted from the adsorbent with the aid of a suitable strong solvent, the suspension is centrifuged, and the supernatant layer is subjected to appropriate methods of quantitative analysis.

The second method involves the use of a scanning densitometer. This is a spectrophotometric device that directs a beam of monochromatic radiation across the surface of the plate. After interaction with the solutes in the adsorbent layer, the radiation is detected as transmitted or reflected light and a recording of light intensity versus distance traveled is produced. The concentration of a particular species is proportional to the area under its peak and can be determined accurately by comparison with standards.

Gas Chromatography

The distinguishing features of gas chromatography are a gaseous mobile phase and a solid or immobilized liquid stationary phase. Liquid stationary phases are available in packed or capillary columns. In the packed columns, the liquid phase is deposited on a finely divided, inert solid support, such as diatomaceous earth or porous polymer, which is packed into a column that typically has a 2-mm to 4-mm id and is 1–3 m long. In capillary columns, which contain no particles, the liquid phase is deposited on the inner surface of the fused silica column and may be chemically bonded to it. In gas–solid chromatography, the solid phase is an active adsorbent, such as alumina, silica, or carbon, packed into a column. Polyaromatic porous resins, which are sometimes used in packed columns, are not coated with a liquid phase.

When a volatile compound is introduced into the carrier gas and carried into the column, it is partitioned between the gas and stationary phases by a dynamic countercurrent distribution process. The compound is carried down the column by the carrier gas, retarded to a greater or lesser extent by sorption and desorption in the stationary phase. The elution of the compound is characterized by the partition ratio, k, a dimensionless quantity also called the capacity factor. It is equivalent to the ratio of the time required for the compound to flow through the column (the retention time) to the retention time of a nonretarded compound. The value of the capacity factor depends on the chemical nature of the compound; the nature, amount, and surface area of the liquid phase; and the column temperature. Under a specified set of experimental conditions, a characteristic capacity factor exists for every compound. Separation by gas chromatography occurs only if the compounds concerned have different capacity factors.

APPARATUS

A gas chromatograph consists of a carrier gas source, an injection port, column, detector, and recording device. The injection port, column, and detector are carefully temperature controlled. The typical carrier gas is helium or nitrogen, depending on the column and detector in use. The gas is supplied from a high-pressure cylinder and passes through suitable pressure-reducing valves to the injection port and column. Compounds to be chromatographed, either in solution or as gases, are injected into the gas stream at the injection port. Depending on the configuration of the apparatus, the test mixture may be injected directly into the column or be vaporized in the injection port and mixed into the flowing carrier gas before entering the column.

Once in the column, compounds in the test mixture are separated by virtue of differences in their capacity factors, which in turn depend on their vapor pressure and degree of interaction with the stationary phase. The capacity factor, which governs resolution and retention times of components of the test mixture, is also temperature dependent. The use of temperature-programmable column ovens takes advantage of this dependence to achieve efficient separation of compounds differing widely in vapor pressure.

As resolved compounds emerge from the column, they pass through a detector, which responds to the amount of each compound present. The type of detector to be used depends on the nature of the compounds to be analyzed, and is specified in the individual monograph. Detectors are heated above the maximum column operating temperature to prevent condensation of the eluting compounds.

Detector output is recorded as a function of time, producing a chromatogram, which consists of a series of peaks on a time axis. Each peak represents a compound in the vaporized test mixture, although some peaks may overlap. The elution time is characteristic of the individual compounds (qualitative analysis), and the peak area is a function of the amount present (quantitative analysis).

Injectors: Sample injection devices range from simple syringes to fully programmable automatic injectors. The amount of sample that can be injected into a capillary column without overloading is small compared with the amount that can be injected into a packed column, and may be less than the smallest amount that can be manipulated satisfactorily by syringe. Capillary columns are therefore used with injectors able to split samples into two fractions, a small one that enters the

column and a large one that goes to waste (split injector). Such injectors may also be used in a splitless mode for analyses of trace or minor components.

Purge and trap injectors are equipped with a sparging device by which volatile compounds in solution are carried into a low-temperature trap. When sparging is complete, trapped compounds are thermally desorbed into the carrier gas by rapid heating of the temperature-programmable trap.

Headspace injectors are equipped with a thermostatically controlled sample-heating chamber. Solid or liquid samples in tightly closed containers are heated in the chamber for a fixed period of time, allowing the volatile components in the sample to reach an equilibrium between the nongaseous phase and the gaseous or headspace phase.

After this equilibrium has been established, the injector automatically introduces a fixed amount of the headspace in the sample container into the gas chromatograph.

Columns: Capillary columns, which are usually made of fused silica, have a 0.2-mm to 0.53-mm id and are 5–30 m long. The liquid or stationary phase is 0.1–1.0 µm thick, although nonpolar stationary phases may be up to 5 µm thick.

Packed columns, made of glass or metal, are 1–3 m long, with a 2-mm to 4-mm id. Those used for analysis typically have liquid phase loadings of about 5% (w/w) on a solid support.

Supports for analysis of polar compounds on low-capacity, low-polarity liquid phase columns must be inert to avoid peak tailing. The reactivity of support materials can be reduced by silanizing before coating with liquid phase. Acid-washed, flux-calcined diatomaceous earth is often used for drug analysis. Support materials are available in various mesh sizes, with 80- to 100-mesh and 100- to 120-mesh being more commonly used with 2-mm to 4-mm columns. Because of the absence of a solid support, capillary compounds are much more inert than packed columns.

Retention time and the peak efficiency depend on the carrier gas flow rate; retention time is also directly proportional to column length, while resolution is proportional to the square root of the column length. For packed columns, the carrier gas flow rate is usually expressed in mL/min at atmospheric pressure and room temperature. It is measured at the detector outlet with a soap film flow meter while the column is at operating temperature. Unless otherwise specified in the individual monograph, flow rates for packed columns are 60–75 mL/min for 4-mm id columns and ~30 mL/min for 2-mm id columns.

For capillary columns, linear flow velocity is often used instead of flow rate. This is conveniently determined from the length of the column and the retention time of a dilute methane sample, provided a flame-ionization detector is in use. Typical linear velocities are 20–60 cm/s for helium. At high operating temperatures there is sufficient vapor pressure to result in a gradual loss of liquid phase, a process called "bleeding."

Detectors: Flame-ionization detectors are used for most analyses, with lesser use made of thermal conductivity, electron-capture, nitrogen–phosphorus, and mass spectrometric detectors. For quantitative analyses, detectors must have a wide linear dynamic range: the response must be directly proportional to the amount of compound present in the detector over a wide range of concentrations. Flame-ionization detectors have a wide linear range (~10^6) and are sensitive to organic compounds. Unless otherwise specified in individual monographs, flame-ionization detectors with either helium or nitrogen carrier gas are to be used for packed columns, and helium is used for capillary columns.

The thermal conductivity detector detects changes in the thermal conductivity of the gas stream as solutes are eluted. Although its linear dynamic range is smaller than that of the flame-ionization detector, it is quite rugged and occasionally used with packed columns, especially for compounds that do not respond to flame-ionization detectors.

The alkali flame-ionization detector, sometimes called an NP or nitrogen–phosphorus detector, contains a thermionic source, such as an alkali-metal salt or a glass element containing rubidium or other metal, that results in the efficient ionization of organic nitrogen and phosphorus compounds. It is a selective detector that shows little response to hydrocarbons.

The electron-capture detector contains a radioactive source (usually ^{63}Ni) of ionizing radiation. It exhibits an extremely high response to compounds containing halogens and nitro groups but little response to hydrocarbons. The sensitivity increases with the number and atomic weight of the halogen atoms.

Data collection devices: Modern data stations receive the detector output, calculate peak areas, and print chromatograms, complete with run parameters and peak data. Chromatographic data may be stored and reprocessed, with integration and other calculation variables being changed as required. Data stations are used also to program the chromatograph, controlling most operational variables and providing for long periods of unattended operation.

Data can also be collected for manual measurement on simple recorders or on integrators whose capabilities range from those providing a printout of peak areas to those providing chromatograms with peak areas and peak heights calculated and data stored for possible reprocessing.

PROCEDURE

Capillary columns must be tested to ensure that they comply with the manufacturers' specifications before they are used. These tests consist of the following injections: a dilute methane sample to determine the linear flow velocity; a mixture of alkanes (e.g., C_{14}, C_{15}, and C_{16}) to determine resolution; and a polarity test mixture to check for active sites on the column. The latter mixture may include a methyl ester, an unsaturated compound, a phenol, an aromatic amine, a diol, a free carboxylic acid, and a polycyclic aromatic compound, depending on the samples to be analyzed.

Packed columns must be conditioned before use until the baseline and other characteristics are stable. This may be done by operation at a temperature above that called for by the method or by repeated injections of the compound or mixture to be chromatographed. A suitable test for support inertness should be done. Very polar molecules (like free fatty acids) may require a derivatization step.

Before any column is used for assay purposes, a calibration curve should be constructed to verify that the instrumental response is linear over the required range and that the curve passes through the origin. If the compound to be analyzed is adsorbed within the system, the calibration curve will intersect the abscissa at a nonzero value. This may result in error, particularly for compounds at low concentrations determined by a procedure based on a single reference point. At high concentrations, the liquid phase may be overloaded, leading to loss of peak height and symmetry.

Assays require quantitative comparison of one chromatogram with another. A major source of error is irreproducibility in the amount of sample injected, notably when manual injections are made with a syringe. The effects of variability can be minimized by addition of an internal standard, a noninterfering compound present at the same concentration as in the sample and standard solutions. The ratio of peak response of the analyte to that of the internal standard is compared from one chromatogram to another. Where the internal standard is chemically similar to the substance being determined, there is also compensation for minor variations in column and detector characteristics. In some cases, the internal standard may be carried through the sample preparation procedure before gas chromatography to control other quantitative aspects of the assay. Automatic injectors greatly improve the reproducibility of sample injections and reduce the need for internal standards.

Many monographs require that system suitability requirements be met before samples are analyzed, see *System Suitability* below.

High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) is a separation technique based on a solid stationary phase and a liquid mobile phase. Separations are achieved by partition, adsorption, exclusion, or ion-exchange processes, depending on the type of stationary phase used. HPLC has distinct advantages over gas chromatography for the analysis of nonvolatile organic compounds. Compounds to be analyzed are dissolved in a liquid, and most separations take place at room temperature.

As in gas chromatography, the elution time of a compound can be described by the capacity factor, k, which depends on the chemical nature of the composition and flow rate of the mobile phase, and the composition and surface area of the stationary phase. Column length is an important determinant of resolution. Only compounds having different capacity factors can be separated by HPLC.

APPARATUS

A liquid chromatograph consists of one, two, or more reservoirs containing the mobile phase, a pump to force the mobile phase through the system at high pressure, an injector to introduce the sample into the mobile phase, a chromatographic column, a detector, and a data collection device such as a computer, integrator, or recorder. Short, 3-cm, 5-cm, 10-cm, and 25-cm small-bore columns containing densely packed particles of stationary phase provide for the rapid exchange of compounds between the mobile and stationary phases. In addition to receiving and reporting detector output, computers are used to control chromatographic settings and operations, thus providing for long periods of unattended operation.

Pumping systems: HPLC pumping systems deliver metered amounts of mobile phase from the solvent reservoirs to the column through high-pressure tubing and fittings. Modern systems consist of one or more computer-controlled metering pumps that can be programmed to vary the ratio of mobile phase components, as is required for gradient chromatography, or to mix isocratic mobile phases (i.e., mobile phases having a fixed ratio of solvents). However, the proportion of ingredients in premixed isocratic mobile phases can be more accurately controlled than in those delivered by most pumping systems. Operating pressures up to 5000 psi with delivery rates up to about 10 mL/min are typical. Pumps used for quantitative analysis should be constructed of materials inert to corrosive mobile phase components and be capable of delivering the mobile phase at a constant rate with minimal fluctuations over extended periods of time.

Injectors: After dissolution in mobile phase or other suitable solution, compounds to be chromatographed are injected into the mobile phase, either manually by syringe or loop injectors, or automatically by autosamplers. The latter consist of a carousel or rack to hold sample vials with tops that have a pierceable septum or stopper and an injection device to transfer sample from the vials to a calibrated, fixed-volume loop from which it is loaded into the chromatograph. Some autosamplers can be programmed to control sample volume, the number of injections and loop rinse cycles, the interval between injections, and other operating variables.

Some valve systems incorporate a calibrated sample loop that is filled with test solution for transfer to the column in the mobile phase. In other systems, test solution is transferred to a cavity by syringe and then switched into the mobile phase.

Columns: For most analyses, separation is achieved by partition of compounds in the test solution between the mobile and stationary phases. Systems consisting of polar stationary phases and nonpolar mobile phases are described as normal phase, while the opposite arrangement, polar mobile phases and nonpolar stationary phases, is called reversed-phase chromatography. Partition chromatography is almost always used for hydrocarbon-soluble compounds of a molecular weight that is less than 1000. The affinity of a compound for the stationary phase, and thus its retention time on the column, is controlled by making the mobile phase more or less polar. Mobile phase polarity can be varied by the addition of a second, and sometimes a third or even a fourth, component.

Stationary phases for modern, reversed-phase liquid chromatography typically consist of an organic phase chemically bound to silica or other materials. Particles are usually 3 µm, 5 µm, or 10 µm in diameter, but sizes may range up to 50 µm for preparative columns. Small particles thinly coated with organic phase allow fast mass transfer and, hence, rapid transfer of compounds between the stationary and mobile phases. Column polarity depends on the polarity of the bound functional groups, which range from relatively nonpolar octadecyl silane to very polar nitrile groups.

Columns used for analytical separations usually have internal diameters of 2–4.6 mm; larger diameter columns are used for preparative chromatography. Columns may be heated to give more efficient separations, but only rarely are they used at temperatures above 60° because of potential stationary phase degradation or mobile phase volatility. Unless otherwise specified in the individual monograph, columns are used at an ambient temperature.

Ion-exchange chromatography is used to separate water-soluble, ionizable compounds of molecular weights that are less than 2000. The stationary phases are usually synthetic organic resins; cation-exchange resins contain negatively charged active sites and are used to separate basic substances such as amines; while anion-exchange resins have positively charged active sites for separation of compounds with negatively charged groups such as phosphate, sulfonate, or carboxylate groups. Water-soluble ionic or ionizable compounds are attracted to the resins, and differences in affinity bring about the

chromatographic separation. The pH of the mobile phase, temperature, ion type, ionic concentration, and organic modifiers affect the equilibrium, and these variables can be adjusted to obtain the desired degree of separation.

In size-exclusion chromatography, columns are packed with a porous stationary phase. Molecules of the compounds being chromatographed are filtered according to size. Those too large to enter the pores pass unretained through the column (total exclusion). Smaller molecules enter the pores and are increasingly retained as molecular size decreases. These columns are typically used to remove high molecular weight matrices or to characterize the molecular weight distribution of a polymer.

Detectors: Many compendial HPLC methods require the use of spectrophotometric detectors. Such a detector consists of a flow-through cell mounted at the end of the column. A beam of ultraviolet radiation passes through the flow cell and into the detector. As compounds elute from the column, they pass through the cell and absorb the radiation, resulting in measurable energy level changes.

Fixed, variable, and photodiode array (PDA) detectors are widely available. Fixed wavelength detectors operate at a single wavelength, typically 254 nm, emitted by a low-pressure mercury lamp. Variable wavelength detectors contain a continuous source, such as a deuterium or high-pressure xenon lamp, and a monochromator or an interference filter to generate monochromatic radiation at a wavelength selected by the operator. Modern variable wavelength detectors can be programmed to change wavelength while an analysis is in progress. Multi-wavelength detectors measure absorbance at two or more wavelengths simultaneously. In diode array multi-wavelength detectors, continuous radiation is passed through the sample cell, then resolved into its constituent wavelengths, which are individually detected by the photodiode array. These detectors acquire absorbance data over the entire UV-visible range, thus providing the analyst with chromatograms at multiple, selectable wavelengths and spectra of the eluting peaks. Diode array detectors usually have lower signal-to-noise ratios than fixed or variable wavelength detectors, and thus are less suitable for analysis of compounds present at low concentrations.

Differential refractometer detectors measure the difference between the refractive index of the mobile phase alone and that of the mobile phase containing chromatographed compounds as it emerges from the column. Refractive index detectors are used to detect non-UV absorbing compounds, but they are less sensitive than UV detectors. They are sensitive to small changes in solvent composition, flow rate, and temperature, so that a reference column may be required to obtain a satisfactory baseline.

Fluorometric detectors are sensitive to compounds that are inherently fluorescent or that can be converted to fluorescent derivatives either by chemical transformation of the compound or by coupling with fluorescent reagents at specific functional groups. If derivatization is required, it can be done before chromatographic separation or, alternatively, the reagent can be introduced into the mobile phase just before its entering the detector.

Potentiometric, voltammetric, or polarographic electrochemical detectors are useful for the quantitation of species that can be oxidized or reduced at a working electrode. These detectors are selective, sensitive, and reliable, but require conducting mobile phases free of dissolved oxygen and reducible metal ions. A pulseless pump must be used, and care must be taken to ensure that the pH, ionic strength, and temperature of the mobile phase remain constant. Working electrodes are prone to contamination by reaction products with consequent variable responses.

Electrochemical detectors with carbon-paste electrodes may be used advantageously to measure nanogram quantities of easily oxidized compounds, notably phenols and catechols.

Data collection devices: Modern data stations receive and store detector output and print out chromatograms complete with peak heights, peak areas, sample identification, and method variables. They are also used to program the liquid chromatograph, controlling most variables and providing for long periods of unattended operation.

Data also may be collected on simple recorders for manual measurement or on stand-alone integrators, which range in complexity, from those providing a printout of peak areas to those providing a printout of peak areas and peak heights calculated and data stored for possible subsequent reprocessing.

PROCEDURE

The mobile phase composition significantly influences chromatographic performance and the resolution of compounds in the mixture being chromatographed. Composition has a much greater effect than temperature on the capacity factor, k.

In partition chromatography, the partition coefficient, and hence the separation, can be changed by addition of another component to the mobile phase. In ion-exchange chromatography, pH and ionic strength as well as changes in the composition of the mobile phase affect capacity factors. The technique of continuously increasing mobile phase strength during the chromatographic run is called gradient elution or solvent programming. It is sometimes used to chromatograph complex mixtures of components differing greatly in their capacity factors. Detectors that are sensitive to change in solvent composition, such as the differential refractometer, are more difficult to use with the gradient elution technique.

For accurate quantitative work, high-purity, "HPLC-grade" solvents and reagents must be used. The detector must have a broad linear dynamic range, and compounds to be measured must be resolved from any interfering substances. The linear dynamic range of a compound is the range over which the detector signal response is directly proportional to the amount of the compound. For maximum flexibility in quantitative work, this range should be about three orders of magnitude. HPLC systems are calibrated by plotting peak responses in comparison with known concentrations of a reference standard, using either an external or an internal standardization procedure.

Reliable quantitative results are obtained by external calibration if automatic injectors or autosamplers are used. This method involves direct comparison of the peak responses obtained by separately chromatographing the test and reference standard solutions. If syringe injection, which is irreproducible at the high pressures involved, must be used, better quantitative results are obtained by the internal calibration procedure where a known amount of a noninterfering compound, the internal standard, is added to the test and reference standard solutions, and the ratios of peak responses of the analyte and internal standard are compared.

Because of normal variations in equipment, supplies, and techniques, a system suitability test is required to ensure that a given operating system may be generally applicable. The main features of *System Suitability* tests are described below.

For information on the interpretation of results, see the section *Interpretation of Chromatograms*.

INTERPRETATION OF CHROMATOGRAMS

Figure 1 represents a typical chromatographic separation of two substances, 1 and 2, in which $t_{R(1)}$ and $t_{R(2)}$ are the respective retention times; h, h/2, and $W_{h/2}$ are the height, the half-height, and the width at half-height, respectively, for peak 1; and W_1 and W_2 are the respective widths of peaks 1 and 2 at the baseline. Air peaks are a feature of gas chromatograms and correspond to the solvent front in liquid chromatography.

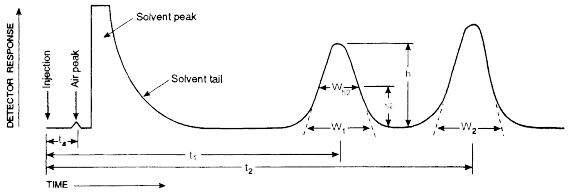


Figure 1. Chromatographic Separation of Two Substances

Chromatographic retention times are characteristic of the compounds they represent but are not unique. Coincidence of retention times of a test and a reference substance can be used as a feature in construction of an identity profile but is insufficient on its own to establish identity. Absolute retention times of a given compound vary from one chromatogram to the next. Comparisons are normally made in terms of relative retention, which is calculated by the equation:

$$\alpha = (t_{R(2)} - t_{R(0)})/(t_{R(1)} - t_O)$$

in which $t_{R(2)}$ and $t_{R(1)}$ are the retention times, measured from the point of injection, of the test and reference substances, respectively, determined under identical experimental conditions on the same column, and t_O is the retention time of a nonretained substance, such as methane in this case, of gas chromatography.

In this and the following expressions, the corresponding retention volumes or linear separations on the chromatogram, both of which are directly proportional to retention time, may be substituted in the equations. Where the value of t_O is small, R_r may be estimated from the retention times measured from the point of injection ($t_{R(2)}/t_{R(1)}$).

The number of theoretical plates, N, is a measure of column efficiency. For Gaussian peaks, it is calculated by the equations:

$$N = 16(t_R/W)^2 \text{ or } N = 5.54(t_R/W_{½})^2$$

in which t_R is the retention time of the substance and W is the width of the peak at its base, obtained by extrapolating the relatively straight sides of the peak to the baseline. $W_{½}$ is the peak width at half-height, obtained directly by electronic integrators. The value of N depends on the substance being chromatographed as well as the operating conditions such as mobile phase or carrier gas flow rates and temperature, the quality of the packing, the uniformity of the packing within the column, and for capillary columns, the thickness of the stationary phase film and the internal diameter and length of the column.

The separation of two components in a mixture, the resolution, R, is determined by the equation:

$$R = 2(t_{R(2)} - t_{R(1)})/(W_2 + W_1)$$

in which $t_{R(2)}$ and $t_{R(1)}$ are the retention times of the two components, and W_2 and W_1 are the corresponding widths at the bases of the peaks obtained by extrapolating the relatively straight sides of the peaks to the baseline.

Peak areas and peak heights are usually proportional to the quantity of compound eluting. These are commonly measured by electronic integrators but may be determined by more classical approaches. Peak areas are generally used but may be less accurate if peak interference occurs. For manual measurements, the chart should be run faster than usual, or a comparator should be used to measure the width at half-height and the width at the base of the peak, to minimize error in these measurements. For accurate quantitative work, the components to be measured should be separated from any interfering components. Peak tailing and fronting and the measurement of peaks on solvent tails are to be avoided (*see Figure 2*). The relative standard deviation is expressed by the equation:

$$S_R(\%) = (100/\overline{X})\left\{\left[\sum_{i=1}^{N}(X_i - \overline{X})^2\right]\Big/(N-1)\right\}^{1/2}$$

in which S_R is the relative standard deviation in percent, $\overline{X}$ is the mean of the set of N measurements, and X_i is an individual measurement. When an internal standard is used, the measurement X_i usually refers to the measurement of relative area, A_s:

$$X_i = A_s = a_r/a_i$$

in which a_r is the area of the peak corresponding to the standard substance and a_i is the area of the peak corresponding to the internal standard. When peak heights are used, the measurement X_i refers to the measurement of relative heights, H_s:

$$X_i = H_s = h_r/h_i$$

in which h_r is the height of the peak corresponding to the standard substance and h_i is the height of the peak corresponding to the internal standard.

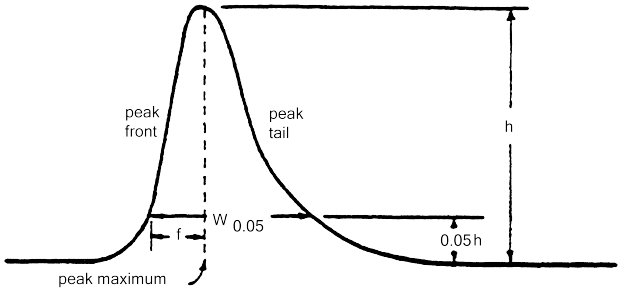


Figure 2. Asymmetrical Chromatographic Peak

SYSTEM SUITABILITY

Such tests are an integral part of gas and liquid chromatographic methods. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. The tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as such.

The resolution, R, is a function of column efficiency, N, and is specified to ensure that closely eluting compounds are resolved from each other, to establish the general resolving power of the system, and to ensure that internal standards are resolved from the analyte. Column efficiency may be specified also as a system suitability requirement, especially if there is only one peak of interest in the chromatogram; however, it is a less reliable means to ensure resolution than direct measurement. Column efficiency is a measure of peak sharpness, which is important for the detection of trace components.

Replicate injections of a standard preparation used in the assay or other standard solution are compared to ascertain whether requirements for precision are met. Unless otherwise specified in the individual monograph, data from five replicate injections of the analyte are used to calculate the relative standard deviation if the requirement is 2.0% or less; data from six replicate injections are used if the relative standard deviation requirement is more than 2.0%.

The tailing factor, T, a measure of peak symmetry, is unity for perfectly symmetrical peaks, and its value increases as tailing becomes more pronounced. In some cases, values less than unity may be observed. As peak asymmetry increases, integration, and hence precision, becomes less reliable. The calculation is expressed by the equation:

$$\text{tailing factor} = T = W_{0.05}/2f$$

These tests are performed by collecting data from replicate injections of standard or other solutions as specified in the individual monograph. The specification of definitive parameters in a monograph does not preclude the use of other suitable operating conditions (see *Procedures* under *Tests and Assays* in *General Provisions*). Adjustments of operating conditions to meet system suitability requirements may be necessary.

Unless otherwise directed in the monograph, system suitability parameters are determined from the analyte peak.

To ascertain the effectiveness of the final operating system, it should be subjected to a suitability test before use and during testing whenever there is a significant change in equipment or in a critical reagent or when a malfunction is suspected.

B. PHYSICOCHEMICAL PROPERTIES

Distillation Range

SCOPE

This method is to be used for determining the distillation range of pure or nearly pure compounds or mixtures having a relatively narrow distillation range of about 40° or less. The result so determined is an indication of purity, not necessarily of identity. Products having a distillation range of greater than 40° may be determined by this method if a wide-range thermometer, such as ASTM E1, 1C, 2C, or 3C, is specified in the individual monograph.

DEFINITIONS

Distillation range: The difference between the temperature observed at the start of a distillation and that observed at which a specified volume has distilled, or at which the dry point is reached.
Initial boiling point: The temperature indicated by the distillation thermometer at the instant the first drop of condensate leaves the end of the condenser tube.
Dry point: The temperature indicated at the instant the last drop of liquid evaporates from the lowest point in the distillation flask, disregarding any liquid on the side of the flask.

APPARATUS

Distillation flask: A 200-mL round-bottom distilling flask of heat-resistant glass is preferred when sufficient sample (in excess of 100 mL) is available for the test. If a sample of less than 100 mL must be used, a smaller flask having a capacity of at least double the volume of the liquid taken may be employed. The 200-mL flask has a total length of 17–19 cm, and the inside diameter of the neck is 20–22 mm. Attached about midway on the neck, approximately 12 cm from the bottom of the flask, is a side arm 10–12.7 cm long and 5 mm in internal diameter, which forms an angle of 70°–75° with the lower portion of the neck.
Condenser: Use a straight glass condenser of heat-resistant tubing, 56–60 cm long and equipped with a water jacket so that about 40 cm of the tubing is in contact with the cooling medium. The lower end of the condenser may be bent to provide a delivery tube or it may be connected to a bent adapter that serves as the delivery tube.

[NOTE—All-glass apparatus with standard-taper ground joints may be used alternatively if the assembly employed provides results equal to those obtained with the flask and condenser described above.]

Receiver: The receiver is a 100-mL cylinder that is graduated in 1-mL subdivisions and calibrated "to contain." It is used for measuring the sample as well as for receiving the distillate.
Thermometer: An accurately standardized partial-immersion thermometer having the smallest practical subdivisions (not greater than 0.2°) is recommended to avoid the necessity for an emergent stem correction. Suitable thermometers are available as the ASTM E1 Series 37C through 41C, and 102C through 107C, or as the MCA types R-1 through R-4 (see *Thermometers, Appendix I*).
Source of heat: A Bunsen burner is the preferred source of heat. An electric heater may be used, however, if it is shown to give results comparable to those obtained with the gas burner.
Shield: The entire burner and flask assembly should be protected from external air currents. Any efficient shield may be employed for this purpose.
Flask support: A heat-resistant board, 5–7 mm in thickness and having a 10-cm circular hole, is placed on a suitable ring or platform support and fitted loosely inside the shield to ensure that hot gases from the source of heat do not come in contact with the sides or neck of the flask. A second 5–7-mm thick heat-resistant board, 14–16-cm square and provided with a 30–40-mm circular hole, is placed on top of the first board. This board is used to hold the 200-mL distillation flask, which should be fitted firmly on the board so that direct heat is applied to the flask only through the opening in the board.

PROCEDURE

[NOTE—For materials boiling below 50°, cool the liquid to below 10° before sampling, receive the distillate in a water bath cooled to below 10°, and use water cooled to below 10° in the condenser.]

Measure 100 ± 0.5 mL of the liquid in the 100-mL graduate, and transfer the sample, together with an efficient antibumping device, into the distilling flask. Do not use a funnel in the transfer or allow any of the sample to enter the side arm of the flask. Place the flask on the heat-resistant boards, which are supported on a ring or platform, and position the shield for the flask and burner. Connect the flask and condenser, place the graduate under the outlet of the condenser tube, and insert the thermometer. The thermometer should be located in the center of the neck so that the top of the contraction chamber (or bulb, if 37C or 38C is used) is level with the bottom of the outlet to the side arm. Regulate the heating so that the first drop of liquid is collected within 5–10 min. Read the thermometer at the instant the first drop of distillate falls from the end of the condenser tube, and record as the initial boiling point. Continue the distillation at the rate of 4 or 5 mL/min of distillate, noting the temperature as soon as the last drop of liquid evaporates from the bottom of the flask (dry point) or when the specified percentage has distilled over. Correct the observed temperature readings for any variation in the barometric pressure from the normal (760 mm) by allowing 0.1° for each 2.7 mm of variation, adding the correction if the pressure is lower, or subtracting if higher, than 760 mm.

When a total-immersion thermometer is used, correct for the temperature of the emergent stem:

$$\text{Result} = 0.00015 \times N(T - t)$$

in which N represents the number of degrees of emergent stem from the bottom of the stopper, T represents the observed temperatures of the distillation, and t represents the temperature registered by an auxiliary thermometer, the bulb of which is placed midway of the emergent stem, adding the correction to the observed readings of the main thermometer.

Melting Range or Temperature Determination

For purposes of the *FCC*, the melting range or temperature of a solid is defined as those points of temperature within which or the point at which the solid coalesces and is completely melted when determined as directed below. Any apparatus

or method capable of equal accuracy may be used. The accuracy should be checked frequently by the use of one or more of the six USP Melting Point Reference Standards, preferably the one that melts nearest the melting temperature of the compound to be tested.

Five procedures for the determination of melting range or temperature are given herein, varying in accordance with the nature of the substance. When no class is designated in the monograph, use the procedure for *Class I*.

The procedure known as the mixed melting point determination, whereby the melting range of a solid under test is compared with that of an intimate mixture of equal parts of the solid and an authentic specimen of it, may be used as a confirmatory identification test. Agreement of the observations on the original and the mixture usually constitutes reliable evidence of chemical identity.

APPARATUS

The melting range apparatus consists of a glass container for a bath of colorless fluid, a suitable stirring device, an accurate thermometer (see *Appendix I*), and a controlled source of heat. The bath fluid is selected consistent with the temperature required, but light paraffin is used generally, and certain liquid silicones are well adapted to the higher temperature ranges. The fluid is deep enough to permit immersion of the thermometer to its specified immersion depth so that the bulb is still about 2 cm above the bottom of the bath. The heat may be supplied electrically or by an open flame. The capillary tube is about 10 cm long, with an internal diameter of 0.8–1.2 mm, and with walls 0.2–0.3 mm thick.

The thermometer is preferably one that conforms to the specifications provided under *Thermometers, Appendix I*, selected for the desired accuracy and range of temperature.

PROCEDURE FOR CLASS I

Reduce the sample to a very fine powder, and unless otherwise directed, render it anhydrous when it contains water of hydration by drying it at the temperature specified in the monograph, or when the substance contains no water of hydration, dry it over a suitable desiccant for 16–24 h.

Charge a capillary glass tube, one end of which is sealed, with a sufficient amount of the dry powder to form a column in the bottom of the tube 2.5–3.5 mm high when packed down as closely as possible by moderate tapping on a solid surface.

Heat the bath until a temperature approximately 30° below the expected melting point is reached, attach the capillary tube to the thermometer, and adjust its height so that the material in the capillary is level with the thermometer bulb. Return the thermometer to the bath, continue the heating, with constant stirring, at a rate of rise of approximately 3°/min until a temperature 3° below the expected melting point is attained, then carefully regulate the rate to about 1°–2°/min until melting is complete.

The temperature at which the column of the sample is observed to collapse definitely against the side of the tube at any point is defined as the beginning of melting, and the temperature at which the sample becomes liquid throughout is defined as the end of melting. The two temperatures fall within the limits of the melting range.

PROCEDURE FOR CLASS IA

Prepare the sample and charge the capillary glass tube as directed for *Class I*. Heat the bath until a temperature 10 ± 1° below the expected melting range is reached, then introduce the charged tube, and heat at a rate of rise of 3 ± 0.5°/min until melting is complete. Record the melting range as for *Class I*.

PROCEDURE FOR CLASS IB

Place the sample in a closed container, and cool to 10° or lower for at least 2 h. Without previous powdering, charge the cooled material into the capillary tube as directed for *Class I*, immediately place the charged tube in a vacuum desiccator, and dry at a pressure not exceeding 20 mm Hg for 3 h. Immediately upon removal from the desiccator, fire-seal the open end of the tube. As soon as is practicable, proceed with the determination of the melting range as follows: Heat the bath until a temperature of 10 ± 1° below the expected melting range is reached, then introduce the charged tube, and heat at a rate of rise of 3 ± 0.5°/min until melting is complete. Record the melting range as directed in *Class I*.

If the particle size of the material is too large for the capillary, precool the sample as directed above, then with as little pressure as possible, gently crush the particles to fit the capillary, and immediately charge the tube.

PROCEDURE FOR CLASS II

Carefully melt the material to be tested at as low a temperature as possible, and draw it into a capillary tube that is left open at both ends to a depth of about 10 mm. Cool the charged tube at 10°, or lower, for 24 h, or in contact with ice for at least 2 h. Then attach the tube to the thermometer by means of a rubber band, adjust it in a water bath so that the upper edge of the material is 10 mm below the water level, and heat as directed for *Class I*, except within 5° of the expected melting temperature, regulate the rate of rise of temperature to 0.5°–1.0°/min. The temperature at which the material is observed to rise in the capillary tube is the melting temperature.

PROCEDURE FOR CLASS III

Melt a quantity of the substance slowly, while stirring, until it reaches a temperature of 90°–92°. Remove the source of heat, and allow the molten substance to cool to a temperature of 8°–10° above the expected melting point. Chill the bulb of

an ASTM 14C thermometer (see *Appendix I*) to 5°, wipe it dry, and while it is still cold, dip it into the molten substance so that approximately the lower half of the bulb is submerged. Withdraw it immediately, and hold it vertically away from the heat until the wax surface dulls, then dip it for 5 min into a water bath having a temperature not higher than 16°.

Fix the thermometer securely in a test tube so that the lower point is 15 mm above the bottom of the test tube. Suspend the test tube in a water bath adjusted to about 16°, and raise the temperature of the bath at the rate of 2°/min to 30°, then change to a rate of 1°/min, and note the temperature at which the first drop of melted substance leaves the thermometer. Repeat the determination twice on a freshly melted portion of the sample. If the variation of three determinations is less than 1°, take the average of the three as the melting point. If the variation of three determinations is greater than 1°, make two additional determinations and take the average of the five.

Optical (Specific) Rotation

Many chemicals in a pure state or in solution are optically active in the sense that they cause incident polarized light to emerge in a plane forming a measurable angle with the plane of the incident light. When this effect is large enough for precise measurement, it may serve as the basis for an assay or an identity test. In this connection, the optical rotation is expressed in degrees, as either *angular rotation* (observed) or *specific rotation* (calculated with reference to the specific concentration of 1 g of solute in 1 mL of solution, measured under stated conditions).

Specific rotation of a liquid substance usually is expressed:

$$[\alpha]^t_x = a/ld$$

For solutions of solid substances, is expressed:

$$[\alpha]^t_x = 100a/lpd = 100a/lc$$

t = temperature

x = wavelength of the light used

a = corrected observed rotation (°)

l = length of the polarimeter cell (dm)

d = specific gravity of the liquid or solution at the temperature of observation

p = concentration of the solution (number of grams of substance in 100 g of solution)

c = concentration of the solution (number of grams of substance in 100 mL of solution)

The concentrations p and c should be calculated on the dried or anhydrous basis, unless otherwise specified. Spectral lines most frequently employed are the D line of sodium (doublet at 589.0 nm and 589.6 nm) and the yellow-green line of mercury at 546.1 nm. The specific gravity and the rotatory power vary appreciably with the temperature.

The accuracy and precision of optical rotatory measurements will be increased if they are carried out with due regard for the following general considerations.

Supplement the source of illumination with a filtering system capable of transmitting light of a sufficiently monochromatic nature. Precision polarimeters generally are designed to accommodate interchangeable disks to isolate the D line from sodium light or the 546.1-nm line from the mercury spectrum. With polarimeters not thus designed, cells containing suitably colored liquids may be employed as filters.[1]

Pay special attention to temperature control of the solution and of the polarimeter. Make accurate and reproducible observations to the extent that differences between replicates, or between observed and true values of rotation (the latter value having been established by calibration of the polarimeter scale with suitable standards), calculated in terms of either specific rotation or angular rotation, whichever is appropriate, do not exceed one-fourth of the range given in the individual monograph for the rotation of the article being tested. Generally, a polarimeter accurate to 0.05° of angular rotation, and capable of being read with the same precision, suffices for *FCC* purposes; in some cases, a polarimeter accurate to 0.01°, or less, of angular rotation, and read with comparable precision, may be required.

Fill polarimeter tubes in such a way as to avoid creating or leaving air bubbles, which interfere with the passage of the beam of light. Interference from bubbles is minimized with tubes in which the bore is expanded at one end. However, tubes of uniform bore, such as semimicro- or micro-tubes, require care for proper filling. At the time of filling, the tubes and the liquid or solution should be at a temperature not higher than that specified for the determination to guard against the formation of a bubble upon cooling and contraction of the contents.

In closing tubes having removable end plates fitted with gaskets and caps, the latter should be tightened only enough to ensure a leak-proof seal between the end plate and the body of the tube. Excessive pressure on the end plate may set up strains that result in interference with the measurements. In determining the specific rotation of a substance of low rotatory power, loosen the caps and tighten them again between successive readings in the measurement of both the rotation and the zero point. Differences arising from end plate strain thus generally will be revealed and appropriate adjustments to eliminate the cause may be made.

[1] See also A. Weissberger and B. W. Rossiter, *Techniques of Chemistry*, Vol. I: *Physical Methods of Chemistry*, Part 3, Wiley-Interscience, New York, 1972.

PROCEDURE

In the case of a solid, dissolve the substance in a suitable solvent, reserving a separate portion of the latter for a blank determination. Make at least five readings of the rotation of the solution, or of the substance itself if liquid, at 25° or the temperature specified in the individual monograph. Replace the solution with the reserved portion of the solvent (or, in the case of a liquid, use the empty tube), make the same number of readings, and use the average as the zero point value. Subtract the zero point value from the average observed rotation if the two figures are of the same sign, or add if opposite in sign, to obtain the corrected observed rotation.

[NOTE—Where a photoelectric polarimeter is used, a single measurement, corrected for the solvent blank, is made.]

CALCULATION

Calculate the specific rotation of a liquid substance, or of a solid in solution, by application of one of the following formulas:

(1) for liquid substances:

$$[\alpha]_x^t = a/ld$$

(2) for solutions of solids:

$$[\alpha]_x^t = 100\,a/lpd = 100\,a/lc$$

t = temperature

x = wavelength of the light used

a = corrected observed rotation (°)

l = length of the polarimeter cell (dm)

d = specific gravity of the liquid or solution at the temperature of observation

p = concentration of the solution (number of grams of substance in 100 g of solution)

c = concentration of the solution (number of grams of substance in 100 mL of solution)

The concentrations p and c should be calculated on the dried or anhydrous basis, unless otherwise specified.

pH Determination

PRINCIPLE

The definition of pH is the negative log of the hydrogen ion concentration in moles per liter of aqueous solutions. Measure pH potentiometrically by using a pH meter or colorimetrically by using pH indicator paper.

SCOPE

This method is suitable to determine the pH of aqueous solutions. While pH meters, calibrated with aqueous solutions, are sometimes used to make measurements in semiaqueous solutions or in nonaqueous polar solutions, the value obtained is the apparent pH value only and should not be compared with the pH of aqueous solutions. For nonpolar solutions, pH has no meaning, and pH electrodes may be damaged by direct contact with these solutions. References to the pH of nonpolar solutions or liquids usually indicate the pH of a water extract of the nonpolar liquid or the apparent pH of a mixture of the nonpolar liquid in a polar liquid such as alcohol or alcohol–water mixtures.

PROCEDURE [POTENTIOMETRIC METHOD (pH METER)]

Calibration: Select two standard buffers to bracket, if possible, the anticipated pH of the unknown substances. These commercially available standards and the sample should be at the same temperature, within 2°. Set the temperature compensator of the pH meter to the temperature of the samples and standards. Follow the manufacturer's instructions for setting temperature compensation and for adjusting the output during calibration. Rinse the electrodes with distilled or deionized water, and blot them dry with clean, absorbent laboratory tissue. Place the electrode(s) in the first standard buffer solution, and adjust the standardization control so that the pH reading matches the stated pH of the standard buffer. Repeat this procedure with fresh portions of the first buffer solution until two successive readings are within ±0.02 pH units with no further adjustment. Rinse the electrodes, blot them dry, and place them in a portion of the second standard buffer solution. Following the manufacturer's instructions, adjust the slope control (not the standardization control) until the output displays the pH of the second standard buffer.

Repeat the sequence of standardization with both buffers until pH readings are within ± 0.02 pH units for both buffers without adjustments to either the slope or standardization controls. The pH of the unknown may then be measured, using

either a pH electrode in combination with a reference electrode or a single combination electrode. Select electrodes made of chemically resistant glass when measuring samples of either low or high pH.

pH Indicator paper: Test papers impregnated with acid–base indicators, although less accurate than pH meters, offer a convenient way to determine the pH of an aqueous solution. They may be purchased in rolls or strips covering all or part of the pH range; papers covering a narrow part of the pH range can be sensitive to differences of 0.2 pH units. Some test papers comprise a plastic strip with small squares of test paper attached. The different squares are sensitive to different pH ranges. When using this type of test paper, wet all of the squares with the test sample to ensure a correct pH reading.

Test paper can contaminate the sample being tested; therefore, do not dip it into the sample. Either use a clean glass rod to remove a drop of the test solution and place it on the test paper, or transfer a small amount of the sample to a small container, dip the test paper into this portion, and compare the developed color with the color comparison chart provided with the test paper to determine the pH of the sample.

Readily Carbonizable Substances

REAGENTS

Sulfuric acid, 95%: Add a quantity of sulfuric acid of known concentration to sufficient water to adjust the final concentration to 94.5%–95.5% of H_2SO_4. Because the acid concentration may change upon standing or upon intermittent use, check the concentration frequently and either adjust solutions assaying more than 95.5% or less than 94.5% by adding either diluted or fuming sulfuric acid, as required, or discard them.

Cobaltous chloride CS: Dissolve about 65 g of cobaltous chloride ($CoCl_2 \cdot 6H_2O$) in enough of a mixture of 25 mL of hydrochloric acid and 975 mL of water to make 1000 mL. Pipet 5 mL of this solution into a 250-mL iodine flask, add 5 mL of hydrogen peroxide TS (3%) and 15 mL of a solution of sodium hydroxide (1:5), boil for 10 min, cool, and add 2 g of potassium iodide and 20 mL of sulfuric acid (1:4). When the precipitate has dissolved, titrate the liberated iodine with 0.1 N sodium thiosulfate. The titration is sensitive to air oxidation and should be blanketed with carbon dioxide. Each mL of 0.1 N sodium thiosulfate is equivalent to 23.79 mg of $CoCl_2 \cdot 6H_2O$. Adjust the final volume of the solution by adding enough of the mixture of hydrochloric acid and water so that each mL contains 59.5 mg of $CoCl_2 \cdot 6H_2O$.

Cupric sulfate CS: Dissolve about 65 g of cupric sulfate ($CuSO_4 \cdot 5H_2O$) in enough of a mixture of 25 mL of hydrochloric acid and 975 mL of water to make 1000 mL. Pipet 10 mL of this solution into a 250-mL iodine flask; add 40 mL of water, 4 mL of acetic acid, and 3 g of potassium iodide; and titrate the liberated iodine with 0.1 N sodium thiosulfate, adding starch TS as the indicator. Each mL of 0.1 N sodium thiosulfate is equivalent to 24.97 mg of $CuSO_4 \cdot 5H_2O$. Adjust the final volume of the solution by adding enough of the mixture of hydrochloric acid and water so that each mL contains 62.4 mg of $CuSO_4 \cdot 5H_2O$.

Ferric chloride CS: Dissolve about 55 g of ferric chloride ($FeCl_3 \cdot 6H_2O$) in enough of a mixture of 25 mL of hydrochloric acid and 975 mL of water to make 1000 mL. Pipet 10 mL of this solution into a 250-mL iodine flask; add 15 mL of water, 5 mL of hydrochloric acid, and 3 g of potassium iodide; and allow the mixture to stand for 15 min. Dilute with 100 mL of water, and titrate the liberated iodine with 0.1 N sodium thiosulfate, adding starch TS as the indicator. Perform a blank determination with the same quantities of the same reagents and in the same manner, and make any necessary correction. Each mL of 0.1 N sodium thiosulfate is equivalent to 27.03 mg of $FeCl_3 \cdot 6H_2O$. Adjust the final volume of the solution by adding the mixture of hydrochloric acid and water so that each mL contains 45.0 mg of $FeCl_3 \cdot 6H_2O$.

Platinum–cobalt CS: Transfer 1.246 g of potassium chloroplatinate (K_2PtCl_6) and 1.00 g of crystallized cobaltous chloride ($CoCl_2 \cdot 6H_2O$) into a 1000-mL volumetric flask, dissolve in about 200 mL of water and 100 mL of hydrochloric acid, dilute with water to volume, and mix. This solution has a color of 500 APHA units. [NOTE—Use this solution only when specified in an individual monograph.]

PROCEDURE

Unless otherwise directed, add the specified quantity of the substance, finely powdered if in solid form, in small portions to the comparison container, which is made of colorless glass resistant to the action of sulfuric acid and contains the specified volume of *95% Sulfuric Acid*.

Stir the mixture with a glass rod until solution is complete, allow the solution to stand for 15 min, unless otherwise directed, and compare the color of the solution with that of the specified matching fluid in a comparison container that also is of colorless glass and has the same internal and cross-section dimensions, viewing the fluids transversely against a background of white porcelain or white glass.

When heat is directed to effect solution of the substance in the *95% Sulfuric Acid*, mix the sample and the acid in a test tube, heat as directed, cool, and transfer the solution to the comparison container for matching.

MATCHING FLUIDS

For purposes of comparison, a series of 20 matching fluids, each designated by a letter of the alphabet, is provided, the composition of each being as indicated in the accompanying table. To prepare the matching fluid specified, pipet the prescribed volumes of the colorimetric test solutions (CS) and water into one of the matching containers, and mix the solutions in the container.

Matching Fluids[a]

Matching Fluid	Parts of Cobaltous Chloride CS	Parts of Ferric Chloride CS	Parts of Cupric Sulfate CS	Parts of Water
A	0.1	0.4	0.1	4.4
B	0.3	0.9	0.3	8.5
C	0.1	0.6	0.1	4.2
D	0.3	0.6	0.4	3.7
E	0.4	1.2	0.3	3.1
F	0.3	1.2	0.0	3.5
G	0.5	1.2	0.2	3.1
H	0.2	1.5	0.0	3.3
I	0.4	2.2	0.1	2.3
J	0.4	3.5	0.1	1.0
K	0.5	4.5	0.0	0.0
L	0.8	3.8	0.1	0.3
M	0.1	2.0	0.1	2.8
N	0.0	4.9	0.1	0.0
O	0.1	4.8	0.1	0.0
P	0.2	0.4	0.1	4.3
Q	0.2	0.3	0.1	4.4
R	0.3	0.4	0.2	4.1
S	0.2	0.1	0.0	4.7
T	0.5	0.5	0.4	3.6

[a] Solutions A–D, very light brown-yellow. Solutions E–L, yellow through red-yellow. Solutions M–O, green-yellow. Solutions P–T, light pink.

Refractive Index

The refractive index of a transparent substance is the ratio of the velocity of light in air to its velocity in that material under like conditions. It is equal to the ratio of the sine of the angle of incidence made by a ray in air to the sine of the angle of refraction made by the ray in the material being tested. The refractive index values specified in this Codex are for the D line of sodium (589 nm) unless otherwise specified. The determination should be made at the temperature specified in the individual monograph, or at 25° if no temperature is specified. This physical constant is used as a means for identification of, and detection of impurities in, volatile oils and other liquid substances. The Abbé refractometer, or other refractometers of equal or greater accuracy, may be employed at the discretion of the operator.

Solidification Point

SCOPE

This method is designed to determine the solidification point of food-grade chemicals having appreciable heats of fusion. It is applicable to chemicals having solidification points between –20° and +150°. Necessary modifications will be noted in individual monographs.

DEFINITION

Solidification Point is an empirical constant defined as the temperature at which the liquid phase of a substance is in approximate equilibrium with a relatively small portion of the solid phase. It is measured by noting the maximum temperature reached during a controlled cooling cycle after the appearance of a solid phase.

The solidification point is distinguished from the freezing point in that the latter term applies to the temperature of equilibrium between the solid and liquid state of pure compounds.

Some chemical compounds have more than one temperature at which there may be an equilibrium between the solid and liquid state depending on the crystal form of the solid that is present.

APPARATUS

The apparatus illustrated in *Figures 3* and *4* consists of the components described in the following paragraphs.

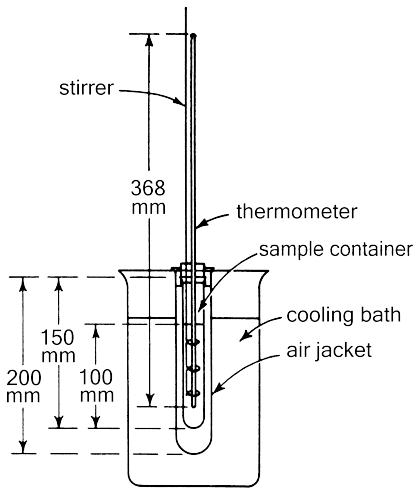


Figure 3. Apparatus for Determination of Solidification Point

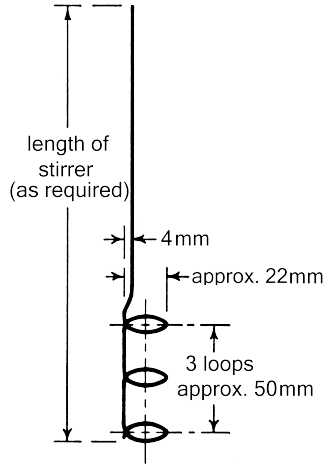


Figure 4. Stirrer for Solidification Point Determination

Thermometer: A thermometer having a range not exceeding 30°, graduated in 0.1° divisions, and calibrated for 76-mm immersion should be employed. A satisfactory series of thermometers, covering a range from −20° to +150°, is available as ASTM-E1 89C through 96C (see *Thermometers, Appendix I*). A thermometer should be chosen such that the solidification point is not obscured by the cork stopper of the sample container.
Sample container: Use a standard glass 25-mm × 150-mm test tube with a lip, fitted with a two-hole cork stopper to hold the thermometer in place and to allow adequate stirring with a stirrer.
Air jacket: For the air jacket, use a standard glass 38-mm × 200-mm test tube with a lip and fitted with a cork or rubber stopper bored with a hole into which the sample container can easily be inserted up to the lip.
Cooling bath: Use a 2000-mL beaker or a similar, suitable container as a cooling bath. Fill it with an appropriate cooling medium such as glycerin, mineral oil, water, water and ice, or alcohol–dry ice.
Stirrer: The stirrer (*Figure 4*) consists of a 1-mm in diameter (B & S gauge 18), corrosion-resistant wire bent into a series of three loops about 25 mm apart. It should be made so that it will move freely in the space between the thermometer and the inner wall of the sample container. The shaft of the stirrer should be of a convenient length designed to pass loosely through a hole in the cork holding the thermometer. Stirring may be hand operated or mechanically activated at 20–30 strokes/min.
Assembly: Assemble the apparatus in such a way that the cooling bath can be heated or cooled to control the desired temperature ranges. Clamp the air jacket so that it is held rigidly just below the lip, and immerse it in the cooling bath to a depth of 160 mm.

SAMPLE PREPARATION

The solidification point of chemicals is usually determined as they are received. Some may be hygroscopic, however, and will require special drying. If this is necessary, it will be noted in the individual monographs.

Products that are normally solid at room temperature must be carefully melted at a temperature about 10° above the expected solidification point. Care should be observed to avoid heating in such a way as to decompose or distill any portion of a sample.

PROCEDURE

Adjust the temperature of the cooling bath to about 5° below the expected solidification point. Fit the thermometer and stirrer with a cork stopper so that the thermometer is centered and the bulb is about 20 mm from the bottom of the sample container. Transfer a sufficient amount of the sample, previously melted if necessary, into the sample container to fill it to a depth of about 90 mm when in the molten state. Place the thermometer and stirrer in the sample container, and adjust the thermometer so that the immersion line will be at the surface of the liquid and so that the end of the bulb is 20 ± 4 mm from the bottom of the sample container. When the temperature of the sample is about 5° above the expected solidification point, place the assembled sample tube in the air jacket.

Allow the sample to cool while stirring, at the rate of 20–30 strokes/min, in such a manner that the stirrer does not touch the thermometer. Stir the sample continuously during the remainder of the test.

The temperature at first will gradually fall, then will become constant as crystallization starts and continues under equilibrium conditions, and finally will start to drop again. Some chemicals may supercool slightly below (0.5°) the solidification point; as crystallization begins, the temperature will rise and remain constant as equilibrium conditions are established. Other products may cool more than 0.5° and cause deviation from the normal pattern of temperature change. If the temperature rise exceeds 0.5° after the initial crystallization begins, repeat the test, and seed the melted compound with small crystals of the sample at 0.5° intervals as the temperature approaches the expected solidification point. Crystals for seeding may be obtained by freezing a small sample in a test tube directly in the cooling bath. It is preferable that seed of the stable phase be used from a previous determination.

Observe and record the temperature readings at regular intervals until the temperature rises from a minimum, due to supercooling, to a maximum and then finally drops. The maximum temperature reading is the solidification point. Readings 10 s apart should be taken to establish that the temperature is at the maximum level and should continue until the drop in temperature is established.

Viscosity Determination

Viscosity is a fluid's measured internal resistance to flow. Thick, slow-moving fluids have higher viscosities than thin, free-flowing fluids. The basic unit of measure for viscosity is the poise or Pascal second, Pa · s, in SI units. The relationship between poise and Pa · s is 1 poise = 0.1 Pa · s. Since commonly encountered viscosities are often fractions of 1 poise, viscosities are commonly expressed as centipoises (one centipoise = 0.01 poise). Poise or centipoise is the unit of measure for absolute viscosity. Kinematic viscosity also is commonly used and is determined by dividing the absolute viscosity of the test liquid by the density of the test liquid at the same temperature as the viscosity measurement and is expressed as stokes or centistokes (poise/density = stokes). The specified temperature is important: viscosity varies greatly with temperature, generally decreasing with increasing temperature.

Absolute viscosity can be determined directly if accurate dimensions of the measuring instruments are known. It is common practice to calibrate an instrument with a fluid of known viscosity and to determine the unknown viscosity of another fluid by comparison with that of the known viscosity.

Many substances, such as gums, have a variable viscosity, and most of them are less resistant to flow at higher flow (more correctly, shear) rates. In such cases, select a given set of conditions for measurement, and consider the measurement obtained to be an apparent viscosity. Since a change in the conditions of measurement would yield a different value for the apparent viscosity of such substances, the operator must closely adhere to the instrument dimensions and conditions for measurement.

MEASURING VISCOSITY

Several common methods are available for measuring viscosity. Two very common ones are the use of capillary tubes such as Ubbelohde, Ostwald, or Cannon-Fenske viscometer tubes and the use of a rotating spindle such as the Brookfield viscometer.

Determine the viscosity in capillary tubes by measuring the amount of time it takes for a given volume of liquid to flow through a calibrated capillary tube. Calibrate the capillary tube by using liquids of known viscosity. The calibration may be supplied with the viscometer tube when purchased along with specific instructions for its use. Many types of capillary viscometer tubes are available, and exact procedures will vary with the type of tube chosen. Examples of procedures are in the following sections: *Viscosity of Dimethylpolysiloxane* and *Viscosity of Methylcellulose*. In general, calibrate capillary viscometers by filling the viscometers per the manufacturer's instructions and allowing the filled tube to equilibrate to the given temperature in a constant-temperature bath. Draw the liquid to the top graduation line, and measure the time, in seconds, it takes for the liquid to flow from the upper mark to the lower mark in the capillary tube. Calculate the viscometer constant, k:

$$k = v/dt$$

in which v is the known viscosity, in centipoises, of the standard liquid; d is the density, at the specified temperature, of the liquid; and t is the time, in seconds, for the liquid to pass from the upper mark to the lower mark. It is not necessary to recalibrate the tube unless changes or repairs are made to it. To measure viscosity, introduce the unknown liquid into the viscometer tube in the same way as the calibration standard was introduced, and measure the time, in seconds, it takes for the liquid to flow from the upper mark to the lower mark. Calculate viscosity:

$$v = kdt$$

in which v is the viscosity to be determined, k is the viscometer constant, and d is the density of the liquid being measured.

Using rotational viscometers provides a particularly rapid and convenient method for determining viscosity. They employ a rotating spindle or cup immersed in the liquid, and they measure the resistance of the liquid to the rotation of the spindle or cup. A wide range of viscosities can be measured with one instrument by using spindles or cups of different sizes and by rotating them at different speeds. The manufacturer supplies the calibration of viscosity versus the spindle size and speed, which can be checked by using fluids of known viscosity. Take a measurement by allowing the sample to come to the desired temperature in a constant-temperature bath and immersing the spindle or cup to the depth specified by the manufacturer. Allow the spindle or cup to rotate until a constant reading is obtained. Multiply the reading by a factor supplied by the manufacturer for a given spindle or cup and given rotational speed to obtain the viscosity. The exact procedures will vary with the particular instrument. An example is given in the section on *Viscosity of Cellulose Gum*.

Another method to determine viscosity uses the falling-ball viscometer. Determine viscosity by noting the time it takes for a ball to fall through the distance between two marks on a tube filled with the unknown liquid (the tube is generally in a constant-temperature bath). Use balls of different weights to measure a wide range of viscosities. Calculate the viscosity by using manufacturer-supplied constants for the ball used. These instruments can be quite precise for Newtonian liquids, that is, liquids that do not have viscosities that vary with flow (more correctly, shear) rate.

Three specific methods are described below:

VISCOSITY OF DIMETHYLPOLYSILOXANE

Apparatus: The Ubbelohde suspended level viscometer, shown in *Figure 5* is preferred to determine the viscosity of dimethylpolysiloxane. Alternatively, a Cannon-Ubbelohde viscometer may be used.

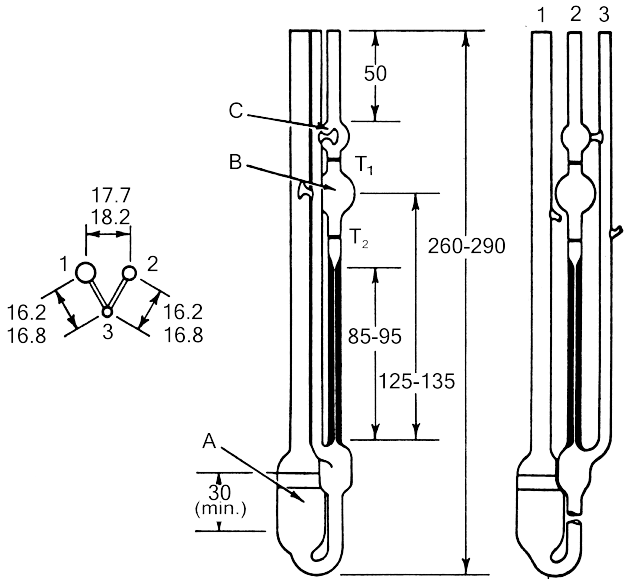


Figure 5. Ubbelohde Viscometer for Dimethylpolysiloxane (all dimensions are in mm)

Select a viscometer having a minimum flow time of at least 200 s. Use a No. 3 size Ubbelohde, or a No. 400 size Cannon-Ubbelohde, viscometer for the range of 300–600 centistokes. The viscometer should be fitted with holders that satisfy the dimensional positions of the separate tubes as shown in the diagram and that hold the viscometer vertically. Filling lines in bulb A indicate the minimum and maximum volumes of liquid to be used for convenient operation. The volume of bulb B is approximately 5 mL.

Calibration of the viscometer: Determine the viscosity constant, C, for each viscometer by using an oil of known viscosity.[2] Charge the viscometer by tilting the instrument about 30 degrees from the vertical, with bulb A below the capillary, and then introduce enough of the sample into tube I to bring the level up to the lower filling line. The level should not be above the upper filling line when the viscometer is returned to the vertical position and the sample has drained from tube I. Charge the viscometer in such a manner that the U-tube at the bottom fills completely without trapping air.

[2] Oils of known viscosities may be obtained from the Cannon Instrument Co., P.O. Box 812, State College, PA 16801. For determining the viscosity of dimethylpolysiloxane, choose an oil with a viscosity as close as possible to that of the type of sample to be tested.

After the viscometer has been in a constant-temperature bath (25 ± 0.2°) long enough for the sample to reach temperature equilibrium, place a finger over tube 3, and apply suction to tube 2 until the liquid reaches the center of bulb C. Remove suction from tube 2, then remove the finger from tube 3, and place it over tube 2 until the sample drops away from the lower end of the capillary. Remove the finger from tube 2, and measure the time, to the nearest 0.1 s, required for the meniscus to pass from the first timing mark (T_1) to the second (T_2).

Calculate the viscometer constant, C:

$$C = cs/t_1$$

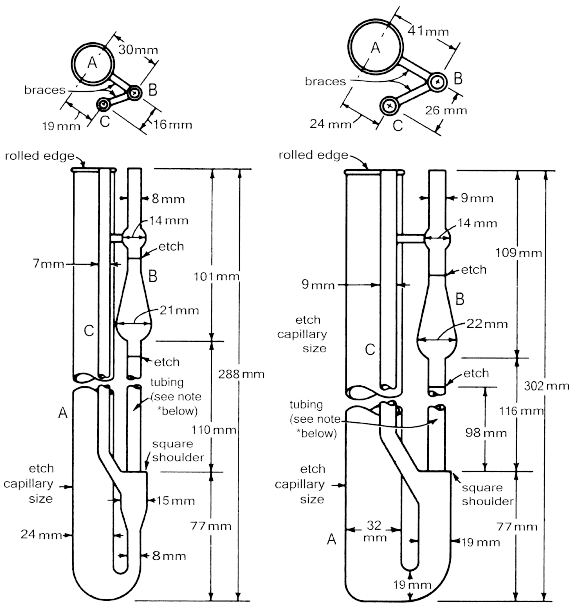
in which cs is the viscosity, in centistokes, and t_1 is the efflux time, in seconds, for the standard liquid.

Determination of the viscosity of dimethylpolysiloxane: Charge the viscometer with the sample in the same manner as described for the calibration procedure; determine the efflux time, t_2; and calculate the viscosity of the dimethylpolysiloxane:

$$V = C \times t_2$$

VISCOSITY OF METHYLCELLULOSE

Apparatus: Viscometers used to determine the viscosity of methylcellulose and some related compounds are illustrated in *Figure 6* and consist of three parts: a large filling tube, A; an orifice tube, B; and an air vent to the reservoir, C.



Viscometer for Low Viscosity

*Precision bore capillary tubing 1.5 mm id for 15 cps, 1.8 mm id for 25 cps, 2.4 mm id for 100 cps, and 3.2 mm id for 400 cps viscosities.

Viscometer for High Viscosity

*Precision bore capillary tubing 5.0 mm id for 1500 cps and 6.0 mm id for 4000 cps viscosities.

Figure 6. Methylcellulose Viscometers

There are two basic types of methylcellulose viscometers—one for cellulose derivatives of a range between 1500 and 4000 centipoises, and the other for less viscous ones. Each type of viscometer is modified slightly for the different viscosities.

Calibration of the viscometer: Determine the viscometer constant, K, for each viscometer by using an oil of known viscosity.[2] Place an excess of the liquid that is to be tested (adjusted to 20 ± 0.1°) in the filling tube, A, and transfer it to the orifice tube, B, by gentle suction, taking care to keep the liquid free from air bubbles by closing the air vent tube, C. Adjust the column of liquid in tube B so it is even with the top graduation line. Open both tubes B and C to permit the liquid to flow into the reservoir against atmospheric pressure.

[NOTE—Failure to open air vent tube C before determining the viscosity will yield false values.]

Record the time, in seconds, for the liquid to flow from the upper mark to the lower mark in tube B.

Calculate the viscometer constant, K:

$$K = V/dt$$

in which V is the viscosity, in centipoises, of the liquid; K is the viscometer constant; d is the specific gravity of the liquid tested at 20°/20°; and t is the time, in seconds, for the liquid to pass from the upper to the lower mark.

For the calibration, all values in the equation are known or can be determined except K, which must be solved. If a tube is repaired, it must be recalibrated to avoid obtaining significant changes in the value of K.

Determination of the viscosity of methylcellulose: Prepare a 2% solution of methylcellulose or other cellulose derivative, by weight, as directed in the monograph. Place the solution in the proper viscometer and determine the time, t, required for the solution to flow from the upper mark to the lower mark in orifice tube B. Separately determine the specific gravity, d, at 20°/20°. Viscosity, V = Kdt.

VISCOSITY OF CELLULOSE GUM

Apparatus: Use a Brookfield Model LV series viscometer, analog or digital, or equivalent type viscometer for the determination of viscosity of aqueous solutions of cellulose gum within the range of 25–10,000 centipoises at 25°. Rotational viscometers of this type have spindles for use in determining the viscosity of different viscosity types of cellulose gum. The spindles and speeds for determining viscosity within different ranges are tabulated below.

Viscometer Spindles Required for Given Speeds

Viscosity Range (centi-poises)	Spindle No.	Speed (rpm)	Scale	Factor
10–100	1	60	100	1
100–200	1	30	100	2
200–1000	2	30	100	10
1000–4000	3	30	100	40
4000–10,000	4	30	100	200

Mechanical stirrer: Use an agitator, essentially as shown in *Figure 7*, that can be attached to a variable-speed motor capable of operating at 900 ± 100 rpm under varying load conditions.

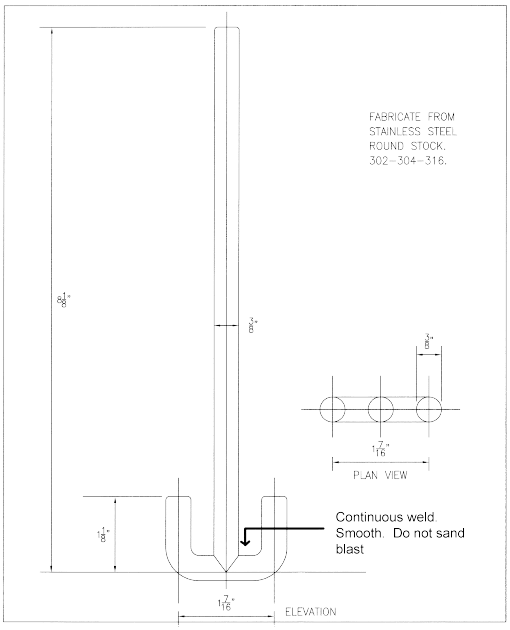


Figure 7. Agitator for Viscosity of Cellulose Gum

[NOTE—The agitator may be fabricated from stainless steel (Hercules, Inc., Wilmington, Delaware, or equivalent.) or glass as shown in *Figure 7*. Where this procedure is specified for viscosity measurements by reference in other monographs, equivalent three-blade agitators may be used.]

Sample container: Use a glass jar about 152 mm deep having an od of approximately 64 mm and a capacity of about 340 g.

Water bath: Use a water bath capable of maintaining a constant temperature. Set the temperature to 25°, and maintain it within ±0.2°.

Thermometer: Use an ASTM Saybolt Viscosity Thermometer having a range from 19° to 27° and conforming to the requirements for Thermometer 17C as described in ASTM Specification E1.

Sample preparation: Accurately weigh an amount of sample equivalent to 4.8 g of cellulose gum on the dried basis, and record the actual quantity required, in grams, as S. Transfer an accurately measured volume of water equivalent to 240 – S g into the sample container. Position the stirrer in the sample container, allowing minimal clearance between the stirrer and

the bottom of the container. Begin stirring, and slowly add the sample. Adjust the stirring speed to approximately 900 ± 100 rpm. Mix for exactly 2 h. Do not allow the stirring speed to exceed 1200 rpm. Remove the stirrer, cap the sample container, and transfer the sample container into a constant-temperature water bath, maintained at 25 ± 0.2°, for 1 h. Check the sample temperature with a thermometer at the end of 1 h to ensure that the test temperature has been reached.
Procedure: Remove the sample container from the water bath, shake vigorously for 10 s, and measure the viscosity with the Brookfield viscometer, using the proper spindle and speed indicated in the accompanying table. Be sure to use the viscometer guard, and allow the spindle to rotate for 3 min before taking the reading. Calculate the viscosity, in centipoises, by multiplying the reading observed by the appropriate factor from the table.

Water Determination

METHOD I (KARL FISCHER TITRIMETRIC METHOD)

Determine the water by *Method Ia*, unless otherwise specified in the individual monograph.

METHOD Ia (DIRECT TITRATION)

Principle: The titrimetric determination of water is based on the quantitative reaction of water with an anhydrous solution of sulfur dioxide and iodine in the presence of a buffer that reacts with hydrogen ions.

In the original titrimetric solution, known as *Karl Fischer Reagent*, the sulfur dioxide and iodine are dissolved in pyridine and methanol. Pyridine-free reagents are more commonly used now. The test specimen may be titrated with the *Karl Fischer Reagent* directly, or the analysis may be carried out by a residual titration procedure. The stoichiometry of the reaction is not exact, and the reproducibility of the determination depends on such factors as the relative concentrations of the *Karl Fischer Reagent* ingredients, the nature of the inert solvent used to dissolve the test specimen, the apparent pH of the final mixture, and the technique used in the particular determination. Therefore, an empirically standardized technique is used to achieve the desired accuracy. Precision in the method is governed largely by the extent to which atmospheric moisture is excluded from the system. The titration of water is usually carried out with the use of anhydrous methanol as the solvent for the test specimen; however, other suitable solvents may be used for special or unusual test specimens.

Substances that may interfere with the test results are ferric ion, chlorine, and similar oxidizing agents, as well as significant amounts of strong acids or bases, phosgene, or anything that will reduce iodide to iodine, poison the reagent, and show the sample to be bone dry when water may be present (false negative). 8-Hydroxyquinoline may be added to the vessel to eliminate interference from ferric ion. Chlorine interference can be eliminated with sulfur dioxide or unsaturated hydrocarbon. Excess pyridine or other amines may be added to the vessel to eliminate the interference of strong acids. Excess acetic acid or other carboxylic acid can be added to reduce the interference of strong bases. Aldehydes and ketones may react with the solution, showing the sample to be wet while the detector never reaches an endpoint (false positive).
Apparatus: Any apparatus may be used that provides for adequate exclusion of atmospheric moisture and for determination of the endpoint. In the case of a colorless solution that is titrated directly, the endpoint may be observed visually as a change in color from canary yellow to amber. The reverse is observed in the case of a test specimen that is titrated residually. More commonly, however, the endpoint is determined electrometrically with an apparatus employing a simple electrical circuit that serves to impress about 200 mV of applied potential between a pair of platinum electrodes (about 5 mm^2 in area and about 2.5 cm apart) immersed in the solution to be titrated. At the endpoint of the titration, a slight excess of the reagent increases the flow of current to 50–150 microamperes for 30 s to 30 min, depending on the solution being titrated. The time is shortest for substances that dissolve in the reagent. The longer times are required for solid materials that do not readily go into solution in the *Karl Fischer Reagent*. With some automatic titrators, the abrupt change in current or potential at the endpoint serves to close a solenoid-operated valve that controls the buret delivering the titrant. A commercially available apparatus generally comprises a closed system consisting of one or two automatic burets and a tightly covered titration vessel fitted with the necessary electrodes and a magnetic stirrer. The air in the system is kept dry with a suitable desiccant such as phosphorus pentoxide, and the titration vessel may be purged by means of a stream of dry nitrogen or a current of dry air.
Reagent: The *Karl Fischer Reagent* may be prepared as follows: Add 125 g of iodine to a solution containing 670 mL of methanol and 170 mL of pyridine, and cool. Place 100 mL of pyridine in a 250-mL graduated cylinder, and keeping the pyridine cold in an ice bath, pass in dry sulfur dioxide until the volume reaches 200 mL. Slowly add this solution, with shaking, to the cooled iodine mixture. Shake to dissolve the iodine, transfer the solution to the apparatus, and allow the solution to stand overnight before standardizing. One mL of this solution, when freshly prepared, is equivalent to approximately 5 mg of water, but it deteriorates gradually; therefore, standardize it within 1 h before use, or daily in continual use. Protect the solution from light while in use. Store any bulk stock of the solution in a suitably sealed, glass-stoppered container, fully protected from light and under refrigeration.

A commercially available, stabilized solution of a Karl Fischer-type reagent may be used. Commercially available reagents containing solvents or bases other than pyridine and/or alcohols other than methanol also may be used. These may be single solutions or reagents formed in situ by combining the components of the reagents present in two discrete solutions. The diluted *Karl Fischer Reagent* called for in some monographs should be diluted as directed by the manufacturer. Either methanol, or another suitable solvent such as ethylene glycol monomethyl ether, may be used as the diluent.
Test preparation: Unless otherwise specified in the individual monograph, use an accurately weighed or measured amount of the specimen under test estimated to contain 10–250 mg of water.

Where the monograph specifies that the specimen under test is hygroscopic, accurately weigh a sample of the specimen into a suitable container. Use a dry syringe to inject an appropriate volume of methanol, or other suitable solvent, accurately measured, into the container and shake to dissolve the specimen. Dry the syringe, and use it to remove the solution from the container and transfer it to a titration vessel prepared as directed under *Procedure*. Repeat the procedure with a second

portion of methanol, or other suitable solvent, accurately measured; add this washing to the titration vessel; and immediately titrate. Determine the water content, in milligrams, of a portion of solvent of the same total volume as that used to dissolve the specimen and to wash the container and syringe, as directed under *Standardization of water solution for residual titration*, and subtract this value from the water content, in mg, obtained in the titration of the specimen under test.

Standardization of the reagent: Place enough methanol or other suitable solvent in the titration vessel to cover the electrodes, and add sufficient *Karl Fischer Reagent* to give the characteristic color or 100 ± 50 microamperes of direct current at about 200 mV of applied potential. Pure methanol can make the detector overly sensitive, particularly at low ppm levels of water, causing it to deflect to dryness and slowly recover with each addition of reagent. This slows down the titration and may allow the system to actually pick up ambient moisture during the resulting long titration. Adding chloroform or a similar nonconducting solvent will retard this sensitivity and can improve the analysis.

For determination of trace amounts of water (less than 1%), quickly add 25 µL (25 mg) of pure water, using a 25- or 50-µL syringe, and titrate to the endpoint. The water equivalence factor F, in mg of water per mL of reagent, is given below:

$$\text{Result} = 25/V$$

in which V is the volume, in mL, of the *Karl Fischer Reagent* consumed in the second titration.

For the precise determination of significant amounts of water (more than 1%), quickly add 25–250 mg (25–250 µL) of pure water, accurately weighed by difference from a weighing pipet or from a precalibrated syringe or micropipet, the amount of water used being governed by the reagent strength and the buret size, as referred to under *Volumetric Apparatus*. Titrate to the endpoint. Calculate the water equivalence factor, F, in mg of water per mL of reagent:

$$\text{Result} = W/V$$

in which W is the weight, in mg, of the water, and V is the volume, in mL, of the *Karl Fischer Reagent* required.

Procedure: Unless otherwise specified, transfer 35–40 mL of methanol or other suitable solvent to the titration vessel, and titrate with the *Karl Fischer Reagent* to the electrometric or visual endpoint to consume any moisture that may be present. (Disregard the volume consumed because it does not enter into the calculations.) Quickly add the *Test Preparation*, mix, and again titrate with the *Karl Fischer Reagent* to the electrometric or visual endpoint. Calculate the water content of the specimen, in mg:

$$\text{Result} = SF$$

in which S is the volume, in mL, of the *Karl Fischer Reagent* consumed in the second titration, and F is the water equivalence factor of the *Karl Fischer Reagent*.

METHOD Ib (RESIDUAL TITRATION)

Principle: See the information in the section entitled *Principle* under *Method Ia*. In the residual titration, add excess *Karl Fischer Reagent* to the test specimen, allow sufficient time for the reaction to reach completion, and titrate the unconsumed *Karl Fischer Reagent* with a standard solution of water in a solvent such as methanol. The residual titration procedure is generally applicable and avoids the difficulties that may be encountered in the direct titration of substances from which the bound water is released slowly.

Apparatus, Reagent, and Test preparation: Use those in *Method Ia*.

Standardization of water solution for residual titration: Prepare a *Water solution* by diluting 2 mL of pure water to 1000 mL with methanol or another suitable solvent. Standardize this solution by titrating 25.0 mL with the *Karl Fischer Reagent*, previously standardized as directed under *Standardization of the reagent*. Calculate the water content, in mg/mL, of the *Water solution*:

$$\text{Result} = VF/25$$

in which V is the volume of the *Karl Fischer Reagent* consumed, and F is the water equivalence factor of the *Karl Fischer Reagent*. Determine the water content of the *Water solution* weekly, and standardize the *Karl Fischer Reagent* against it periodically as needed. Store the *Water solution* in a tightly capped container.

Procedure: Where the individual monograph specifies the water content is to be determined by *Method Ib*, transfer 35–40 mL of methanol or other suitable solvent into the titration vessel, and titrate with the *Karl Fischer Reagent* to the electrometric or visual endpoint. Quickly add the *Test preparation*, mix, and add an accurately measured excess of the *Karl Fischer Reagent*. Allow sufficient time for the reaction to reach completion, and titrate the unconsumed *Karl Fischer Reagent* with standardized *Water solution* to the electrometric or visual endpoint. Calculate the water content of the specimen, in mg:

$$\text{Result} = F(X' - XR)$$

in which F is the water equivalence factor of the *Karl Fischer Reagent*; X′ is the volume, in mL, of the *Karl Fischer Reagent* added after introduction of the specimen; X is the volume, in mL, of standardized *Water solution* required to neutralize the unconsumed *Karl Fischer Reagent*; and R is the ratio V/25 (mL of *Karl Fischer Reagent*/mL of *Water solution*), determined from the *Standardization of water solution for residual titration*.

METHOD Ic (COULOMETRIC TITRATION)

Principle: Use the Karl Fischer reaction in the coulometric determination of water. In this determination, iodine is not added in the form of a volumetric solution, but is produced in an iodide-containing solution by anodic oxidation. The reaction cell

usually consists of a large anode compartment and a small cathode compartment that are separated by a diaphragm. Other suitable types of reaction cells (e.g., without diaphragms) may be used. Each compartment has a platinum electrode that conducts current through the cell. Iodine, which is produced at the anode electrode, immediately reacts with the water present in the compartment. When all the water has been consumed, an excess of iodine occurs, which can be detected potentiometrically, thus indicating the endpoint. Pre-electrolysis, which can take several hours, eliminates moisture from the system. Therefore, changing the *Karl Fischer Reagent* after each determination is not practical. Individual determinations may be carried out in succession in the same reagent solution. A requirement for this method is that each component of the test specimen be compatible with the other components and that no side reactions take place. Samples may be transferred into the vessel as solids or as solutions by means of injection through a septum. Gases can be introduced into the cell by means of a suitable gas inlet tube. For the water determination of solids, another common technique is to dissolve the solid in a suitable solvent and then inject a portion of this solution into the cell. In the case of insoluble solids, water may be extracted using suitable solvents, and then the extracts injected into the coulometric cell. Alternatively, an evaporation technique may be used in which the sample is heated in a tube and the water is evaporated and carried into the cell by means of a stream of dry, inert gas. Precision in the method is predominantly governed by the extent to which atmospheric moisture is excluded from the system. Control of the system may be monitored by measuring the amount of baseline drift. The titration of water in solid test specimens is usually carried out with the use of anhydrous methanol as the solvent. Other suitable solvents may be used for special or unusual test specimens. This method is particularly suited to chemically inert substances such as hydrocarbons, alcohols, and ethers. In comparison with the volumetric Karl Fischer titration, coulometry is a micro-method. The method uses extremely small amounts of current. It is predominantly used for substances with a very low water content (0.1%–0.0001%).

Apparatus: Any commercially available apparatus consisting of an absolutely tight system fitted with the necessary electrodes and a magnetic stirrer is appropriate. The instrument's microprocessor controls the analytical procedure and displays the results. Calibration of the instrument is not necessary as the current consumed can be measured absolutely. Proper operation of the instrument can be confirmed by injecting 1 μL of water into the vessel. The instrument should read 1000 μg of water on reaching the endpoint.

Reagent: See *Reagent* under *Method Ia*.

Test preparation: Using a dry syringe, inject an appropriate volume of test specimen estimated to contain 0.5–5 mg of water, accurately measured, into the anolyte solution. The sample may also be introduced as a solid, accurately weighed, into the anolyte solution. Perform coulometric titration, and determine the water content of the specimen under test.

Alternatively, when the specimen is a suitable solid, dissolve an appropriate quantity, accurately weighed, in anhydrous methanol or another suitable solvent, and inject a suitable portion into the anolyte solution.

When the specimen is an insoluble solid, extract the water by using a suitable anhydrous solvent from which an appropriate quantity, accurately weighed, may be injected into the anolyte solution. Alternatively use an evaporation technique.

Procedure: Quickly inject the *Test preparation*, or transfer the solid sample, into the anolyte, mix, and perform the coulometric titration to the electrometric endpoint. Read the water content of the *Test preparation* directly from the instrument's display, and calculate the percent that is present in the substance.

METHOD II (TOLUENE DISTILLATION METHOD)

Principle: This method determines water by distillation of a sample with an immiscible solvent, usually toluene.

Apparatus: Use a glass distillation apparatus (see *Figure 8*) provided with 24/40 ground-glass connections. The components consist of a 500-mL short-neck, round-bottom flask connected by means of a trap to a 400-mm water-cooled condenser. The lower tip of the condenser should be about 7 mm above the surface of the liquid in the trap after distillation conditions have been established (see *Procedure*).

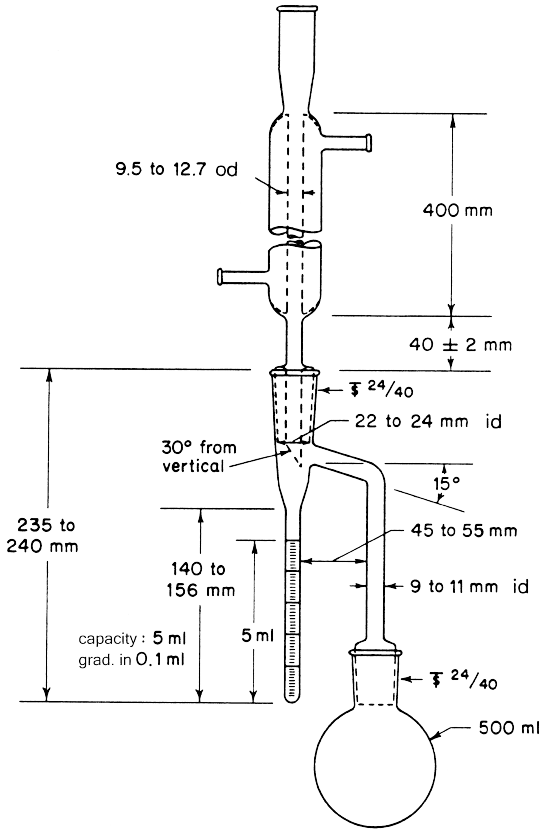


Figure 8. Moisture Distillation Apparatus

The trap should be constructed of well-annealed glass, the receiving end of which is graduated to contain 5 mL and subdivided into 0.1-mL divisions, with each 1-mL line numbered from 5 mL beginning at the top. Calibrate the receiver by adding 1 mL of water, accurately measured, to 100 mL of toluene contained in the distillation flask. Conduct the distillation, and calculate the volume of water obtained as directed in the *Procedure*. Add another mL of water to the cooled apparatus, and repeat the distillation. Continue in this manner until five 1-mL portions of water have been added. The error at any indicated capacity should not exceed 0.05 mL. The source of heat is either an oil bath or an electric heater provided with a suitable means of temperature control. The distillation may be better controlled by insulating the tube leading from the flask to the receiver. It is also advantageous to protect the flask from drafts. Clean the entire apparatus with potassium dichromate-sulfuric acid cleaning solution, rinse thoroughly, and dry completely before using.

Procedure: Place in the previously cleaned and dried flask a quantity of the substance, weighed accurately to the nearest 0.01 g, that is expected to yield 1.5–4 mL of water. If the substance is of a pastelike consistency, weigh it in a boat of metal foil that will pass through the neck of the flask. If the substance is likely to cause bumping, take suitable precautions to prevent it. Transfer about 200 mL of ACS reagent-grade toluene into the flask, and swirl to mix it with the sample. Assemble the apparatus, fill the receiver with toluene by pouring it through the condenser until it begins to overflow into the flask, and insert a loose cotton plug in the top of the condenser. Heat the flask so that the distillation rate will be about 200 drops/min, and continue distilling until the volume of water in the trap remains constant for 5 min. Discontinue the heating, use a copper or nichrome wire spiral to dislodge any drops of water that may be adhering to the inside of the condenser tube or receiver, and wash down with about 5 mL of toluene. Disconnect the receiver, immerse it in water at 25° for at least 15 min or until the toluene layer is clear, and then read the volume of water. Conduct a blank determination using the same volume of toluene as used when distilling the sample mixture, and make any necessary correction (see *General Provisions*).

C. OTHERS

Ash (Acid-Insoluble)

Boil the ash obtained as directed under *Ash (Total)*, below, with 25 mL of 2.7 N hydrochloric acid for 5 min, collect the insoluble matter on a tared, porous-bottom porcelain filter crucible or ashless filter, wash it with hot water, ignite to constant weight at 675 ± 25°, and weigh. Calculate the percent acid-insoluble ash from the weight of the sample taken.

[NOTE—Avoid exposing the crucible to sudden temperature changes.]

Ash (Total)

Unless otherwise directed, accurately weigh about 3 g of the sample in a tared crucible, ignite it at a low temperature (about 550°), not to exceed a very dull redness, until it is free from carbon, cool it in a desiccator, and weigh. If a carbon-free ash is not obtained, wet the charred mass with hot water, collect the insoluble residue on an ashless filter paper, and ignite the residue and filter paper until the ash is white or nearly so. Finally, add the filtrate, evaporate it to dryness, and heat the whole to a dull redness. If a carbon-free ash is still not obtained, cool the crucible, add 15 mL of ethanol, break up the ash with a glass rod, then burn off the ethanol, again heat the whole to a dull redness, cool it in a desiccator, and weigh.

Hydrochloric Acid Table

°Bé	Sp. Gr.	Percent HCl
1.00	1.0069	1.40
2.00	1.0140	2.82
3.00	1.0211	4.25
4.00	1.0284	5.69
5.00	1.0357	7.15
5.25	1.0375	7.52
5.50	1.0394	7.89
5.75	1.0413	8.26
6.00	1.0432	8.64
6.25	1.0450	9.02
6.50	1.0469	9.40
6.75	1.0488	9.78
7.00	1.0507	10.17
7.25	1.0526	10.55
7.50	1.0545	10.94
7.75	1.0564	11.32
8.00	1.0584	11.71
8.25	1.0603	12.09
8.50	1.0623	12.48
8.75	1.0642	12.87
9.00	1.0662	13.26
9.25	1.0681	13.65
9.50	1.0701	14.04
9.75	1.0721	14.43
10.00	1.0741	14.83
10.25	1.0761	15.22
10.50	1.0781	15.62
10.75	1.0801	16.01
11.00	1.0821	16.41
11.25	1.0841	16.81
11.50	1.0861	17.21
11.75	1.0881	17.61
12.00	1.0902	18.01
12.25	1.0922	18.41
12.50	1.0943	18.82
12.75	1.0964	19.22
13.00	1.0985	19.63

°Bé	Sp. Gr.	Percent HCl
13.25	1.1006	20.04
13.50	1.1027	20.44
13.75	1.1048	20.86
19.2	1.1526	30.00
19.3	1.1535	30.18
19.4	1.1544	30.35
19.5	1.1554	30.53
19.6	1.1563	30.71
19.7	1.1572	30.90
19.8	1.1581	31.08
19.9	1.1590	31.27
20.0	1.1600	31.45
20.1	1.1609	31.64
20.2	1.1619	31.82
20.3	1.1628	32.01
20.4	1.1637	32.19
20.5	1.1647	32.38
20.6	1.1656	32.56
20.7	1.1666	32.75
20.8	1.1675	32.93
20.9	1.1684	33.12
21.0	1.1694	33.31
21.1	1.1703	33.50
21.2	1.1713	33.69
21.3	1.1722	33.88
21.4	1.1732	34.07
21.5	1.1741	34.26
21.6	1.1751	34.45
21.7	1.1760	34.64
21.8	1.1770	34.83
21.9	1.1779	35.02
22.0	1.1789	35.21
22.1	1.1798	35.40
22.2	1.1808	35.59
22.3	1.1817	35.78
22.4	1.1827	35.97
22.5	1.1836	36.16
22.6	1.1846	36.35
22.7	1.1856	36.54
22.8	1.1866	36.73
22.9	1.1875	36.93
23.0	1.1885	37.14
23.1	1.1895	37.36
23.2	1.1904	37.58
23.3	1.1914	37.80
23.4	1.1924	38.03

°Bé	Sp. Gr.	Percent HCl
23.5	1.1934	38.26
23.6	1.1944	38.49
23.7	1.1953	38.72
23.8	1.1963	38.95
23.9	1.1973	39.18
24.0	1.1983	39.41
24.1	1.1993	39.64
24.2	1.2003	39.86
24.3	1.2013	40.09
24.4	1.2023	40.32
24.5	1.2033	40.55
24.6	1.2043	40.78
24.7	1.2053	41.01
24.8	1.2063	41.24
24.9	1.2073	41.48
25.0	1.2083	41.72
25.1	1.2093	41.99
25.2	1.2103	42.30
25.3	1.2114	42.64
25.4	1.2124	43.01
25.5	1.2134	43.40

Specific gravity determinations were made at 60°F, compared with water at 60°F.
From the specific gravities, the corresponding degrees Baumé were calculated by the following formula:

$$\text{degrees Baumé} = 145 - (145/\text{sp. gr.})$$

Baumé hydrometers for use with this table must be graduated by the above formula, which should always be printed on the scale.

ALLOWANCE FOR TEMPERATURE

10°–15°Bé: 1/40 °Bé or 0.0002 sp. gr. for 1°F
15°–22°Bé: 1/30 °Bé or 0.0003 sp. gr. for 1°F
22°–25°Bé: 1/28 °Bé or 0.00035 sp. gr. for 1°F

Insoluble Foreign Matter in Amino Acids

This method was developed to detect insoluble foreign contaminants such as fibers, metal, glass, insects, hair, and other foreign matter that may be present in amino acid ingredients.

REAGENTS

Diluent: 2.5 N sodium hydroxide unless otherwise specified in the individual monograph. [NOTE—Filter before use (100 μm or less).]
Apparatus: Assemble a vacuum filter flask apparatus[3] with a 100-μm nylon net filter as described in *Figure 9*.

[3] Sartorius Type 16510 (Sartorius AG), or equivalent.

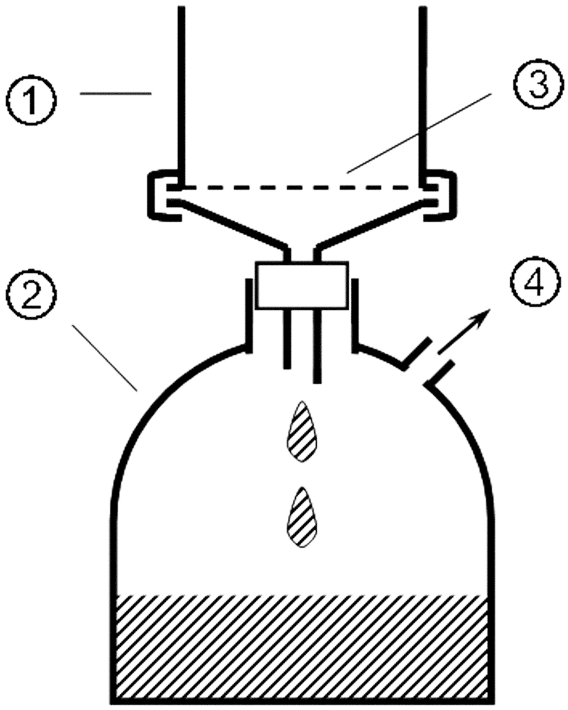


Figure 9. Vacuum Filter Apparatus (1. Clamped funnel, diameter about 5 cm, and clamp; 2. suction flask attached to clamped funnel with a rubber stopper/sleeve with one hole; 3. 100-µm nylon net filter[4] pre-dried and cooled (see *Procedure*); 4. vacuum line connection.)

SAMPLING

Composite sample: Combine and thoroughly mix the samples collected as prescribed below in the *Sampling plan*. Use a 100 g aliquot of this mixture as the test *Composite sample*, unless otherwise indicated in the monograph.
Sampling plan: For consignments comprising less than or equal to 3 containers, collect representative samples from each container in the consignment. For consignments comprising greater than or equal to 4 containers, take a representative sample from each of the n containers in the consignment where:

$$n = 1.5 \times \text{squareroot}(N)$$

N = total number of containers comprising the consignment

[NOTE—Appropriate measures should be taken to ensure that samples withdrawn from containers are representative of the entire container, especially for containers much larger than 50 kg. The *USP* general chapter *Bulk Powder Sampling Procedures* <1097> is one guidance standard that may be useful for this purpose. Additionally, for consignments far exceeding 100 containers, other applicable *Codex Alimentarius* and ISO standards may be consulted for appropriate sampling procedures.]

Sample solution: Quantitatively transfer the *Composite sample* into a glass beaker, add 500 mL *Diluent*, and stir until the sample is completely dissolved. [NOTE—*Diluent* can be used to aid in the quantitative transfer of the *Composite sample* into the glass beaker. Do not use a magnetic stirrer as it may trap ferric and/or ferrous contaminants.]

PROCEDURE

[NOTE—Operation shall be done on a clean bench or a booth. To avoid contamination during the preparation, use clean utensils.]

Pre-dry the 100-µm nylon net filter[4] in a glass beaker at 90° for 1 h. Cool the dried nylon net filter in a glass beaker to room temperature in a desiccator for 1 h and weigh the initial nylon net filter to the nearest 0.1 mg (X_1).

Filter the *Sample solution* through the nylon net filter with the *Vacuum Filter Apparatus* (see *Figure 9*). To ensure quantitative transfer of the *Sample solution* and any insoluble matter on the inner wall of the glass beaker, rinse the glass beaker twice with 200 mL of *Diluent* and pour the rinsing through the nylon net filter. Perform a final washing with a spray of water on the inner wall of the beaker and simultaneously pour this volume into the funnel. Wash the inner wall of the clamped funnel and the nylon net filter once with 200 mL of *Diluent* and twice with 250 mL water to ensure no insoluble matter remains on the inner wall of the funnel and no crystal residues of the tested amino acid are found on the nylon net

[4] Millipore catalog number NY1H04700, or equivalent.

filter. Observe the residue on the nylon net filter with a magnifying glass (4×) with the aid of light to detect presence of any fibers. Measure the length of any fibers present, in mm. Carefully transfer the nylon net filter with any foreign matter that it may hold into a glass beaker that has been previously dried at 90° for 1 h and cooled in a desiccator for 1 h. Dry the beaker and nylon net filter at 90° for 1 h, and cool in a desiccator for 1 h. Weigh the nylon net filter with any foreign matter to the nearest 0.1 mg (X_2). Calculate the foreign matter weight:

$$\text{Result} = X_2 - X_1$$

X_1 = initial filter membrane weight (mg)

X_2 = final filter membrane weight (mg)

Loss on Drying

This procedure is used to determine the amount of volatile matter expelled under the conditions specified in the monograph. Because the volatile matter may include material other than adsorbed moisture, this test is designed for compounds in which the loss on drying may not definitely be attributable to water alone. For substances appearing to contain water as the only volatile constituent, the *Direct (Karl Fischer) Titration Method*, provided under *Water, Appendix IIB*, is usually appropriate.

PROCEDURE

Unless otherwise directed in the monograph, conduct the determination on 1–2 g of the substance, previously mixed and accurately weighed. If the sample is in the form of large crystals, reduce the particle size to about 2 mm, quickly crushing the sample to avoid absorption or loss of moisture. Tare a glass-stoppered, shallow weighing bottle that has been dried for 30 min under the same conditions to be used in the determination. Transfer the sample to the bottle, replace the cover, and weigh the bottle and its contents. By gentle sideways shaking, distribute the sample as evenly as possible to a depth of about 5 mm for most substances and not over 10 mm in the case of bulky materials. Place the loaded bottle in the drying chamber, removing the stopper and leaving it also in the chamber, and dry at the temperature and for the length of time specified in the monograph. Upon opening the chamber, close the bottle promptly and allow it to come to room temperature, preferably in a desiccator, before weighing.

Where drying in vacuum is specified in the monograph, use a pressure as low as that obtainable by an aspirating water pump (NMT 20 mm Hg).

If the test substance melts at a temperature lower than that specified for the determination, preheat the bottle and its contents for 1–2 h at a temperature 5°–10° below the melting range, then continue drying at the specified temperature for the determination. When drying the sample in a desiccator, ensure that the desiccant is kept fully effective by replacing it frequently.

Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) spectroscopy is an analytical procedure based on the magnetic properties of certain atomic nuclei. It is similar to other types of spectroscopy in that absorption or emission of electromagnetic energy at characteristic frequencies provides analytical information. NMR differs in that the discrete energy levels between which the transitions take place are created artificially by placing the nuclei in a magnetic field.

Atomic nuclei are charged and behave as if they were spinning on the nuclear axis, thus creating a magnetic dipole of moment μ along this axis. The angular momentum of the spinning nucleus is characterized by a spin quantum number (I). If the mass number is odd, I is ½ or an integer plus ½; otherwise, it has a value of 0 or a whole number.

Nuclei having a spin quantum number $I \neq 0$, when placed in an external uniform static magnetic field of strength, H_0, align with respect to the field in $(2I + 1)$ possible orientations. Thus, for nuclei with I = ½, which include most isotopes of analytical significance, as shown in the table below, there are two possible orientations, corresponding to two different energy states. A nuclear resonance is the transition between these states, by absorption or emission of the corresponding amount of energy. In a static magnetic field the nuclear magnetic axis precesses (Larmor precession) about the external field axis. The precessional angular velocity, ω_0, is related to the external magnetic field strength through the equation:

$$\omega_0 = \gamma H_0$$

in which γ is the magnetogyric ratio and is a constant for all nuclei of a given isotope. If energy from an oscillating radio-frequency field is introduced, the absorption of radiation takes place according to the relationship:

$$\Delta E = h\nu = \mu H_O/I$$

where h is Planck's constant, and

$$\nu = \omega_0/2\pi = \gamma H_0/2\pi$$

Thus, when the frequency (ν_0) of the external energy field ($E = h\nu$) is the same as the precessional angular velocity, resonance is achieved.

The energy difference between the two levels corresponds to electromagnetic radiation in the radio-frequency range. It is a function of γ, which is a property of the nucleus, and H_0, the external field strength. As shown in the table below, the resonance frequency of a nucleus increases with the increase of the magnetic field strength.

NMR is a technique of high specificity but relatively low sensitivity. The basic reason for the low sensitivity is the comparatively small difference in energy between the excited and the ground states (0.02 calories at 15–20 kilogauss field strength), which results in a population difference between the two levels of only a few ppm. Another important aspect of the NMR phenomenon, with negative effects on the sensitivity, is the long lifetime of most nuclei in the excited state, which affects the design of the NMR analytical test, especially in pulsed repetitive experiments. Simultaneous acquisition of the entire spectrum instead of frequency-swept spectra can give sensitivity enhancement.

Properties of Some Nuclei Amenable to NMR Study

				Resonance Frequency (MHZ) at		
Nucleus	**I**	**Natural Abundance, %**	**Sensitivity**	**1.4093 T***	**2.3488 T**	**4.6975 T**
^{1}H	½	99.980	1.000	60.000	100.000	200.000
^{13}C	½	1.108	0.0159	15.087	25.144	50.288
^{19}F	½	100.000	0.830	56.446	94.077	188.154
^{31}P	½	100.000	0.0663	24.289	40.481	80.961
^{11}B	$(^{3}/_{2})$	80.420	0.170	19.250	32.084	64.167

* T = tesla, 1 T = 10,000 Gauss.

APPARATUS

The distinctive components of an NMR spectrometer are a magnet and a source of radio frequency. The instruments are described by the approximate resonance frequency of the analytical nucleus, e.g., ^{1}H NMR. More recently, instruments are being referred to by their field strengths. Some spectrometers are dedicated to the analysis of one type of nucleus; others are designed to obtain spectra of different nuclei.

There are two types of commercial NMR spectrometers: the classical continuous wave (CW) instruments and the more modern pulse Fourier-transform (FT) instruments. The CW spectrometers use a technique similar to that of classical optical spectrometers: a slow scan of the radio frequency (at fixed magnetic field) or the magnetic field (at fixed radio frequency) over a domain corresponding to the resonance of the nuclei being studied. The signal generated by the absorption of energy is detected, amplified, and recorded.

Various instrument configurations are possible. The arrangement of a typical double-coil spectrometer, as one might see in the lower resolution 60-MHz and 100-MHz CW instruments, is illustrated in *Figure 10*.

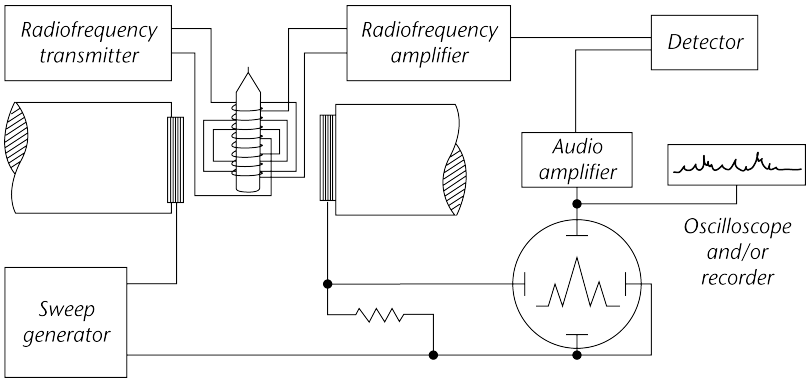


Figure 10. Block Diagram of a Typical NMR Spectrometer

The limitations of the CW spectrometers are low sensitivity and long analysis time. In pulsed NMR spectrometers, a single pulse of radio frequency energy is used to simultaneously activate all nuclei. The excited nuclei returning to the lower energy level generate a free induction decay (FID) signal that contains in a time domain all the information obtained in a frequency domain with a CW spectrometer. The time domain and the frequency domain responses form a pair of FTs; the mathematical operation is performed by a computer after analog-to-digital conversion. After a delay allowing for relaxation of the excited nuclei, the pulse experiment (transient) may be repeated and the response coherently added in the computer memory, with random noise being averaged out. (A similar signal-to-noise increase can be obtained by combining CW spectrometers with computers that average transients.)

The block diagram of a typical high-resolution pulsed spectrometer is shown in *Figure 11*.

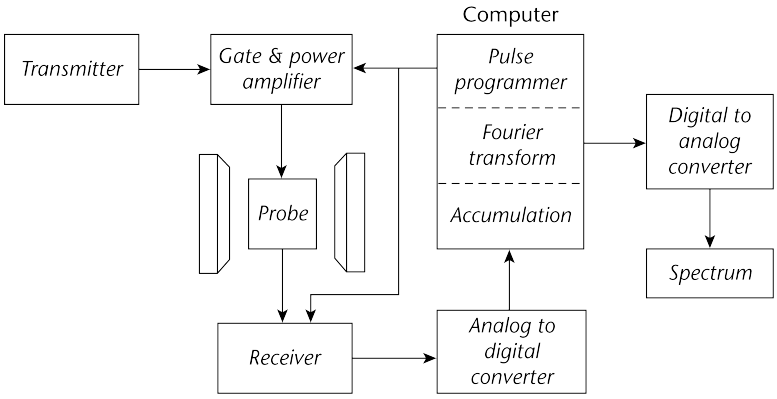


Figure 11. Block Diagram of a Typical Pulsed FT-NMR Spectrometer

It is a typical configuration of the high-resolution spectrometer that uses a superconducting (cryogenic) solenoid as the source of the magnetic field. Introduction of the pulsed NMR spectrometer has made the acquisition of spectra of many nuclei, other than protons, routine. It has also allowed proton spectra to be obtained in much less time, and with smaller amounts of specimen, as compared to CW techniques.

NMR spectrometers have strict stability and homogeneity requirements. Stability is often achieved by a field-frequency locking system that "locks" the magnetic field to the resonance frequency of a reference signal. The lock signal can be homonuclear or heteronuclear. In the latter case, the reference resonance is usually a deuterium signal from a deuterated solvent. On older spectrometers, using deuterium as a locking nucleus permits noise decoupling of protons to be carried out while studying nuclei like ^{13}C. While internal homonuclear locks are still used in CW proton spectrometers (where tetramethylsilane at about 0.5% provides a convenient lock), they are hardly ever used in pulsed FT spectrometers.

No type of magnet is capable of producing a homogeneous field over the space occupied by the specimen. Two techniques are usually employed to compensate for this lack of homogeneity: specimen spinning and the use of additional (shim) coils. Because of design, particularly probe design, the spinning in the case of the electromagnet or permanent magnet is perpendicular to the basic field. In the superconducting magnet, the axis of rotation can only be parallel to the basic magnetic field. The spin rate should be sufficient to produce averaging of the field, but not fast enough to produce an extended vortex in the specimen tube. A vortex extended near the region exposed to the radio-frequency coils decreases resolution. The shim coils are adjusted by the operator until instrumental contributions to the observed line width are minimized.

An electronic integrator is a feature of most NMR spectrometers. On a CW instrument (^1H and ^{19}F) the integrator, connected to the spectrometer output stage, determines the relative areas of the resonance peaks and presents these areas as a series of stepped horizontal lines when a sweep is made in the integration mode. On FT-NMR spectrometers, an integration algorithm is included in the spectrometer software, and the resonance peak areas may be presented graphically as stepped lines or tabulated as numeric values. The use of computer-generated tabulated/numeric integration data should not be accepted without a specific demonstration of precision and accuracy on the spectrometer in question.

THE SPECTRUM

The signals (peaks) in an NMR spectrum are characterized by four attributes: resonance frequency, multiplicity, line width, and relative intensity. The analytical usefulness of the NMR technique resides in the fact that the same types of nuclei, when located in different molecular environments, exhibit different resonance frequencies. The reason for this difference is that the effective field experienced by a particular nucleus is a composite of the external field provided by the instrument and the field generated by the circulation of the surrounding electrons. (The latter is generally opposed to the external field and the phenomenon is called "shielding.") In contrast with other spectroscopic methods, it is not possible to measure accurately the absolute values of transition frequencies. However, it is possible to measure accurately the difference in frequencies between two resonance signals. The position of a signal in an NMR spectrum is described by its separation from another resonance signal arbitrarily taken as standard. This separation is called chemical shift.

The chemical shift, being the difference between two resonance frequencies, is directly proportional to the magnetic field strength (or to the frequency of the oscillator). However, the ratio between the chemical shift, in frequency units, and the instrument frequency is constant. This allows definition of a dimensionless chemical shift parameter (δ) that is independent of the instrument frequency:

$$\delta = (\nu_s - \nu_r)/\nu_o + \delta_r$$

in which ν_s is the test substance line frequency, ν_r is the reference line frequency, ν_o is the instrument frequency, in mHz, and δ_r is the chemical shift of the reference.

By employing the above equation, it is possible to use (with appropriate caution) the chemical shift of any known species (such as the residual ^1H-containing species in deuterated solvent) as a chemical shift reference. The above equation, now in common use, is applicable to nearly all methods except in the relatively rare cases where extremely precise chemical shift values must be determined, and is readily adaptable to nuclei where non-zero reference standards are the only practical method of chemical shift determinations.

For CW instruments, tetramethylsilane (TMS) is the most widely used chemical shift reference for proton and carbon spectra. It is chemically inert, exhibits only one line, which is at a higher field than most signals, and is volatile, thus allowing for ready specimen recovery. Sodium 3-(trimethylsilyl)propionate (TSP) or sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) are used as NMR references for aqueous solutions. The resonance frequency of the TSP or DSS methyl groups closely approximate that of the TMS signal; however, DSS has the disadvantage of showing a number of methylene multiplets that may interfere with signals from the test substance. Where the use of an internal NMR reference material is not desirable, an external reference may be used.

Conventional NMR spectra are shown with the magnetic field strength increasing from left to right. Nuclei that resonate at high magnetic field strengths (to the right) are said to be more shielded (greater electron density) than those that resonate at lower magnetic field strengths: these are said to be de-shielded (lower electron density).

Figure 12 shows the proton NMR spectrum of 2,3-dimethyl-2-butenyl methyl ether. This compound contains protons in a methylene group (marked *d* in the graphic formula) and in four methyl groups (*a*, *a*, *b*, and *c*). Methyl groups *b* and *c* are situated in distinctly different molecular environments than the two *a* methyl groups. Three different methyl proton resonances are observed as spectral peaks in addition to the peak corresponding to methylene proton resonance. The two *a* methyl groups, being in very similar environments, have the same chemical shift. Interaction between magnetically active nuclei situated within a few bond lengths of each other leads to coupling, which results in a mutual splitting of the respective signals into sets of peaks or multiplets.

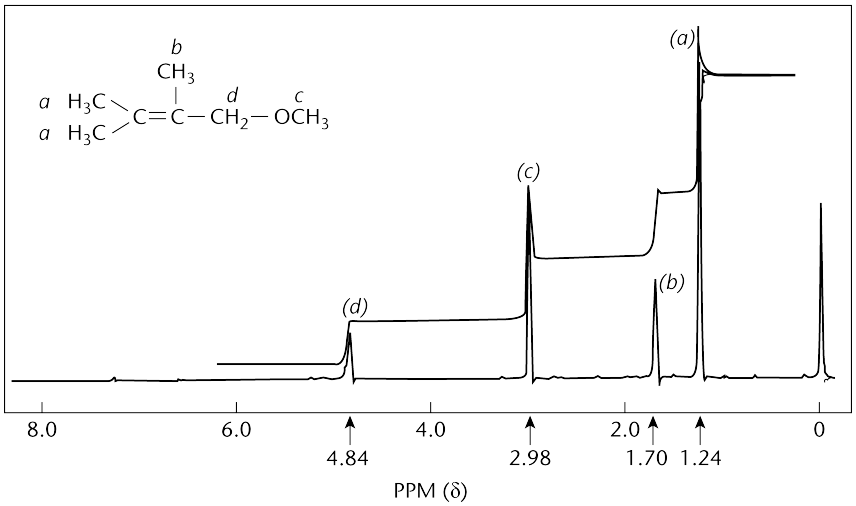


Figure 12. NMR Spectrum of 2,3-Dimethyl-2-butenyl methyl ether (15% in CCl_4) showing four nonequivalent, apparently uncoupled protons with a normal integral trace (peak area ratio from low H_0 to high H_0 of 2:3:3:6). (Tetramethylsilane, the NMR Reference, appears at 0 ppm.) The system of units represented by δ is defined under *The Spectrum*, in this section.

The coupling between two nuclei may be described in terms of the spin-spin coupling constant, J, which is the separation (in hertz) between the individual peaks of the multiplet. Where two nuclei interact and cause reciprocal splitting, the measured coupling constants in the two resulting mutiplets are equal. Furthermore, J is independent of magnetic field strength.

In a first-order, comparatively noncomplex spin system, the number of individual peaks that are expected to be present in a multiplet and the relative peak intensities are predictable. The number of peaks is determined by 2 nI + 1, where n is the number of nuclei on adjacent groups that are active in splitting. For protons this becomes (n + 1) peaks. In general, the relative intensity of each peak in the multiplet follows the coefficient of the binomial expansion $(a + b)^n$. These coefficients may conveniently be found by use of Pascal's triangle, which produces the following relative areas for the specified multiplets: doublet, 1:1; triplet, 1:2:1; quartet, 1:3:3:1; quintet, 1:4:6:4:1; sextet, 1:5:10:10:5:1; and septet, 1:6:15:20:15:6:1. This orderly arrangement, generally referred to as first-order behavior, may be expected when the ratio of Dν to J is greater than about 10; Dν is the chemical shift difference between two nuclei or two groups of equivalent nuclei. Two examples of idealized spectra arising from first-order coupling are shown in *Figure 13*.

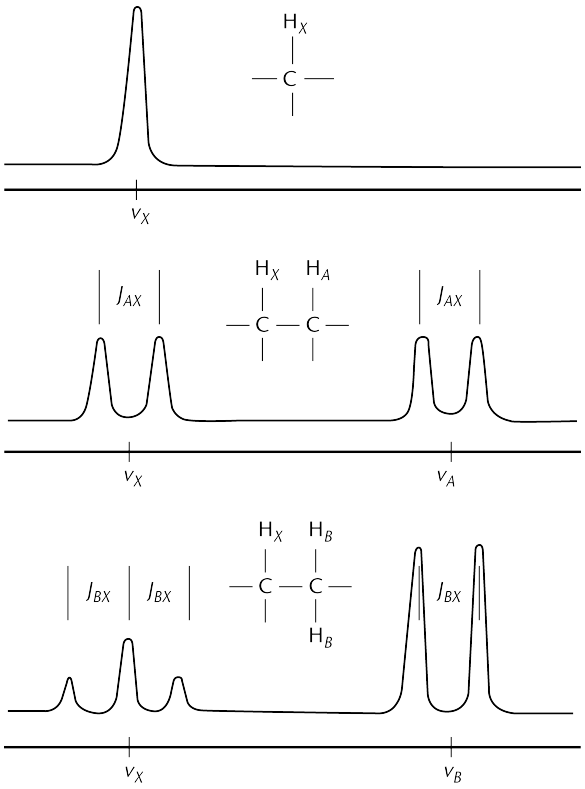


Figure 13. Diagrammatic Representation of Simple First-Order Coupling of Adjacent Protons

Figure 14 shows a spectrum displaying triplet signals resulting from the mutual splitting of two adjacent methylene groups.

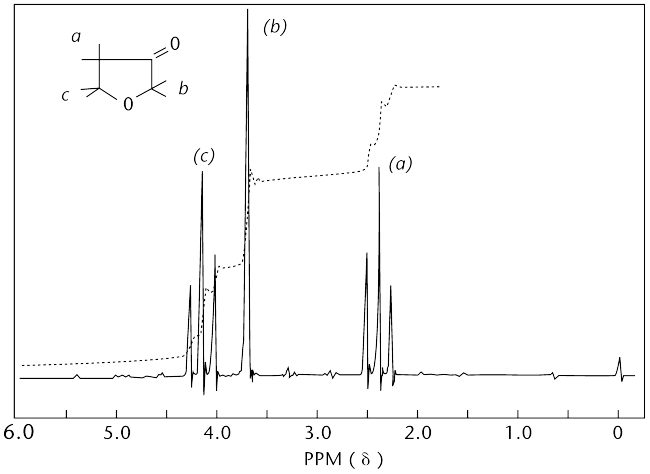


Figure 14. NMR Spectrum of 3-Keto-tetrahydrofuran (10% in CCl_4) showing three nonequivalent protons, with a normal integral trace (peak area ratio from low H_0 to high H_0 of 1:1:1). Note two sets of methylene groups coupled to each other at 4.2 and 2.4 ppm. (Tetramethylsilane, the NMR Reference, appears at 0 ppm.)

Coupling may occur between ^1H and other nuclei, such as ^{19}F, ^{13}C, and ^{31}P. In some cases, e.g., in the CW mode, the coupling constants may be large enough so that part of the multiplet is off scale at either the upfield or downfield end. This type of coupling may occur over the normal "three-bond distance," as for ^1H-^1H coupling.

Magnetically active nuclei with $I \geq 1$, such as ^{14}N, possess an electrical quadrupole moment, which produces line-broadening of the signal due to neighboring nuclei.

Another characteristic of the signal, its relative intensity, has wide analytical applications. In carefully designed experiments (see *General Method*, below), the area or intensity of a signal is directly proportional to the number of protons giving rise to the signal. As a result, it is possible to determine the relative ratio of the different kinds of protons or other nuclei in a specimen or to perform NMR assays with the aid of an internal standard.

The NMR spectra may contain extraneous signals due to the inhomogeneity of the magnetic field throughout the specimen. These artifacts, called spinning side bands, appear as minor lines symmetrically located around each signal. The presence of large spinning side bands indicates that the non-spinning shims require adjustment. The separation is equal to the frequency of the specimen tube spin rate or some integral multiple of that frequency. Thus, spinning side bands are readily identifiable.

GENERAL METHOD

Inadequate specimen preparation or incorrect instrumental adjustments and parameters may lead to poor resolution, decreased sensitivity, spectral artifacts, and erroneous data. It is preferable that the operator be familiar with the basic theory of NMR, the properties of the specimen, and the operating principles of the instruments. Strict adherence to the instruction manuals provided by the manufacturer and frequent checks of the performance of the instrument are essential.

The method and procedures discussed here refer specifically to ^{1}H (proton) and ^{19}F NMR. They are applicable, with modification, to other nuclei. The discussion presumes that the NMR spectra are obtained from liquid test substances or solutions in suitable solvents.

SELECTION OF SOLVENT

In addition to having good solubility properties, suitable solvents do not exhibit resonance peaks that obscure resonance peaks of the specimen being analyzed. The most commonly used solvents for proton and carbon NMR are listed in the table below. Deuterated solvents also provide the signal for the heteronuclear system lock. If solvent peaks might interfere with any signals from the specimen, then the isotopic purity of the solvent should be as high as possible. Deuterium (I = 1) does not exhibit resonance under ^{1}H conditions but may cause J-coupling to be observed. The residual protons generate solvent peaks whose chemical shifts are shown in the table below.

Solvents Commonly Used for Proton NMR

Solvent	Residual Proton Signal, δ[a]
CCl_4[b]	—
CS_2[b]	—
SO_2 (liquid)	—
$(CF_3)_2CO$	—
$CDCl_3$	7.27
CD_3OD	3.35, 4.8[c]
$(CD_3)_2CO$	2.05
D_2O	4.7[c]
DMSO-d_6[d]	2.50
C_6D_6	7.20
p-Dioxane-d_8	3.55
CD_3CO_2D	2.05, 8.5[c]
DMF-d_7[e]	2.77, 2.93, 8.05

[a] δ in ppm relative to tetramethylsilane arbitrarily taken as 0δ or 0 ppm.
[b] Spectrophotometric grade.
[c] Highly variable; depends on solute and temperature.
[d] Dimethyl sulfoxide-d_6.
[e] *N*,*N*-Dimethylformamide-d_7 per Aldrich, Alfa, Fluka, and Sigma catalogs.

Some solvents (e.g., D_2O or CD_3OD) enter into fast exchange reactions with protons and may eliminate resonance signals from –COOH, –OH, and $–NH_2$ structural groups. The protons in alcohols and amines do not take part in rapid exchange unless catalyzed by small concentrations of acid or base, except in the presence of D_2O and some other solvents (e.g., CD_3OD).

For ^{19}F NMR, most solvents used in proton NMR may be employed, the most common ones being $CHCl_3$, CCl_4, H_2O, CS_2, aqueous acids and bases, and dimethylacetamide. In general, any nonfluorinated solvent may be used, provided that it is of spectral quality. Obviously, there is no interference from the protonated functional groups of the solvent. However, unless they are decoupled, protonated functional groups on the ^{19}F-containing specimen will provide J-coupling.

SPECIMEN PREPARATION

Directions are usually given in individual monographs. The solute concentration depends on the objective of the experiment and on the type of instrument. Detection of minor contaminants may require higher concentrations. The solutions are prepared in separate vials and transferred to the NMR specimen tube. The volume required depends on the size of the specimen tube and on the geometry of the instrument. The level of the solution in the tube must be high enough to extend beyond the coils when the tube is inserted in the instrument probe and spun.

The NMR specimen tubes must meet narrow tolerance specifications in diameter, wall thickness, concentricity, and camber. The most widely used tubes have a 5-mm or 10-mm outside diameter and a length of 15–20 cm. Microtubes are available for the analysis of small amounts of specimen.

PROCEDURE

The specimen tube is placed in a probe located in the magnetic field. The probe contains electronic circuitry including the radio-frequency coil(s), and is provided with attachments for the air supply that spins the specimen tubes.

Instrument adjustments are made before each experiment. The spinning rate of the specimen tube is adjusted so that spinning side bands do not interfere with the peaks of interest and the vortex does not extend beyond the coils in the probe. To optimize the instrument performance, the magnetic shim gradients on FT-NMR spectrometers are adjusted. In adjusting resolution on CW spectrometers, a good indicator is the definite "ringing" of the TMS peak. The phenomenon of ringing is the oscillation of the recorder trace after the magnetic field has passed through a resonance frequency. Ringing, evident on a number of the peaks in *Figures 14* and *15*, arises during rapid scans and decays exponentially to the baseline value.

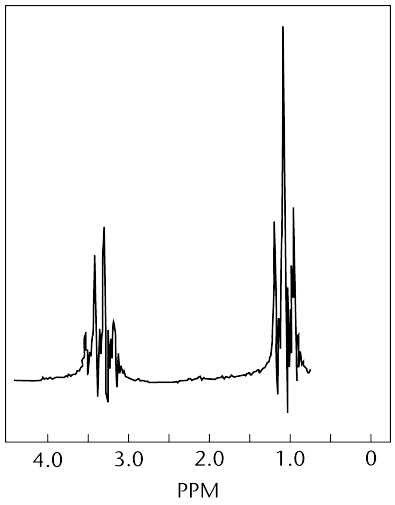


Figure 15. Continuous Wave Proton Spectrum of Ethyl Ether

Figure 16 clearly indicates the absence, in an FT experiment, of the ringing phenomenon. Ringing will not appear because the spectrum obtained is the result of analysis of the FID by Fourier transformation and not a magnetic field or frequency sweep through the individual resonance positions.

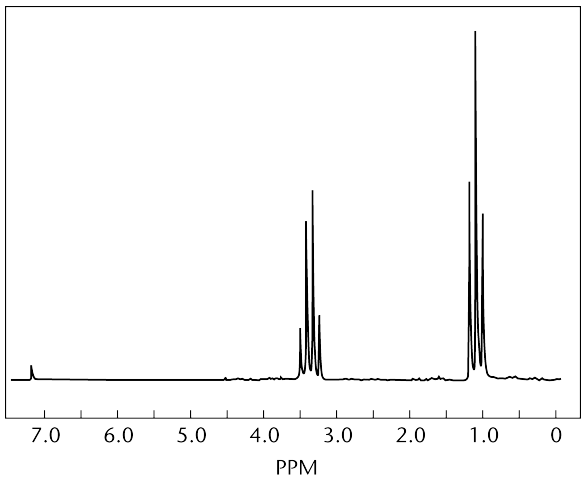


Figure 16. Proton NMR Spectrum of Ethyl Ether in Deuterated Chloroform

With proton CW instruments the spectrum is scanned from 0 ppm to about 10 ppm with a scan time of about 1–5 min. The amplification is adjusted so that all peaks remain on scale. If the response is low at reasonable amplitude, the radio-frequency power is increased to obtain the highest possible peak response without peak broadening. After the initial scan, the presence of peaks downfield of 10 ppm is quickly checked by off-setting the instrument response by about 5 ppm. With CW instrumentation, it is common for the TMS peak to shift slightly during an extended scan. The extent of the shift is usually obtained by comparing the relative positions of another peak in the initial scan with the same peak in the offset scan.

The operation of an FT-NMR spectrometer is a much more elaborate experiment. The computer serves to control the spectrometer, to program the experiment, and to store and process the data. Programming the experiment involves setting values for a large number of variables including the spectral width to be examined, the duration ("width") of the excitation pulse, the time interval over which data will be acquired, the number of transients to be accumulated, and the delay between one acquisition and the next. The analysis time for one transient is in the order of seconds. The number of transients is a function of the specimen concentration, the type of nucleus, and the objective of the experiment. At the end of the experiment, the FID signal is stored in digitized form in the computer memory and is displayed on the video screen. The signal can be processed mathematically to enhance either the resolution or the sensitivity, and it can be Fourier-transformed into a frequency-domain spectrum. The instrument provides a plot of the spectrum. The integration routine, accessed through keyboard commands, results in a stepped-line plot. Considerably more accurate integrals are obtained if the signals or regions of interest are separately integrated.

FT-NMR spectrometers may yield qualitative and quantitative data from the same experiment, but this is seldom done in practice. In quantitative FT experiments, special precautions must be taken for the signal areas to be proportional to the number of protons. The delays between pulses must be long enough to allow complete relaxation of all excited nuclei. This results in a considerable increase in analysis time and in some loss of resolution. Qualitative analysis is usually performed in nonquantitative conditions, with the design of the experiment directed to fast analysis with maximum resolution or sensitivity.

QUALITATIVE AND QUANTITATIVE ANALYSIS

NMR spectroscopy has been used for a wide range of applications such as structure elucidation; thermodynamic, kinetic, and mechanistic studies; and quantitative analysis. Some of these applications are beyond the scope of compendial methods.

All five characteristics of the signal (chemical shift, multiplicity, line width, coupling constants, and relative intensity) contribute analytical information.

QUALITATIVE APPLICATIONS

Comparison of a spectrum from the literature or from an authentic specimen with that of a test specimen may be used to confirm the identity of a compound and to detect the presence of impurities that generate extraneous signals. The NMR spectra of simple structures can be adequately described by the numeric value of the chemical shifts and coupling constants, and by the number of protons under each signal. (The software of modern instruments includes programs that generate simulated spectra using these data.) Experimental details, such as the solvent used, the specimen concentration, and the chemical shift reference, must also be provided.

For unknown specimens, NMR analysis, usually coupled with other analytical techniques, is a powerful tool for structure elucidation. Chemical shifts provide information on the chemical environment of the nuclei. Extensive literature is available with correlation charts and rules for predicting chemical shifts. The multiplicity of the signals provides important stereochemical information. Mutual signal splitting of functional groups indicates close proximity. The magnitude of the coupling constant, J, between residual protons on substituted aromatic, olefinic, or cycloalkyl structures is used to identify the relative position of the substituents.

Several special techniques (double resonance, chemical exchange, use of shift reagents, two-dimensional analysis, etc.) are available to simplify some of the more complex spectra, to identify certain functional groups, and to determine coupling correlations.

Double resonance, or spin decoupling, is a technique that removes the coupling between nuclei and thus simplifies the spectrum and identifies the components in a coupling relationship. For example, in a simple two-proton system, generally designated an AX system (see *Figure 13*), each proton appears as a doublet. If a strong radio-frequency field is introduced at the frequency of X, while the normal radio-frequency field is maintained at the frequency that causes A to resonate, the coupling between A and X is removed (homonuclear decoupling). A is no longer split, but instead appears as a singlet. Routine ^{13}C spectra are obtained under proton decoupling conditions that remove all heteronuclear ^{13}C-^{1}H couplings. As a result of this decoupling, the carbon signals appear as singlets, unless other nuclei that are not decoupled are present (e.g., ^{19}F, ^{31}P).

Functional groups containing exchangeable protons bound to hetero-atoms such as –OH, $–NH_2$, or –COOH groups may be identified by taking advantage of the rapid exchange of these protons with D_2O. To determine the presence and position of these groups, scan the test substance in $CDCl_3$ or DMSO-d_6, then add a few drops of D_2O to the specimen tube, shake, and scan again. The resonance peaks from these groups collapse in the second scan and are replaced by the HDO singlet between 4.7 and 5.0 ppm.

This chemical exchange is an example of the effect of intermolecular and intramolecular rate processes on NMR spectra. If a proton can experience different environments by virtue of such a process (tautomerism, rotation about a bond, exchange equilibria, ring inversion, etc.), the appearance of the spectrum will be a function of the rate of the process. Slow processes (on an NMR time scale) result in more than one signal, fast processes average these signals to one line, and intermediate processes produce broad signals.

The software of modern FT-NMR spectrometers allows for sequences of pulses much more complex than the repetitive accumulation of transients described above. Such experiments include homonuclear or heteronuclear two-dimensional analysis, which determines the correlation of couplings and may simplify the interpretation of otherwise complex spectra.

QUANTITATIVE APPLICATIONS

If appropriate instrument settings for quantitative analysis have been made, the areas (or intensities) of two signals are proportional to the total number of protons generating the signals.

$$A_1/A_2 = N_1/N_2 \quad (1)$$

If the two signals originate from two functional groups of the same molecule, the equation can be simplified to:

$$A_1/A_2 = n_1/n_2 \quad (2)$$

in which n_1 and n_2 are the number of protons in the respective functional groups.

If the two signals originate from different molecular species,

$$A_1/A_2 = n_1m_1/n_2m_2 = (n_1W_1/M_1)/(n_2W_2/M_2) \quad (3)$$

in which m_1 and m_2 are the numbers of moles; W_1 and W_2 are the masses; and M_1 and M_2 are the molecular weights of compounds 1 and 2, respectively.

Examination of Equations 2 and 3 shows that NMR quantitative analysis can be performed in an absolute or relative manner. In the absolute method, an internal standard is added to the specimen and a resonance peak area arising from the test substance is compared with a resonance peak area from the internal standard. If both test substance and internal standard are accurately weighed, the absolute purity of the substance may be calculated. A good internal standard has the following properties: it presents a reference resonance peak, preferably a singlet, at a field position removed from all specimen peaks; it is soluble in the analytical solvent; its proton equivalent weight, i.e., the molecular weight divided by the number of protons giving rise to the reference peak, is low; and it does not interact with the compound being tested. Typical examples of useful standards are 1,2,4,5-tetrachlorobenzene, 1,4-dinitrobenzene, benzyl benzoate, and maleic acid. The choice of a standard will be dictated by the spectrum of the specimen.

The relative method may be used to determine the molar fraction of an impurity in a test substance (or of the components in a mixture) as calculated by Equation 3.

Quantitative analysis, as well as detection of trace impurities, is markedly improved with modern instrumentation. Stronger magnetic fields and the ability to accumulate and/or average signals over long periods of time greatly enhance the sensitivity of the method.

ABSOLUTE METHOD OF QUANTITATION

Where the individual monograph directs that the *Absolute Method of Quantitation* be employed, proceed as follows.

Solvent, Internal standard, and NMR reference: Use as directed in the individual monograph.

Test preparation: Transfer an accurately weighed quantity of the test substance, containing about 4.5 proton mEq, to a glass-stoppered, graduated centrifuge tube. Add about 4.5 proton mEq of *Internal standard*, accurately weighed, and 3.0 mL of *Solvent*, insert the stopper, and shake. When dissolution is complete, add about 30 µL (30 mg if a solid) of *NMR Reference*, provided that it does not interfere with subsequent measurements, and shake.

Procedure: Transfer an appropriate amount (0.4–0.8 mL) of *Test preparation* to a standard 5-mm NMR spinning tube, and record the spectrum, adjusting the spin rate so that no spinning side bands interfere with the peaks of interest. Measure the area under each of the peaks specified in the individual monograph by integrating not fewer than five times. Record the average area of the *Internal standard* peak as A_S and that of the *Test preparation* peak as A_U.

Calculate the quantity, in mg, of the analyte in the *Test preparation*:

$$W_S(A_U/A_S)(E_U/E_S)$$

in which W_S is the weight, in mg, of *Internal standard* taken; and E_U and E_S are the proton equivalent weights (i.e., the molecular weights divided by the number of protons giving rise to the reference peak) of the analyte and the *Internal standard*, respectively.

RELATIVE METHOD OF QUANTITATION

Where the individual monograph directs that the *Relative Method of Quantitation* be employed, proceed as follows.

Solvent, NMR reference, and Test preparation: Use as directed under *Absolute Method of Quantitation*.

Procedure: Transfer an appropriate amount (0.4–0.8 mL) of *Test preparation* to a standard 5-mm NMR spinning tube, and record the spectrum, adjusting the spin rate so that no spinning side bands interfere with the peaks of interest. Measure the area or intensity under each of the peaks specified in the individual monograph by integrating not fewer than five times. Record the average area or intensity resulting from the resonances of the groups designated in the individual monograph as A_1 and A_2.

Calculate the quantity, in mole percent, of the analyte in the *Test preparation*:

$$(100 \times (A_1/n_1)/[(A_1/n_1) + (A_2/n_2)]$$

in which n_1 and n_2 are, respectively, the numbers of protons in the designated groups.

Oil Content of Synthetic Paraffin

APPARATUS

Filter stick: Use either a 10-mm diameter sintered-glass filter stick of 10–15-µm maximum pore diameter, or a filter stick made of stainless steel and having a 0.5-in. disk of 10–15-µm maximum pore diameter. Determine conformance with the pore diameter specified as follows: Clean sintered-glass filter sticks by soaking in hydrochloric acid, or stainless steel sticks by soaking in nitric acid, wash with water, rinse with acetone, and dry in air followed by drying in an oven at 105° for 30 min.

Thoroughly wet the clean filter stick by soaking in water, and then connect it with an apparatus (see *Figure 17*) consisting of a mercury-filled manometer, readable to 0.5 mm; a clean and filtered air supply; a drying bulb filled with silica gel; and a needle-valve type air pressure regulator. Apply pressure slowly from the air source, and immerse the filter just below the surface of water contained in a beaker.

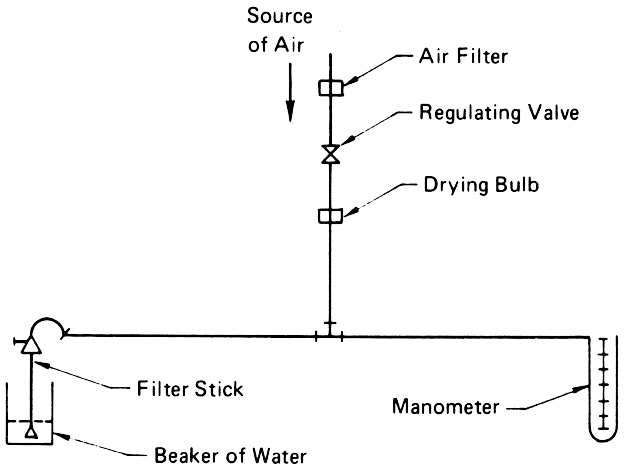


Figure 17. Assembly for Checking Pore Diameter of Filter Sticks

[NOTE—If a head of liquid is noted above the surface of the filter after it is inserted into the water, the back pressure thus produced should be subtracted from the observed pressure when the pore diameter is calculated as directed below.]

Increase the air pressure to 10 mm below the acceptable pressure limit, and then increase the pressure at a slow, uniform rate of about 3 mm Hg per minute until the first bubble passes through the filter. This can be conveniently observed by placing the beaker over a mirror. Read the manometer when the first bubble passes off the underside of the filter. Calculate the pore diameter, in µm:

$$\text{Result} = 2180/p$$

in which p is the observed pressure, in mm, corrected for any back pressure as mentioned above.

Filtration assembly: Connect the *Filter Stick* with an air pressure inlet tube and delivery nozzle and ground-glass joint to fit a 25-mm × 170-mm test tube as shown in *Figure 18*. If a stainless steel *Filter Stick* is used, make the connection to the test tube by means of a cork.

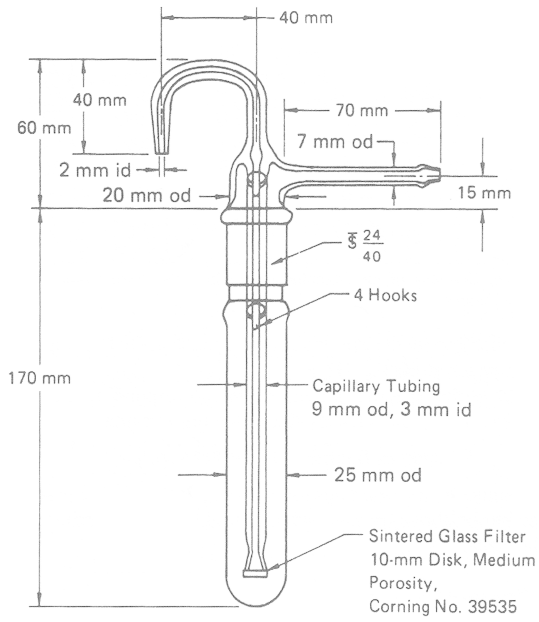


Figure 18. Filtration Assembly for Determination of Oil Content

Cooling bath: Use a suitable insulated box having 1-in. holes in the center to accommodate any desired number of test tubes. The bath may be filled with a suitable medium such as kerosene and may be cooled by circulating a refrigerant through coils, or by using solid carbon dioxide, to produce a temperature of 30 ± 2°F.
Air pressure regulator: Use a suitable pressure-reduction valve, or other suitable regulator, that will supply air to the *Filtration Assembly* at the volume and pressure required to give an even flow of filtrate (see *Procedure*). Connect the regulator with rubber tubing to the end of the *Filter Stick* in the *Filtration Assembly*.
Thermometer: Use an ASTM Oil in Wax Thermometer having the range of –35° to +70°F and conforming to the requirements for an ASTM 71F thermometer (see *Thermometers, Appendix I*).
Weighing bottles: Use glass-stoppered conical bottles having a capacity of 15 mL. The bottles are used as evaporating flasks in the *Procedure*.
Evaporation assembly: The assembly consists of an evaporating cabinet capable of maintaining a temperature of 95 ± 2°F around the evaporation flasks, and air jets (4 ± 0.2 mm id) for delivering a stream of clean, dry air vertically downward into the flasks. In the *Procedure* below, support each jet so that the tip is 15 ± 5 mm above the surface of the liquid at the start of the evaporation. Supply the air (purified by passage through a tube of 1-cm bore packed loosely to a height of 20 cm with absorbent cotton) at the rate of 2 to 3 L/min per jet. The cleanliness of the air should be checked periodically to ensure that NMT 0.1 mg of residue is obtained when 4 mL of methyl ethyl ketone is evaporated as directed in the *Procedure*.
Wire stirrer: Use a 250-mm length of stiff iron or nichrome wire of about No. 20 B & S gauge. Form a 10-mm diameter loop at each end, and bend the loop at the bottom end so that the plane of the loop is perpendicular to the length of the wire.

SAMPLE SELECTION

If the sample weighs about 1 kg or less, obtain a representative portion by melting the entire sample and stirring thoroughly. For samples heavier than about 1 kg, exercise special care to ensure that a truly representative portion is obtained, noting that the oil may not be distributed uniformly throughout the sample and that mechanical operations may have expressed some of the oil.

PROCEDURE

Melt a representative portion of the sample in a beaker, using a water bath or oven maintained at 160°–210°F. As soon as the sample is completely melted, thoroughly mix it by stirring. Preheat a dropper pipet, provided with a rubber bulb and calibrated to deliver 1 ± 0.05 g of molten sample, and withdraw a 1-g portion of the sample as soon as possible after it has melted. Hold the pipet in a vertical position, and carefully transfer its contents into a clean, dry test tube previously weighed to the nearest milligram. Evenly coat the bottom of the tube by swirling, allow the tube to cool, and weigh to the nearest milligram. Calculate the sample weight, in grams, and record it as B (see *Calculation*). Pipet 15 mL of methyl ethyl ketone (ASTM Specification D 740, or equivalent) into the tube, and immerse the tube up to the top of the liquid in a hot water or steam bath. Stir with an up-and-down motion with the wire stirrer, and continue heating and stirring until a homogeneous solution is obtained, exercising care to avoid loss of solvent by prolonged boiling.

[NOTE—If it appears that a clear solution will not be obtained, stir until any undissolved material is well dispersed so as to produce a slightly cloudy solution.]

After the sample solution is prepared, plunge the test tube into an 800-mL beaker of ice water, and continue to stir until the contents are cold. Remove the stirrer, then remove the test tube from the bath, dry the outside of the tube with a cloth, and weigh to the nearest 100 mg. Calculate the weight, in grams, of solvent in the test tube, and record it as C (see *Calculation*). Place the tube in the cooling bath, maintained at –30 ± 2°F, and stir continuously with the thermometer until the temperature reaches –25 ± 0.5°F, maintaining the slurry at a uniform consistency and taking precautions to prevent the sample from setting up on the walls of the tube or forming crystals.

Place the filter stick in a test tube and cool at –30 ± 2°F in the cooling bath for a minimum of 10 min. Immerse the cooled filter stick in the sample, then connect the filtration assembly, seating the ground-glass joint of the filter so as to make an airtight seal. Place an unstoppered weighing bottle, previously weighed together with the glass stopper to the nearest 0.1 mg, under the delivery nozzle of the filtration assembly.

[NOTE—Suitable precautions and proper analytical technique should be applied to ensure the accuracy of the weight of the bottle. Before determining its weight, the bottle and its stopper should have been cleaned and dried, then rinsed with methyl ethyl ketone, wiped dry on the outside, dried in the evaporation assembly for about 5 min, and cooled. Then allow it to stand for about 10 min near the balance before weighing.]

Apply air pressure to the filtration assembly, immediately collect about 4 mL of filtrate in the weighing bottle, and release the air pressure to permit the liquid to drain back slowly from the delivery nozzle. Stopper the bottle, and weigh it to the nearest 10 mg without waiting for it to come to room temperature. Remove the stopper, transfer the bottle to the evaporation assembly maintained at 95 ± 2°F, and place it under an air jet centered inside the neck, with the tip 15 ± 5 mm above the surface of the liquid. After the solvent has evaporated (usually less than 30 min), stopper the bottle, and allow it to stand near the balance for about 10 min before it is weighed to the nearest 0.1 mg. Repeat the evaporation procedure for 5-min periods until the loss between successive weighings is NMT 0.2 mg. Determine the weight of the oil residue, in grams, by subtracting the weight of the empty stoppered bottle from the weight of the stoppered bottle plus the oil residue after the evaporation procedure, and record the results as A (see *Calculation*). Determine the weight of solvent evaporated, in grams, by subtracting the weight of the bottle plus oil residue from the weight of the bottle plus filtrate, and record the result as D (see *Calculation*).

CALCULATION

Calculate the percent, by weight, of oil in the sample:

$$\text{Result} = (100\ AC/BD) - 0.15$$

in which 0.15 is a factor to correct for solubility of the sample in the solvent at –25°F.

Plasma Spectrochemistry

Plasma-based instrumental techniques that are useful for food ingredient analyses fall into two major categories: those based on the inductively coupled plasma, and those where a plasma is generated at or near the surface of the sample. An inductively coupled plasma (ICP) is a high-temperature excitation source that desolvates, vaporizes, and atomizes aerosol samples and ionizes the resulting atoms. The excited analyte ions and atoms can then subsequently be detected by observing their emission lines, a method termed inductively coupled plasma–atomic emission spectroscopy (ICP–AES), also known as inductively coupled plasma–optical emission spectroscopy (ICP–OES); or the excited or ground state ions can be determined by a technique known as inductively coupled plasma–mass spectrometry (ICP–MS). ICP–AES and ICP–MS may be used for either single- or multi-element analysis, and they provide good general-purpose procedures for either sequential or simultaneous analyses over an extended linear range with good sensitivity.

An emerging technique in plasma spectrochemistry is laser-induced breakdown spectroscopy (LIBS). In LIBS, a solid, liquid, or gaseous sample is heated directly by a pulsed laser, or indirectly by a plasma generated by the laser. As a result, the sample is volatilized at the laser beam contact point, and the volatilized constituents are reduced to atoms, molecular fragments, and larger clusters in the plasma that forms at or just above the surface of the sample. Emission from the atoms and ions in the sample is collected, typically using fiber optics or a remote viewing system, and is measured using an array detector such as a charge-coupled device (CCD). LIBS can be used for qualitative analysis or against a working standard curve for quantitative analysis. Although LIBS is not currently in wide use, it might be suited for at-line or on-line measurements in a production setting, as well as in the laboratory. Because of its potential, it should be considered a viable technique for plasma spectrochemistry in the laboratory. However, because LIBS is still an emerging technique, details will not be further discussed here.[5]

SAMPLE PREPARATION

Sample preparation is critical to the success of plasma-based analysis and is the first step in performing any analysis via ICP–AES or ICP–MS. Plasma-based techniques are heavily dependent on sample transport into the plasma, and because ICP–AES and ICP–MS share the same sample introduction system, the means by which samples are prepared may be applicable to either technique. The most conventional means by which samples are introduced into the plasma is via solution nebulization. If solution nebulization is employed, solid samples must be dissolved in order to be presented into the plasma for analysis. Samples may be dissolved in any appropriate solvent. There is a strong preference for the use of aqueous or

[5] Yueh F-Y, Singh JP, Zhang H. Laser-induced breakdown spectroscopy, elemental analysis. In: *Encyclopedia of Analytical Chemistry: Instrumentation and Applications.* New York: Wiley; 2000:2066–2087.

dilute nitric acid solutions, because there are minimal interferences with these solvents compared to other solvent choices. Hydrogen peroxide, hydrochloric acid, sulfuric acid, perchloric acid, combinations of acids, or various concentrations of acids can all be used to dissolve the sample for analysis. Dilute hydrofluoric acid may also be used, but great care must be taken to ensure the safety of the analyst, as well as to protect the quartz sample introduction equipment when using this acid; specifically, the nebulizer, spray chamber, and inner torch tube should be manufactured from hydrofluoric acid-tolerant materials. Additionally, alternative means of dissolving the sample can be employed. These include, but are not limited to, the use of dilute bases, straight or diluted organic solvents, combinations of acids or bases, and combinations of organic solvents.

When samples are introduced into the plasma via solution nebulization, it is important to consider the potential matrix effects and interferences that might arise from the solvent. The use of an appropriate internal standard and/or matching the standard matrix with samples should be applied for ICP–AES and ICP–MS analyses in cases where accuracy and precision are not adequate. In either event, the selection of an appropriate internal standard should consider the analyte in question, ionization energy, wavelengths or masses, and the nature of the sample matrix.

Where a sample is found not to be soluble in any acceptable solvent, a variety of digestion techniques can be employed. These include hot-plate digestion and microwave-assisted digestions, including open- and closed-vessel approaches. The decision regarding the type of digestion technique to use depends on the nature of the sample being digested, as well as on the analytes of interest.

Open-vessel digestion is generally not recommended for the analysis of volatile metals, e.g., selenium and mercury. The suitability of a digestion technique, whether open- or closed-vessel, should be supported by spike recovery experiments in order to verify that, within an acceptable tolerance, volatile metals have not been lost during sample preparation. Use acids, bases, and hydrogen peroxide of ultra-high purity, especially when ICP–MS is employed. Deionized water must be at least 18 megaohm. Check diluents for interferences before they are used in an analysis. Because it is not always possible to obtain organic solvents that are free of metals, use organic solvents of the highest quality possible with regard to metal contaminants.

It is important to consider the selection of the type, material of construction, pretreatment, and cleaning of analytical labware used in ICP–AES and ICP–MS analyses. The material must be inert and, depending on the specific application, resistant to caustics, acids, and/or organic solvents. For some analyses, diligence must be exercised to prevent the adsorption of analytes onto the surface of a vessel, particularly in ultra-trace analyses. Contamination of the sample solutions from metal and ions present in the container can also lead to inaccurate results.

The use of labware that is not certified to meet Class A tolerances for volumetric flasks is acceptable if the linearity, accuracy, and precision of the method have been experimentally demonstrated to be suitable for the purpose at hand.

SAMPLE INTRODUCTION

There are two ways to introduce the sample into the nebulizer: by a peristaltic pump and by self-aspiration. The peristaltic pump is preferred, and serves to ensure that the flow rate of sample and standard solution to the nebulizer is the same, irrespective of sample viscosity. In some cases, where a peristaltic pump is not required, self-aspiration can be used.

A wide variety of nebulizer types is available, including pneumatic (concentric and cross-flow), grid, and ultrasonic nebulizers. Micronebulizers, high-efficiency nebulizers, direct-injection high-efficiency nebulizers, and flow-injection nebulizers are also available. The selection of the nebulizer for a given analysis should consider the sample matrix, analyte, and desired sensitivity. Some nebulizers are better suited for use with viscous solutions or those containing a high concentration of dissolved solids, whereas others are better suited for use with organic solutions.

Note that the self-aspiration of a fluid is due to the Bernoulli or Venturi effect. Not all types of nebulizers will support self-aspiration. The use of a concentric nebulizer, for example, is required for self-aspiration of a solution.

Once a sample leaves the nebulizer as an aerosol, it enters the spray chamber, which is designed to permit only the smallest droplets of sample solution into the plasma; as a result, typically only 1%–2% of the sample aerosol reaches the ICP, although some special-purpose nebulizers have been designed that permit virtually all of the sample aerosol to enter the ICP. As with nebulizers, there is more than one type of spray chamber available for use with ICP–AES or ICP–MS. Examples include the Scott double-pass spray chamber, as well as cyclonic spray chambers of various configurations. The spray chamber must be compatible with the sample and solvent, and must equilibrate and wash out in as short a time as possible. When a spray chamber is selected, the nature of the sample matrix, the nebulizer, the desired sensitivity, and the analyte should all be considered.

Gas and liquid chromatography systems can be interfaced with ICP–AES and ICP–MS for molecular speciation, ionic speciation, or other modes of separation chemistry, based on elemental emission or mass spectrometry.

Ultimately, the selection of sample introduction hardware should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision of the analysis at hand.

In addition to solution nebulization, it is possible to analyze solid samples directly via laser ablation (LA). In such instances, the sample enters the torch as a solid aerosol. LA–ICP–AES and LA–ICP–MS are better suited for qualitative analyses of compounds, because of the difficulty in obtaining appropriate standards. Nonetheless, quantitative analyses can be performed if it can be demonstrated through appropriate method validation that the available standards are adequate.[6]

STANDARD PREPARATION

Single- or multi-element standard solutions, which have concentrations traceable to primary reference standards, such as those of the National Institute of Standards and Technology (NIST), can be purchased for use in the preparation of working standard solutions. Alternatively, standard solutions of elements can be accurately prepared from standard materials and

[6] For additional information on laser ablation, see Russo R, Mao X, Borisov O, Liu H. Laser ablation in atomic spectrometry. In: *Encyclopedia of Analytical Chemistry: Instrumentation and Applications.* New York: Wiley; 2000.

their concentrations, determined independently, as appropriate. Working standard solutions, especially those used for ultra-trace analyses, may have limited shelf life. As a general rule, working standard solutions should be retained for no more than 24 h, unless stability is demonstrated experimentally. The selection of the standard matrix is of fundamental importance in the preparation of element standard solutions. Spike recovery experiments should be conducted with specific sample matrices in order to determine the accuracy of the method. If sample matrix effects cause excessive inaccuracies, standards, blanks, and sample solutions should be matrix matched, if possible, in order to minimize matrix interferences.

In cases where matrix matching is not possible, an appropriate internal standard or the method of standard additions should be used for ICP–AES or ICP–MS. Internal standards can also be introduced through a T connector into the sample uptake tubing. In any event, the selection of an appropriate internal standard should consider the analytes in question, their ionization and excitation energies, their chemical behavior, their wavelengths or masses, and the nature of the sample matrix. Ultimately, the selection of an internal standard should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision of the analysis at hand.

The method of standard additions involves adding a known concentration of the analyte element to the sample at no fewer than two concentration levels plus an unspiked sample preparation. The instrument response is plotted against the concentration of the added analyte element, and a linear regression line is drawn through the data points. The absolute value of the x-intercept multiplied by any dilution factor is the concentration of the analyte in the sample.

The presence of dissolved carbon at concentrations of a small percentage in aqueous solutions enhances ionization of selenium and arsenic in an inductively coupled argon plasma. This phenomenon frequently results in a positive bias for ICP–AES and ICP–MS selenium and arsenic quantification measurements, which can be remedied by using the method of standard additions or by adding a small percentage of carbon, such as analytically pure glacial acetic acid, to the linearity standards.

ICP

The components that make up the ICP excitation source include the argon gas supply, torch, radio frequency (RF) induction coil, impedance-matching unit, and RF generator. Argon gas is almost universally used in an ICP. The plasma torch consists of three concentric tubes designated as the inner, the intermediate, and the outer tube. The intermediate and outer tubes are almost universally made of quartz. The inner tube can be made of quartz or alumina if the analysis is conducted with solutions containing hydrofluoric acid. The nebulizer gas flow carries the aerosol of the sample solution into and through the inner tube of the torch and into the plasma. The intermediate tube carries the intermediate (sometimes referred to as the auxiliary) gas. The intermediate gas flow helps to lift the plasma off the inner and intermediate tubes to prevent their melting and the deposition of carbon and salts on the inner tube. The outer tube carries the outer (sometimes referred to as the plasma or coolant) gas, which is used to form and sustain the toroidal plasma. The tangential flow of the coolant gas through the torch constricts the plasma and prevents the ICP from expanding to fill the outer tube, keeping the torch from melting. An RF induction coil, also called the load coil, surrounds the torch and produces an oscillating magnetic field, which in turn sets up an oscillating current in the ions and electrons produced from the argon. The impedance-matching unit serves to efficiently couple the RF energy from the generator to the load coil. The unit can be of either the active or passive type. An active matching unit adjusts the impedance of the RF power by means of a capacitive network, whereas the passive type adjusts the impedance directly through the generator circuitry. Within the load coil of the RF generator, the energy transfer between the coil and the argon creates a self-sustaining plasma. Collisions of the ions and electrons liberated from the argon ionize and excite the analyte atoms in the high-temperature plasma. The plasma operates at temperatures of 6,000 to 10,000 K, so most covalent bonds and analyte-to-analyte interactions have been eliminated.

ICP-AES

An inductively coupled plasma can use either an optical or a mass spectral detection system. In the former case, ICP–AES, analyte detection is achieved at an emission wavelength of the analyte in question. Because of differences in technology, a wide variety of ICP–AES systems are available, each with different capabilities, as well as different advantages and disadvantages. Simultaneous-detection systems are capable of analyzing multiple elements at the same time, thereby shortening analysis time and improving background detection and correction. Sequential systems move from one wavelength to the next to perform analyses, and often provide a larger number of analytical lines from which to choose. Array detectors, including charge-coupled devices and charge-injection devices, with detectors on a chip, make it possible to combine the advantages of both simultaneous and sequential systems. These types of detection devices are used in the most powerful spectrometers, providing rapid analysis and a wide selection of analytical lines.

The ICP can be viewed in either axial or radial (also called lateral) mode. The torch is usually positioned horizontally in axially viewed plasmas and is viewed end on, whereas it is positioned vertically in radially viewed plasmas and is viewed from the side. Axial viewing of the plasma can provide higher signal-to-noise ratios (better detection limits and precision); however, it also incurs greater matrix and spectral interferences. Methods validated on an instrument with a radial configuration will probably not be completely transferable to an instrument with an axial configuration, and vice versa.

Additionally, dual-view instrument systems are available, making it possible for the analyst to take advantage of either torch configuration. The selection of the optimal torch configuration will depend on the sample matrix, analyte in question, analytical wavelength(s) used, cost of instrumentation, required sensitivity, and type of instrumentation available in a given laboratory.

Regardless of torch configuration or detector technology, ICP–AES is a technique that provides a qualitative and/or quantitative measurement of the optical emission from excited atoms or ions at specific wavelengths. These measurements are then used to determine the analyte concentration in a given sample. Upon excitation, an atom or atomic ion emits an array of different frequencies of light that are characteristic of the distinct energy transition allowed for that element. The intensity of the light is generally proportional to the analyte concentration. It is necessary to correct for the background emission from the plasma. Sample concentration measurements are usually determined from a working curve of known

standards over the concentration range of interest. It is, however, also possible to perform a single-point calibration under certain circumstances, such as with limit tests, if the methodology has been validated for sufficient specificity, sensitivity, linearity, accuracy, precision, ruggedness, and robustness.

Because there are distinct transitions between atomic energy levels, and because the atoms in an ICP are rather dilute, emission lines have narrow bandwidths. However, because the emission spectra from the ICP contain many lines, and because "wings" of these lines overlap to produce a nearly continuous background on top of the continuum that arises from the recombination of argon ions with electrons, a high-resolution spectrometer is required in ICP–AES. The decision regarding which spectral line to measure should include an evaluation of potential spectral interferences. All atoms in a sample are excited simultaneously; however, the presence of multiple elements in some samples can lead to spectral overlap. Spectral interference can also be caused by background emission from the sample or plasma. Modern ICPs usually have background correction available, and a number of background correction techniques can be applied. Simple background correction typically involves measuring the background emission intensity at some point away from the main peak and subtracting this value from the total signal being measured. Mathematical modeling to subtract the interfering signal as a background correction can also be performed with certain types of ICP–AES spectrometers.

The selection of the analytical spectral line is critical to the success of an ICP–AES analysis, regardless of torch configuration or detector type. Though some wavelengths are preferred, the final choice must be made in the context of the sample matrix, the type of instrument being used, and the sensitivity required. Analysts might choose to start with the wavelengths recommended by the manufacturer of their particular instrument, and select alternative wavelengths based on manufacturer recommendations or published wavelength tables.[7,8,9,10,11] Ultimately, the selection of analytical wavelengths should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision of the analysis at hand.

Forward power, gas flow rates, viewing height, and torch position can all be optimized to provide the best signal. However, it must also be kept in mind that these same variables can influence matrix and spectral interferences.

In general, it is desirable to operate the ICP under robust conditions, which can be gauged on the basis of the MgII/MgI line pair at (280.270 nm/285.213 nm). If that ratio of intensities is above 6.0 in an aqueous solution, the ICP is said to be *robust*, and is less susceptible to matrix interferences. A ratio of about 10.0 is generally what is sought. Note that the term *robust conditions* is unrelated to *robustness* as applied to analytical method validation. Operation of an instrument with an MgII/MgI ratio greater than 6.0 is not mandated, but is being suggested as a means of optimizing instrument parameters in many circumstances.

The analysis of the Group I elements can be an exception to this strategy. When atomic ions are formed from elements in this group, they assume a noble gas electron configuration, with correspondingly high excitation energy. Because the first excited state of these ions is extremely high, few are excited, so emission intensity is correspondingly low. This situation can be improved by reducing the fractional ionization, which can in turn be achieved by using lower forward power settings in combination with adjusted viewing height or nebulizer gas flow, or by adding an ionization suppression agent to the samples and standards.

When organic solvents are used, it is often necessary to use a higher forward power setting, higher intermediate and outer gas flows, and a lower nebulizer gas flow than would be employed for aqueous solutions, as well as a reduction in the nebulizer gas flow. When using organic solvents, it may also be necessary to bleed small amounts of oxygen into the torch to prevent carbon buildup in the torch.

Calibration: The wavelength accuracy for ICP–AES detection must comply with the manufacturer's applicable operating procedures. Because of the inherent differences among the types of instruments available, there is no general system suitability procedure that can be employed. Calibration routines recommended by the instrument manufacturer for a given ICP–AES instrument should be followed. These might include, but are not limited to, use of a multi-element wavelength calibration with a reference solution, internal mercury (Hg) wavelength calibration, and peak search. The analyst should perform system checks in accordance with the manufacturer's recommendations.

Standardization: The instrument must be standardized for quantification at time of use. However, because ICP–AES is a technique generally considered to be linear over a range of 6–8 orders of magnitude, it is not always necessary to continually demonstrate linearity by the use of a standard curve composed of multiple standards. Once a method has been developed and is in routine use, it is possible to calibrate with a blank and a single standard. One-point standardizations are suitable for conducting limit tests on production materials and final products if the methodology has been rigorously validated for sufficient specificity, sensitivity, linearity, accuracy, precision, ruggedness, and robustness. The use of a single-point standardization is also acceptable for qualitative ICP–AES analyses, where the purpose of the experiment is to confirm the presence or absence of elements without the requirement of an accurate quantification.

An appropriate blank solution and standards that bracket the expected range of the sample concentrations should be assayed and the detector response plotted as a function of analyte concentration, as in the case where the concentration of a known component is being determined within a specified tolerance. However, it is not always possible to employ a bracketing standard when an analysis is performed at or near the detection limit. This lack of use of a bracketing standard is acceptable for analyses conducted to demonstrate the absence or removal of elements below a specified limit. The number and concentrations of standard solutions used should be based on the purpose of the quantification, the analyte in question, the desired sensitivity, and the sample matrix. Regression analysis of the standard plot should be employed to evaluate the linearity of detector response, and individual monographs may set criteria for the residual error of the regression line. Optimally, a correlation coefficient of NLT 0.99, or as indicated in the individual monograph, should be demonstrated for the working curve. Here, too, however, the nature of the sample matrix, the analyte(s), the desired sensitivity, and the type

---

7 Payling R, Larkins P. *Optical Emission Lines of the Elements.* New York: Wiley; 2000.

8 Harrison GR. *Massachusetts Institute of Technology Wavelength Tables* [also referred to as *MIT Wavelength Tables*]. Cambridge, MA: MIT Press; 1969.

9 Winge RK, Fassel VA, Peterson VJ, Floyd MA. *Inductively Coupled Plasma Atomic Emission Spectroscopy: An Atlas of Spectral Information.* New York: Elsevier; 1985.

10 Boumans PWJM. *Spectrochim Acta A.* 1981;36B:169.

11 Boumans PWJM. *Line Coincidence Tables for Inductively Coupled Plasma Atomic Emission Spectrometry.* 2nd ed.; Oxford, UK: Pergamon; 1984.

of instrumentation available may dictate a correlation coefficient lower than 0.99. The analyst should use caution when proceeding with such an analysis, and should employ additional working standards.

To demonstrate the stability of the system's initial standardization, a solution used in the initial standard curve must be reassayed as a check standard at appropriate intervals throughout the analysis of the sample set. The reassayed standard should agree with its expected value to within ±10%, or as specified in an individual monograph, for single-element analyses when analytical wavelengths are 200–500 nm, or concentrations are >1 µg/mL. The reassayed standard should agree with its theoretical value to within ±20%, or as specified in an individual monograph, for multi-element analyses, when analytical wavelengths are <200 nm or >500 nm, or at concentrations of <1 µg/mL. In cases where an individual monograph provides different guidance regarding the reassayed check standard, the requirements of the monograph take precedence.

Procedure: Follow the procedure as directed in the individual monograph for the instrumental parameters. Because of differences in manufacturers' equipment configurations, the manufacturer's suggested default conditions may be used and modified as needed. The specification of definitive parameters in a monograph does not preclude the use of other suitable operating conditions, and adjustments of operating conditions may be necessary. Alternative conditions must be supported by suitable validation data, and the conditions in the monograph will take precedence for official purposes. Data collected from a single sample introduction are treated as a single result. This result might be the average of data collected from replicate sequential readings from a single solution introduction of the appropriate standard or sample solution. Sample concentrations are calculated versus the working curve generated by plotting the detector response versus the concentration of the analyte in the standard solutions. This calculation is often performed directly by the instrument.

ICP-MS

When an inductively coupled plasma uses a mass spectral detection system, the technique is referred to as inductively coupled plasma–mass spectrometry (ICP–MS). In this technique, analytes are detected directly at their atomic masses. Because these masses must be charged to be detected in ICP–MS, the method relies on the ability of the plasma source to both atomize and ionize sample constituents. As is the case with ICP–AES, a wide variety of ICP–MS instrumentation systems are available.

The systems most commonly in use are quadrupole-based systems. Gaining in interest is time-of-flight ICP–MS. Although still not in widespread use, this approach may see greater use in the future. Additionally, high-resolution sector field instruments are available.

Regardless of instrument design or configuration, ICP–MS provides both a qualitative and a quantitative measurement of the components of the sample. Ions are generated from the analyte atoms by the plasma. The analyte ions are then extracted from the atmospheric-pressure plasma through a sampling cone into a lower-pressure zone, ordinarily held at a pressure near 1 Torr. In this extraction process, the sampled plasma gases, including the analyte species, form a supersonic beam, which dictates many of the properties of the resulting analyte ions. A skimmer cone, located behind the sampling cone, "skims" the supersonic beam of ions as they emerge from the sampling cone. Behind the skimmer cone is a lower-pressure zone, often held near a milliTorr. Lastly, the skimmed ions pass a third-stage orifice to enter a zone held near a microTorr, where they encounter ion optics and are passed into the mass spectrometer. The mass spectrometer separates the ions according to their mass-to-charge (m/z) ratios. The ICP–MS has a mass range up to 240 atomic mass units (amu). Depending on the equipment configuration, analyte adducts can form with diluents, with argon, or with their decomposition products. Also formed are oxides and multiply-charged analyte ions, which can increase the complexity of the resulting mass spectra. Interferences can be minimized by appropriate optimization of operational parameters, including gas flows (central, intermediate, and outer gas flow rates), sample-solution flow, RF power, extraction-lens voltage, etc., or by the use of collision or reaction cells, or cool plasma operation, if available on a given instrument. Unless a laboratory is generating or examining isotopes that do not naturally occur, a list of naturally occurring isotopes will provide the analyst with acceptable isotopes for analytical purposes. Isotopic patterns also serve as an aid to element identification and confirmation. Additionally, tables of commonly found interferences and polyatomic isobaric interferences and correction factors can be used.

ICP–MS generally offers considerably lower (better) detection limits than ICP–AES, largely because of the extremely low background that it generates. This ability is a major advantage of ICP–MS for determination of very low analyte concentrations or when elimination of matrix interferences is required. In the latter case, some interferences can be avoided simply by additional dilution of the sample solution. In some applications, analytes can be detected below the parts per trillion (ppt) level using ICP–MS. As a general rule, ICP–MS as a technique requires that samples contain significantly less total dissolved solids than does ICP–AES.

The selection of the analytical mass to use is critical to the success of an ICP–MS analysis, regardless of instrument design. Though some masses are often considered to be the primary ones, because of their high natural abundance, an alternative mass for a given element is often used to avoid spectral overlaps (isobaric interferences). Selection of an analytical mass must always be considered in the context of the sample matrix, the type of instrument being used, and the concentrations to be measured. Analysts might choose to start with masses recommended by the manufacturer of their particular instrument, and select alternate masses based on manufacturer's recommendations or published tables of naturally occurring isotopes.[12]

Optimization of an ICP–MS method is also highly dependent on the plasma parameters and means of sample introduction. Forward power, gas flow rates, and torch position may all be optimized to provide the best signal. When organic solvents are used, it is often necessary to use a higher forward power setting and a lower nebulizer flow rate than would be used for aqueous solutions. Additionally, when organic solvents are used, it might be necessary to introduce small amounts of oxygen into the central or intermediate gas to prevent carbon buildup in the torch or on the sampler cone orifice. The use of a platinum-tipped sampling or skimmer cone may also be required in order to reduce cone degradation with some organic solvents.

---

[12] Horlick G, Montaser A. Analytical characteristics of ICPMS. In: Montaser A, Editor. *Inductively Coupled Plasma Mass Spectrometry.* New York: Wiley-VCH; 1998:516–518.

Calibration: The mass spectral accuracy for ICP–MS detection must be in accordance with the applicable operating procedures. Because of the inherent differences between the types of instruments available, there is no general system suitability procedure that can be employed. Analysts should refer to the tests recommended by the instrument manufacturer for a given ICP–MS instrument. These may include, but are not limited to, tuning on a reference mass or masses, peak search, and mass calibration. The analyst should perform system checks recommended by the instrument manufacturer.
Standardization: The instrument must be standardized for quantification at the time of use. Because the response (signal vs. concentration) of ICP–MS is generally considered to be linear over a range of 6–8 orders of magnitude, it is not always necessary to continually demonstrate linearity by the use of a working curve. Once a method has been developed and is in routine use, it is common practice to calibrate with a blank and a single standard. One-point standardizations are suitable for conducting limit tests on production materials and final products, provided that the methodology has been rigorously validated for sufficient specificity, sensitivity, linearity, accuracy, precision, ruggedness, and robustness. An appropriate blank solution and standards that bracket the expected range of the sample concentrations should be assayed and the detector response plotted as a function of analyte concentration. The number and concentration of standard solutions used should be based on the analyte in question, the expected concentrations, and the sample matrix, and should be left to the discretion of the analyst. Optimally, a correlation coefficient of NLT 0.99, or as indicated in the individual monograph, should be demonstrated for the working standard curve. Here, too, however, the nature of the sample matrix, the analyte, the desired sensitivity, and the type of instrumentation available might dictate a correlation coefficient lower than 0.99. The analyst should use caution when proceeding with such an analysis and should employ additional working standards.

To demonstrate the stability of the system since initial standardization, a solution used in the initial standard curve must be reassayed as a check standard at appropriate intervals throughout the analysis of the sample set. Appropriate intervals may be established as occurring after every fifth or tenth sample, or as deemed adequate by the analyst, on the basis of the analysis being performed. The reassayed standard should agree with its expected value to within ±10% for single-element analyses when analytical masses are free of interferences and when concentrations are >1 ng/mL. The reassayed standard should agree with its expected value to within ±20% for multi-element analyses, or when concentrations are <1 ng/mL. In cases where an individual monograph provides different guidance regarding the reassayed check standard, the requirements of the monograph take precedence.

The method of standard additions should be employed in situations where matrix interferences are expected or suspected. This method involves adding a known concentration of the analyte element to the sample solution at no fewer than two concentration levels. The instrument response is plotted against the concentration of the added analyte element, and a linear regression line is drawn through the data points. The absolute value of the x-intercept multiplied by any dilution factor is the concentration of the analyte in the sample.
Procedure: Follow the procedure as directed in the individual monograph for the detection mode and instrument parameters. The specification of definitive parameters in a monograph does not preclude the use of other suitable operating conditions, and adjustments of operating conditions may be necessary. Alternative conditions must be supported by suitable validation data, and the conditions in the monograph will take precedence for official purposes. Because of differences in manufacturers' equipment configurations, the analyst may wish to begin with the manufacturer's suggested default conditions and modify them as needed. Data collected from a single sample introduction are treated as a single result. Data collected from replicate sequential readings from a single introduction of the appropriate standard or sample solutions are averaged as a single result. Sample concentrations are calculated versus the working curve generated by plotting the detector response versus the concentration of the analyte in the standard solutions. With modern instruments, this calculation is often performed by the instrument.

GLOSSARY

Auxiliary gas: See *Intermediate (or auxiliary) gas.*
Axial viewing: A configuration of the plasma for AES in which the plasma is directed toward the spectrometer optical path, also called "end-on viewing."
Central (or nebulizer) gas: One of three argon gas flows in an ICP torch. The central gas is used to help create a fine mist of the sample solution when solution nebulization is employed. This fine mist is then directed through the central tube of the torch and into the plasma.
Collision cell: A design feature of some ICP–MS instruments. Collision cells are used to reduce interferences from argon species or polyatomic ions and to facilitate the analysis of elements that might be affected by those interferences.
Cool plasma: Plasma conditions used for ICP–MS that result in a plasma that is cooler than that normally used for an analysis. This condition is achieved by using a lower forward power setting and higher central-gas flow rate, and is used to help reduce isotopic interferences caused by argon and some polyatomic ions.
Coolant gas: See *Outer (or coolant or plasma) gas.*
Forward power: The number of watts used to ignite and sustain the plasma during an analysis. Forward power requirements may vary, depending on sample matrix and analyte.
Intermediate (or auxiliary) gas: Gas used to "lift" the plasma off the surface of the torch, thereby preventing melting of the intermediate tube and the formation of carbon and salt deposits on the inner tube.
Internal standard: An element added to or present in the same concentration in blanks, standards, and samples to act as an intensity reference for the analysis. An internal standard should be used for ICP–AES work and must always be used for quantitative ICP–MS analyses.
m: The ion mass of interest
Lateral viewing: See *Radial viewing.*
Multiply-charged ions: Atoms that, when subjected to the high-ionization temperature of the ICP, can form doubly or triply charged ions (X^{++}, X^{+++}, etc.). When detected by MS, the apparent mass of these ions will be half or one-third that of the atomic mass.
Nebulizer: Used to form a consistent sample aerosol that mixes with the argon gas, which is subsequently sent into the ICP.

Published August 17, 2020

Outer (or coolant or plasma) gas: The main gas supply for the plasma.
Plasma gas: See *Outer (or coolant or plasma) gas.*
Radial viewing: A configuration of the plasma for AES in which the plasma is viewed orthogonal to the spectrometer optic path. Also called "side-on viewing." See also *Lateral viewing.*
Reaction cell: Similar to *Collision cell,* but operating on a different principle. Designed to reduce or eliminate spectral interferences.
Sampling cone: A metal cone (usually nickel-, aluminum-, or platinum-tipped) with a small opening, through which ionized sample material flows after leaving the plasma.
Sequential: A type of detector configuration for AES or MS in which discrete emission lines or isotopic peaks are observed by scanning or hopping across the spectral range by means of a monochromator or scanning mass spectrometer.
Simultaneous: A type of detector configuration for AES or MS in which all selected emission lines or isotopic peaks are observed at the same time by using a polychromator or simultaneous mass spectrometer, offering increased analysis speed for analyses of multi-element samples.
Skimmer cone: A metal cone through which ionized sample flows after leaving the sampling cone and before entering the high-vacuum region of an ICP–MS.
Standard additions: A method used to determine the actual analyte concentration in a sample when viscosity or matrix effects might cause erroneous results.
Torch: A series of three concentric tubes, usually manufactured from quartz, in which the ICP is formed.

Residue on Ignition (Sulfated Ash)

METHOD I (FOR SOLIDS)

Transfer the quantity of the sample directed in the individual monograph onto a tared 50-mL to 100-mL platinum dish or other suitable container, and add sufficient 2 N sulfuric acid to moisten the entire sample. Heat gently, using a hot plate, an Argand burner, or an infrared heat lamp, until the sample is dry and thoroughly charred, then continue heating until all of the sample has been volatilized or nearly all of the carbon has been oxidized, and cool. Moisten the residue with 0.1 mL of sulfuric acid, and heat in the same manner until the remainder of the sample and any excess sulfuric acid have been volatilized. To promote volatilization of sulfuric acid, add a few pieces of ammonium carbonate just before completing ignition. Finally, ignite to constant weight in a muffle furnace at 800 ± 25° for 15 min, or longer if necessary to complete ignition, cool in a desiccator, and weigh.

METHOD II (FOR LIQUIDS)

Unless otherwise directed, transfer the required weight of the sample onto a tared 75-mL to 100-mL platinum dish. Heat gently, using an Argand or Meker burner, until the sample ignites, then allow the sample to burn until it self-extinguishes. Cool, then wet the residue with 2 mL of concentrated sulfuric acid, and heat the sample over a low flame until dry. Ignite to constant weight in a muffle furnace at 800 ± 25° for 30 min, or longer if necessary for complete ignition, cool in a desiccator, and weigh.

Sieve Analysis of Granular Metal Powders (Based on ASTM Designation: B 214)[13]

APPARATUS

Sieves: Use a set of standard sieves, ranging from 80-mesh to 325-mesh, conforming to the specifications in ASTM Designation: E 11 (Sieves for Testing Purposes).
Sieve shaker: Use a mechanically operated sieve shaker that imparts to the set of sieves a horizontal rotary motion of 270–300 rotations/min and a tapping action of 140–160 taps/min. The sieve shaker is fitted with a plug to receive the impact of the tapping device. The entire apparatus is rigidly mounted—bolted to a solid foundation, preferably of concrete. Preferably a time switch is provided to ensure the accuracy of test duration.

PROCEDURE

Assemble the sieves in consecutive order by opening size, with the coarsest sieve (80-mesh) at the top, and place a solid-collecting pan below the bottom sieve (325-mesh). Place 100.0 g of the test sample, W, on the top sieve, and close the sieve with a solid cover. Securely fasten the assembly to the sieve shaker, and operate the shaker for 15 min. Remove the most coarse sieve from the nest, gently tap its contents to one side, and pour the contents onto a tared, glazed paper. Using a soft brush, transfer onto the next finer sieve any material adhering to the bottom of the sieve and frame. Place the sieve just removed upside down on the paper containing the retained portion, and tap the sieve. Accurately weigh the paper and its contents, and record the net weight of the fraction, F, obtained. Repeat this process for each sieve in the nest and for the portion of the sample that has been collected in the bottom pan. Record the total of the fractions retained on the sieves as T and that portion collected in the pan as t. The combined total, S, of T + t is the amount of the sample, W, recovered in the test. Calculate the percent recovery:

[13] Adapted from ASTM B214 Standard Test Method for Sieve Analysis of Metal Powders. The original ASTM method is available in its entirety from ASTM International, 100 Barr Harbor Drive, West Conshohocken, PA 19428. Phone: 610-832-9585, Fax: 610-832-9555, Email: service@astm.org, Website: www.astm.org.

$$\text{Result} = S/W \times 100$$

If the percent recovery is less than 99.0%, check the condition of the sieves and for possible errors in weighing, and repeat the test. If the percent recovery is NLT 99.0%, calculate the percent retained on each sieve:

$$\text{Result} = F/W \times 100$$

Calculate the percent through the smallest mesh sieve from the portion collected in the pan:

$$\text{Result} = [(100 - t)/W] \times 100$$

Sulfuric Acid Table

°Bé	Sp. Gr.	Percent H_2SO_4
0	1.0000	0.00
1	1.0069	1.02
2	1.0140	2.08
3	1.0211	3.13
4	1.0284	4.21
5	1.0357	5.28
6	1.0432	6.37
7	1.0507	7.45
8	1.0584	8.55
9	1.0662	9.66
10	1.0741	10.77
11	1.0821	11.89
12	1.0902	13.01
13	1.0985	14.13
14	1.1069	15.25
15	1.1154	16.38
16	1.1240	17.53
17	1.1328	18.71
18	1.1417	19.89
19	1.1508	21.07
20	1.1600	22.25
21	1.1694	23.43
22	1.1789	24.61
23	1.1885	25.81
24	1.1983	27.03
25	1.2083	28.28
26	1.2185	29.53
27	1.2288	30.79
28	1.2393	32.05
29	1.2500	33.33
30	1.2609	34.63
31	1.2719	35.93
32	1.2832	37.26
33	1.2946	38.58
34	1.3063	39.92
35	1.3182	41.27
36	1.3303	42.63

°Bé	Sp. Gr.	Percent H_2SO_4
37	1.3426	43.99
38	1.3551	45.35
39	1.3679	46.72
40	1.3810	48.10
41	1.3942	49.47
42	1.4078	50.87
43	1.4216	52.26
44	1.4356	53.66
45	1.4500	55.07
46	1.4646	56.48
47	1.4796	57.90
48	1.4948	59.32
49	1.5104	60.75
50	1.5263	62.18
51	1.5426	63.66
52	1.5591	65.13
53	1.5761	66.63
54	1.5934	68.13
55	1.6111	69.65
56	1.6292	71.17
57	1.6477	72.75
58	1.6667	74.36
59	1.6860	75.99
60	1.7059	77.67
61	1.7262	79.43
62	1.7470	81.30
63	1.7683	83.34
64	1.7901	85.66
64.25	1.7957	86.33
64.50	1.8012	87.04
64.75	1.8068	87.81
65	1.8125	88.65
65.25	1.8182	89.55
65.50	1.8239	90.60
66	1.8354	93.19

Specific gravity determinations were made at 60°F, compared with water at 60°F. The values given above for aqueous sulfuric acid solutions were adopted as standard in 1904 by the Manufacturing Chemists' Association of the United States.

From the specific gravities, the corresponding degrees Baumé were calculated by the following equation:

$$°\text{Baumé} = 145 - (145/\text{sp. gr.})$$

Baumé hydrometers for use with this table must be graduated by the above formula, which should always be printed on the scale. Acids stronger than 66°Bé should have their percentage compositions determined by chemical analysis.

Water-Insoluble Matter

SAMPLE PREPARATION

Add 5 g of sample (if a different amount of sample is specified in the individual monograph, use that amount) to 100 mL of water, and stir until the sample is dissolved.

PROCEDURE

Dry a membrane filter (cellulose nitrate, 0.45-µm porosity) at 110°C for 1 h, allow to cool in a desiccator, and weigh to the nearest 0.1 mg. Pass the *Sample preparation* through the dried membrane filter and wash with three successive 10-mL portions of water. Dry the membrane filter at 110°C for 1 h. Cool in a desiccator, and weigh the membrane filter to the nearest 0.1 mg. Calculate the insoluble matter as percentage.

Published August 17, 2020

Appendix III: Chemical Tests and Determinations

A. IDENTIFICATION TESTS

The identification tests described in section *A* of this Appendix are frequently referred to in the *Food Chemicals Codex* for the presumptive identification of FCC-grade chemicals taken from labeled containers. These tests are not intended to be applied to mixtures unless so specified.

Acetate: Acetic acid or acetates, when warmed with sulfuric acid and alcohol, form ethyl acetate, recognizable by its characteristic odor. With neutral solutions of acetates, ferric chloride TS produces a deep red color that is destroyed by the addition of a mineral acid.

Aluminum: Solutions of aluminum salts yield with 6 N ammonia a white, gelatinous precipitate that is insoluble in an excess of the 6 N ammonia. The same precipitate is produced by 1 N sodium hydroxide, but it dissolves in an excess of this reagent.

Ammonium: Ammonium salts are decomposed by 1 N sodium hydroxide with the evolution of ammonia, recognizable by its alkaline effect on moistened red litmus paper. The decomposition is accelerated by warming.

Benzoate: Neutral solutions of benzoates yield a salmon colored precipitate with ferric chloride TS. From moderately concentrated solutions of benzoate, 2 N sulfuric acid precipitates free benzoic acid, which is readily soluble in ether.

Bicarbonate: *See Carbonate.*

Bisulfite: *See Sulfite.*

Bromide: Free bromine is liberated from solutions of bromides upon the dropwise addition of chlorine TS. When shaken with chloroform, the bromine dissolves, coloring the chloroform red to red-brown. A yellow-white precipitate, which is insoluble in nitric acid and slightly soluble in 6 N ammonia, is produced when solutions of bromides are treated with silver nitrate TS.

Calcium: Insoluble oxalate salts are formed when solutions of calcium salts are treated in the following manner: using 2 drops of methyl red TS as the indicator, neutralize a 1:20 solution of a calcium salt with 6 N ammonia, then add 2.7 N hydrochloric acid, dropwise, until the solution is acid. A white precipitate of calcium oxalate forms upon the addition of ammonium oxalate TS. This precipitate is insoluble in acetic acid but dissolves in hydrochloric acid.

Calcium salts moistened with hydrochloric acid impart a transient yellow-red color to a nonluminous flame.

Carbonate: Carbonates and bicarbonates effervesce with acids, yielding a colorless gas that produces a white precipitate immediately when passed into calcium hydroxide TS. Cold solutions of soluble carbonates are colored red by phenolphthalein TS, whereas solutions of bicarbonates remain unchanged or are slightly changed.

Chloride: Solutions of chlorides yield with silver nitrate TS a white, curdy precipitate that is insoluble in nitric acid but soluble in a slight excess of 6 N ammonia.

Citrate: To 15 mL of pyridine add a few mg of a citrate salt, dissolved or suspended in 1 mL of water, and shake. Add 5 mL of acetic anhydride to this mixture, and shake. A light red color appears.

Cobalt: Solutions of cobalt salts (1:20) in 2.7 N hydrochloric acid yield a red precipitate when heated on a steam bath with an equal volume of a hot, freshly prepared 1:10 solution of 1-nitroso-2-naphthol in 9 N acetic acid. Solutions of cobalt salts yield a yellow precipitate when saturated with potassium chloride and treated with potassium nitrite and acetic acid.

Copper: When solutions of cupric compounds are acidified with hydrochloric acid, a red film of metallic copper is deposited on a bright untarnished surface of metallic iron. An excess of 6 N ammonia, added to a solution of a cupric salt, produces first a blue precipitate and then a deep blue colored solution. Solutions of cupric salts yield with potassium ferrocyanide TS a red-brown precipitate, insoluble in diluted acids.

Hypophosphite: Hypophosphites evolve spontaneously flammable phosphine when strongly heated. Solutions of hypophosphites yield a white precipitate with mercuric chloride TS. This precipitate becomes gray when an excess of hypophosphite is present. Hypophosphite solutions, acidified with sulfuric acid and warmed with copper sulfate TS, yield a red precipitate.

Iodide: Solutions of iodides, upon the dropwise addition of chlorine TS, liberate iodine, which colors the solution yellow to red. Chloroform is colored violet when shaken with this solution. The iodine thus liberated gives a blue color with starch TS. In solutions of iodides, silver nitrate TS produces a yellow, curdy precipitate that is insoluble in nitric acid and in 6 N ammonia.

Iron: Solutions of ferrous and ferric compounds yield a black precipitate with ammonium sulfide TS. This precipitate is dissolved by cold 2.7 N hydrochloric acid with the evolution of hydrogen sulfide.

Ferric Salts: Potassium ferrocyanide TS (10%) produces a dark blue precipitate in acid solutions of ferric salts. With an excess of 1 N sodium hydroxide, a red-brown precipitate is formed. Solutions of ferric salts produce with ammonium thiocyanate TS (1.0 N) a deep red color that is not destroyed by diluted mineral acids.

Ferrous Salts: Potassium ferricyanide TS (10%) produces a dark blue precipitate in solutions of ferrous salts. This precipitate, which is insoluble in dilute hydrochloric acid, is decomposed by 1 N sodium hydroxide. Solutions of ferrous salts yield with 1 N sodium hydroxide a green-white precipitate, the color rapidly changing to green and then to brown when shaken.

Lactate: When solutions of lactates are acidified with sulfuric acid, potassium permanganate TS (0.1 N) is added, and the mixture is heated, acetaldehyde is evolved. This can be detected by allowing the vapor to come into contact with a filter paper that has been moistened with a freshly prepared mixture of equal volumes of 20% aqueous morpholine and sodium nitroferricyanide TS. A blue color is produced.

Magnesium: Solutions of magnesium salts in the presence of ammonium chloride yield no precipitate with ammonium carbonate TS, but a white crystalline precipitate, which is insoluble in 6 N ammonium hydroxide, is formed on the subsequent addition of sodium phosphate TS (6%).

Manganese: Solutions of manganous salts yield with ammonium sulfide TS a salmon colored precipitate that dissolves in acetic acid.

Nitrate: When a solution of a nitrate is mixed with an equal volume of sulfuric acid, the mixture cooled, and a solution of ferrous sulfate superimposed, a brown color is produced at the junction of the two liquids. Brown-red fumes are evolved when a nitrate is heated with sulfuric acid and metallic copper. Nitrates do not decolorize acidified potassium permanganate TS (0.1 N) (distinction from nitrites).

Nitrite: Nitrites yield brown-red fumes when treated with diluted mineral acids or acetic acid. A few drops of potassium iodide TS (15%) and a few drops of 2 N sulfuric acid added to a solution of nitrite liberate iodine, which colors starch TS blue.

Peroxide: Solutions of peroxides slightly acidified with sulfuric acid yield a deep blue color on the addition of potassium dichromate TS. On shaking the mixture with an equal volume of diethyl ether and allowing the liquids to separate, the blue color is transferred to the ether layer.

Phosphate: Neutral solutions of orthophosphates yield with silver nitrate TS (0.1 N) a yellow precipitate, which is soluble in 1.7 N nitric acid or in 6 N ammonium hydroxide. Acidified solutions of orthophosphates yield a yellow precipitate with ammonium molybdate TS, which is soluble in 6 N ammonium hydroxide.

Potassium: Potassium compounds impart a violet color to a nonluminous flame if not masked by the presence of small quantities of sodium. In neutral, concentrated or moderately concentrated solutions of potassium salts, sodium bitartrate TS (10%) slowly produces a white, crystalline precipitate that is soluble in 6 N ammonium hydroxide and in solutions of alkali hydroxides or carbonates. The precipitation may be accelerated by stirring or rubbing the inside of the test tube with a glass rod or by the addition of a small amount of glacial acetic acid or alcohol.

Sodium: Dissolve 0.1 g of the sodium compound in 2 mL of water. Add 2 mL of 15% potassium carbonate, and heat to boiling. No precipitate is formed. Add 4 mL of potassium pyroantimonate TS, and heat to boiling. Allow to cool in ice water, and if necessary, rub the inside of the test tube with a glass rod. A dense precipitate is formed. Sodium compounds impart an intense yellow color to a nonluminous flame.

Sulfate: Solutions of sulfates yield with barium chloride TS a white precipitate that is insoluble in hydrochloric and nitric acids. Sulfates yield with lead acetate TS a white precipitate that is soluble in ammonium acetate solution. Hydrochloric acid produces no precipitate when added to solutions of sulfates (distinction from thiosulfates).

Sulfite: When treated with 2.7 N hydrochloric acid, sulfites and bisulfites yield sulfur dioxide, recognizable by its characteristic odor. This gas blackens filter paper moistened with mercurous nitrate TS.

Tartrate: When a few mg of a tartrate are added to a mixture of 15 mL of pyridine and 5 mL of acetic anhydride, an emerald green color is produced.

Thiosulfate: With hydrochloric acid, solutions of thiosulfates yield a white precipitate that soon turns yellow, liberating sulfur dioxide, recognizable by its odor. The addition of ferric chloride TS to solutions of thiosulfates produces a dark violet color that quickly disappears.

Zinc: Zinc salts, in the presence of sodium acetate, yield a white precipitate with hydrogen sulfide. This precipitate, which is insoluble in acetic acid, is dissolved by 2.7 N hydrochloric acid. A similar precipitate is produced by ammonium sulfide TS in neutral or alkaline solutions. Solutions of zinc salts yield with potassium ferrocyanide TS (10%) a white precipitate that is insoluble in 2.7 N hydrochloric acid.

B. LIMIT TESTS

ALUMINUM LIMIT TEST

[NOTE—The *Standard solutions* and *Sample solution* may be modified, if necessary, to obtain solutions of suitable concentrations adaptable to the linear or working range of the instrument.]

Nitric acid diluent: Dilute 40 mL of nitric acid with water to 1000 mL.

Standard aluminum solutions: Treat a quantity of aluminum wire with 6 N hydrochloric acid at 80° for a few min. Dissolve 100 mg of the treated wire in a mixture consisting of 10 mL of hydrochloric acid and 2 mL of nitric acid, by heating at 80° for about 30 min. Continue heating until the volume is reduced to about 4 mL. Cool to room temperature, and add 4 mL of water. Evaporate to about 2 mL by heating. Cool, and transfer this solution, with the aid of water, to a 100-mL volumetric flask, and dilute with water to volume (1 mg/mL aluminum). Transfer 10.0 mL of this solution to a second 100-mL volumetric flask, and dilute with water to volume (100 µg/mL aluminum). Transfer 1.0 mL of this solution to a third 100-mL volumetric flask, and dilute with water to volume (1 µg/mL aluminum). [NOTE—If more diluted *Standard aluminum solutions* are required, transfer 1.0-mL, 2.0-mL, and 4.0-mL portions of the 1 µg/mL *Standard aluminum solution* to separate 100-mL volumetric flasks, dilute with *Nitric acid diluent* to volume, and mix. These solutions contain 0.01 µg/mL, 0.02 µg/mL, and 0.04 µg/mL of aluminum, respectively.]

Sample solution: Transfer the amount of sample specified in the monograph to a plastic 100-mL volumetric flask. Add 50 mL of water, and sonicate for 30 min. Add 4 mL of nitric acid, and dilute with water to volume.

Procedure: Determine the absorbances of the *Standard aluminum solutions* and the *Sample solution* at the aluminum emission line at 309.3 nm with a suitable atomic absorption spectrophotometer equipped with an aluminum hollow-cathode lamp and a flameless electrically heated furnace, using the *Nitric acid diluent* as the blank. Plot the absorbances of the *Standard solutions* versus the concentration of aluminum, in µg/mL, drawing a straight line best fitting the three points. From the graph so obtained, determine the concentration, in µg/mL, of aluminum in the *Sample solution*.

Calculate the amount of aluminum in the sample taken, in µg/g:

$$\text{Result} = C_A/C_S$$

in which C_A is the concentration of aluminum in the *Sample solution*, in µg/mL, obtained from the standard curve; and C_S is the concentration of the *Sample solution*, in g/mL.

ARSENIC LIMIT TEST

• Silver Diethyldithiocarbamate Colorimetric Method

[NOTE—All reagents used in this test should be very low in arsenic content.]

Apparatus: Use the general apparatus shown in *Figure 1* unless otherwise specified in an individual monograph. It consists of a 125-mL arsine generator flask (*a*) fitted with a scrubber unit (*c*) and an absorber tube (*e*), with a 24/40 standard-taper joint (*b*) and a ball-and-socket joint (*d*), secured with a No. 12 clamp, connecting the units. The tubing between *d* and *e* and between *d* and *c* is a capillary having an id of 2 mm and an od of 8 mm. Alternatively, an apparatus embodying the principle of the general assembly described and illustrated may be used.

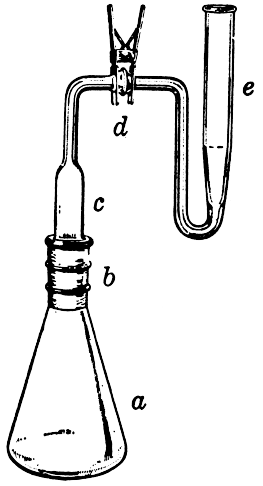


Figure 1. General Apparatus for Arsenic Limit Test (Courtesy of the Fisher Scientific Co., Pittsburgh, PA.)

[NOTE—The special assemblies shown in *Figures 2*, *3*, and *4* are to be used only when specified in certain monographs.]

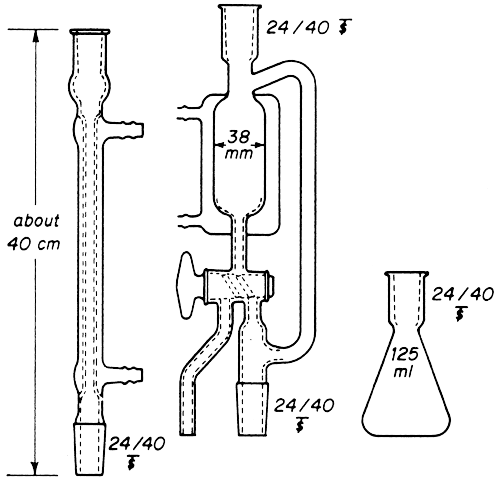


Figure 2. Modified Bethge Apparatus for the Distillation of Arsenic Tribromide

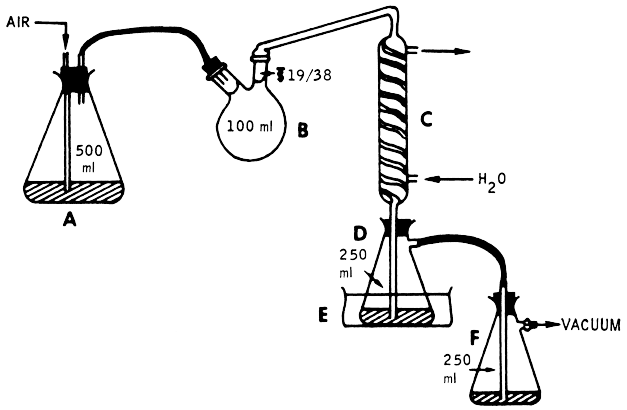


Figure 3. Special Apparatus for the Distillation of Arsenic Trichloride (Flask *A* contains 150 mL of hydrochloric acid; flasks *D* and *F* contain 20 mL of water. Flask *D* is placed in an ice water bath, *E*.)

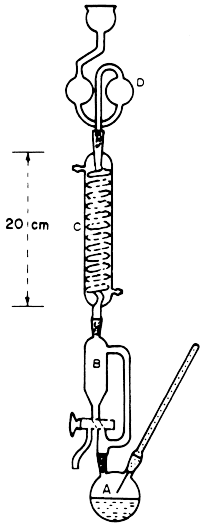


Figure 4. Special Apparatus for the Determination of Inorganic Arsenic (*A*, 250-mL distillation flask; *B*, receiver chamber, approximately 50-mL capacity; *C*, reflux condenser; *D*, splash head.)

Standard arsenic solution: Accurately weigh 132.0 mg of arsenic trioxide that has been previously dried at 105° for 1 h, and dissolve it in 5 mL of a 1:5 sodium hydroxide solution. Neutralize the solution with 2 N sulfuric acid, add 10 mL in excess, and dilute with recently boiled water to 1000.0 mL. Transfer 10.0 mL of this solution into a 1000-mL volumetric flask, add 10 mL of 2 N sulfuric acid, dilute with recently boiled water to volume, and mix. Use this final solution, which contains 1 μg of arsenic in each mL, within 3 days.

Silver diethyldithiocarbamate solution: Dissolve 1 g of ACS reagent-grade silver diethyldithiocarbamate in 200 mL of recently distilled pyridine. Store this solution in a light-resistant container and use within 1 month.

Stannous chloride solution: Dissolve 40 g of stannous chloride dihydrate ($SnCl_2 \cdot 2H_2O$) in 100 mL of hydrochloric acid. Store the solution in glass containers and use within 3 months.

Lead acetate-impregnated cotton: Soak cotton in a saturated solution of lead acetate trihydrate, squeeze out the excess solution, and dry in a vacuum at room temperature.

Sample solution: Use directly as the *Sample solution* in the *Procedure* the solution obtained by treating the sample as directed in an individual monograph. Prepare Sample solutions of organic compounds in the generator flask (*a*), unless otherwise directed, according to the following general procedure:

[**CAUTION**—Some substances may react unexpectedly with explosive violence when digested with hydrogen peroxide. Use appropriate safety precautions at all times.]

[NOTE—If halogen-containing compounds are present, use a lower temperature while heating the sample with sulfuric acid; do not boil the mixture; and add the peroxide, with caution, before charring begins to prevent loss of trivalent arsenic.]

Transfer 1.0 g of the sample into the generator flask, add 5 mL of sulfuric acid and a few glass beads, and digest at a temperature not exceeding 120° until charring begins, preferably using a hot plate in a fume hood. (Additional sulfuric acid may be necessary to completely wet some samples, but the total volume added should not exceed about 10 mL.) After the acid has initially decomposed the sample, cautiously add, dropwise, hydrogen peroxide

(30%), allowing the reaction to subside and reheating the sample between drops. Add the first few drops very slowly with sufficient mixing to prevent a rapid reaction, and discontinue heating if foaming becomes excessive. Swirl the solution in the flask to prevent unreacted substance from caking on the walls or bottom of the flask during digestion.

[NOTE—Maintain oxidizing conditions at all times during the digestion by adding small quantities of the peroxide whenever the mixture turns brown or darkens.]

Continue the digestion until the organic matter is destroyed, gradually raising the temperature of the hot plate to 250° to 300° until fumes of sulfur trioxide are copiously evolved and the solution becomes colorless or retains only a light straw color. Cool, cautiously add 10 mL of water, heat again to strong fuming, and cool. Cautiously add 10 mL of water, mix, wash the sides of the flask with a few mL of water, and dilute to 35 mL.

Procedure: If the *Sample solution* was not prepared in the generator flask, transfer to the flask a volume of the solution, prepared as directed, equivalent to 1.0 g of the substance being tested, and add water to make 35 mL. Add 20 mL of 1:5 sulfuric acid, 2 mL of *potassium iodide TS*, 0.5 mL of *Stannous chloride solution*, and 1 mL of isopropyl alcohol, and mix. Allow the mixture to stand for 30 min at room temperature. Pack the scrubber unit (*c*) with two plugs of *Lead acetate-impregnated cotton*, leaving a small air space between the two plugs, lubricate joints *b* and *d* with stopcock grease, if necessary, and connect the scrubber unit with the absorber tube (*e*). Transfer 3.0 mL of *Silver diethyldithiocarbamate solution* to the absorber tube, add 3.0 g of granular zinc (20-mesh) to the mixture in the flask, and immediately insert the standard-taper joint (*b*) into the flask. Allow the evolution of hydrogen and color development to proceed at room temperature (25 ± 3°) for 45 min, swirling the flask gently at 10-min intervals. Disconnect the absorber tube from the generator and scrubber units, and transfer the *Silver diethyldithiocarbamate solution* to a 1-cm absorption cell. Determine the absorbance at the wavelength of maximum absorption between 535 nm and 540 nm, with a suitable spectrophotometer or colorimeter, using *Silver diethyldithiocarbamate solution* as the blank. The absorbance due to any red color from the solution of the sample does not exceed that produced by 3.0 mL of *Standard arsenic solution* (3 µg As) when treated in the same manner and under the same conditions as the sample. The room temperature during the generation of arsine from the standard should be held to within ±2° of that observed during the determination of the sample.

Interferences: Metals or salts of metals such as chromium, cobalt, copper, mercury, molybdenum, nickel, palladium, and silver may interfere with the evolution of arsine. Antimony, which forms stibine, is the only metal likely to produce a positive interference in the color development with the silver diethyldithiocarbamate. Stibine forms a red color with silver diethyldithiocarbamate that has a maximum absorbance at 510 nm, but at 535–540 nm, the absorbance of the antimony complex is so diminished that the results of the determination would not be altered significantly.

CADMIUM LIMIT TEST

Spectrophotometer: Use any suitable atomic absorption spectrophotometer equipped with a Boling-type burner, an air–acetylene flame, and a hollow-cathode cadmium lamp. The instrument should be capable of operating within the sensitivity necessary for the determination.

Standard solution: Transfer 100 mg of cadmium chloride crystals ($CdCl_2 \cdot 2½H_2O$), accurately weighed, into a 1000-mL volumetric flask, dissolve in and dilute with water to volume, and mix. Pipet 25 mL of this solution into a 100-mL volumetric flask, add 1 mL of hydrochloric acid, dilute with water to volume, and mix. Each mL contains 12.5 µg of cadmium.

Sample solution: Transfer 10 g of sample, accurately weighed, into a 50-mL volumetric flask, dissolve in and dilute with water to volume, and mix.

Test solutions: Transfer 5.0 mL of the *Sample solution* into each of five separate 25-mL volumetric flasks. Dilute the contents of *Flask 1* with water to volume, and mix. Add 1.00 mL, 2.00 mL, 3.00 mL, and 4.00 mL of *Standard solution* to *Flasks 2*, *3*, *4*, and *5*, respectively; then dilute each flask with water to volume; and mix. The *Test solutions* contain, respectively, 0 µg/mL, 0.5 µg/mL, 1.0 µg/mL, 1.5 µg/mL, and 2.0 µg/mL of cadmium.

Procedure: Determine the absorbance of each *Test solution* at 228.8 nm, setting the instrument to previously established optimum conditions, using water as a blank. Plot the absorbance of the *Test solutions* versus their contents of cadmium, in µg/mL. Draw the straight line best fitting the five points, and extrapolate the line until it intercepts the concentration axis. From the intercept, determine the amount, in µg, of cadmium in each mL of the *Test solution* containing 0 mL of the *Standard preparation*. Calculate the quantity, in mg/kg, of cadmium in the sample by multiplying this value by 25.

CHLORIDE AND SULFATE LIMIT TESTS

Where limits for chloride and sulfate are specified in the individual monograph, compare the *Sample solution* and control in appropriate glass cylinders of the same dimensions and matched as closely as practicable with respect to their optical characteristics.

If the solution is not perfectly clear after acidification, pass it through filter paper that has been washed free of chloride and sulfate. Add identical quantities of the precipitant (*silver nitrate TS* or *barium chloride TS*) in rapid succession to both the *Sample solution* and the control solution.

Experience has shown that visual turbidimetric comparisons are best made between solutions containing from 10 to 20 µg of chloride (Cl) ion or from 200 to 400 µg of sulfate (SO_4) ion in 50 mL. Weights of samples are specified on this basis in the individual monographs in which these limits are included.

• **Chloride Limit Test**

Standard chloride solution: Dissolve 165 mg of sodium chloride in water and dilute to 100.0 mL. Transfer 10.0 mL of this solution into a 1000-mL volumetric flask, dilute with water to volume, and mix. Each mL of the final solution contains 10 µg of chloride (Cl) ion.

Procedure: Unless otherwise directed, dissolve the specified amount of the test substance in 30–40 mL of water; neutralize to litmus external indicator with nitric acid, if necessary; and add 1 mL in excess. Add 1 mL of *silver nitrate TS* to the clear solution or filtrate, dilute with water to 50 mL, mix, and allow to stand for 5 min protected

from direct sunlight. Compare the turbidity, if any, with that produced similarly in a control solution containing the required volume of *Standard chloride solution* and the quantities of the reagents used for the sample.

- **Sulfate Limit Test**

Standard sulfate solution: Dissolve 148 mg of anhydrous sodium sulfate in water, and dilute to 100.0 mL. Transfer 10.0 mL of this solution to a 1000-mL volumetric flask, dilute with water to volume, and mix. Each mL of the final solution contains 10 µg of sulfate (SO_4).

Procedure: Unless otherwise directed, dissolve the specified amount of the test substance in 30–40 mL of water; neutralize to litmus external indicator with hydrochloric acid, if necessary; then add 1 mL of 2.7 N hydrochloric acid. Add 3 mL of *barium chloride TS* to the clear solution or filtrate, dilute with water to 50 mL, and mix. After 10 min compare the turbidity, if any, with that produced in a solution containing the required volume of *Standard sulfate solution* and the quantities of the reagents used for the sample.

COPPER LIMIT TEST

- **Flame Atomic Absorption Spectrometric Method**

[NOTE—Soak all glassware in 10% nitric acid for over 24 h, then rinse them thoroughly with water, followed by deionized water. Dry all glassware before usage.]

Dilution solution: Dilute 0.5 mL concentrated nitric acid with water to 100 mL

Copper stock solution (1000 µg/mL): Dissolve 1000.0 mg of copper (Cu, 99.99%) in 40% nitric acid (total amount NMT 37 mL) in a 1000-mL volumetric flask, and dilute with water to volume.

Diluted standard copper solutions: Transfer 10 mL of *Copper stock solution* into a 100-mL volumetric flask, and dilute with *Dilution solution* to volume. Transfer 10 mL of the obtained solution into a 100-mL volumetric flask, and dilute with *Dilution solution* to volume. The obtained solution is further diluted with the same method to create a working solution with a copper concentration of 1 µg/mL. Then, further dilute the working solution (again, with *Dilution solution*) to create "Diluted standard copper solutions" containing 0.10 µg/mL, 0.20 µg/mL, 0.40 µg/mL, 0.60 µg/mL, 0.80 µg/mL and 1.00 µg/mL of copper.

Sample preparation: Transfer 2.0 g of sample to a quartz or porcelain crucible, add 5 mL concentrated nitric acid, and let the mixture stand for 30 min. Heat the mixture to dry using soft fire, then continue heating to carbonize the mixture. Transfer the carbonized material to a muffle furnace, and continue the carbonization at 500° ± 25° for 1 h. Remove the carbonized sample from the furnace, and cool at room temperature. Add 1 mL concentrated nitric acid to wet the ash in the crucible, and then evaporate it carefully until dry. Carbonize the material in a muffle furnace again at 500° for 30 min, then remove it from the furnace and allow it to cool to room temperature. To the ash obtained, add 1 mL of 20% nitric acid, transfer the solution to a 10-mL volumetric flask, repeat it 4 times, and then dilute with water to volume.

Blank preparation: Prepare as directed in the *Sample preparation*, replacing the sample with the same amount of concentrated nitric acid.

Procedure: Determine the absorbance of the *Blank preparation*, the *Diluted standard copper solutions*, and the *Sample preparation* at the copper emission line of 324.8 nm, using a slit-width of 0.5 nm. Use a suitable atomic absorption spectrophotometer equipped with a copper electrodeless discharge lamp (EDL), an air–acetylene flame, and a 4-in burner head. Use water as the blank.

Calculations: Determine the corrected absorbance values by subtracting the *Blank preparation* absorbance from each of the *Diluted standard copper solutions* and from the *Sample preparation* absorbance. Prepare a standard curve by plotting the corrected *Diluted standard copper solutions* absorbance values versus their corresponding concentrations expressed as µg/mL. Determine the copper concentration in the *Sample preparation* by reference to the calibration curve. Calculate the quantity of copper, in mg/kg, in the sample taken:

$$\text{Result} = 10C/W_S$$

C = concentration of copper from the Standard curve (µg/mL)

W_S = weight of the sample taken (g)

- **Atomic Absorption Spectrophotometric Graphite Furnace Method**

[NOTE—Soak all glassware in 10% nitric acid for over 24 h, then rinse them thoroughly with water, followed by deionized water. Dry all glassware before usage.]

Apparatus: Use a suitable graphite furnace atomic absorption spectrophotometer set at 324.8 nm and equipped with an autosampler, pyrolytically coated graphite tubes, solid pyrolytic graphite platforms, and an adequate means of background correction. Zeeman effect or Smith-Hieftje background correction is preferred, but deuterium arc background correction should be acceptable. (This method was developed on a Perkin-Elmer Model Z5100, 0.7-nm slit, HGA-600 furnace, AS-60 autosampler with Zeeman background correction.) If the instrument does not have a well-defined calibration function, a separate calculator or computer is required for linear least squares, nonlinear, or quadratic calibrations. Set up the instrument according to the manufacturer's specifications with consideration of current good GFAAS practices—addressing such factors as line voltage, cooling water temperature, graphite part specifications, and furnace temperature. If an optical pyrometer or thermocouple is not available to check the furnace controller temperature calibration, dim the room lights, and observe the furnace emission through the sample introduction port while increasing the furnace temperature. A characteristic cherry red glow should begin to appear at 800°. If it glows at a lower temperature, then the furnace is hotter, and temperatures must be adjusted downward accordingly.

Use acid-cleaned [in a mixture of 5% sub-boiling, distilled nitric acid and 5% sub-boiling, distilled hydrochloric acid made up in deionized, distilled water (18 megohm), and thoroughly rinsed with deionized, distilled water

(18 megohm)] autosampler cups (PE B008-7600 Teflon, or equivalent) to avoid contamination. Use micropipets with disposable tips free of contamination for dilution. Ensure accuracy and precision of micropipets and tips by dispensing and weighing 5–10 replicate portions of water onto a microbalance. Use acid-cleaned volumetric glassware to prepare standards and dilute samples to a final volume. For digestion, use acid-cleaned, high-density polyethylene tubes, polypropylene tubes, Teflon tubes, or quartz tubes. Store final diluted samples in plastic tubes.

Air ashing: The furnace controller must be able to handle two gas flows to facilitate air ashing. Oxygen ashing is used to avoid build up of residue during the char step. Argon is used as the purge gas for the furnace for all steps but the char. Breathing quality air can be used as the alternate gas for the air ashing.

Dilution solution: Dilute 0.5 mL concentrated nitric acid to 100 mL with water

Copper stock solution (1000 µg/mL): Dissolve 1000.0 mg of copper (Cu, 99.99%) in 40% nitric acid (total amount NMT 37 mL) in a 1000-mL volumetric flask, and dilute with water to volume.

Diluted standard copper solutions: Transfer 10 mL of *Copper stock solution* into a 100-mL volumetric flask, and dilute with *Dilution solution* to volume. Transfer 10 mL of the obtained solution into a 100-mL volumetric flask, and dilute with *Dilution solution* to volume. The obtained solution is further diluted with the same method to create a working solution with a copper concentration of 0.1 µg/mL. Then further dilute the working solution (again, with *Dilution solution*) to create "Diluted standard copper solutions" containing 0.01 µg/mL, 0.02 µg/mL, 0.04 µg/mL, 0.06 µg/mL, 0.08 µg/mL, and 0.10 µg/mL of copper.

Sample preparation: Transfer 2.0 g of sample to a quartz or porcelain crucible, add 5 mL concentrated nitric acid, and let the mixture stand for 30 min. Heat the mixture carefully until dry, then continue heating to carbonize the mixture. Transfer the carbonized material to a muffle furnace, and continue the carbonization at 500° ± 25° for 1 h. Remove the carbonized sample from the furnace, and cool at room temperature. Add 1 mL concentrated nitric acid to wet the ash in the crucible, and then evaporate it carefully until dry. Carbonize the material in a muffle furnace again at 500° for 30 min, then remove it from the furnace and allow it to cool to room temperature. To the ash obtained, add 1 mL of 20% nitric acid, transfer the solution to a 10-mL volumetric flask, repeat it 4 times, and then dilute with water to volume.

Blank preparation: Prepare as directed in the *Sample preparation*, replacing the sample with the same amount of concentrated nitric acid.

Procedure: Inject 10–20 µL *Sample preparation*, *Blank preparation*, and *Diluted standard copper solutions* into a graphite furnace atomizer. The furnace program is as follows: (1) Dry at 90°, using a 20-s hold and a 1500-mL/min argon flow (or other inert gas); (2) char the sample at 800°, using a 20-s hold and a constant speed of air flow; [NOTE—Air ashing is a critical step to avoid build up of residue during the char step.] (3) cool down, and purge the air from the furnace for 60 s, using a temperature set to 20° and a 1500-mL/min argon flow (or other inert gas); (4) atomize at 2300°, using a 4-s hold with the argon flow (or other inert gas) stopped. Determine the absorbance at the copper emission line of 324.8 nm, using a slit-width of 0.5 nm.

[NOTE—If the test has interference from sodium chloride or other materials, either dilute the *Sample preparation* with 1 mg/mL ammonium nitrate or ammonium dihydrogen phosphate before injection, or add the same quantity of ammonium nitrate or ammonium dihydrogen as a chemical modifier after injection (into graphite furnace).]

Calculations: Determine the corrected absorbance values by subtracting the *Blank preparation* absorbance from each of the *Diluted standard copper solutions* and from the *Sample preparation* absorbance. Prepare a Standard curve by plotting the corrected *Diluted standard copper solutions* absorbance values versus their corresponding concentrations expressed as µg/mL. Determine the copper concentration in the *Sample preparation* by reference to the calibration curve. Calculate the quantity of copper, in mg/kg, in the sample taken:

$$\text{Result} = 10C/W_S$$

C = concentration of copper from the standard curve (µg/mL)

W_S = weight of the sample taken (g)

1,4-DIOXANE LIMIT TEST

Vacuum distillation apparatus: Assemble a closed-system vacuum distillation apparatus employing glass vacuum stopcocks (*A*, *B*, and *C*), as shown in *Figure 5*.

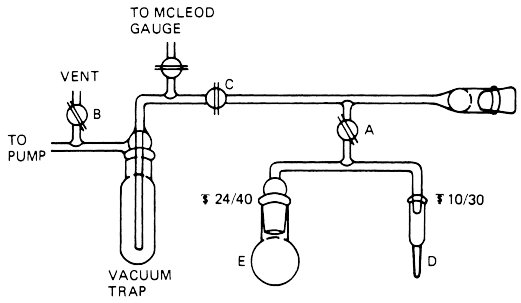


Figure 5. Closed-System Vacuum Distillation Apparatus for 1,4-Dioxane

The concentrator tube (*D*) is made of borosilicate or quartz (not flint) glass, graduated precisely enough to measure the 0.9 mL or more of distillate and marked so that the analyst can accurately dilute to 2.0 mL (available as Chromaflex concentrator tube, Kontes Glass Co., Vineland, NJ, Catalog No. K42560-0000).

Standard preparation: Prepare a solution of 1,4-dioxane in water containing 100 µg/mL. Keep the solution refrigerated, and prepare fresh weekly.

Sample preparation: Transfer 20 g of the sample, accurately weighed, into a 50-mL round-bottom flask (*E*) having a 24/40 ground-glass neck. Semisolid or waxy samples should be liquefied by heating on a steam bath before making the transfer. Add 2.0 mL of water to the flask for crystalline samples, and 1.0 mL for liquid, semisolid, or waxy samples. Place a small Teflon-covered stirring bar in the flask, stopper, and stir to mix. Immerse the flask in an ice bath, and chill for about 1 min.

Wrap heating tape around the tube connecting the Chromaflex tube (*D*) and the round-bottom flask (*E*), and apply about 10 V to the tape. Apply a light coating of high-vacuum silicone grease to the ground-glass joints, and connect the Chromaflex tube to the 10/30 joint and the round-bottom flask to the 24/40 joint. Immerse the vacuum trap in a Dewar flask filled with liquid nitrogen, close stopcocks *A* and *B*, open stopcock *C*, and begin evacuating the system with a vacuum pump. Prepare a slush bath from powdered dry ice and methanol, and raise the bath to the neck of the round-bottom flask. After freezing the contents of the flask for about 10 min, and when the vacuum system is operating at 0.05 mm pressure or lower, open stopcock *A* for 20 s, and then close it. Remove the slush bath, and allow the flask to warm in air for about 1 min. Immerse the flask in a water bath at 20°–25°, and after about 5 min warm the water in the bath to 35°–40° (sufficient to liquefy most samples) while stirring slowly but constantly with the magnetic bar. Cool the water in the bath by adding ice, and chill for about 2 min. Replace the water bath with the slush bath, freeze the contents of the flask for about 10 min, then open stopcock *A* for 20 s, and close it. Remove the slush bath, and repeat the heating steps as before, this time reaching a final temperature of 45°–50° or a temperature necessary to melt the sample completely. If there is any condensation in the tube connecting the round-bottom flask to the Chromaflex tube, slowly increase the voltage to the heating tape and heat until condensation disappears.

Stir with the magnetic stirrer throughout the following steps: very slowly immerse the Chromaflex tube in the Dewar flask containing liquid nitrogen. [**CAUTION**—When there is liquid distillate in the Chromaflex tube, the tube must be immersed in the nitrogen very slowly, or the tube will break.]

Water will begin to distill into the tube. As ice forms in the tube, raise the Dewar flask to keep the liquid nitrogen level only slightly below the level of ice in the tube. When water begins to freeze in the neck of the 10/30 joint, or when liquid nitrogen reaches the 2.0-mL graduation mark on the Chromaflex tube, remove the Dewar flask and let the ice melt without heating. After the ice has melted, check the volume of water that has distilled, and repeat the sequence of chilling and thawing until at least 0.9 mL of water has been collected. Freeze the tube once again for about 2 min, and release the vacuum first by opening stopcock *B*, followed by stopcock *A*. Remove the Chromaflex tube from the apparatus, close it with a greased stopper, and let the ice melt without heating. Mix the contents of the tube by swirling, note the volume of distillate, and dilute with water to 2.0 mL, if necessary. Use this *Sample preparation* as directed under *Chromatography*.

Chromatography: (See *Chromatography, Appendix IIA*.) Use a gas chromatograph equipped with a flame-ionization detector. Under typical conditions, the instrument contains a 4-mm (id) × 6-ft glass column, or equivalent, packed with 80-/100- or 100-/120-mesh Chromosorb 104, or equivalent. The column is maintained isothermally at about 140°, the injection port at 200°, and the detector at 250°. Nitrogen is the carrier gas, flowing at a rate of about 35 mL/min. Install an oxygen scrubber between the carrier gas line and the column. The column should be conditioned for about 72 h at 250° with 30–40 mL/min carrier flow.

[NOTE—Chromosorb 104 is oxygen sensitive. Both new and used columns should be flushed with carrier gas for 30–60 min before heating each time they are installed in the gas chromatograph.]

Inject a volume of the *Standard preparation*, accurately measured, to give about 20% of maximum recorder response. Where possible, keep the injection volume in the range of 2–4 µL, and use the solvent-flush technique to minimize errors associated with injection volumes. In the same manner, inject an identical volume of the *Sample preparation*. The height of the peak produced by the *Sample preparation* does not exceed that produced by the *Standard preparation*.[1]

FLUORIDE LIMIT TEST

• Method I (Thorium Nitrate Colorimetric Method)

Use this method unless otherwise directed in the individual monograph.

[**CAUTION**—When applying this test to organic compounds, rigidly control at all times the temperature at which the distillation is conducted to the recommended range of 135°–140° to avoid the possibility of explosion.]

[NOTE—To minimize the distillation blank resulting from fluoride leached from the glassware, treat the distillation apparatus as follows: treat the glassware with hot 10% sodium hydroxide solution, followed by flushing with tap water and rinsing with distilled water. At least once daily, treat in addition by boiling down 15–20 mL of 1:2 sulfuric acid until the still is filled with fumes; cool, pour off the acid, treat again with 10% sodium hydroxide solution, and rinse thoroughly. For further details, see AOAC method 944.08.]

Unless otherwise directed, place a 5.0-g sample and 30 mL of water in a 125-mL Pyrex distillation flask with a side arm and trap. The flask is connected with a condenser and carries a thermometer and a capillary tube, both of which must extend into the liquid. Slowly add, with continuous stirring, 10 mL of 70% perchloric acid, and then add 2 or 3 drops of a 1:2 solution of silver nitrate and a few glass beads. Connect a small dropping funnel or a steam generator

[1] If the sample fails the test because of known or suspected interference, another aliquot may be run on a 6-ft × 2-mm (id) column, or equivalent, of 0.2% Carbowax 1500 on Carbopak C, operating at 100° isothermal, with 20 mL/min of helium carrier flow. Under these conditions, the 1,4-dioxane elutes in about 4 min.

to the capillary tube. Support the flask on a flame-resistant mat or shielding board, with a hole that exposes about one-third of the flask to the low, "clean" flame of a Bunsen burner.

[NOTE—The shielding is essential to prevent the walls of the flask from overheating above the level of its liquid contents.]

Distill until the temperature reaches 135°. Add water from the funnel or introduce steam through the capillary, maintaining the temperature between 135° and 140° at all times. Continue the distillation until 100 mL of distillate has been collected. After the 100-mL portion (*Distillate A*) is collected, collect an additional 50-mL portion of distillate (*Distillate B*) to ensure that all of the fluorine has been volatilized.

Place 50 mL of *Distillate A* in a 50-mL Nessler tube. In another, similar Nessler tube, place 50 mL of water distilled through the apparatus as a control. Add to each tube 0.1 mL of a filtered 1:1000 solution of sodium alizarinsulfonate and 1 mL of a freshly prepared 1:4000 solution of hydroxylamine hydrochloride, and mix well. Add, dropwise and with stirring, either 1 N or 0.05 N sodium hydroxide, depending on the expected volume of volatile acid distilling over, to the tube containing the distillate until its color just matches that of the control, which is faintly pink. Then add to each tube 1.0 mL of 0.1 N hydrochloric acid, and mix well. From a buret, graduated in 0.05 mL, add slowly to the tube containing the distillate enough of a 1:4000 solution of thorium nitrate so that, after mixing, the color of the liquid just changes to a faint pink. Note the volume of the solution added, then add exactly the same volume to the control, and mix. Now add to the control solution sodium fluoride TS [10 µg of fluoride (F) per mL] from a buret to make the colors of the two tubes match after dilution to the same volume. Mix well, and allow all air bubbles to escape before making the final color comparison. Check the endpoint by adding 1 or 2 drops of sodium fluoride TS to the control. A distinct change in color should take place. Note the volume of sodium fluoride TS added.

Dilute *Distillate B* to 100 mL, and mix well. Place 50 mL of this solution in a 50-mL Nessler tube, and follow the procedure used for *Distillate A*. The total volume of sodium fluoride TS required for the solutions from both *Distillate A* and *Distillate B* should not exceed 2.5 mL.

• **Method II (Ion-Selective Electrode Method A)**

Buffer solution: Dissolve 36 g of cyclohexylenedinitrilotetraacetic acid (CDTA) in sufficient 1 N sodium hydroxide to make 200 mL. Transfer 20 mL of this solution (equivalent to 4 g of disodium CDTA) into a 1000-mL beaker containing 500 mL of water, 57 mL of glacial acetic acid, and 58 g of sodium chloride, and stir to dissolve. Adjust the pH of the solution to 5.0–5.5 by the addition of 5 N sodium hydroxide, then cool to room temperature, dilute with water to 1000 mL, and mix.

Fluoride standard: Transfer 2.2108 g of sodium fluoride, previously dried at 200° for 4 h, into a 1000-mL volumetric flask, and dilute with water to volume. The resulting solution contains 1000 µg of fluoride per mL.

Fluoride standard solution: Transfer 1 mL of the *Fluoride standard* to a 10-mL volumetric flask, and dilute with water to volume. The resulting solution contains 100 µg of fluoride (F) ion per mL.

Electrode calibration: Pipet 50 mL of the *Buffer solution* into a plastic beaker. Place the fluoride ion and reference electrodes (or a combination fluoride electrode) into the plastic beaker, and stir. At 5-min intervals, add 100 µL and 1000 µL of the *Fluoride standard* and read the potential, in mV, after each addition. The difference between the two readings is the slope of the fluoride electrode and should typically be in the range of 54–60 mV at 25° for the standards in the *Buffer solution*. If the difference in potential is not within this range, check, and, if necessary, replace the electrode, instrument, or solutions. Alternatively, the electrode calibration should be performed according to the manufacturer's instructions and should comply with the manufacturer's calibration range at 25°. If the difference in potential is not within this range, evaluate the system and equipment as necessary.

Procedure: Unless otherwise directed in the individual monograph, transfer 8.0 g of sample and 20 mL of water into a 250-mL distilling flask, cautiously add 20 mL of perchloric acid, and then add 2 or 3 drops of a 1:2 solution of silver nitrate and a few glass beads.

[**CAUTION**—Handle perchloric acid in an appropriate fume hood.]

Following the directions, and observing the *Caution* and *Notes*, as given under *Method I*, distill the solution until 200 mL of distillate has been collected.

Transfer a 25.0-mL aliquot of the distillate into a 250-mL plastic beaker, and dilute with the *Buffer solution* to 100 mL. Place the fluoride ion and reference electrodes (or a combination fluoride electrode) of a suitable ion-selective electrode apparatus in the solution. Allow sufficient time for equilibration (about 20 min), stirring constantly during the equilibration period and throughout the remainder of the procedure, and record the initial equilibrated reading in mV. Pipet 1.0 mL of the *Fluoride standard solution* into the beaker, allow the electrode to come to equilibrium, and record the final reading in mV.

[NOTE—Follow the instrument manufacturer's instructions regarding precautions and interferences, electrode filling and check, temperature compensation, and calibration.]

Calculations: Calculate the fluoride content, in mg/kg, of the sample taken:

$$\text{Result} = (800/W)/\{1.01 \times 10^{[(E2 - E1)/S]} - 1\}$$

800 = factor that corrects for the sample dilutions

W = original weight of the sample (g)

1.01 = correction factor that is the relationship between the volume of standard used for standard addition and the volume of the sample dilution on which the standard addition is performed (V_S/V)

E2 = final equilibrated reading (mV)

E1 = initial equilibrated reading (mV)

S = electrode slope

- **Method III (Ion-Selective Electrode Method B)**

Sodium fluoride solution (5 µg F/mL): Transfer 2.210 g of sodium fluoride, previously dried at 200° for 4 h and accurately weighed, into a 400-mL plastic beaker, add 200 mL of water, and stir until dissolved. Quantitatively transfer this solution into a 1000-mL volumetric flask with the aid of water, dilute with water to volume, and mix. Store this stock solution in a plastic bottle. On the day of use, transfer 5.0 mL of the stock solution into a 1000-mL volumetric flask, dilute with water to volume, and mix.

Calibration curve: Transfer 1.0, 2.0, 3.0, 5.0, 10.0, and 15.0 mL of the *Sodium fluoride solution* into separate 250-mL plastic beakers; add 50 mL of water, 5 mL of 1 N hydrochloric acid, 10 mL of 1 M sodium citrate, and 10 mL of 0.2 M disodium EDTA to each beaker; and mix. Transfer each solution into separate 100-mL volumetric flasks, dilute with water to volume, and mix. Transfer a 50-mL portion of each solution into separate 125-mL plastic beakers, and measure the potential of each solution with a suitable ion-selective electrode apparatus (such as the Orion Model No. 94-09, with solid-state membrane), using a suitable reference electrode (such as the Orion Model No. 90-01, with single junction). Plot the Calibration curve on two-cycle semilogarithmic paper (such as K & E No. 465130) or with the use of a suitable graphing calculator or spreadsheet program, with µg of fluoride (F) per 100 mL of solution on the logarithmic scale.

Procedure: Transfer 1.00 g of sample into a 150-mL glass beaker, add 10 mL of water, and, while stirring continuously, slowly add 20 mL of 1 N hydrochloric acid to dissolve the sample. Boil rapidly for 1 min, then transfer into a 250-mL plastic beaker, and cool rapidly in ice water. Add 15 mL of 1 M sodium citrate and 10 mL of 0.2 M disodium EDTA, and mix. Adjust the pH to 5.5 ± 0.1 with 1 N hydrochloric acid or 1 N sodium hydroxide, if necessary; transfer into a 100-mL volumetric flask; dilute with water to volume; and mix. Transfer a 50-mL portion of this solution into a 125-mL plastic beaker, and measure the potential of the solution with the apparatus described under *Calibration curve*. Determine the fluoride content, in µg, of the sample from the *Calibration curve*.

Determine the percentage of fluoride in the sample taken:

$$\text{Result} = (C/W_S) \times 0.000001 \times 100\%$$

C = content of fluoride in the sample, determined from the *Calibration curve* (µg)

W_S = sample weight (g)

0.000001 = conversion factor (µg to grams)

- **Method IV (Ion-Selective Electrode Method C)**

[NOTE—Unless directed otherwise by the individual monograph, use *Buffer solution A* for samples with a neutral to higher pH, and use *Buffer solution B* for samples with a neutral to lower pH.]

Buffer solution A: Add 2 volumes of 6 N acetic acid to 1 volume of water, and adjust the pH to 5.0 with 50% potassium hydroxide solution.

Buffer solution B: Dissolve 150 g of sodium citrate dihydrate and 10.3 g of disodium EDTA dihydrate in 800 mL of water, adjust the pH to 8.0 with 50% sodium hydroxide solution, and dilute with water to 1000 mL.

Fluoride standard solutions

1000 mg/kg Fluoride standard: Transfer 2.2108 g of sodium fluoride, previously dried at 200° for 4 h, into a 1000-mL volumetric flask, and dissolve in and dilute with water to volume. The resulting solution contains 1000 µg of fluoride per mL.

50 mg/kg Fluoride standard: Pipet 50 mL of the *1000 mg/kg Fluoride standard* into a 1000-mL volumetric flask. Dilute with water to volume.

10 mg/kg Fluoride standard: Pipet 100 mL of the *50 mg/kg Fluoride standard* into a 500-mL volumetric flask. Dilute with water to volume.

Fluoride limit solutions (for a 1-g sample)

50 mg/kg Fluoride limit solution (1 mg/kg Fluoride standard): Pipet 50 mL of the *10 mg/kg Fluoride standard* into a 500-mL volumetric flask, and dilute with water to volume.

10 mg/kg Fluoride limit solution (0.2 mg/kg Fluoride standard): Pipet 10 mL of the *10 mg/kg Fluoride standard* into a 500-mL volumetric flask, and dilute with water to volume.

Fluoride limit solutions (for a 2-g sample)

50 mg/kg Fluoride limit solution (2 mg/kg Fluoride standard): Pipet 100 mL of the *10 mg/kg Fluoride standard* into a 500-mL volumetric flask, and dilute with water to volume.

10 mg/kg Fluoride limit solution (0.4 mg/kg Fluoride standard): Pipet 20 mL of the *10 mg/kg Fluoride standard* into a 500-mL volumetric flask, and dilute with water to volume.

[NOTE—Store all standard and limit solutions in plastic containers.]

Sample preparation: Accurately weigh the amount of sample specified in the monograph, transfer it into a 100-mL volumetric flask, and dissolve it in a minimal amount of water. Add 50.0 mL of the appropriate *Buffer solution*, dilute with water to volume, and mix.

Electrode calibration: Pipet 50 mL of the appropriate *Buffer solution* into a plastic beaker. Place the fluoride ion and reference electrodes (or a combination fluoride electrode) into the plastic beaker and stir. At 5-min intervals, add 100 µL and 1000 µL of the *1000 mg/kg Fluoride standard* and read the potential, in mV, after each addition. The difference between the two readings is the slope of the fluoride electrode and should typically be in the range of 63–

70 mV at 25° for *Buffer solution A* and in the range of 54–60 mV at 25° for *Buffer solution B*. If the difference in potential is not within this range, check, and, if necessary, replace the electrode, instrument, or solutions.

Alternatively, the electrode calibration should be performed according to the manufacturer's instructions and should comply with the manufacturer's calibration range at 25°. If the difference in potential is not within this range, evaluate the system and equipment as necessary.

Procedure: Transfer the entire sample into a plastic beaker. Place the electrode into the beaker, allow the solution to equilibrate for 5 min with stirring, and read the potential, in mV. Remove and rinse the electrode(s) with water. In another beaker, using a pipet, add 50 mL of the appropriate *Buffer solution* followed by 50 mL of the *Fluoride limit solution* that best reflects the fluoride limit of the sample. Place the electrode in the beaker, equilibrate for 3 min, and read the potential in mV. If the potential of the *Fluoride limit solution* is less than that of the sample, the sample passes the test criteria for maximum acceptable fluoride level limit.

- **Method V**

Lime suspension: Carefully shake about 56 g of low-fluorine calcium oxide [about 2 mg/kg of fluoride (F)] with 250 mL of water, and while stirring, slowly add 250 mL of 60% perchloric acid. Add a few glass beads, and boil until copious fumes of perchloric acid evolve, then cool, add 200 mL of water, and boil again.

[**CAUTION**—Handle perchloric acid in an appropriate fume hood.]

Repeat the dilution and boiling once more, cool, dilute considerably, and if precipitated silicon dioxide forms, pass through a fritted-glass filter. While stirring, pour the clear solution into 1000 mL of a 1:10 solution of sodium hydroxide, allow the precipitate to settle, and siphon off the supernatant liquid. Remove the sodium salts from the precipitate by washing five times with water in large centrifuge bottles, shaking the mass thoroughly each time. Finally, shake the precipitate into a suspension, and dilute with water to 2000 mL. Store in paraffin-lined bottles, and shake well before use.

[NOTE—100 mL of this suspension should give no appreciable fluoride blank when evaporated, distilled, and titrated as directed under *Method I*.]

Procedure: Assemble the distilling apparatus as described under *Method I*, and add 1.67 g of sample, accurately weighed, and 25 mL of 1:2 sulfuric acid to the distilling flask. Distill until the temperature reaches 160°, then maintain at 160°–165° by adding water from the funnel, collecting 300 mL of distillate. Oxidize the distillate by cautiously adding 2 or 3 mL of fluorine-free 30% hydrogen peroxide (to remove sulfates), allow to stand for a few minutes, and evaporate in a platinum dish with an excess of *Lime suspension*. Ignite briefly at 600°, then cool and wet the ash with about 10 mL of water. Cover the dish with a watch glass, and cautiously introduce under the watch glass just sufficient 60% perchloric acid to dissolve the ash. Add the contents of the dish through the dropping funnel of a freshly prepared distilling apparatus (the distilling flask should contain a few glass beads), using a total of 20 mL of the 60% perchloric acid to dissolve the ash and transfer the solution. Add 10 mL of water and a few drops of a 1:2 solution of silver perchlorate through the dropping funnel, and continue as directed under *Method I*, beginning with "Distill until the temperature reaches 135°".

- **Method VI (Ion-Selective Electrode Method D)**

Sample: 3 g

Buffer solution: Transfer 36 g of cyclohexylenedinitrilotetraacetic acid (CDTA) in a 200-mL volumetric flask and dissolve in 1 N sodium hydroxide to volume. Transfer 20 mL of this solution (equivalent to 4 g of disodium CDTA) into a 1000-mL beaker containing 500 mL of water, 57 mL of glacial acetic acid, and 58 g of sodium chloride, and stir to dissolve. Adjust the pH of the solution to 5.0–5.5 by the addition of 5 N sodium hydroxide, then cool to room temperature, dilute with water to 1000 mL, and mix.

Fluoride standard: Transfer 2.2108 g of sodium fluoride, previously dried at 200° for 4 h, into a 1000-mL volumetric flask, and dilute with water to volume. The resulting solution contains 1000 µg of fluoride per mL.

Fluoride standard solution: Transfer 10 mL of the *Fluoride standard* to a 100-mL volumetric flask, and dilute with water to volume. The resulting solution contains 100 µg of fluoride (F) ion per mL.

Electrode calibration: Pipet 50 mL of the *Buffer solution* and 50 mL of water into a plastic beaker. Place the fluoride ion and reference electrodes (or a combination fluoride electrode) into the plastic beaker, and stir. At 5-min intervals, add 1 mL and 10 mL of the *Fluoride standard solution* and read the potential, in mV, after each addition. The difference between the two readings is the slope of the fluoride electrode and should typically be in the range of 54–60 mV at 25° for the standards in the *Buffer solution*. If the difference in potential is not within this range, check, and, if necessary, replace the electrode, instrument, or solutions. Alternatively, the electrode calibration should be performed according to the manufacturer's instructions and should comply with the manufacturer's calibration range at 25°. If the difference in potential is not within this range, evaluate the system and equipment as necessary.

Analysis: Transfer the *Sample* into a 100-mL volumetric flask, and dissolve it in a minimal amount of water. Add 50.0 mL of the *Buffer solution*, dilute with water to volume, and mix. Transfer the entire solution to a plastic beaker. Place the fluoride ion and reference electrode (or fluoride combination electrode) into the beaker, allow the solution to equilibrate for 5 min with stirring, and read the potential in mV (E_1). Pipet 0.25 mL of the *Fluoride standard solution* into the beaker, allow the electrode to come to equilibrium, and record the final potential in mV (E_2). Measure a blank fluoride level by repeating this procedure without sample and using 0.1 mL of the *Fluoride standard solution*.

Calculate the change in mV (ΔE) for the blank and the sample taken:

$$\Delta E = E_1 - E_2$$

E_1 = reading before addition of the *Fluoride standard solution* (mV)

E_2 = reading after addition of the *Fluoride standard solution* (mV)

Calculate the diluted fluoride level, in µg/mL, in the blank (C_B) and in the sample taken (C_S):

$$C_S = 0.25/(1.0025 \times 10^{\Delta E/S} - 1)$$

$$C_B = 0.1/(1.001 \times 10^{\Delta E/S} - 1)$$

0.25, 1.0025, 0.1, and 1.001 = formula constants

ΔE = change in mV calculated above

S = absolute value of the electrode as determined in *Electrode Calibration*

Calculate the fluoride content, in mg/kg, in the sample taken:

$$\text{Result} = 100 \times (C_S - C_B)/W$$

C_S = diluted fluoride level in the *Sample* calculated above (µg/mL)

C_B = diluted fluoride level in blank calculated above (µg/mL)

W = weight of the *Sample* (g)

LEAD LIMIT TEST

[NOTE—Unless otherwise specified in the monograph, use the *Dithizone Method* to determine lead levels.]

• **Dithizone Method**

Special reagents: Select reagents having as low a lead content as practicable, and store all solutions in containers of borosilicate glass. Rinse all glassware thoroughly with warm, 1:2 nitric acid followed by water.

Ammonia–cyanide solution: Dissolve 2 g of potassium cyanide in 15 mL of ammonium hydroxide, and dilute with water to 100 mL.

Ammonium citrate solution: Dissolve 40 g of citric acid in 90 mL of water, add 2 or 3 drops of phenol red TS, then cautiously add ammonium hydroxide until the solution acquires a red color. Extract it with 20-mL portions of *Dithizone extraction solution* until the dithizone solution retains its green color or remains unchanged.

Diluted standard lead solution (1 µg Pb in 1 mL)

Lead nitrate stock solution: Dissolve 159.8 mg of ACS Reagent-Grade Lead Nitrate [$Pb(NO_3)_2$] in 100 mL of water containing 1 mL of nitric acid, dilute with water to 1000.0 mL, and mix. Prepare and store this solution in glass containers that are free from lead salts.

Standard lead solution: On the day of use, dilute 10.0 mL of *Lead nitrate stock solution* with water to 100.0 mL. Each mL of *Standard lead solution* contains the equivalent of 10 µg of lead (Pb) ion.

Diluted standard lead solution: Immediately before use, transfer 10.0 mL of *Standard lead solution* into a 100-mL volumetric flask, dilute with 1:100 nitric acid to volume, and mix.

Dithizone extraction solution: Dissolve 30 mg of dithizone in 1000 mL of chloroform, add 5 mL of alcohol, and mix. Store in a refrigerator. Before use, shake a suitable volume of the solution with about half its volume of 1:100 nitric acid, discarding the nitric acid.

Hydroxylamine hydrochloride solution: Dissolve 20 g of hydroxylamine hydrochloride in sufficient water to make about 65 mL, transfer the solution into a separator, add a few drops of thymol blue TS, then add ammonium hydroxide until the solution assumes a yellow color. Add 10 mL of a 1:25 solution of sodium diethyldithiocarbamate, mix, and allow to stand for 5 min. Extract the solution with successive 10- to 15-mL portions of chloroform until a 5-mL test portion of the chloroform extract does not assume a yellow color when shaken with cupric sulfate TS. Add 2.7 N hydrochloric acid until the extracted solution is pink, adding 1 or 2 drops more of thymol blue TS if necessary, then dilute with water to 100 mL, and mix.

Potassium cyanide solution: Dissolve 50 g of potassium cyanide in sufficient water to make 100 mL. Remove the lead from the solution by extraction with successive portions of *Dithizone extraction solution* as described under *Ammonium citrate solution*, then extract any dithizone remaining in the cyanide solution by shaking with chloroform. Finally, dilute the cyanide solution with sufficient water so that each 100 mL contains 10 g of potassium cyanide.

Standard dithizone solution: Dissolve 10 mg of dithizone in 1000 mL of chloroform, keeping the solution in a glass-stoppered, lead-free bottle suitably wrapped to protect it from light and stored in a refrigerator.

Sample solution: Use the solution obtained by treating the sample as directed in an individual monograph as the *Sample solution* in the *Procedure*. Sample solutions of organic compounds are prepared, unless otherwise directed, according to the following general method: [**CAUTION**—Some substances may react unexpectedly with explosive violence when digested with hydrogen peroxide. Use appropriate safety precautions at all times.]

Transfer 1.0 g of sample into a suitable flask, add 5 mL of sulfuric acid and a few glass beads, and digest at a temperature not exceeding 120° until charring begins, using preferably a hot plate in a fume hood. (Additional sulfuric acid may be necessary to completely wet some samples, but the total volume added should not exceed about 10 mL.) After the sample has initially been decomposed by the acid, add with caution, dropwise, hydrogen peroxide (30%), allowing the reaction to subside and reheating between drops. The first few drops must be added very slowly with sufficient mixing to prevent a rapid reaction, and heating should be discontinued if foaming becomes excessive. Swirl the solution in the flask to prevent unreacted substance from caking on the walls or bottom of the flask during the digestion.

[NOTE—Add small quantities of the peroxide when the solution begins to darken.]

Continue the digestion until the organic matter is destroyed, gradually raising the temperature of the hot plate to 250°–300° until fumes of sulfur trioxide are copiously evolved and the solution becomes colorless or retains only a light straw color. Cool, cautiously add 10 mL of water, again evaporate to strong fuming, and cool. Quantitatively transfer the solution into a separator with the aid of small quantities of water.

Procedure: Transfer the *Sample solution*, prepared as directed in the individual monograph, into a separator, and unless otherwise directed, add 6 mL of *Ammonium citrate solution* and 2 mL of *Hydroxylamine hydrochloride solution*. (Use 10 mL of the citrate solution when determining lead in iron salts.) Add 2 drops of phenol red TS to the separator, and make the solution just alkaline (red in color) by the addition of ammonium hydroxide. Cool the solution, if necessary, under a stream of tap water, then add 2 mL of *Potassium Cyanide Solution*. Immediately extract the solution with 5-mL portions of *Dithizone extraction solution*, draining each extract into another separator, until the dithizone solution retains its green color. Shake the combined dithizone solutions for 30 s with 20 mL of 1:100 nitric acid, discard the chloroform layer, add 5.0 mL of *Standard Dithizone Solution* and 4 mL of *Ammonia–cyanide solution* to the acid solution, and shake for 30 s. The purple hue in the chloroform solution of the sample caused by any lead dithizonate present does not exceed that in a control, containing the volume of *Diluted Standard lead solution* equivalent to the amount of lead specified in the monograph, when treated in the same manner as the sample.

• **Flame Atomic Absorption Spectrophotometric Method**

Select reagents having as low a lead content as practicable, and store all solutions in high-density polyethylene containers. Rinse all plastic and glassware thoroughly with warm 1:2 nitric acid followed by water.

Lead nitrate stock solution (100 µg/mL): Dissolve 159.8 mg of reagent-grade lead nitrate [$Pb(NO_3)_2$] in 100 mL of water containing 1 mL of nitric acid in a 1000-mL volumetric flask, and dilute with water to volume.

Standard lead solution (10 µg/mL): On the day of use, transfer 10 mL of *Lead nitrate stock solution* into a 100-mL volumetric flask, and dilute with water to volume.

Diluted standard lead solutions: On the day of use, prepare a set of Standard lead solutions that corresponds to the lead limit specified in the monograph:

1 mg/kg Lead limit (0.5 µg/mL, 1.0 µg/mL, and 1.5 µg/mL standards): On the day of use, transfer 5.0 mL, 10.0 mL, and 15.0 mL of *Standard lead solution* into three separate 100-mL volumetric flasks, add 10 mL of 3 N hydrochloric acid to each, and dilute with water to volume.

5 mg/kg Lead limit (1.0 µg/mL, 5.0 µg/mL, and 10.0 µg/mL standards): On the day of use, transfer 10.0 mL and 50.0 mL of *Standard lead solution* into two separate 100-mL volumetric flasks, add 10 mL of 3 N hydrochloric acid to each, and dilute with water to volume. The final standard, 10.0 µg/mL, is taken directly from the *Standard lead solution*.

10 mg/kg Lead limit (5.0 µg/mL, 10.0 µg/mL, and 15.0 µg/mL standards): On the day of use, transfer 5.0 mL, 10.0 mL, and 15.0 mL of *Lead nitrate stock solution* into three separate 100-mL volumetric flasks, add 10 mL of 3 N hydrochloric acid to each, and dilute with water to volume.

25% Sulfuric acid solution (by volume): Cautiously add 100 mL of sulfuric acid to 300 mL of water with constant stirring while cooling in an ice bath.

Sample preparation: Transfer the sample weight as specified in the monograph, weighed to the nearest 0.1 mg, into an evaporating dish. Add a sufficient amount of *25% Sulfuric acid solution*, and distribute the sulfuric acid uniformly through the sample. Within a hood, place the dish on a steam bath to evaporate most of the water. Place the dish on a burner, and slowly pre-ash the sample by expelling most of the sulfuric acid. Place the dish in a muffle furnace that has been set at 525°, and ash the sample until the residue appears free from carbon. Prepare a *Sample Blank* by ashing 5 mL of 25% sulfuric acid. Cool, and cautiously wash down the inside of each evaporation dish with water.

Add 5 mL of 1 N hydrochloric acid. Place the dish on a steam bath, and evaporate to dryness. Add 1.0 mL of 3 N hydrochloric acid and approximately 5 mL of water, and heat briefly on a steam bath to dissolve any residue. Transfer each solution quantitatively to a 10-mL volumetric flask, dilute to volume, and mix.

Procedure: Concomitantly determine the absorbances of the *Sample Blank*, the *Diluted standard lead solutions*, and the *Sample preparation* at the lead emission line of 283.3 nm, using a slit-width of 0.7 nm. Use a suitable atomic absorption spectrophotometer equipped with a lead electrodeless discharge lamp (EDL), an air–acetylene flame, and a 4-in burner head. Use water as the blank.

Calculations: Determine the corrected absorbance values by subtracting the *Sample Blank* absorbance from each of the *Diluted standard lead solutions* and from the *Sample preparation* absorbances. Prepare a Standard curve by plotting the corrected *Diluted standard lead solutions* absorbance values versus their corresponding concentrations expressed as µg/mL. Determine the lead concentration in the *Sample preparation* by reference to the calibration curve. Calculate the quantity of lead, in mg/kg, in the sample taken:

$$\text{Result} = 10C/W_S$$

C = concentration of lead from the standard curve (µg/mL)

W_S = weight of the sample taken (g)

• **Atomic Absorption Spectrophotometric Graphite Furnace Method**

The following methods are primarily intended for the analysis of applicable substances containing less than 1 mg/kg of lead.

Method I

This method is intended for the quantitation of lead in substances that are soluble in water, such as sugars and sugar syrups, at levels as low as 0.03 mg/kg. The method detection limit is approximately 5 ng/kg.

Apparatus: Use a suitable graphite furnace atomic absorption spectrophotometer set at 283.3 nm and equipped with an autosampler, pyrolytically coated graphite tubes, solid pyrolytic graphite platforms, and an adequate means of background correction. Zeeman effect or Smith-Hieftje background correction is preferred, but deuterium arc background correction should be acceptable. (This method was developed on a Perkin-Elmer Model Z5100, 0.7-nm slit, HGA-600 furnace, AS-60 autosampler with Zeeman background correction.) If the instrument does not have a well-defined calibration function, a separate calculator or computer is required for linear least squares, nonlinear, or quadratic calibrations. Set up the instrument according to the manufacturer's specifications with consideration of current good GFAAS practices—addressing such factors as line voltage, cooling water temperature, graphite part specifications, and furnace temperature. If an optical pyrometer or thermocouple is not available to check the furnace controller temperature calibration, dim the room lights, and observe the furnace emission through the sample introduction port while increasing the furnace temperature. A characteristic cherry red glow should begin to appear at 800°. If it glows at a lower temperature, then the furnace is hotter, and temperatures must be adjusted downward accordingly.

Use acid-cleaned [in a mixture of 5% sub-boiling, distilled nitric acid and 5% sub-boiling, distilled hydrochloric acid made up in deionized, distilled water (18 megohm), and thoroughly rinsed with deionized, distilled water (18 megohm)] autosampler cups (PE B008-7600 Teflon, or equivalent) to avoid contamination. Use micropipets with disposable tips free of lead contamination for dilution. Ensure accuracy and precision of micropipets and tips by dispensing and weighing 5–10 replicate portions of water onto a microbalance. Use acid-cleaned volumetric glassware to prepare standards and dilute samples to a final volume. For digestion, use acid-cleaned, high-density polyethylene tubes, polypropylene tubes, Teflon tubes, or quartz tubes. Store final diluted samples in plastic tubes.

Air ashing: The furnace controller must be able to handle 2 gas flows to facilitate air ashing. Oxygen ashing is used to avoid build up of residue during the char step. Argon is used as the purge gas for the furnace for all steps but the char. Breathing quality air can be used as the alternate gas for the air ashing.

Standard solutions: Prepare all lead solutions in 5% sub-boiling distilled nitric acid. Use a single-element 1000- or 10,000-µg/mL lead stock to prepare (weekly) an intermediate 10-µg/mL standard in 5% nitric acid. Prepare (daily) a *Lead standard solution* (1 µg/mL) by diluting the intermediate 10-µg/mL stock solution 1:10. Prepare *Working Calibration Standards* of 100.0 ng/mL, 50.0 ng/mL, 25.0 ng/mL, and 10.0 ng/mL from this, using appropriate dilutions. Store standards in acid-cleaned polyethylene test tubes or bottles. If the GFAAS autosampler is used to automatically dilute standards, ensure calibration accuracy by pipetting volumes of 3 µL or greater.

Modifier stock solution: Weigh 20 g of ultrapure magnesium nitrate hexahydrate, and dilute to 100 mL. Just before use, prepare a *Modifier working solution* by diluting stock solution 1:10. A volume of 5 µL will provide 0.06 mg of magnesium nitrate.

Sample digestion

[**CAUTION**—Perform the procedure in a fume hood, and wear safety glasses.]

Obtain a representative subsample to be analyzed. For liquid samples such as sugar syrups, ultrasonicate and/or vortex mix before weighing. For solid samples such as crystalline sucrose, make a sugar solution using equal weights of sample (5-g minimum) and deionized, distilled (18 megohm) water. Mix samples until completely dissolved. Transfer approximately 1.5 g (record to nearest mg) of sample (or 3.0 g of sugar solution), accurately weighed, into a digestion tube. Run a *Sample preparation blank* of 1.5 g of deionized, distilled (18 megohm) water through the entire procedure with each batch of samples. Add 0.75 mL of sub-boiling, distilled nitric acid. Heat plastic tubes in a water bath, quartz tubes in a water bath or heating block, warming slowly to 90°–95° to avoid spattering. Monitor the temperature by using a "dummy" sample. Heat until all brown vapors have dissipated and any rust-colored tint is gone (20–30 min). Cool. Add 0.5 mL of 50% hydrogen peroxide dropwise, heat at 90°–95° for 5 min, and cool. Add a second 0.5-mL portion of 50% hydrogen peroxide, dropwise, and heat at 90°–100° for 5–10 min or until clear. Cool, and dilute with water to a final volume of 10 mL.

Procedure: The furnace program is as follows: (1) Dry at 200°, using a 20-s ramp and a 30-s hold and a 300-mL/min argon flow; (2) char the sample at 750°, using a 40-s ramp and a 40-s hold and a 300-mL/min air flow [NOTE—Air ashing is a critical step to avoid build up of residue during the char step.] ; (3) cool down, and purge the air from the furnace for 60 s, using a 20° set temperature and a 300-mL/min argon flow; (4) atomize at 1800°, using a 0-s ramp and a 10-s hold with the argon flow stopped; (5) clean out at 2600°, with a 1-s ramp and a 7-s hold; (6) cool down the furnace (if necessary) at 20°, with a 1-s ramp and a 5-s hold with a 300-mL/min argon flow.

Program the autosampler to add 5 µL of *Modifier working solution* separately into 20 µL each of blanks, calibration standards, and sample solutions while introducing the solutions into the graphite furnace. Inject each respective solution in triplicate, and average the results. Use peak area measurements for all quantitation. After ensuring that the furnace is clean by running a 5% nitric acid blank, check the instrument sensitivity according to manufacturer's specifications by running the 25-ng/mL calibration standard. Calculate the characteristic mass (m_o) (mass of Pb pg necessary to produce an integrated absorbance of 0.0044 abs-sec) as follows:

$$m_o = (0.0044 \text{ abs-sec})(25 \text{ pg/µL})(20 \text{ µL})/(\text{measured } 25 \text{ pg/µL abs-sec})$$

Record and track the integrated absorbance and m_o for reference and quality assurance.

Standard curve: Inject each calibration standard in triplicate and determine the instrument linearity according to manufacturer's instructions. Use the calibration algorithms provided in the instrument software. Recheck calibration periodically (≤15 samples) by running a 25- or 50-ng/mL calibration standard interspersed with samples. If recheck differs from calibration by >10%, recalibrate the instrument. The instrumental detection limit (DL) and quantitation limit (QL), in picograms, may be based on 7–10 replicates of the *Sample preparation blank* and calculated as follows:

DL = (3)(s.d. blank abs-sec)(10 pg/µL)(20 µL)/(abs-sec 10 ng/mL std)

QL = (10)(s.d. blank abs-sec)(10 pg/µL)(20 µL)/(abs-sec 10 ng/mL std)

During method development, detection limits were typically 10–14 pg, corresponding to 0.5–0.7 ng/mL for 20 µL. This corresponds to a method detection limit of 3.3–4.7 ng/g of sugar.

Sample analyses: Inject each sample digest in triplicate, and record the integrated absorbance. If instrument response exceeds that of the calibration curve, dilute with 5% nitric acid to bring the sample response into working range, and note the dilution factor (DF). Sample solutions having a final concentration beyond the linearity range should be diluted 1:10 to facilitate analysis in the linear range for systems not equipped with nonlinear calibration. All sample analyses should be blank corrected using the *Sample preparation blank*. This can typically be done automatically by the software after identifying and running a representative *Sample preparation blank*. Use the calibration algorithm provided in the instrument software to calculate a blank-corrected, digest lead concentration (in ng/mL).

Calculation of lead content: Calculate the lead level in the original sample as follows:

Pb (ng/g) = (blank-corrected Pb ng/mL)(DF)[sample vol (10 mL)]/[sample wt (approx. 1.5 g)][2]

Quality assurance: To ensure analytical accuracy, an appropriate trace elements in water reference standard with a certified lead content or a similar material should be analyzed before the unknown samples are.[3] If the concentration determined is not within 10% of the mean reference value, the reason for inaccuracy should be evaluated, and unknown samples should not be analyzed until acceptable accuracy is achieved. Also prepare an in-house control solution made from uncontaminated table sugar or reagent-grade sucrose (or other appropriate substance with a Pb content <5 ng/g as received) mixed with an equal volume of water. Spike this solution with Pb to produce a concentration of 100 ng/g. Analyze with each batch of samples. Recoveries should be 100 ± 20%, and the precision for complete replicate digestions should be <5% RSD. Periodically, a sample digest should be checked using the method of standard additions to ensure that there are no multiplicative or chemical interferences. Spiking samples and checking recoveries is always a good practice.

Method II

This method is primarily intended for the determination of lead at levels of less than 1 mg/kg in substances immiscible with water, such as edible oils.

Apparatus: Use a suitable atomic absorption spectrophotometer (Perkin-Elmer Model 3100 or equivalent) fitted with a graphite furnace (Perkin-Elmer HGA 600 or equivalent). Use a lead hollow-cathode lamp (Perkin-Elmer or equivalent) with argon as the carrier gas. Follow the manufacturers' directions for setting the appropriate instrument parameters for lead determination.

[NOTE—For this test, use reagent-grade chemicals with as low a lead content as is practicable, as well as high-purity water and gases. Before use in this analysis, rinse all glassware and plasticware twice with 10% nitric acid and twice with 10% hydrochloric acid, and then rinse them thoroughly with high-purity water, preferably obtained from a mixed-bed strong-acid strong-base, ion-exchange cartridge capable of producing water with an electrical resistivity of 12–15 megohms.]

Hydrogen peroxide–nitric acid solution: Dissolve equal volumes of 10% hydrogen peroxide and 10% nitric acid.

[NOTE—Use caution.]

Lead nitrate stock solution: Dissolve 159.8 mg of ACS Reagent-Grade Lead Nitrate (alternatively, use NIST Standard Reference Material, containing 10 mg of lead per kg, or equivalent) in 100 mL of *Hydrogen peroxide–nitric acid solution*. Dilute with *Hydrogen peroxide–nitric acid solution* to 1000.0 mL, and mix. Prepare and store this solution in glass containers that are free from lead salts. Each mL of this solution contains the equivalent of 100 µg of lead (Pb) ion.

Standard lead solution: On the day of use, dilute 10.0 mL of *Lead nitrate stock solution* with *Hydrogen peroxide–nitric acid solution* to 100.0 mL, and mix. Each mL of *Standard lead solution* contains the equivalent of 10 µg of lead (Pb) ion.

Butanol–nitric acid solution: Slowly add 50 mL of nitric acid to approximately 500 mL of butanol contained in a 1000-mL volumetric flask. Dilute with butanol to volume, and mix.

Standard solutions: Prepare a series of Lead standard solutions serially diluted from the *Standard lead solution* in *Butanol–nitric acid solution*. Pipet into separate 100-mL volumetric flasks 0.2 mL, 0.5 mL, 1 mL, and 2 mL, respectively, of *Standard lead solution*, dilute with *Butanol–nitric acid solution* to volume, and mix. The *Standard solutions* contain, respectively, 0.02 µg, 0.05 µg, 0.1 µg, and 0.2 µg of lead per mL. (For lead limits greater than 1 mg/kg, prepare a series of Standard solutions in a range encompassing the expected lead concentration in the sample.)

Sample solution

[**CAUTION**—Perform this procedure in a fume hood, and wear safety glasses.]

Transfer 1 g of sample, accurately weighed, into a large test tube. Add 1 mL of nitric acid. Place the test tube in a rack in a boiling water bath. As soon as the rusty tint is gone, add 1 mL of 30% hydrogen peroxide dropwise to avoid a vigorous reaction, and wait for bubbles to form. Stir with an acid-washed plastic spatula if necessary. Remove

[2] If a Sample solution was prepared initially to ensure sample homogeneity, this is the weight of the original sugar digested (not the weight of the solution).
[3] NIST SRM 1643 - Trace Elements in Water (National Institute of Standards and Technology, Gaithersburg, MD, USA), or equivalent.

the test tube from the water bath, and let it cool. Transfer the solution into a 10-mL volumetric flask, and dilute with *Butanol–nitric acid solution* to volume, and mix. Use this solution for analysis.

Procedure

Tungsten solution: Transfer 0.1 g of tungstic acid (H_2WO_4) and 5 g of sodium hydroxide pellets into a 50-mL plastic bottle. Add 5.0 mL of high-purity water, and mix. Heat the mixture in a hot water bath until a complete solution is achieved. Cool, and store at room temperature.

Procedure: Place the graphite tube in the furnace. Inject a 20-µL aliquot of the *Tungsten solution* into the graphite tube, using a 300-mL/min argon flow and the following sequence of conditions: dry at 110° for 20 s, char at 700°–900° for 20 s, and with the argon flow stopped, atomize at 2700° for 10 s; repeat this procedure once more using a second 20-µL aliquot of the *Tungsten solution*. Clean the quartz windows.

Standard curve: [NOTE—The sample injection technique is the most crucial step in controlling the precision of the analysis; the volume of the sample must remain constant. Rinse the µL pipet tip (Eppendorf or equivalent) three times with either the *Standard solutions* or *Sample solution* before injection. Use a fresh pipet tip for each injection, and start the atomization process immediately after injecting the sample. Between injections, flush the graphite tube of any residual lead by purging at a high temperature as recommended by the manufacturer.]

With the hollow-cathode lamp properly aligned for maximum absorbance and the wavelength set at 283.3 nm, atomize 20-µL aliquots of the four *Standard solutions*, using a 300-mL/min argon flow and the following sequence of conditions: dry at 110° for 30 s, with a 20-s ramp period and a 10-s hold time; then char at 700° for 42 s, with a 20-s ramp period and a 22-s hold time; and then, with the argon flow stopped, atomize at 2300° for 7 s.

Plot a Standard curve using the concentration, in µg/mL, of each *Standard solution* versus its maximum absorbance value compensated for background correction as directed for the particular instrument, and draw the best straight line.

Atomize 20 µL of the *Sample solution* under identical conditions, and measure its corrected maximum absorbance. From the *Standard curve*, determine the concentration, C, in µg/mL, of the *Sample solution*. Calculate the quantity, in mg/kg, of lead in the sample:

$$\text{Result} = 10C/W$$

in which W is the weight, in grams, of the sample taken.

• **APDC Extraction Method**

Select reagents having as low a lead content as practicable, and store all solutions in high-density polyethylene containers. Rinse all plastic and glassware thoroughly with warm, 1:2 nitric acid followed by water.

2% APDC solution: Dissolve 2.0 g of ammonium pyrrolidinedithiocarbamate (APDC) in 100 mL of water. Filter any slight residue of insoluble APDC from the solution before use.

Lead nitrate stock solution (100 µg/mL): Dissolve 159.8 mg of reagent-grade lead nitrate [$Pb(NO_3)_2$] in 100 mL of water containing 1 mL of nitric acid in a 1000-mL volumetric flask, and dilute with water to volume.

Standard lead solutions

2 mg/kg Lead standard: On the day of use, transfer 2.0 mL of *Lead nitrate stock solution* into a 100-mL volumetric flask, and dilute with water to volume. The resulting solution contains 2 µg of lead per mL.

3 mg/kg Lead standard: On the day of use, transfer 3.0 mL of *Lead nitrate stock solution* into a 100-mL volumetric flask, and dilute with water to volume. The resulting solution contains 3 µg of lead per mL.

4 mg/kg Lead standard: On the day of use, transfer 4.0 mL of *Lead nitrate stock solution* into a 100-mL volumetric flask, and dilute with water to volume. The resulting solution contains 4 µg of lead per mL.

10 mg/kg Lead standard: On the day of use, transfer 10.0 mL of *Lead nitrate stock solution* into a 100-mL volumetric flask, and dilute with water to volume. The resulting solution contains 10 µg of lead per mL.

Sample preparation: Transfer a 10.0-g sample to a clean 150-mL beaker. Add 30 mL of water and the minimum amount of hydrochloric acid needed to dissolve the sample, plus an additional 1 mL of hydrochloric acid to ensure the dissolution of any lead present. Heat to boiling, and boil for several minutes. Allow to cool, and dilute with deionized water to about 100 mL. Adjust the pH of the resulting solution to 1.0–1.5 with 25% NaOH. Quantitatively transfer the pH-adjusted solution to a clean 250-mL separatory funnel, and dilute with water to about 200 mL. Add 2 mL of *2% APDC Solution*, and mix. Extract with two 20-mL portions of chloroform, collecting the extracts in a clean 50-mL beaker. Evaporate to dryness on a steam bath. Add 3 mL of nitric acid to the residue, and heat to near dryness. Then add 0.5 mL of nitric acid and 10 mL of deionized water to the beaker, and heat until the volume is reduced to about 3–5 mL. Transfer the digested extract to a clean 10-mL volumetric flask, and dilute with water to volume.

Reagent blank: Prepare as *Sample preparation*, except do not add the 10 g of sample, and replace 10 g of sample with water.

Procedure: Zero the instrument with water, and concomitantly determine the absorbances of the appropriate *Standard lead solution*, the *Sample preparation*, and the *Reagent blank* at the lead emission line of 283.3 nm, using a slit-width of 0.7 nm. Use a suitable atomic absorption spectrophotometer equipped with a lead electrodeless discharge lamp (EDL), or equivalent; an air–acetylene flame; and a 4-in burner head. Correct the absorbance of the *Sample preparation* with the absorbance obtained from the *Reagent blank*. The absorbance of the corrected *Sample preparation* is not greater than that of the *Standard lead solution*.

MANGANESE LIMIT TEST

Manganese detection instrument: Use any suitable atomic absorption spectrophotometer equipped with a fast-response recorder or other readout device and capable of measuring the radiation absorbed by manganese atoms at the manganese resonance line of 279.5 nm.

Standard preparations: Transfer 1000 mg, accurately weighed, of manganese metal powder into a 1000-mL volumetric flask, dissolve by warming in a mixture of 10 mL of water and 10 mL of 0.5 N hydrochloric acid, cool, dilute with water to volume, and mix. Pipet 5.0 mL of this solution into a 50-mL volumetric flask, dilute with water to volume, and mix. Finally, pipet 5.0 mL, 10.0 mL, 15.0 mL, and 25.0 mL of this solution into separate 1000-mL volumetric flasks, dilute each flask with water to volume, and mix. The final solutions contain 0.5 mg/kg, 1.0 mg/kg, 1.5 mg/kg, and 2.5 mg/kg of Mn, respectively.

Sample preparation: Transfer 10.000 g of the sample into a 200-mL Kohlrausch volumetric flask, previously rinsed with 0.5 N hydrochloric acid, add 140 mL of 0.5 N hydrochloric acid, and shake vigorously for 15 min, preferably with a mechanical shaker. Dilute with 0.5 N hydrochloric acid to volume, and shake. Centrifuge approximately 100 mL of the sample mixture in a heavy-walled centrifuge tube at 2000 rpm for 5 min, and use the clear supernatant liquid in the following *Procedure*.

Procedure: Aspirate 0.5 N hydrochloric acid through the air–acetylene burner for 5 min, and obtain a baseline reading at 279.5 nm, following the manufacturer's instructions for operating the atomic absorption spectrophotometer being used for the analysis. Aspirate a portion of each *Standard preparation* in the same manner, note the readings, then aspirate a portion of the *Sample preparation*, and note the reading. Prepare a Standard curve by plotting the mg/kg of Mn in each *Standard preparation* against the respective readings. From the graph determine the mg/kg of Mn in the *Sample preparation*, and multiply this value by 20 to obtain the mg/kg of Mn in the original sample taken for analysis.

MERCURY LIMIT TEST

• **Method I**

Mercury detection instrument: Use any suitable atomic absorption spectrophotometer equipped with a fast-response recorder and capable of measuring the radiation absorbed by mercury vapors at the mercury resonance line of 253.6 nm. A simple mercury vapor meter or detector equipped with a variable span recorder also is satisfactory.

[NOTE—Wash all glassware associated with the test with nitric acid, and rinse thoroughly with water before use.]

Aeration apparatus: The apparatus, shown in *Figure 6*, consists of a flowmeter (*a*), capable of measuring flow rates from 500 to 1000 mL/min, connected via a three-way stopcock (*b*), with a Teflon plug, to 125-mL gas washing bottles (*c* and *d*), followed by a drying tube (*e*), and finally a suitable quartz liquid absorption cell (*f*), terminating with a vent (*g*) to a fume hood.

[NOTE—The absorption cell will vary in optical pathlength depending on the type of mercury detection instrument used.]

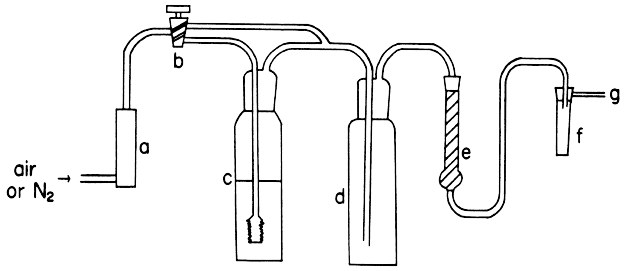


Figure 6. Aeration apparatus for Mercury Limit Test

Bottle *c* is fitted with an extra-coarse fritted bubbler (Corning 31770 125 EC, or equivalent), and the bottle is marked with a 60-mL calibration line. The drying tube *e* is lightly packed with magnesium perchlorate. Bottle *c* is used for the test solution, and bottle *d*, which remains empty throughout the procedure, is used to collect water droplets.

Alternatively, an apparatus embodying the principle of the assembly described and illustrated may be used. The aerating medium may be either compressed air or compressed nitrogen.

Standard preparation: Transfer 1.71 g of mercuric nitrate [$Hg(NO_3) \cdot H_2O$] into a 1000-mL volumetric flask, dissolve in a mixture of 100 mL of water and 2 mL of nitric acid, dilute with water to volume, and mix. Discard after 1 month. Transfer 10.0 mL of this solution into a second 1000-mL volumetric flask, acidify with 5 mL of a 1:5 sulfuric acid solution, dilute with water to volume, and mix. Discard after 1 week. On the day of use, transfer 10.0 mL of the second solution into a 100-mL volumetric flask, acidify with 5 mL of 1:5 sulfuric acid, dilute with water to volume, and mix. Each mL of this solution contains 1 µg of mercury. Transfer 2.0 mL of this solution (2 µg Hg) into a 50-mL beaker, and add 20 mL of water, 1 mL of a 1:5 sulfuric acid solution, and 1 mL of a 1:25 solution of potassium permanganate. Cover the beaker with a watch glass, boil for a few seconds, and cool.

Sample preparation: Prepare as directed in the individual monograph.

Procedure: Assemble the aerating apparatus as shown in *Figure 6*, with bottles *c* and *d* empty and stopcock *b* in the bypass position. Connect the apparatus to the absorption cell (*f*) in the instrument, and adjust the air or nitrogen flow rate so that in the following procedure, maximum absorption and reproducibility are obtained without excessive foaming in the Test solution. Obtain a baseline reading at 253.6 nm, following the manufacturer's instructions for operating the instrument.

Treat the *Standard preparation* as follows: destroy the excess permanganate by adding a 1:10 solution of hydroxylamine hydrochloride, dropwise, until the solution is colorless. Immediately wash the solution into bottle *c* with water, and dilute with water to the 60-mL mark. Add 2 mL of 10% Stannous chloride solution (prepared

fresh each week by dissolving 10 g of $SnCl_2 \cdot 2H_2O$ in 20 mL of warm hydrochloric acid and diluting with 80 mL of water), and immediately reconnect bottle *c* to the aerating apparatus. Turn stopcock *b* from the bypass to the aerating position, and continue the aeration until the absorption peak has been passed and the recorder pen has returned to the baseline. Disconnect bottle *c* from the aerating apparatus, discard the *Standard preparation* mixture, wash bottle *c* with water, and repeat the foregoing procedure using the *Sample preparation*; any absorbance produced by the *Sample preparation* does not exceed that produced by the *Standard preparation*.

- **Method II**

Dithizone extraction solution: Dissolve 30 mg of dithizone in 1000 mL of chloroform, add 5 mL of alcohol, and mix. Store in a refrigerator. Before use, shake a suitable volume of the solution with about half its volume of 1:100 nitric acid, discarding the nitric acid. Discard the solution after 1 month.

Diluted dithizone extraction solution: Just before use, dilute 5 mL of *Dithizone extraction solution* with 25 mL of chloroform.

Hydroxylamine hydrochloride solution: Dissolve 20 g of hydroxylamine hydrochloride in sufficient water to make about 65 mL, transfer the solution into a separator, add a few drops of *thymol blue TS*, and then add ammonium hydroxide until a yellow color develops. Add 10 mL of a 1:25 solution of sodium diethyldithiocarbamate, mix, and allow to stand for 5 min. Extract the solution with successive 10- to 15-mL portions of chloroform until a 5-mL test portion of the chloroform extract does not develop a yellow color when shaken with a dilute solution of cupric sulfate. Add 2.7 N hydrochloric acid until the extracted solution is pink, adding one or two more drops of *thymol blue TS*, if necessary, then dilute with water to 100 mL, and mix.

Mercury stock solution: Transfer 135.4 mg of mercuric chloride, accurately weighed, into a 100-mL volumetric flask, dissolve in and dilute with 1 N sulfuric acid to volume, and mix. Dilute 5.0 mL of this solution with 1 N sulfuric acid to 500.0 mL. Each mL contains the equivalent of 10 µg of mercury.

Diluted standard mercury solution: On the day of use, transfer 10.0 mL of *Mercury stock solution* into a 100-mL volumetric flask, dilute with 1 N sulfuric acid to volume, and mix. Each mL contains the equivalent of 1 µg of mercury.

Sodium citrate solution: Dissolve 250 g of sodium citrate dihydrate in 1000 mL of water.

Sample solution: Dissolve 1 g of sample in 30 mL of 1.7 N nitric acid by heating on a steam bath. Cool to room temperature in an ice bath, stir, and pass through S and S No. 589, or equivalent, filter paper that has been previously washed with 1.7 N nitric acid, followed by water. Add 20 mL of *Sodium citrate solution* and 1 mL of *Hydroxylamine hydrochloride solution* to the filtrate.

Procedure: [NOTE—Because mercuric dithizonate is light sensitive, perform this procedure in subdued light.] Prepare a control containing 3.0 mL of *Diluted standard mercury solution* (3 µg Hg), 30 mL of 1.7 N nitric acid, 5 mL of *Sodium citrate solution*, and 1 mL of *Hydroxylamine hydrochloride solution*. Treat the control and the *Sample solution* as follows: using a pH meter, adjust the pH of each solution to 1.8 with ammonium hydroxide, and transfer the solutions into different separators. Extract each with two 5-mL portions of *Dithizone extraction solution*, and then extract again with 5 mL of chloroform, discarding the aqueous solutions. Transfer the combined extracts from each separator into different separators, add 10 mL of 1:2 hydrochloric acid to each, shake well, and discard the chloroform layers. Extract the acid solutions with about 3 mL of chloroform, shake well, and discard the chloroform layers. Add 0.1 mL of 0.05 M disodium EDTA and 2 mL of 6 N acetic acid to each separator, mix, and then slowly add 5 mL of ammonium hydroxide. Stopper the separators, cool under a stream of cold water, and dry the outside of the separators. To avoid loss, carefully pour the solutions through the tops of the separators into separate beakers, and using a pH meter, adjust the pH of both solutions to 1.8 with 6 N ammonium hydroxide. Return the sample and control solutions to their original separators, add 5.0 mL of *Diluted dithizone extraction solution*, and shake vigorously. Any color developed in the *Sample solution* does not exceed that in the control.

NICKEL LIMIT TEST

[NOTE—Unless otherwise specified in the individual monograph, use *Method I*.]

- **Method I**

Atomic absorption system apparatus: Use a suitable atomic absorption spectrometer equipped with a nickel hollow-cathode lamp and an air–acetylene flame to measure the absorbance of the *Blank preparation*, the *Standard preparations,* and the *Test preparation* as directed under *Procedure*.

Test preparation: Dissolve 20.0 g of sample in dilute acetic acid TS, and dilute with the same solvent to 150.0 mL. Add 2.0 mL of a saturated solution of ammonium pyrrolidinedithiocarbamate (about 10 g/L of water) and 10.0 mL of methyl isobutyl ketone, and shake for 30 s. Protect from bright light. Allow the two layers to separate, and use the methyl isobutyl ketone layer.

Blank preparation: Prepare in the same manner as in the *Test preparation*, but omit the sample.

Standard preparations: Prepare three *Standard preparations* in the same manner as in the *Test preparation,* but add 0.5 mL, 1.0 mL, and 1.5 mL, respectively, of 10 mg/kg *nickel standard solution TS* in addition to 20.0 g of sample.

Procedure: Zero the instrument with the *Blank preparation*. Concomitantly determine the absorbances of each of the *Standard preparations* and of the *Test preparation* at least three times each, and record the average of the steady readings for each. Between each measurement, aspirate the *Blank preparation*, and ascertain that the reading returns to its initial blank value.

Calculation: Calculate the linear equation of the graph using a least-squares fit, and derive from it the concentration of nickel in the *Test preparation*. Alternatively, plot on a graph the mean of the readings against the added quantity of nickel. Extrapolate the line joining the points on the graph until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of nickel in the *Test preparation*.

- **Method II**

[NOTE—All glassware used must be soaked in *1% Nitric acid* for at least 2 h, and then rinsed with water.]

1% Nitric acid: Cautiously add 10 mL of nitric acid to a 1000-mL volumetric flask containing about 500 mL of water. Mix, and dilute with water to volume.

Blank solution: Use *1% Nitric acid*.

Nickel sock standard solution: Immediately before use, dilute an appropriate amount of nickel standard[4] with *1% Nitric acid* to prepare a solution containing the equivalent of 10 µg of nickel per mL.

Standard solutions: Into three identical 100-mL volumetric flasks, introduce respectively 2.0 mL, 5.0 mL, and 10.0 mL of *Nickel stock standard solution*. Dilute with *1% Nitric acid* to volume, and mix. These standards contain 0.2 µg, 0.5 µg, and 1.0 µg of nickel per mL.

Test solution: Weigh accurately a quantity of test specimen containing about 5 g of solids into a 100-mL volumetric flask. Dissolve in and dilute with *1% Nitric Acid* to volume, and mix.

Procedure: Concomitantly determine the absorbances of the *Standard solutions* and the *Test solution* at least three times each, at the wavelength of maximum absorbance at 232.0 nm, with a suitable atomic absorption spectrophotometer equipped with an air–acetylene flame and a nickel hollow-cathode lamp using the *Blank solution* to zero the instrument. Record the average of the steady readings for each of the *Standard solutions* and the *Test solution*. Clear the nebulizer using the *Blank solution* and aspirate each of the *Standard solutions* and the *Test solution* in turn. The standard chosen for reslope should be run every 4 to 5 samples. If there is a significant change in its response, reslope and repeat the previous samples. The standard deviation for the *Standard solution* of 0.2 µg of nickel per mL must be less than 20%. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of nickel, and draw the straight line best fitting the three plotted points. From the graph so obtained, determine the concentration, C, in µg/mL, of nickel in the *Test solution*. Calculate the quantity, in µg, of nickel in each g of test specimen taken:

$$\text{Result} = 100C/W$$

in which W is the weight, in g, of test specimen taken to prepare the *Test solution*.

PHOSPHORUS LIMIT TEST

Reagents

Ammonium molybdate solution (5%): Dissolve 50 g of ammonium molybdate tetrahydrate, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, in 900 mL of warm water, cool to room temperature, dilute with water to 1000 mL, and mix.

Ammonium vanadate solution (0.25%): Dissolve 2.5 g of ammonium metavanadate, NH_4VO_3, in 600 mL of boiling water, cool to 60°–70°, and add 20 mL of nitric acid. Cool to room temperature, dilute with water to 1000 mL, and mix.

Zinc acetate solution (10%): Dissolve 120 g of zinc acetate dihydrate, $Zn(C_2H_3O_2)_2 \cdot 2H_2O$, in 880 mL of water, and pass through Whatman No. 2V or equivalent filter paper before use.

Nitric acid solution (29%): Add 300 mL of nitric acid (sp. gr. 1.42) to 600 mL of water, and mix.

Standard phosphorus solution (100 µg P in 1 mL): Dissolve 438.7 mg of monobasic potassium phosphate, KH_2PO_4, in water in a 1000-mL volumetric flask, dilute with water to volume, and mix.

Standard curve: Pipet 5.0 mL, 10.0 mL, and 15.0 mL of the *Standard phosphorus solution* into separate 100-mL volumetric flasks. To each of these flasks, and to a fourth, blank flask, add in the order stated 10 mL of *Nitric acid solution*, 10 mL of *Ammonium vanadate solution*, and 10 mL of *Ammonium molybdate solution*, mixing thoroughly after each addition. Dilute with water to volume, mix, and allow to stand for 10 min. Determine the absorbance of each standard solution in a 1-cm cell at 460 nm, with a suitable spectrophotometer, using the blank to set the instrument to zero. Prepare a standard curve by plotting the absorbance of each solution versus its concentration, in mg of phosphorus (P) per 100 mL.

Treated sample: Place 20–25 g of the starch sample in a 250-mL beaker, add 200 mL of a 7:3 mixture of methanol and water, disperse the sample, and agitate mechanically for 15 min. Recover the starch by vacuum filtration in a 150-mL medium-porosity fritted-glass or Büchner funnel, and wash the wet cake with 200 mL of the methanol and water mixture. Reslurry the wet cake in the solvent, and wash it a second time in the same manner. Dry the filter cake in an air oven at a temperature below 50°, then grind the sample to 20-mesh or finer, and blend thoroughly. Determine the amount of dry substance by drying a 5-g portion in a vacuum oven, not exceeding 100 mm Hg, at 120° for 5 h.

[NOTE—The treatment outlined above is satisfactory for starch products that are insoluble in cold water. For pregelatinized starch and other water-soluble starches, prepare a 1%–2% aqueous paste, place it in a cellophane tube, and dialyze against running distilled water for 30–40 h. Precipitate the starch by pouring the solution into 4 volumes of acetone per volume of paste while stirring. Recover the starch by vacuum filtration in a medium-porosity fritted-glass or Büchner funnel, and wash the filter cake with absolute ethanol. Dry the filter cake, and determine the amount of dry substance as directed for water-insoluble starches.]

Sample preparation: Transfer about 10 g of the *Treated sample*, calculated on the dry-substance basis and accurately weighed, into a Vycor dish, and add 10 mL of *Zinc acetate solution* in a fine stream, distributing the solution uniformly in the sample. Carefully evaporate to dryness on a hot plate, then increase the heat, and carbonize the sample on the hot plate or over a gas flame. Ignite in a muffle furnace at 550° until the ash is free from carbon (about 1–2 h), and cool. Wet the ash with 15 mL of water, and slowly wash down the sides of the dish with 5 mL of *Nitric acid solution*. Heat to boiling, cool, and quantitatively transfer the mixture into a 200-mL volumetric flask, rinsing the dish with three 20-mL portions of water and adding the rinsings to the flask. Dilute with water to volume, and mix. Transfer an accurately measured aliquot (V, in mL) of this solution, containing not more than 1.5 mg of

[4] Suitable nickel standards are available from e.g. Fisher Scientific, Fair Lawn, NJ (nickel, reference standard solution, 1000 ppm ± 1%, certified, application: for atomic absorption) or RICCA Chemical Company, Arlington, TX (nickel standard, 1000 ppm Ni, for atomic absorption).

phosphorus, into a 100-mL volumetric flask, and add 50 mL of water to a second flask to serve as a blank. To each flask add in the order stated 10 mL of *Nitric acid solution*, 10 mL of *Ammonium vanadate solution*, and 10 mL of *Ammonium molybdate solution*, mixing thoroughly after each addition. Dilute with water to volume, mix, and allow to stand for 10 min.

Procedure: Determine the absorbance of the *Sample preparation* in a 1-cm cell at 460 nm, with a suitable spectrophotometer, using the blank to set the instrument at zero. From the *Standard curve*, determine the mg of phosphorus in the aliquot taken, recording this value as a. Calculate the amount, in mg/kg, of phosphorus (P) in the original sample:

$$\text{mg/kg P} = (a \times 200 \times 1000)/(V \times W)$$

in which W is the weight, in grams, of the sample taken.

SELENIUM LIMIT TEST

Reagents and solutions

2,3-Diaminonaphthalene solution: On the day of use, dissolve 100 mg of 2,3-diaminonaphthalene ($C_{10}H_{10}N_2$) and 500 mg of hydroxylamine hydrochloride ($NH_2OH \cdot HCl$) in sufficient 0.1 N hydrochloric acid to make 100 mL.

Selenium stock solution: Transfer 40.0 mg of powdered metallic selenium into a 1000-mL volumetric flask, and dissolve in 100 mL of 1:2 nitric acid, warming gently on a steam bath to effect solution. Cool, dilute with water to volume, and mix.

Selenium standard solution: Pipet 5.0 mL of *Selenium stock solution* into a 200-mL volumetric flask, dilute with water to volume, and mix. Each mL of this solution contains the equivalent of 1 µg of selenium (Se). Alternatively, the solution may be prepared using a commercially available stock solution diluted to 1 µg/mL.

• **Method I**

Standard preparation: Pipet 6.0 mL of *Selenium standard solution* into a 150-mL beaker, add 50 mL of 0.25 N nitric acid, and mix.

Sample preparation: Using a 1000-mL combustion flask and 25 mL of 0.5 N nitric acid as the absorbing liquid, proceed as directed under *Oxygen flask combustion*, Appendix I, using the amount of sample specified in the individual monograph (and the magnesium oxide or other reagent, where specified).

[NOTE—If the sample contains water of hydration or more than 1% of moisture, dry it at 140° for 2 h before combustion, unless otherwise directed.]

Upon completion of combustion, place a few mL of water in the cup or lip of the combustion flask, loosen the stopper of the flask, and rinse the stopper, sample holder, and sides of the flask with about 10 mL of water. Transfer the solution, with the aid of about 20 mL of water, into a 150-mL beaker, heat gently to boiling, boil for 10 min, and cool.

Procedure: Treat the *Sample preparation*, the *Standard preparation*, and 50 mL of 0.25 N nitric acid, to serve as the blank, similarly and in parallel as follows: add a 1:2 solution of ammonium hydroxide to adjust the pH of the solution to 2.0 ± 0.2. Dilute with water to 60.0 mL, and transfer to a low-actinic separator with the aid of 10.0 mL of water, adding the 10.0 mL of rinsings to the separator. Add 200 mg of hydroxylamine hydrochloride, swirl to dissolve, immediately add 5.0 mL of *2,3-Diaminonaphthalene solution*, insert the stopper, and swirl to mix. Allow the solution to stand at room temperature for 100 min. Add 5.0 mL of cyclohexane, shake vigorously for 2 min, and allow the layers to separate. Discard the aqueous phases, and centrifuge the cyclohexane extracts to remove any traces of water. Determine the absorbance of each extract in a 1-cm cell at the maximum at about 380 nm with a suitable spectrophotometer, using the extract from the blank to set the instrument. The absorbance of the extract from the *Sample preparation* is not greater than that from the *Standard preparation* when a 200-mg sample is tested, or not greater than one-half the absorbance of the extract from the *Standard preparation* when a 100-mg sample is tested.

• **Method II**

Standard preparation: Pipet 6.0 mL of *Selenium standard solution* into a 150-mL beaker, add 50 mL of 2 N hydrochloric acid, and mix.

Sample preparation: Transfer the amount of the sample specified in the individual monograph into a 150-mL beaker, dissolve in 25 mL of 4 N hydrochloric acid, swirling if necessary to effect solution, heat gently to boiling, and digest on a steam bath for 15 min. Remove from heat, add 25 mL of water, and allow to cool to room temperature.

Procedure: Place the beakers containing the *Standard preparation* and the *Sample preparation* in a fume hood, and to a third beaker add 50 mL of 2 N hydrochloric acid to serve as the blank. Cautiously add 5 mL of ammonium hydroxide to each beaker, mix, and allow the solution to cool. Treat each solution, similarly and in parallel, as directed under *Procedure* in *Method I*, beginning with "Add a 1:2 solution of ammonium hydroxide...".

C. OTHERS

ALGINATES ASSAY

In a suitable closed system, liberate the carbon dioxide from the uronic acid groups of about 250 mg of the test sample by heating with hydrochloric acid, and sweep the carbon dioxide, by means of an inert gas, into a titration vessel containing excess standardized sodium hydroxide. Any suitable system may be used as long as it provides precautions against leakage and overheating of the reaction mixture, adequate sweeping time, avoidance of entrainment of hydrochloric acid, and meets the requirements of the *System Suitability Test*. One suitable system, with accompanying procedure, is given below.

Apparatus: The apparatus is shown in *Figure 7*. It consists essentially of a soda lime column, *A*, a mercury valve, *B*, connected through a side arm, *C*, to a reaction flask, *D*, by means of a rubber connection. Flask *D* is a 100-mL round-bottom, long-neck boiling flask, resting in a suitable heating mantle, *E*.

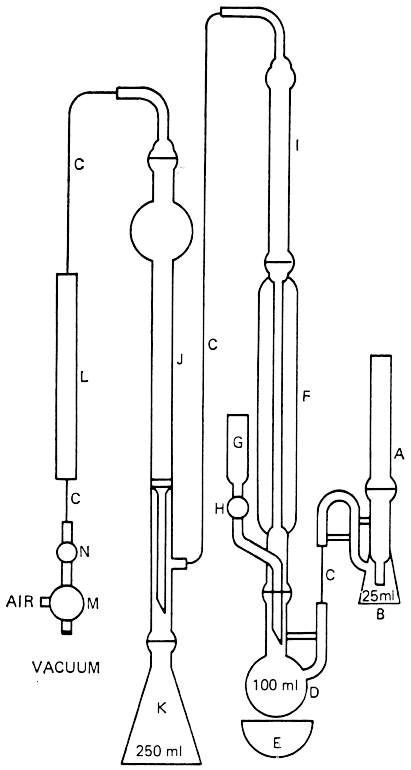


Figure 7. Apparatus for Alginates Assay

The reaction flask is provided with a reflux condenser, *F*, to which is fitted a delivery tube, *G*, of 40-mL capacity, having a stopcock, *H*. The reflux condenser terminates in a trap, *I*, containing 25 g of 20-mesh zinc or tin, which can be connected with an absorption tower, *J*.

The absorption tower consists of a 45-cm tube fitted with a medium-porosity fritted glass disk sealed to the inner part above the side arm and having a delivery tube sealed to it extending down to the end of the tube. A trap, consisting of a bulb of approximately 100-mL capacity, is blown above the fritted disk and the outer portion of a ground spherical joint is sealed on above the bulb. A 250-mL Erlenmeyer flask, *K*, is connected to the bottom of the absorption tower. The top of the tower is connected to a soda lime tower, *L*, which is connected to a suitable pump to provide vacuum and air supply, the choice of which is made by a three-way stopcock, *M*. The volume of air or vacuum is controlled by a capillary-tube regulator or needle valve, *N*.

All joints are a size 35/25 ground spherical type.

Standard D-glucurono-6,3-lactone: This chemical ($C_6H_8O_6$) is available as a reference standard with an assay of 100.0 ± 1.0% (24.99 ± 0.25% CO_2) from Aldrich Chemical Co.

System suitability test: Transfer about 250.0 mg of *Standard D-glucurono-6,3-lactone*, accurately weighed, into the reaction flask, *D*, and carry out the *Procedure* described below. The system is considered suitable when the net titration results in a calculation of %CO_2 in a range of 24.73–25.26, which is equivalent to a range of 98.95%–101.06% D-*glucurono-6,3-lactone*.

Procedure: Transfer about 250 mg of sample, accurately weighed, into the reaction flask, *D*, add 25 mL of 0.1 N hydrochloric acid, insert several boiling chips, and connect the flask to the reflux condenser, *F*, using syrupy phosphoric acid as a lubricant.

[NOTE—Stopcock grease may be used for the other connections.]

Check the system for air leaks by forcing mercury up into the inner tube of the mercury valve, *B*, to a height of about 5 cm. Turn off the pressure using the stopcock, *M*. If the mercury level does not fall appreciably after 1–2 min, the apparatus may be considered to be free from leaks. Draw carbon dioxide-free air through the apparatus at a rate of 3000–6000 mL/h. Raise the heating mantle, *E*, to the flask, heat the sample to boiling, and boil gently for 2 min. Turn off and lower the mantle, and allow the sample to cool for 15 min. Charge the delivery tube, *G*, with 23 mL of hydrochloric acid. Disconnect the absorption tower, *J*, rapidly transfer 25.0 mL of 0.25 N sodium hydroxide into the tower, add 5 drops of *n*-butanol, and reconnect the absorption tower. Draw carbon dioxide-free air through the apparatus at the rate of about 2000 mL/h, add the hydrochloric acid to the reaction flask through the delivery tube, raise the heating mantle, and heat the reaction mixture to boiling. After 2 h, discontinue the current of air and heating. Force the sodium hydroxide solution down into the flask, *K*, using gentle air pressure, and then rinse down the absorption tower with three 15-mL portions of water, forcing each washing into the flask with air pressure. Remove the flask, and add to it 10 mL of a 10% solution of barium chloride ($BaCl_2 \cdot 2H_2O$). Stopper the flask, shake gently for about 2 min, add *phenolphthalein TS*, and titrate with 0.1 N hydrochloric acid. Perform a blank determination (see *General*

Provisions). Each mL of 0.25 N sodium hydroxide consumed is equivalent to 5.5 mg of carbon dioxide (CO_2). Calculate the results on the dried basis.

α-AMINO NITROGEN (AN) DETERMINATION

Transfer 7–25 g of sample, accurately weighed, into a 500-mL volumetric flask with the aid of several 50-mL portions of warm, ammonia-free water, dilute with water to volume, and mix. Neutralize 20.0 mL of the solution with 0.2 N barium hydroxide or 0.2 N sodium hydroxide, using *phenolphthalein TS* as the indicator, and add 10 mL of freshly prepared phenolphthalein–formol solution (50 mL of 40% formaldehyde containing 1 mL of 0.05% phenolphthalein in 50% alcohol neutralized exactly to pH 7 with 0.2 N barium hydroxide or 0.2 N sodium hydroxide). Titrate with 0.2 N barium hydroxide or 0.2 N sodium hydroxide to a distinct red color, add a small, but accurately measured, volume of 0.2 N barium hydroxide or 0.2 N sodium hydroxide in excess, and back titrate to neutrality with 0.2 N hydrochloric acid. Conduct a blank titration using the same reagents, with 20 mL of water in place of the test solution. Each mL of 0.2 N barium hydroxide or 0.2 N sodium hydroxide is equivalent to 2.8 mg of α-amino nitrogen.

AMMONIA NITROGEN (NH3-N) DETERMINATION

[**CAUTION**—Provide adequate ventilation.]

[NOTE—Use nitrogen-free reagents, where available, or reagents very low in nitrogen content.]

Transfer between 700 mg and 2.2 g of sample into a 500- to 800-mL Kjeldahl digestion flask of hard, moderately thick, well-annealed glass. If desired, wrap the sample, if solid or semisolid, in nitrogen-free filter paper to facilitate the transfer.

Add about 200 mL of water, and mix. Add a few granules of zinc to prevent bumping, tilt the flask, and cautiously pour sodium hydroxide pellets, or a 2:5 sodium hydroxide solution, down the inside of the flask so that it forms a layer under the solution, using a sufficient amount (usually about 25 g of solid sodium hydroxide) to make the mixture strongly alkaline. Immediately connect the flask to a distillation apparatus consisting of a Kjeldahl connecting bulb and a condenser that has a delivery tube extending well beneath the surface of a measured excess of 0.5 N hydrochloric or sulfuric acid contained in a 500-mL flask. Add 5 to 7 drops of methyl red indicator (1 g of methyl red in 200 mL of alcohol) to the receiver flask. Rotate the Kjeldahl flask to mix its contents thoroughly, and heat until all of the ammonia has distilled, collecting at least 150 mL of distillate. Wash the tip of the delivery tube, collecting the washings in the receiving flask, and titrate the excess acid with 0.5 N sodium hydroxide. Perform a blank determination (see *General Provisions*), substituting 2 g of sucrose for the sample, and make any necessary correction. Each mL of 0.5 N acid consumed is equivalent to 7.003 mg of ammonia nitrogen.

[NOTE—If it is known that the substance to be determined has a low nitrogen content, 0.1 N acid and alkali may be used, in which case each mL of 0.1 N acid consumed is equivalent to 1.401 mg of nitrogen.]

Calculate the percent ammonia nitrogen:

$$\text{Result} = (NH_3\text{-N}/S) \times 100$$

in which NH_3-N is the weight, in mg, of ammonia nitrogen, and S is the weight, in mg, of the sample.

BENZENE (in Paraffinic Hydrocarbon Solvents)

Apparatus: (See *Chromatography, Appendix IIA*.) Use a suitable gas chromatograph, equipped with a column, or equivalent, that will elute *n*-decane before benzene under the conditions of the *System Suitability Test*. Column materials and conditions that have been found suitable for this method are listed in the accompanying tables. See *Figure 8* for a typical chromatogram obtained with column No. 5.

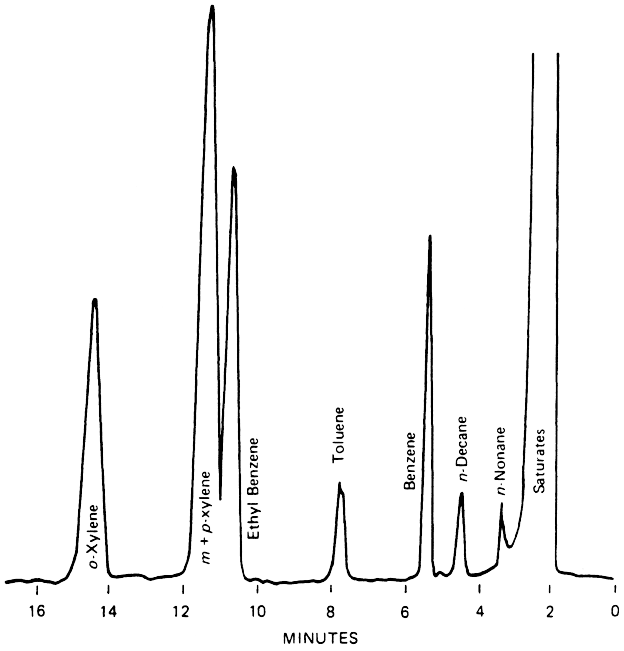


Figure 8. Typical Chromatogram for the Determination of Benzene in Hexanes Using Column No. 5

Reagents

Isooctane: 99 mole percent minimum containing less than 0.05 mole percent aromatic material

Benzene: 99.5 mole percent minimum

Internal standard: *n*-Decane and either *n*-undecane or *n*-dodecane according to the requirement of the *System suitability test*

Reference solution A: Prepare a standard solution containing 0.5% by weight each of the *Internal standard* and of benzene in isooctane.

Reference solution B: Prepare a standard solution containing about 0.5% by weight each of *n*-decane, of *Internal standard*, and of benzene in isooctane.

Calibration: Select the instrument conditions necessary to give the desired sensitivity. Inject a known volume of *Reference solution A*, and change the attenuation, if necessary, so that the benzene peak is measured with a chart deflection of not less than 25% or more than 95% of full scale. When choosing the attenuation, consider all unresolved peaks to represent a single compound. There may be tailing of the nonaromatic peak, but do not use any conditions that lead to a depth of the valley ahead of the benzene peak (A) less than 50% of the weight of the benzene peak (B) as depicted in *Figure 9*.

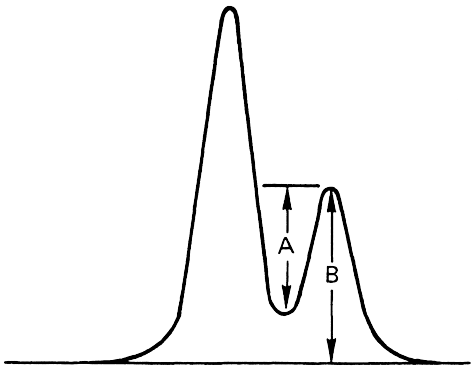


Figure 9. Illustration of A/B Ratio

If there is tailing of the nonaromatic material, construct a baseline by drawing a line from the bottom of the valley ahead of the benzene peak to the point of tangency after the peak (see *Figure 10*). Measure the areas of the benzene peak and the internal standard peak by any of the following means: triangulation, planimeter, paper cutout, or mechanical or electronic integrator. Do not use integrators on peaks without a constant baseline, unless the integrator has provision for making baseline corrections with accuracy at least as good as that of manual methods.

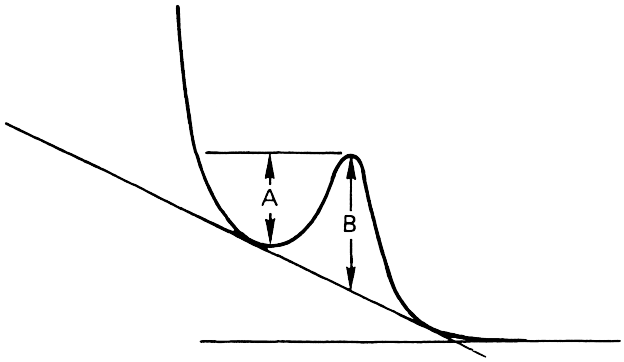


Figure 10. Illustration of A/B Ratio for a Small Component Peak on the Tail of a Large Peak

Calculate a response factor for benzene (R_b) relative to the *Internal standard*:

$$\text{Result} = A_i/W_i \times W_b/A_b$$

in which A_i is the area of the *Internal standard* peak in arbitrary units corrected for attenuation; W_i is the weight percent of *Internal standard* in *Reference solution A*; W_b is the weight percent of benzene in *Reference solution A*; and A_b is the area of the benzene peak in arbitrary units corrected for attenuation.

Procedure: Place approximately 0.1 mL of *Internal standard* into a tared 25-mL volumetric flask, weigh on an analytical balance, and dissolve in and dilute with the sample to be analyzed to volume.

Using the exact instrumental conditions that were used in the calibration, inject the same volume of sample containing the *Internal standard*. Before measuring the area of the *Internal standard* and benzene peaks, change the attenuation to ensure at least 25% chart deflection.

Measure the area of the *Internal standard* and benzene peaks in the same manner as was used for the calibration. Calculate the weight percentage of benzene in the sample (W_B):

$$\text{Result} = (A_b \times R_b \times W_i \times 100)/(A_i \times S)$$

in which A_b is the area of the benzene peak corrected for attenuation; R_b is the relative response factor for benzene; W_i is the weight, in grams, of *Internal standard* added; A_i is the area of the *Internal standard* peak corrected for attenuation; and S is the weight, in grams, of the sample taken.

System suitability test: Inject the same volume of *Reference solution B* as in the *Calibration* and record the chromatogram. *n*-Decane must be eluted before benzene, and the ratio of A to B (*Figure 9*) must be at least 0.5 where A is equal to the depth of the valley between the *n*-decane and benzene peaks and B is equal to the height of the benzene peak.

Column Materials and Conditions for the Determination of Benzene in Hexanes

Column No.	1	2	3	4	5	6	7
Liquid phase	CEF	PEF 200	CEF	DEGS	TCEPE	TCEPE	DEGS
Length, ft	15	6	16	10	15	100	12
m	—	4.5	2	5	3.1	—	313.7
Diameter, in (mm) Inside	0.07 (1.8)	—	0.07	0.18 (4.5)	0.06 (1.5)	0.01 (.254)	—
Outside	⅛ (3.2)	¼ (6.4)	⅛	—	—	—	⅛
Weight, percent	17	30	20	20	10	—	20
Solid support	Chromosorb P	Chromosorb P	Chromosorb P	Chromosorb P	Chromosorb P	Capillary	Chromosorb P
Mesh	60–80	60–80	60–80	80–100	60–80	—	80–100
Treatment	AW	AW	AW	none	AW	none	AW Sil
Inlet, deg	200	210	250	260	250	275	260
Detector, deg	200	155	250	200	175	250	240
Column, deg	115	95	90	100	115	95	65
Carrier gas	N_2	He	He	He	N_2	N_2	He
Flow rate, cm^3/min	30	60	60	60	1	3	52
Detector	FI	TC	FI	FI	FI	FI	FI
Recorder, mV	5	1	1	1	10	1	1

Column Materials and Conditions for the Determination of Benzene in Hexanes *(continued)*

Column No.	1	2	3	4	5	6	7
Sample, 1	5	10	1	2	5	0.8	5
Split	9 + 1	—	—	—	100 + 1	100 – 1	—
Area	Tri	EI	DI	Tri Plan	EI	EI	Tri

Abbreviations Used in Table: AW—Acid washed; CEF—N,N-Bis(2-cyanoethyl)formamide; DEGS—Diethylene Glycol Succinate; DI—Disk integrator; EI—Electronic integrator; FI—Flame ionization; Sil—Silanized; TC—Thermal conductivity; TCEPE—Tetracyanoethylated Pentaerythritrol; Tri—Triangulation.

Retention Times in Minutes for Selected Hydrocarbons under the Conditions for the Determination of Benzene in Hexanes

Column No.	1	2	3	4	5	6	7
Benzene	3.4	2.0	6.5	6.7	5.4	6.1	6.7
Toluene	4.4	3.2	9.0	10.3	7.8	7.0	10.3
Ethylbenzene	5.4	5.2	11.5	14.8	10.8	8.0	14.8
p-*m*-Xylenes	5.8	—	12.5	—	11.4	8.5	—
o-Xylene	7.5	6.8	17.0	16.1	14.5	10.0	—
n-Undecane	3.0	2.8	3.5	—	—	—	—
n-Dodecane	—	—	—	12.8	8.5	6.5	—

Color Determination[5]

Chromium

Standards

Standard chromium solution (1000 mg/kg): Transfer 2.829 g of $K_2Cr_2O_7$, accurately weighed (NIST No. 136) into a 1-L volumetric flask; dissolve in and dilute with water to volume.

Standard colorant solution: Transfer 62.5 g of colorant previously shown to be free of chromium to a 1-L volumetric flask; dissolve in and dilute with water to volume.

Apparatus: Use any suitable atomic absorption spectrophotometer equipped with a fast response recorder and capable of measuring the radiation absorbed at 357.9 nm.

Instrument parameters: *Wavelength setting*: 357.9 nm; *optical passes*: 5; *lamp current*: 8 mA; *lamp voltage*: 500 v; *fuel*: hydrogen; *oxidant*: air; *recorder*: l mv with a scale expansion of 5 or 10. Alternatively, follow the instructions supplied with the instrument.

Procedure: Set the instrument at the optimum conditions for measuring chromium as directed by the manufacturer's instructions. Prepare a series of seven standard chromium solutions containing Cr at approximately 5 mg/kg, 10 mg/kg, 15 mg/kg, 20 mg/kg, 40 mg/kg, 50 mg/kg, and 60 mg/kg by appropriate dilutions of the *Standard chromium solution* into 100-mL volumetric flasks; add 80 mL of the *Standard colorant solution*, and dilute each flask with water to volume.

Transfer 5 g of the colorant to be analyzed to a 100-mL volumetric flask; dissolve in and dilute with water to volume. Prepare a calibration curve using the series of standards, and using this curve, determine the chromium content of the colorant samples.

Ether extracts

[**CAUTION**—Isopropyl ether forms explosive peroxides. To ensure the absence of peroxides, perform the following test: prepare a colorless solution of ferrous thiocyanate by mixing equal volumes of 0.1 N ferrous sulfate and 0.1 N ammonium thiocyanate. Using titanous chloride, carefully discharge any red coloration due to ferric ions. Add 10 mL of ether to 50 mL of the solution, and shake vigorously for 2–3 min. A red color indicates the presence of peroxides. If redistillation is necessary, the usual precautions against peroxide detonation should be observed. Immediately before use, pass the ether through a 30-cm column of chromatography-grade aluminum oxide to remove peroxides and inhibitors.]

Apparatus: Use an upward displacement-type liquid–liquid extractor, as shown in *Figure 11*, with a sintered-glass diffuser and a working capacity of 200 mL. Suspend a piece of bright copper wire through the condenser, and place a small coil of copper wire (about 0.5 g) in the distillation flask.

[5] To be used or sold for use to color food that is marketed in the United States, color additives must be from batches that have been certified by the U.S. Food and Drug Administration (FDA). If color additives are not from FDA-certified batches, they are not permitted color additives for food use in the United States, even if they are compositionally equivalent. The FD&C names can be applied only to FDA-certified batches of these color additives.

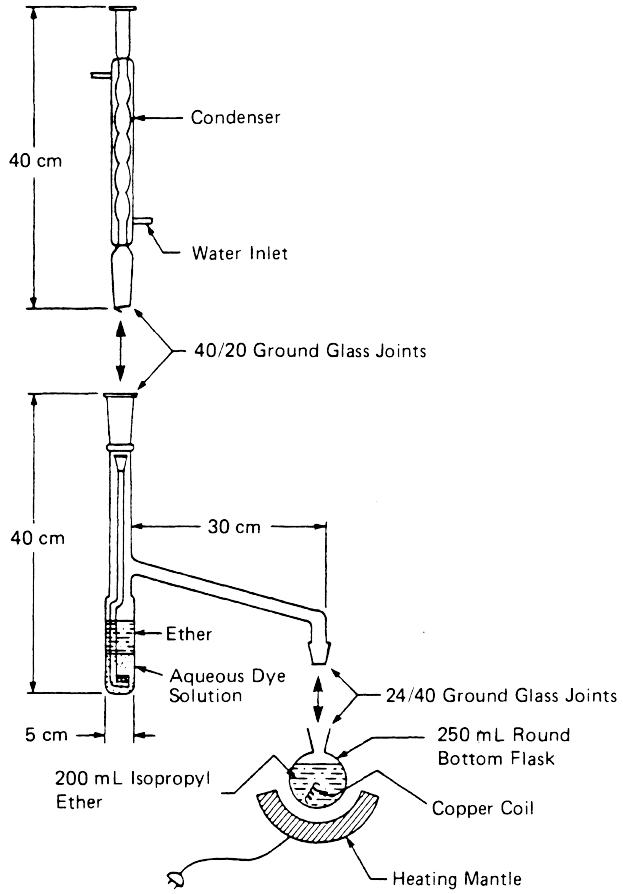


Figure 11. Upward Displacement-Type Liquid–Liquid Extractor with Sintered-Glass Diffuser

Alkaline ether extract: Transfer 5 g of the colorant to a beaker, and dissolve in 150 mL of water. Add 2 mL of 2.5 N NaOH solution, transfer the solution into the extractor, and dilute with water to approximately 200 mL. Add 200 mL of ether to the distillation flask, and extract for 2 h with a reflux rate of about 15 mL/min. Set the extracted colorant solution aside. Transfer the ether extract into a separatory funnel, and wash with two 25-mL portions of 0.1 N NaOH followed by two 25-mL portions of water. Reduce the volume of the ether extract to about 5 mL by distillation (in portions) from a tared flask containing a small piece of clean copper coil.

Acid ether extract: Add 5 mL of 3 N hydrochloric acid to the extracted colorant solution set aside in the alkaline ether extract procedure above, mix, and extract with ether as directed above. Wash the ether extract with two 25-mL portions of 0.1 N hydrochloric acid and water. Transfer the washed ether in portions to the flask containing the evaporated alkaline extract, and carefully remove all the ether by distillation. Dry the residue in an oven at 85° for 20 min. Then allow the flask to cool in a desiccator for 30 min, and weigh. Repeat drying and cooling until a constant weight is obtained. The increase in weight of the tared flask, expressed as a percentage of the sample weight, is the combined ether extract.

Leuco base

Reagents and solutions

Cupric chloride solution: Transfer 10.0 g of $CuCl_2 \cdot 2H_2O$ to a 1-L volumetric flask; dissolve in and dilute with dimethylformamide (DMF) to volume.

Sample solution: Prepare as directed in the individual monograph.

Procedure

Solution 1: Pipet 50 mL of DMF into a 250-mL volumetric flask, cover, and place in the dark.

Solution 2: Pipet 10 mL of the *Sample solution* into a 250-mL volumetric flask, add 50 mL of DMF, and place in the dark.

Solution 3: Pipet 50 mL of *Cupric chloride solution* into a 250-mL volumetric flask, and gently bubble air through the solution for 30 min.

Solutions 4a and 4b: Pipet 10 mL of the *Sample solution* into each of two 250-mL volumetric flasks, add 50 mL of *Cupric chloride solution* to each, and bubble air gently through the solutions for 30 min.

Dilute all of the solutions with water nearly to volume; incubate for 5–10 min, but no longer, in a water bath cooled with tap water; and dilute to volume. Record the spectrum for each solution between 500 nm and 700 nm using an absorbance range of 0 to 1 and a 1-cm pathlength cell; record all spectra on the same spectrogram.

Curve No.	Solution in Sample Cell	Solution in Reference Cell
I	1	1
II	1	2
III	3	3
IVa	3	4a
IVb	3	4b

Calculation

$$\%\,\text{Leuco Base} = \frac{[(\text{IV} - \text{III}) - (\text{II} - \text{I})] \times 2500}{a \times W \times r}$$

in which the Roman numerals I through IV represent the absorbance readings for solutions of the corresponding Arabic numerals (above) at the wavelength maximum; a is the absorptivity (for Fast Green, a = 0.156 at 625 nm; for Brillant Blue, a = 0.164 at 630 nm); W is the weight, in grams, of the sample taken; and r is the ratio of the molecular weights of colorant and leuco base (for Fast Green, r = 0.9712; for Brillant Blue, r = 0.9706).

Mercury

Apparatus: The apparatus used for the direct microdetermination of mercury is shown in *Figure 12*. It consists of a quartz combustion tube designed to hold a porcelain combustion boat (60 × 10 × 8 mm) and a small piece of copper oxide wire. The combustion tube is placed in a heavy-duty hinged combustion tube furnace (Lindburg Type 70T, or equivalent), and it is connected by clamped ball-joints at one end to a source of nitrogen and connected to a series of three traps at the other. The traps are constructed of a linear array of 18- × 2-mm Pyrex tubes connected by clamped ball-joints and extend from the connection at the combustion tube. Trap I contains anhydrous calcium sulfate packed between quartz-wool plugs, trap II contains ascarite packed between cotton plugs, and trap III contains aluminum oxide packed between cotton plugs. The nitrogen flow forces the mercury through the combustion tube, the three traps, and a section of Tygon tube to a mercury vapor meter (Beckman model K-23, or equivalent). The mercury released from a sample during combustion is quantitated by comparing the recorder response with that given by a series of mercury standards.

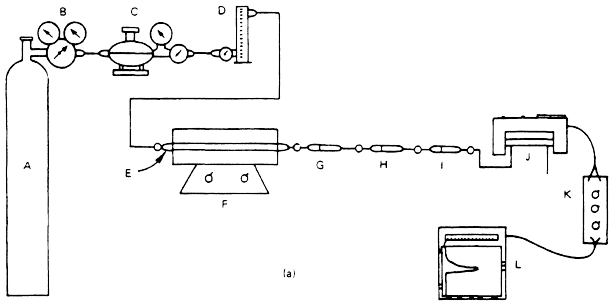


Figure 12. (a) Schematic Diagram of Apparatus for Photometric Mercury Vapor M Method: A. Tank of nitrogen; B. Two-stage pressure regulator; C. Low-pressure regulator; D. Flowmeter; E. Combustion tube; F. Combustion-tube furnace; G. Dehydrite trap; H. Ascarite trap; I. Aluminum oxide trap; J. Mercury vapor meter; K. Atenuator; L. Recorder

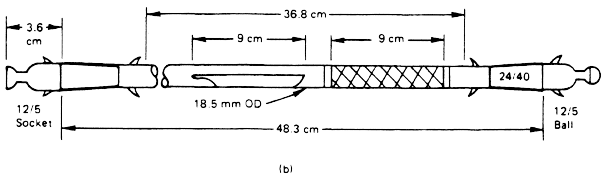


Figure 12 (b) Quartz Combustion Tube with Boat and Copper Oxide Packing

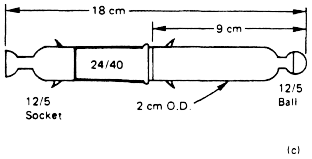


Figure 12 (c) Schematic Diagram of Trap Used to Contain Ascarite, Dehydrite, and Aluminum Oxide

Reagents and equipment

Absorbent cotton

Aluminum oxide: Anhydrous

Calcium sulfate: Anhydrous, dehydrate, or equivalent

Asbestos pads: (1 × 0.5 × 1 cm) Preheated at 800° for 1 h

Ascarite: 20- to 30-mesh

Copper oxide wire: Preheated at 850° for 2 h

Nitrogen: Purified grade

Quartz wool

Sodium carbonate: Anhydrous, fine granular

Standard solution: Transfer approximately 1.35 g of reagent-grade mercurous chloride, accurately weighed, into a 1-L volumetric flask. Dissolve in and dilute with water to volume. When diluted 100-fold, the solution contains 0.01 μg Hg per microliter (*Diluted standard solution*).

Procedure: Preheat the furnace to 650°, and adjust the nitrogen flow to 1 L/min.

Blank analysis: Place a square piece of preheated asbestos pad in the combustion boat, and cover it with sodium carbonate. Stop the nitrogen flow, disconnect the ball-joint, quickly insert the boat into the combustion tube with large forceps, and reconnect the joint. Note the time, allow the boat to sit in the tube with no nitrogen flow for exactly 1 min, and then restart the flow of nitrogen. Mercury elutes almost immediately with the reinstated nitrogen flow; note the recorder response. Allow about 30 s between runs.

Calibration: Determine the recorder response after the application to the asbestos pad of 1 μL, 2 μL, and 3 μL of the *Diluted standard solution*.

Sample analysis: Transfer 25 mg of colorant, accurately weighed, to the combustion boat, and cover the sample completely with sodium carbonate. Follow the procedure used for the *Blank analysis*, and calculate the mercury content using the standard curve.

Trap problems

(1) Some colorants (e.g., Brillant Blue and Fast Green) may give a response that is symmetrically dissimilar to the Hg peak. If such a response "carries over" to the next sample, then the aluminum oxide trap may need to be changed.

(2) If the recorder response is of inadequate sensitivity (peak height induced by 0.01 μg less than 0.5 cm), then the traps are packed too tightly. Remove or redistribute packing first in the aluminum oxide trap, then try the other traps.

(3) The traps will need changing periodically as indicated by a change in the physical appearance of the trap material or by chart responses of different retention times or different symmetry from that of mercury standards.

(4) If two or more standards are run in succession, a later sample might give an erroneous mercury response. Run blanks and then repeat the sample analysis to confirm the validity of the response.

Sodium chloride

Dissolve approximately 2 g of colorant, accurately weighed, in 100 mL of water, and add 10 g of activated carbon that is free of chloride and sulfate. Boil gently for 2–3 min. Cool to room temperature, add 1 mL of 6 N nitric acid, and stir. Dilute with water to volume in a 200-mL volumetric flask, and then filter through dry paper. Repeat the treatment with 2-g portions of carbon until no color is adsorbed onto filter paper dipped into the filtrate.

Transfer 50 mL of filtrate to a 250-mL flask. Add 2 mL of 6 N nitric acid, 5 mL of nitrobenzene, and 10 mL of standardized 0.1 N silver nitrate solution. Shake the flask until the silver chloride coagulates. Prepare a saturated solution of ferric ammonium sulfate, and add just enough concentrated nitric acid to discharge the red color; add 1 mL of this solution to the 250-mL flask to serve as the indicator. Titrate with 0.1 N ammonium thiocyanate solution that has been standardized against the silver nitrate solution until the color persists after shaking for 1 min. Calculate the weight percent of sodium chloride, P:

$$P = [(V \times N)/W] \times 22.79$$

in which V is the net volume, in mL, of silver nitrate solution required; N is the normality of the silver nitrate solution; and W is the weight, in grams, of the sample taken. The factor 22.79 incorporates a total volume of 195 mL because 10 g of activated carbon occupies 5 mL.

Sodium sulfate

Place 25 mL of the decolorized filtrate obtained from the *Sodium Chloride* test into a 125-mL Erlenmeyer flask, and add 1 drop of a 0.5% phenolphthalein solution in 50% ethanol. Add 0.05 N sodium hydroxide, dropwise, until the solution is alkaline to pH paper, and then add 0.002 N hydrochloric acid until the indicator is decolorized.

Add 25 mL of ethanol and about 0.2 g of tetrahydroxyquinone indicator (CAS: 319-89-1)[6] . Titrate with 0.03 N barium chloride solution to a red endpoint. Make a blank determination.

Calculate the weight percent, P, of sodium sulfate:

$$P = [(V - B) \times N/W] \times 55.4$$

in which V is the volume, in mL, of barium chloride solution required to titrate the sample; B is the volume, in mL, of barium chloride solution required for the blank; N is the normality of the barium chloride solution; and W is the weight, in grams, of the sample taken. The factor 55.4 incorporates a total volume of 195 mL because 10 g of activated carbon occupies 5 mL.

Total color

- **Method I (Spectrophotometric)**

Pipet 10.0 mL of the dissolved colorant into a 250-mL Erlenmeyer flask containing 90 mL of 0.04 N ammonium acetate, and mix well. Determine the net absorbance of the solution relative to water at the wavelength maximum given for each color. Calculate the percentage of colorant present:

$$\%\text{total color} = A/(a \times C \times b) \times 100$$

in which A is the absorbance; a is the absorptivity specified in the individual monograph (L/(mg · cm)); C is the concentration of sample in the final test solution (mg/L); and b is the cell pathlength (cm).

- **Method II (Titration with Titanium Chloride)**

Apparatus: The apparatus for determining total color by titration with titanium chloride ($TiCl_3$) is shown in *Figure 13*. It consists of a storage bottle, *A*, of 0.1 N titanium chloride titrant maintained under hydrogen produced by a Kipp generator; an Erlenmeyer flask, *B*, equipped with a source of CO_2 or N_2 to maintain an inert atmosphere in which the reaction takes place; a stirrer; and the buret, *C*.

[6] >99% purity (Catalog Number 099165, Matrix Scientific, Columbia, SC, USA), or equivalent.

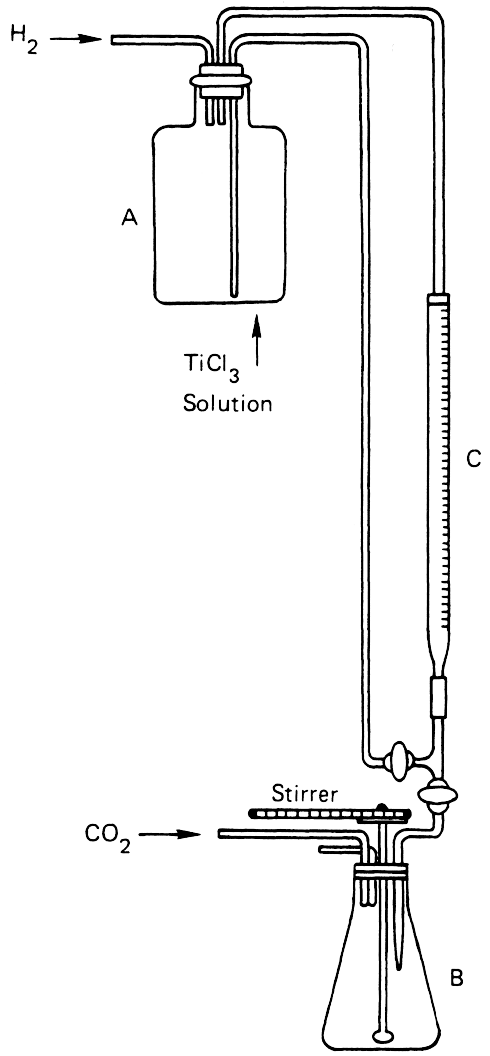


Figure 13. Titanous Chloride Titration Apparatus

Reagents and solutions

Titanium chloride solution (0.1 N): Transfer 73 mL of commercially prepared 20% $TiCl_3$ solution into a storage bottle, and carefully add 82 mL of concentrated HCl per L of final solution. Mix well, and bubble CO_2 or N_2 through the solution for 1 h. Before standardizing, maintain the solution under a hydrogen atmosphere for at least 16 h using a Kipp generator.

Potassium dichromate solution (0.1 N, primary standard): Transfer 4.9032 g of $K_2Cr_2O_7$ (NIST No. 136) to a 1-L volumetric flask; dissolve in and dilute with water to volume.

Ammonium thiocyanate (50%): Transfer 500 g of NH_4SCN, ACS certified, to a 1-L volumetric flask; dissolve in about 600 mL of water, warming if necessary; and dilute to volume.

Ferrous ammonium sulfate: $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, ACS certified

Sodium bitartrate

Standardization of the *Titanium chloride solution*: Drain any standing titanium chloride ($TiCl_3$) from the feed lines and buret, and refill with fresh solution. Add 3.0 g of *Ferrous ammonium sulfate* to a wide-mouth Erlenmeyer flask followed by 200 mL of water, 25 mL of 50% sulfuric acid, 25 mL of 0.1 N *Potassium dichromate solution* (by pipet), and 2 or 3 boiling chips. Boil the solution vigorously on a hot plate for 30 s to remove dissolved air, then quickly transfer the flask to the titration apparatus, securely connect the stopper assembly, and start the carbon dioxide flow and stirrer. Pass carbon dioxide over the solution for 1 min before beginning the titration.

Add the 0.1 N *Titanium chloride solution* at a fast, steady drip to within 1 mL of the estimated endpoint (about 20 mL). Reduce the carbon dioxide flow, remove the solid-glass rod from the stopper assembly, pipet 10 mL of *Ammonium thiocyanate* (50%) into the flask, insert the glass rod, and increase the carbon dioxide flow. Continue titrating slowly until the endpoint: a color change from brown-red to light green is observed. Perform a blank determination using the same reagents and quantities, and calculate the normality, N, of the 0.1 N *Titanium chloride solution* on the basis of three titrations:

$$N = (V_r \times N_r/V_t - V_b)$$

in which V_r is the volume, in mL, of 0.1 N *Potassium dichromate* used; N_r is the normality of the 0.1 N *Potassium dichromate*; V_t is the volume, in mL, of 0.1 N *Titanium chloride solution* used; and V_b is the volume, in mL, of titanium dichloride used in the blank titration.

Procedure: Transfer the quantity of colorant prescribed in the individual monograph into a 500-mL wide-mouth Erlenmeyer flask and add 21–22 g of *Sodium Bitartrate* (sodium citrate for Sunset Yellow), 275 mL of water, and two or three boiling chips. Boil the solution vigorously on a hot plate for 30 s to remove dissolved air, then quickly transfer the flask to the titration apparatus, securely connect the stopper assembly, and start the carbon dioxide flow and stirrer. Pass carbon dioxide over the solution for 1 min before beginning the titration.

Titrate the sample until the color lightens, wait 20 s, and then continue the addition with about 2 s between drops. When the color is almost completely bleached, wait 20 s, and then continue the addition with 5 s between drops. A complete color change indicates the endpoint. Perform a blank determination using the same reagents and quantities, and calculate the total color, T, in percent and on the basis of three titrations:

$$T = [(V_t - V_b)/(W \times F_s)] \times 100 \times N$$

in which V_t is the volume of titrant used; V_b is the volume of titrant required to produce the endpoint in a blank; W is the weight, in grams, of the sample taken; F_s is a factor derived from the stoichiometry of the reaction characteristics of each colorant and is given in the individual monograph; and N is the normality of the titrant.

Method III (Gravimetric)

Transfer approximately 0.5 g of colorant, accurately weighed, to a 400-mL beaker, add 100 mL of water, and heat to boiling. Add 25 mL of 1:50 hydrochloric acid, and bring to a boil. Wash down the sides of the beaker with water, cover, and keep on a steam bath for several hours or overnight. Cool to room temperature, and quantitatively transfer the precipitate into a tared filtering crucible with 1:100 hydrochloric acid. Wash the precipitate with two 15-mL portions of water, and dry the crucible for 3 h at 135°. Cool in a desiccator, and weigh. Calculate the total color, P, in weight percent:

$$P = [(W_p \times F)/W_s] \times 100$$

in which W_p is the weight, in grams, of the precipitate; F is the gravimetric conversion factor given in the individual monograph; and W_s is the original weight, in grams, of the sample taken.

Uncombined intermediates and products of side reactions

- **Method I**

Sample solution: Transfer approximately 2 g of colorant to a 100-mL volumetric flask; dissolve in and dilute with water to volume.

Apparatus: Pack a 2.5-cm × 45-cm glass column with approximately 20 g of cellulose (Whatman CF-11 grade, or equivalent) that has been slurried in the eluant and from which the fines have been removed by decantation. Equilibrate the column thoroughly with the eluant, 35% ammonium sulfate.

Procedure: Pipet 5 mL of the *Sample solution* into a beaker containing 5 g of cellulose that has been slurried in eluant and from which the fines have been removed by decantation. Stir the mixture thoroughly, add 10 g of ammonium sulfate, and stir until uniformly mixed. Mix the slurry with 15 mL of eluant, and apply it to the column. Allow the fluid to enter the column, and wash the beaker with eluant until the sample is quantitatively transferred. Elute the column with approximately 500 mL of 35% ammonium sulfate, and collect a total of eight 60-mL fractions. Divide each collected fraction in half and add 0.5 mL of NH_4OH to one half and 0.5 mL of HCl to the other.

Calculation: After identifying each intermediate and side product by comparing spectra of the fractions with commercial standards, calculate the concentration, C, of each:

$$C = A/(a \times b)$$

in which A is the absorbance at the wavelength of maximal absorption; a is the absorptivity given in the individual monograph; and b is the cell pathlength, in cm.

- **Method II**

Apparatus: Use a suitable high-performance liquid chromatography system (see *Chromatography, Appendix IIA*) equipped with a dual wavelength detector system such that the effluent can be monitored serially at 254 nm and 325–385 nm (wide-band pass). Use a 1-m × 2.1-mm (id) column, or equivalent, packed with a strong anion-exchange resin (Dupont No. 830950405, or equivalent).

Operating conditions: The operating conditions required may vary depending on the system used. The following conditions have been shown to give suitable results for Allura Red, Tartrazine, and Sunset Yellow.

Allura red

Primary eluant: 0.01 M aqueous $Na_2B_4O_7$

Secondary eluant: 0.20 M $NaClO_4$ in aqueous 0.01 M $Na_2B_4O_7$

Sample size: 20 µL of a 0.25% solution

Flow rate: 0.60 mL/min

Gradient: Linear, in two phases: 0%–18% in 40 min, 18%–62% in 8 min more, then hold for 18 min more at 62%

Temperature: 50°

Pressure: 1000 psi

Order of elution: (1) Cresidinesulfonic acid (CSA); (2) unknown; (3) Schaeffer's salt (SS); (4) unknown; (5) 4,4′-diazoaminobis(5-methoxy-2-methylbenzenesulfonic acid) (DMMA); (6) unknown; (7) Allura Red; (8) 6,6′-oxybis(2-naphthalenesulfonic acid) (DONS)

Tartrazine

Primary eluant: 0.01 M aqueous $Na_2B_4O_7$

Secondary eluant: 0.10 M $NaClO_4$ in aqueous 0.01 M $Na_2B_4O_7$

Sample size: 50 µL of a 0.15% solution, prepared within 13 min of injection

Flow rate: 1.00 mL/min

Gradient: Exponential at 4%/min: 0.95%

Temperature: 50°

Pressure: 1000 psi

Order of elution: (1) Phenylhydrazine-*p*-sulfonic acid (PHSA); (2) sulfanilic acid (SA); (3) 1-(4-sulfophenyl)-3-ethylcarboxy-5-hydroxypyrazolone (PY-T); (4) 1-(4-sulfophenyl)-3-carboxy-5-hydroxypyrazolone (EEPT); (5) 4,4′-(diazoamino)-dibenzenesulfonic acid (DAADBSA)

Sunset yellow

Primary eluant: 0.01 M aqueous $Na_2B_4O_7$

Secondary eluant: 0.20 M $NaClO_4$ in aqueous 0.01 M $Na_2B_4O_7$

Sample size: 5 µL of a 1% solution

Flow rate: 0.50 mL/min

Gradient: Linear in four phases: 0%–11% in 10 min; hold 25 min; 11%–38% in 10 min; 38%–42% in 10 min; 42%–98% in 20 min; hold 20 min

Temperature: 50°

Pressure: 1000 psi

Order of elution: (1) Sulfanilic acid (SA); (2) Schaeffer's salt (SS); (3) 4,4′-(diazoamino)-dibenzenesulfonic acid (DAADBSA); (4) *R*-salt dye; (5) Sunset Yellow; (6) 6,6′-oxybis(2-naphthalenesulfonic acid) (DONS)

Standard solutions

Allura red: Prepare a solution containing 0.25 g of colorant, 0.5 mg of CSA, 0.75 mg of SS, 0.25 mg of DMMA, and 1.25 mg of DONS in a 100-mL volumetric flask. Dissolve in and dilute with 0.1 M $Na_2B_4O_7$ to volume.

Tartrazine: Prepare a solution containing 0.15 g of colorant and 0.3 mg each of PHSA, SA, PY-T, EEPT, and DAADBSA in a 100-mL volumetric flask. Dissolve in and dilute with 0.1 M $Na_2B_4O_7$ to volume.

Sunset yellow: Prepare a solution containing 0.25 g of colorant, 0.5 mg of SA, 0.75 mg of SS, 0.25 mg of DAADBSA, and 1.25 mg of DONS in a 100-mL volumetric flask. Dissolve in and dilute with 0.1 M $Na_2B_4O_7$ to volume.

Test solutions: Prepare at least four test solutions, each containing the colorant, and one impurity, accurately weighed, dissolved in 0.1 M $Na_2B_4O_7$, and diluted to volume in a 100-mL volumetric flask. The solutions should encompass the range of concentrations, evenly spaced, given below for each constituent:

Allura red (250 mg): CSA (0.05–0.5 mg); SS (0.05–0.75 mg); DONS (0.5–2.5 mg); DMMA (0.025–0.25 mg). Inject 20 µL of each solution.

Tartrazine (150 mg): SA (7.5 to 300 µg); PY-T (7.5–300 µg); EEPT (7.5–300 µg); DAADBSA (7.5–300 µg). Inject 50 µL of each solution.

Sunset yellow (250 mg): SA (0.05–0.5 mg); SS (0.05–0.75 mg); DONS (0.5–2.5 mg); DAADBSA (0.05–0.25 mg). Inject 20 µL of each solution.

System suitability

Resolution: Elute the column, or equivalent, with the gradient specified under *Operating conditions* until a smooth baseline is obtained. Inject an aliquot of the *Standard solution*. The resolution of the eluted components matches or exceeds that shown for the corresponding colorant (see *Figures 14*, *15*, and *16*). After determining that the column, or equivalent, will give the required resolution, allow it to rest for 2 weeks before use.

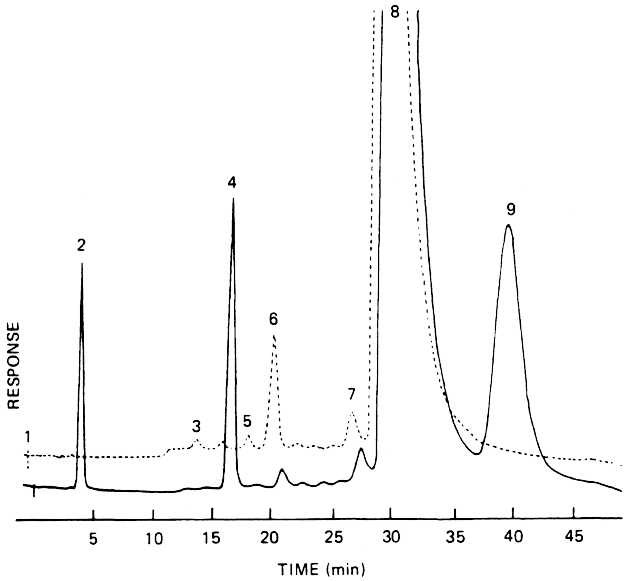


Figure 14. Allura Red–Top Trace: Eluant Monitored at 254 nm; Bottom Trace: Eluant Monitored at 375–385 nm

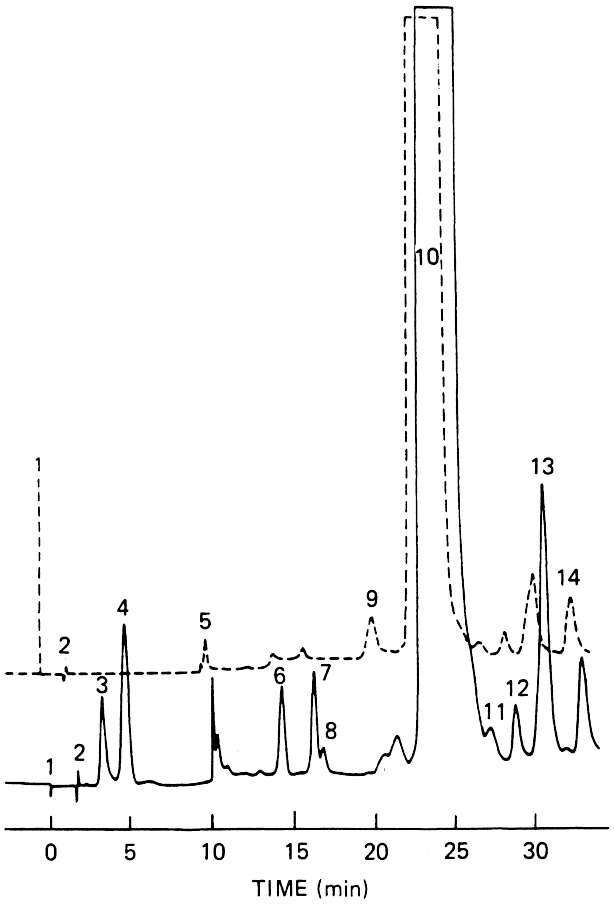


Figure 15. Tartrazine–Top Trace: Eluant Monitored at 254 nm; Bottom Trace: Eluant Monitored at 375–385 nm

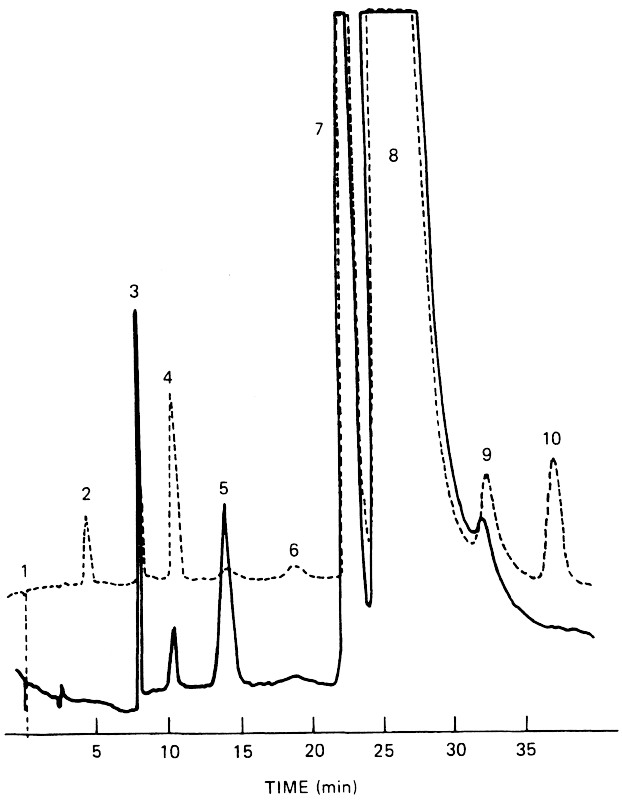


Figure 16. Sunset Yellow–Top Trace: Eluant Monitored at 254 nm; Bottom Trace: Eluant Monitored at 375–385 nm

Calibration: Inject the designated volume of each *Test solution* onto a conditioned column, and prepare a standard curve corresponding to each unreacted intermediate and side reaction product. Determine the area, A, for each peak from the integrator if an automated system is used or by multiplying the peak height by the width at one-half the height. The peak height alone may be used for EEPT, PY-T, and DAADBSA. Calculate the concentration, C_i, of each intermediate or side product:

$$C_i = mA_i + b$$

in which A_i is the area of its corresponding chromatographic peak. Calculate the slope, m, and intercept, b, using the following linear regression equations:

$$m = [N\Sigma C_iA_i - \Sigma C_i\Sigma A_i]/[N\Sigma A_i^2 - (\Sigma A_i)^2]$$

$$b = [\overline{A}]_i - m[\overline{C}]_i$$

in which $\overline{A}$ and $\overline{C}$ are the calculated averages of the peak areas and concentrations, respectively, used to construct the standard curve for one intermediate or side reaction product. Calculate the correlation coefficient, r:

$$r = [\Sigma(C_i - \overline{C})(A_i - A)]/[\Sigma(C_i - \overline{C}^2) \times \Sigma(A_i - \overline{A})^2]$$

Each time the system is calibrated, add the new data to those accumulated from previous analyses. The correlation coefficient must be 0.95–1.00 for any single experiment or from accumulated data.

Recalibrate the system after every 10 determinations or 2 days, whichever occurs first.

Sample preparation: Prepare as directed in the individual monograph.

Procedure: Inject the volume of *Sample preparation* as designated in the monograph into the column. Determine the concentration of intermediates and side reaction products from the peak areas using the slope, m, and intercept, b, calculated under *Calibration*:

$$C_s = mA_s + b$$

in which C_s is the concentration of the unknown in the *Sample preparation* and A_s its corresponding peak area.

Loss on drying (volatile matter)

Transfer 1.5–2.5 g of colorant, accurately weighed, to a tared crucible. Heat in a vacuum oven at 135° for 12–15 h. Lower the pressure in the oven to –125 mm Hg, and continue heating for an additional 2 h. Cover the crucible,

and allow to cool in a desiccator. Reweigh the crucible when cool. The loss of weight is defined as the volatile matter.

Water-insoluble matter

Transfer about 1 g of colorant, accurately weighed, to a 250-mL beaker, and add 200 mL of boiling water. Stir to facilitate dissolution of the color.

Tare a filtering crucible equipped with a glass fiber filter (Reeve Angel, No. 5270, or equivalent). Filter the solution with the aid of suction when it has cooled to ambient temperature. Rinse the beaker three times, pouring the rinsings through the crucible. Wash the filter with water until the filtrate is colorless.

Dry the crucible and filter in an oven at 135° for at least 3 h, cool them in a desiccator, and reweigh to the nearest 0.1 mg. Calculate the percent water-insoluble matter, I:

$$I = (W_c/W_s) \times 100$$

in which W_c is the difference in crucible weight and W_s is the sample weight.

ELEMENTAL IMPURITIES BY ICP

Before the initial use of either of the procedures below, the analyst should ensure that the procedure is appropriate for the instrument and sample used. *Method I* can be used for elemental impurities generally amenable to detection by inductively coupled plasma–atomic (optical) emission spectroscopy (ICP–OES). *Method II* can be used for elemental impurities generally amenable to detection by inductively coupled plasma–mass spectrometry (ICP–MS). If no method is specified in the individual monograph, analysts are instructed to use *Method II* (ICP–MS).

• **Method I: ICP–OES**

Reagents: All reagents used for the preparation of the sample and standard solutions should be free of elemental impurities. Reagents should be commercial elemental stock standards that are NIST-traceable, or equivalent, at a recommended concentration of 100 µg/mL or greater; or appropriate USP Reference Standards, as either single element or multielement.

Aqua regia: Ultra pure nitric acid/hydrochloric acid (1:3) prepared as needed. (A 1%–5% solution of aqua regia is used as a rinsing solution between analyses and as calibration blanks.)

Sample preparation: Use this sample preparation procedure unless otherwise specified in the individual monograph. [NOTE—Weights and volumes provided may be adjusted to meet the requirements of the microwave digestion apparatus used, if proportions remain constant.] Dehydrate and predigest 0.5 g of sample in 5 mL of freshly prepared *Aqua regia*. Sulfuric acid may also be used as a last resort. [NOTE—Sulfuric acid should be used only when absolutely needed because addition of sulfuric acid may cause an extreme exothermic reaction and result in elements being lost and because the viscosity of sulfuric acid is higher than that of other acids, which affects the overall flow of solution.] Allow the sample to sit loosely covered for 30 min in a fume hood. Add an additional 10 mL of *Aqua regia* and digest, using a closed vessel microwave technique. Microwave until digestion or extraction is complete. Repeat if necessary by adding an additional 5 mL of *Aqua regia*. [NOTE—Follow the recommended procedures provided by the manufacturer of the closed vessel microwave digestion apparatus to ensure safe usage. In closed vessel microwave digestion, the use of concentrated hydrofluoric acid (HF) is not recommended; however, when its use is necessary, practice the utmost caution in the preparation of test articles, and review or establish local procedures for safe handling, safe disposal, and HF-tolerant instrumental configurations.]

Sample solution: Allow the digestion vessel containing the *Sample preparation* to cool (for mercury measurements, add an appropriate stabilizer, such as gold at about 0.1 ppm), and dilute with water to 50.0 mL.

Calibration solution 1: 2J of the element of interest in a matched matrix (acid concentrations similar to that of the *Sample solution*), where J is the limit for the specific elemental impurity. [NOTE—Multiple elements of interest may be included in this solution at the same concentration ratio. For mercury analysis, add an appropriate stabilizer, such as gold at about 0.1 ppm.]

Calibration solution 2: 0.1J of the element of interest in a matched matrix (acid concentrations similar to that of the *Sample solution*), where J is the limit for the specific elemental impurity. [NOTE—Multiple elements of interest may be included in this solution at the same concentration ratio. For mercury analysis, add an appropriate stabilizer, such as gold at about 0.1 ppm.]

Check standard solution: 1 ppm of the element of interest in a matched matrix (acid concentrations similar to that of the *Sample solution*). [NOTE—Multiple elements of interest may be included in this solution at the same concentration ratio. For mercury analysis, add an appropriate stabilizer, such as gold at about 0.1 ppm.]

Blank: Matched matrix (acid concentrations similar to that of the *Sample solution*)

Elemental spectrometric system (see *Plasma Spectrochemistry, Appendix IIC***)**

Mode: ICP

Detector: Optical emission spectroscopy

Rinse: 5% *Aqua Regia*

Calibration: Two-point, using *Calibration solution 1*, *Calibration solution 2*, and *Blank*

System suitability

Sample: *Check standard solution*

Suitability requirement: The concentration determined from the resulting chromatogram differs from actual concentration by NMT 20%. [NOTE—If samples are high in mineral content, to minimize sample carryover, rinse system well (60 s) before introducing the *Check Standard Solution*.]

Analysis: Analyze according to manufacturer's suggestions for program and wavelength. Calculate and report results on the basis of the original sample size.

Calculation: Upon completion of the analysis, calculate the final concentration of a given element in the test article (µg/g) from the solution element concentration (µg/mL) as follows:

$$C = [(A \times V_1)/W] \times (V_2/V_3)$$

where C is the concentration of the analyte, µg/g; A is the instrument reading, µg/mL; V_1 is the volume of the initial test article preparation, mL; W is the weight of the test article preparation, g; V_2 is the total volume of any dilution performed, mL; and V_3 is the volume of the aliquot of initial test article preparation used in any dilution performed, mL.

Similarly, calculate the final concentration of a given element in the test article (µg/g) from the solution element concentration (ng/mL) as follows:

$$C = [(A \times V_1)/W] \times (1\ \mu g/1000\ ng)(V_2/V_3)$$

where A is the instrument reading, ng/mL; and the other factors are as defined above.

- **Method II: ICP–MS**

Reagents: All reagents used for the preparation of sample and standard solutions should be free of elemental impurities. Reagents should be commercial elemental stock standards that are NIST-traceable, or equivalent, at a recommended concentration of 100 µg/mL or greater; or appropriate USP Reference Standards, as either single element or multielement.

Aqua regia: Ultra pure nitric acid/hydrochloric acid (1:3) prepared as needed. (A 1%–5% solution of aqua regia is used as a rinsing solution between analyses and as calibration blanks.)

Sample preparation: Proceed as directed under *Method I*.

Sample solution: Allow the digestion vessel containing the *Sample preparation* to cool, and add appropriate internal standards at appropriate concentrations (for mercury measurements, gold should be one of the internal standards). Dilute with water to 50.0 mL.

Calibration solution 1: Proceed as directed under *Method I*.

Calibration solution 2: Proceed as directed under *Method I*.

Blank: Matched matrix (acid concentrations similar to that of the *Sample solution*)

Elemental spectrometric system (see *Plasma Spectrochemistry, Appendix IIC***)**

Mode: ICP. [NOTE—An instrument with a cooled spray chamber is recommended.]

Detector: Mass spectrometer

Rinse: 5% *Aqua regia*

Calibration: *Calibration solution 1*, *Calibration solution 2*, and *Blank*

System suitability

Sample: *Calibration solution 1*

Suitability requirement: The concentration determined from the resulting chromatogram differs from actual concentration by NMT 20%. [NOTE—If samples are high in mineral content, to minimize sample carryover, rinse system well (60 s) before introducing the *Check standard solution*.]

Analysis: Analyze according to the manufacturer's suggestions for the program and m/z. Calculate and report results based on the original sample size. [NOTE—Arsenic is subject to interference from argon chloride. Appropriate measures, including a sample preparation without *Aqua regia*, must be taken to correct for the interference, depending on instrumental capabilities.]

Calculation: Upon completion of the analysis, calculate the final concentration of a given element in the test article (µg/g) from the solution element concentration (µg/mL) as follows:

$$C = [(A \times V_1)/W] \times (V_2/V_3)$$

where C is the concentration of the analyte, µg/g; A is the instrument reading, µg/mL; V_1 is the volume of the initial test article preparation, mL; W is the weight of the test article preparation, g; V_2 is the total volume of any dilution performed, mL; and V_3 is the volume of the aliquot of initial test article preparation used in any dilution performed, mL.

Similarly, calculate the final concentration of a given element in the test article (µg/g) from the solution element concentration (ng/mL) as follows:

$$C = [(A \times V_1)/W] \times (1\ \mu g/1000\ ng)(V_2/V_3)$$

where A is the instrument reading, ng/mL; and the other factors are as defined above.

GLUTAMIC ACID

Apparatus: Use an ion-exchange amino acid analyzer, equipped with sulfonated polystyrene columns, in which the effluent from the sample is mixed with ninhydrin reagent and the absorbance of the resultant color is measured continuously and automatically at 570 nm and 440 nm by a recording photometer.

Standard solution: Transfer 1250 ± 2 mg of reagent-grade glutamic acid, accurately weighed, into a 500-mL volumetric flask. Fill the flask half-full with water, add 5 mL of hydrochloric acid to help dissolve the amino acid, dilute with water to volume, and mix. Prepare the standard for analysis by diluting 1 mL of this solution with 4 mL of 0.2 N sodium citrate, pH 2.2, buffer. This *Standard solution* contains 0.5 mg of glutamic acid per mL (C_S).

Sample preparation: Dilute 5 mg of sample, accurately weighed, to exactly 5 mL with 0.2 N sodium citrate, pH 2.2, buffer. Remove any insoluble material by centrifugation or filtration.

Procedure: Using 2-mL aliquots of the *Standard solution* and *Sample preparation*, proceed according to the apparatus manufacturer's instructions. From the chromatograms thus obtained, match the retention times produced by the *Standard preparation* with those produced by the *Sample Solution*, and identify the peak produced by glutamic acid. Record the area of the glutamic acid peak from the sample as A_U, and that from the standards as A_S.

Calculations: Calculate the concentration, C_A, in mg/mL, of glutamic acid in the *Sample preparation*:

$$\text{Result} = A_U \times C_S/A_S$$

in which C_S is the concentration, in mg/mL, of glutamic acid in the *Standard solution*.

Calculate the percent glutamic acid, on the basis of total protein:

$$\text{Result} = (100 \times C_A)/(6.25 \times N_T)$$

in which 6.25 is the conversion factor for protein and amino acids, and N_T is the percent total nitrogen determined in the monograph *Assay*.

Calculate the percent glutamic acid in the sample:

$$\text{Result} = 100 \times C_A/S_W$$

in which S_W is the weight, in mg, of the sample taken.

HYDROXYPROPOXYL DETERMINATION

Apparatus: The apparatus for hydroxypropoxyl group determination is shown in *Figure 17*.

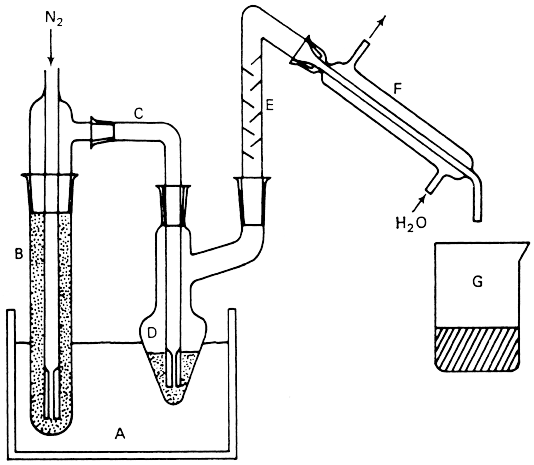


Figure 17. Apparatus for Hydroxypropoxyl Determination

The boiling flask, *D*, is fitted with an aluminum foil-covered Vigreaux column, *E*, on the side arm and with a bleeder tube through the neck and to the bottom of the flask for the introduction of steam and nitrogen. A steam generator, *B*, is attached to the bleeder tube through tube *C*, and a condenser, *F*, is attached to the Vigreaux column. The boiling flask and steam generator are immersed in an oil bath, *A*, equipped with a thermoregulator such that a temperature of 155° and the desired heating rate may be maintained. The distillate is collected in a 150-mL beaker, *G*, or other suitable container.

Procedure: Unless otherwise directed, transfer about 100 mg of the sample, previously dried at 105° for 2 h and accurately weighed, into the boiling flask, and add 10 mL of chromium trioxide solution (60 g in 140 mL of water). Immerse the steam generator and the boiling flask in the oil bath (at room temperature) to the level of the top of the chromium trioxide solution. Start cooling water through the condenser, and pass nitrogen gas through the boiling flask at the rate of one bubble per second. Starting at room temperature, raise the temperature of the oil bath to 155° over a period of not less than 30 min, and maintain this temperature until the end of the determination. Distill until 50 mL of distillate is collected. Detach the condenser from the Vigreaux column, and wash it with water, collecting the washings in the distillate container. Titrate the combined washings and distillate with 0.02 N sodium hydroxide to a pH of 7.0, using a pH meter set at the expanded scale.

[NOTE—phenolphthalein TS may be used for this titration if it is also used for all standards and blanks.]

Record the volume, V_a, of the 0.02 N sodium hydroxide used. Add 500 mg of sodium bicarbonate and 10 mL of 2 N sulfuric acid, and then after evolution of carbon dioxide has ceased, add 1 g of potassium iodide. Stopper the flask, shake the mixture, and allow it to stand in the dark for 5 min. Titrate the liberated iodine with 0.02 N sodium thiosulfate to the sharp disappearance of the yellow color, confirming the endpoint by the addition of a few drops of starch TS. Record the volume of 0.02 N sodium thiosulfate required as Y_a.

Make several reagent blank determinations, using only the chromium trioxide solution in the above procedure. The ratio of the sodium hydroxide titration (V_b) to the sodium thiosulfate titration (Y_b), corrected for variation in normalities, will give the acidity-to-oxidizing ratio, $V_b/Y_b = K$, for the chromium trioxide carried over in the distillation. The factor K should be constant for all determinations.

Make a series of blank determinations using 100 mg of methylcellulose (containing no foreign material) in place of the sample, recording the average volume of 0.02 N sodium hydroxide required as V_m and the average volume of 0.02 N sodium thiosulfate required as Y_m.

Calculate the hydroxypropoxyl content of the sample, in mg:

$$\text{Result} = 75.0 \times [N_1(V_a - V_m) - kN_2(Y_a - Y_m)]$$

in which N_1 is the exact normality of the 0.02 N sodium hydroxide solution, $k = V_bN_1/Y_bN_2$, and N_2 is the exact normality of the 0.02 N sodium thiosulfate solution.

The percentage of substitution, by weight, of hydroxypropoxyl groups, determined as directed above, may be converted to molecular substitution per glucose unit by reference to *Figure 18*.

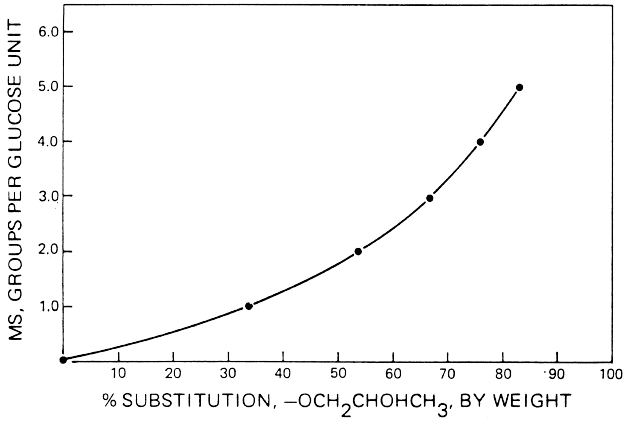


Figure 18. Chart for Converting Percentage of Substitution, by Weight, of Hydroxypropoxyl Groups to Molecular Substitution per Glucose Unit

METHOXYL DETERMINATION

Apparatus: The apparatus for methoxyl determination, as shown in *Figure 19*, consists of a boiling flask, *A*, fitted with a capillary side arm to provide an inlet for carbon dioxide and connected to a column, *B*, which separates aqueous hydriodic acid from the more volatile methyl iodide. After the methyl iodide passes through a suspension of aqueous red phosphorus in the scrubber trap, *C*, it is absorbed in the bromine–acetic acid absorption tube, *D*. The carbon dioxide is introduced from a device arranged to minimize pressure fluctuations and connected to the apparatus by a small capillary containing a small plug of cotton.

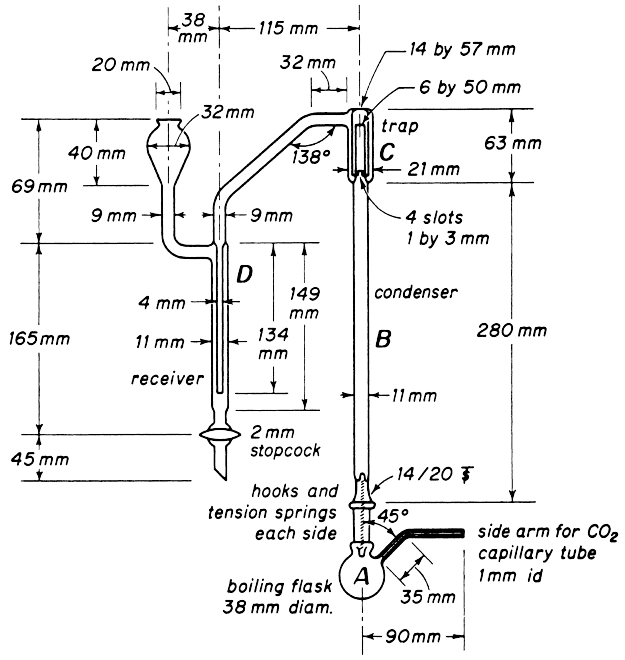


Figure 19. Distillation Apparatus for Methoxyl Determination

Reagents

Acetic potassium acetate: Dissolve 100 g of potassium acetate in 1000 mL of a mixture consisting of 900 mL of glacial acetic acid and 100 mL of acetic anhydride.

Bromine–acetic acid solution: On the day of use, dissolve 5 mL of bromine in 145 mL of the *Acetic potassium acetate* solution.

Hydriodic acid: Use special-grade hydriodic acid suitable for alkoxyl determinations, or purify reagent grade as follows: distill over red phosphorus in an all-glass apparatus, passing a slow stream of carbon dioxide through the apparatus until the distillation is terminated and the receiving flask has completely cooled.

[**CAUTION**—Use a safety shield, and conduct the distillation in a fume hood.]

Collect the colorless, or almost colorless, constant-boiling acid distilling between 126°–127°. Store the acid in a cool, dark place in small, brown, glass-stoppered bottles previously flushed with carbon dioxide and finally sealed with paraffin.

Procedure: Fill trap *C* half-full with a suspension of about 60 mg of red phosphorus in 100 mL of water, introduced through the funnel on tube *D* and the side arm that connects with the trap at *C*. Rinse tube *D* and the side arm with water, collecting the rinsings in trap *C*, then charge absorption tube *D* with 7 mL of *Bromine–acetic acid solution*. Place the sample, previously accurately weighed in a tared gelatin capsule, into the boiling flask *A*, along with a few glass beads or boiling stones, then add 6 mL of *Hydriodic acid*. Connect the flask to the condenser, using a few drops of the acid to seal the junction, and begin passing the carbon dioxide through the apparatus at the rate of about two bubbles per second. Heat the flask in an oil bath at 150°, continue the reaction for 40 min, and drain the contents of absorption tube *D* into a 500-mL Erlenmeyer flask containing 10 mL of a 1:4 solution of sodium acetate. Rinse tube *D* with water, collecting the rinsings in the flask, and dilute with water to about 125 mL. Discharge the red-brown color of bromine by adding formic acid, dropwise with swirling, then add 3 drops in excess. Usually a total of 12 to 15 drops of formic acid is required. Allow the flask to stand for 3 min, add 15 mL of 2 N sulfuric acid and 3 g of potassium iodide, and titrate immediately with 0.1 N sodium thiosulfate, adding starch TS near the endpoint. Perform a blank determination with the same quantities of the same reagents, including the gelatin capsule, and in the same manner, and make any necessary correction. Each mL of 0.1 N sodium thiosulfate is equivalent to 0.517 mg (517 µg) of methoxyl groups ($–OCH_3$).

NITROGEN DETERMINATION (Kjeldahl Method)

[NOTE—All reagents should be nitrogen free, where available, or otherwise very low in nitrogen content.]

• **Method I**

Use this method unless otherwise directed in the individual monograph. It is not applicable to certain nitrogen-containing compounds that do not yield their entire nitrogen upon digestion with sulfuric acid.

Nitrites and nitrates absent

Unless otherwise directed, transfer 1 g of sample into a 500-mL to 800-mL Kjeldahl digestion flask of hard borosilicate, moderately thick, well-annealed glass, wrapping the sample, if solid or semisolid, in nitrogen-free filter paper to facilitate the transfer if desired. Add 10 g of powdered potassium sulfate or anhydrous sodium sulfate, 500 mg of powdered cupric sulfate, and 20 mL of sulfuric acid. Place the flask in an inclined position (about 45°), and heat gently keeping the temperature below the boiling point until frothing ceases, adding a small amount of paraffin, if necessary, to reduce frothing.

[**CAUTION**—The digestion should be conducted in a fume hood, or the digestion apparatus should be equipped with a fume exhaust system.]

Increase the heat until the acid boils briskly and continue the heating process until the solution clears, and then continue boiling for 30 min longer (or for 2 h for samples containing organic material). Cool, add about 150 mL of water, mix, and then cool to below 25°. Add cautiously 100 mL of a 2:5 sodium hydroxide solution, in such a manner as to cause the solution to flow down the inner side of the flask to form a layer under the acid solution, to make the mixture strongly alkaline. Add a few granules of zinc to prevent bumping, and immediately connect the flask to a distillation apparatus consisting of a Kjeldahl connecting bulb and a condenser, the delivery tube of which extends well beneath the surface of 100 mL of a 1:25 boric acid solution contained in a conical flask or a wide-mouth bottle of about 500-mL capacity. Gently, rotate the Kjeldahl flask to mix its contents thoroughly, and then heat until all of the ammonia has distilled, collecting at least 150 mL of distillate (about 80% of the contents of the flask). Wash the tip of the delivery tube, collecting the washings in the receiving flask, and titrate with 0.5 N sulfuric acid, determining the endpoint potentiometrically. Perform a blank determination, substituting 2 g of sucrose for the sample, and make any necessary correction (see *General Provisions*). Each mL of 0.5 N acid is equivalent to 7.003 mg of nitrogen.

[NOTE—An indicator solution can also be used to determine the titration endpoint. For example, dissolve 0.2 g of methyl red in 100 mL of 95% ethanol, 1 g of bromocresol green in 500 mL of 95% ethanol, then combine 1 part of the methyl red solution and 5 parts of the bromocresol green solution. Add 3 mL of methyl red/bromocresol green indicator solution per L of boric acid solution. Then, titrate the sample to the first trace of pink. Also, a bromocresol green–methyl red solution can be used as an alternative. To make the solution, dissolve 0.15 g of bromocresol green and 0.1 g of methyl red in 180 mL of alcohol, and dilute with water to a final volume of 200 mL.]

[NOTE—If the substance to be determined is known to have a low nitrogen content, 0.1 N acid and alkali may be used, in which case each mL of 0.1 N acid consumed is equivalent to 1.401 mg of nitrogen.]

[NOTE—Nitrogen recovery verification can be run to check for accuracy of the procedure and the equipment.

(1) *Nitrogen loss:* Use 0.12 g of ammonium sulfate and 0.85 g of sucrose. Add all other reagents, digest, and distill under the same conditions as for the sample. Recoveries should be NLT 99%.

(2) *Digestion efficiency:* Use 0.16 g of lysine hydrochloride or 0.18 g of tryptophan, with 0.67 g of sucrose per flask. Add all other reagents, digest, and distill under the same conditions as for the sample. Recoveries should be NLT 98%.]

Nitrites and nitrates present

[NOTE—This procedure is not applicable to liquids or to materials having a high chlorine-to-nitrate ratio.]

Unless otherwise directed, transfer a quantity of sample, accurately weighed, corresponding to about 150 mg of nitrogen into a Kjeldahl flask, and add 25 mL of 93%–98% sulfuric acid containing 1 g of salicylic acid. Mix thoroughly by shaking, and then allow to stand for 30 min or more, with frequent shaking. Add 5 g of $Na_2S_2O_3 \cdot 5H_2O$ (as an impalpable powder, not granules or filings), mix, then add 500 mg of powdered cupric sulfate, and proceed as directed under *Nitrates and Nitrites Absent*, beginning with "Incline the flask at an angle of about 45°". When the nitrogen content of the substance is known to exceed 10%, 500 mg to 1 g of benzoic acid may be added, prior to digestion, to facilitate the decomposition of the substance.

• **Method II (Semimicro)**

[NOTE—Automated instruments may be used in place of this manual method, provided the automated equipment has been properly calibrated.]

Transfer an accurately weighed or measured quantity of the sample, equivalent to about 2 or 3 mg of nitrogen, into the digestion flask of a semimicro Kjeldahl apparatus. Add 1 g of a 10:1 powdered mixture of potassium sulfate and cupric sulfate, using a fine jet of water to wash down any material adhering to the neck of the flask, and then pour 7 mL of sulfuric acid down the inside wall of the flask to rinse it. Cautiously add down the inside of the flask 1 mL of 30% hydrogen peroxide, swirling the flask during the addition.

[**CAUTION**—Do not add any peroxide during the digestion.]

Heat over a free flame or an electric heater until the solution has attained a clear blue color and the walls of the flask are free from carbonized material. Cautiously add 20 mL of water, cool, then add through a funnel 30 mL of a 2:5 solution of sodium hydroxide, and rinse the funnel with 10 mL of water. Connect the flask to a steam distillation apparatus, and immediately begin the distillation with steam. Collect the distillate in 15 mL of a 1:25 solution of boric acid to which has been added 3 drops of *methyl red–methylene blue TS* and enough water to cover the end of the condensing tube. Continue passing the steam until 80–100 mL of distillate has been collected, then remove the absorption flask, rinse the end of the condenser tube with a small quantity of water, and titrate with 0.01 N sulfuric acid. Each mL of 0.01 N acid is equivalent to 140 µg of nitrogen.

When more than 2 or 3 mg of nitrogen is present in the measured quantity of the substance to be determined, 0.02 or 0.1 N sulfuric acid may be used in the titration if at least 15 mL of titrant is required. If the total dry weight of the material taken is greater than 100 mg, increase proportionately the quantities of sulfuric acid and sodium hydroxide added before distillation.

SELENIUM(IV) AND SELENIUM(VI) SPECIATION

Principle: As concentrations slightly above those required for health can induce toxicity, it is highly important that selenium determination be precise and specific. The following method utilizes an HPLC-based separation of the sample hyphenated with ICP-MS for the determination of Se(IV) and Se(VI) in sodium selenate. An anion exchange column is used to separate Se(IV) from Se(VI), and an ICP-MS equipped with a collision-reaction cell technology (CCT) was applied to provide sensitive and specific analysis of different oxidation states of selenium by removing the matrix-based polyatomic interferences.

[NOTE—Use high-purity deionized water (>18MΩ · cm at 25°) to prepare all solutions. Use solvents that are of sufficient purity for analysis of trace contaminants.]

Mobile phase (11 mM ammonium citrate buffer, pH 5.0): In a 1000-mL volumetric flask, dissolve 2.10 g of citric acid in about 900 mL of water, adjust the pH of the resulting solution with ammonium hydroxide solution (28%–30% ammonia) to 5.0, and dilute with water to volume.

Standard stock solution A: Use commercially available standard solution containing 1000 mg/L Se(IV).[7] Dilute an aliquot of this solution with water to obtain *Standard stock solution A* containing 11.58 mg/L Se(IV).

Standard stock solution B: Use commercially available standard solution containing 878 mg/L of Se(VI) and 157 mg/L of Se(IV)[8]. Dilute an aliquot of this solution and water to obtain *Standard stock solution B* containing 8.78 mg/L Se(VI) with 1.57 mg/L Se(IV).

Standard solutions: Dilute individual aliquots of *Standard stock solution A* and *Standard stock solution B* with water to obtain *Standard solutions* containing a range of 2–250 µg/L Se(IV).

Sample solution: Dilute a portion of sample to a final concentration within the calibration range with water (0.2 mg/mL Se(IV)).

Chromatographic system, *Appendix IIA*

Mode: HPLC (coupled to ICP-MS with collision-reaction cell technology)

Detector: ICP-MS with collision-reaction cell technology. [NOTE—The instrument and its operational parameters are described in the *Elemental spectrometric system* section.]

Column: 4.1-mm × 250-mm, 10-µm particle size anion exchange; with microporous substrate, fully functionalized with trimethylamine groups.[9]

Column temperature: Ambient

Injection volume: 100 µL

Flow rate: 1.0 mL/min

Elemental spectrometric system, ***Appendix IIIC***

[7] Available from Inorganic Ventures, catalog numbers: CGSE(4)1-1, CGSE(4)1-2, or CGSE(4)1-5, Christiansburg, VA 24073; or equivalent.

[8] Available from Inorganic Ventures, catalog numbers: CGSE(6)1-1 or CGSE(6)1-5, Christiansburg, VA 24073; or equivalent.

[9] Hamilton model PRP-X100 Anion Exchange, PN 79433, available from Hamilton Company, 4970 Energy Way, Reno, NV 89502 USA; or equivalent.

Mode: ICP using collision-reaction cell technology (CCT) mode. [NOTE—The spray chamber of the instrument should be cooled to 2° with a Peltier-type cooling device.]
Detector: Mass spectrometer
ICP RF power: 1400 W
Flow rates
Plasma argon: 13 L/min
Auxiliary argon: 0.7 L/min
Nebulizer gas: 0.84 L/min
CCT gas: 7% hydrogen in helium; 5.35 mL/min
Extraction lens voltage: −161 V
Pole bias: −16 V
Hexapole bias: −20 V
Data mode: Time-resolved analysis
Calibration: Separately introduce each of the *Standard solutions* to the ICP-MS according to the instructions supplied with the instrument. [NOTE—Chromatography is not to be performed on the *Standard solutions*.] Use an aliquot of water as a standard solution with a Se(IV) and Se(VI) concentration of 0 µg/L. Record the results and construct a calibration curve with the integrated peak area (of the species intensity) on the *y*-axis and the concentration of Se(IV) or Se(VI) in the *Standard solutions* on the *x*-axis (µg/L).
Analysis: Inject the *Sample solution* into the chromatograph and record the resulting chromatogram. The approximate retention times for Se(IV) and Se(VI) are 187,000 ms (3.1 min) and 437,000 ms (7.3 min). Record the results of the ICP-MS analysis of Se(IV) and Se(VI), in µg/mL, according to the calibration curve previously generated. Calculate the amount of Se(IV) or Se(VI), as needed, in the sample taken (µg/g):

$$\text{Result} = r_U/C_U$$

r_U = concentration of Se(IV) or Se(VI) in the *Sample solution*, as determined from comparison of the ICP-MS intensity of the *Sample solution* to the calibration curve (µg/L)

C_U = concentration of the *Sample solution* (mg/mL)

SPECTROPHOTOMETRIC IDENTIFICATION TESTS

Spectrophotometric tests contribute meaningfully toward the identification of many compendial chemical substances. The test procedures that follow are applicable to substances that absorb IR and/or UV radiation. The IR absorption spectrum of a substance, compared with that obtained concomitantly for the corresponding USP Reference Standard, provides perhaps the most conclusive evidence of the identity of the substance that can be realized from any single test. The UV absorption spectrum, on the other hand, does not exhibit a high degree of specificity. Conformance with both IR absorption and UV absorption test specifications leaves little doubt, if any, regarding the identity of the specimen under examination.

• **Infrared Spectra**

This test is used for comparison of an IR spectrum for a sample specimen with a reference spectrum provided in the individual monograph.

Sample specimens should be prepared using the same technique as that used for the provided spectra. Unless otherwise noted in the individual monograph or spectrum caption, the spectra for liquid substances were obtained on neat liquids contained in fixed-volume sodium chloride cells or between salt plates. Spectra for solid substances were obtained on a potassium bromide pellet or a mineral oil (Nujol or equivalent) dispersion, as indicated in individual monographs or spectrum caption.

• **Infrared Absorption**

This test is used for comparison of the IR spectrum of a sample specimen with that of a physical USP Reference Standard.

Sample and USP Reference Standard specimens should both be prepared for analysis using the same technique, as directed in the individual monographs, which use the below letter designations. Sample and USP Reference Standard specimens should be used as either dried or undried specimens as directed on the Reference Standard Label.

Record the spectra of the sample and USP Reference Standard specimens over the range of about 2.6–15 µm (3800 cm^{-1} to 650 cm^{-1}) unless otherwise specified in the individual monograph.

Designation	Specimen Preparation Technique
A	Intimately in contact with an internal reflection element for attenuated total reflectance (ATR) analysis
E	Pressed as a thin sample against a suitable plate for IR microscopic analysis
F	Suspended neat between suitable (for example sodium chloride or potassium bromide) plates
K	Mixed intimately with potassium bromide and compressed into a translucent pellet
M	Finely ground and dispersed in mineral oil
S	A solution of designated concentration, prepared as directed in the individual monograph, and examined in 0.1-mm cells (unless otherwise specified in the individual monograph)

[NOTE—*A* and *E* techniques can be used as alternative methods for *K*, *M*, *F*, and *S* where testing is performed qualitatively and the Reference Standard spectra are similarly obtained.]

Differences that may be observed in the IR spectra obtained for the analyte and the standard sometimes are attributed to the presence of polymorphs, which are not always acceptable. Polymorphism gives rise to variations in the IR spectra of many compounds in the solid state. Frequently, small differences in structure result in significant differences in the spectra. Unless otherwise directed in the individual monograph, therefore, continue as follows. If a difference appears in the IR spectra of the analyte and the standard, dissolve equal portions of the test specimen and the Reference Standard in equal volumes of a suitable solvent, evaporate the solution to dryness in similar containers under identical conditions, and repeat the test on the residues.

• **Ultraviolet Absorption**

The test is used for comparison of the UV spectrum of a sample specimen with that of a physical USP Reference Standard.

Sample and USP Reference Standard specimens should be prepared using the solvents and concentrations specified in the individual monograph.

Record the spectra of the sample and USP Reference Standard solutions concomitantly using 1-cm cells over the spectral range of 200–400 nm, unless otherwise indicated in the individual monograph. Calculate absorptivities and/or absorbance ratios where these criteria are included in an individual monograph. Unless otherwise specified, absorbances indicated for these calculations are those measured at the maximum absorbance at the wavelength specified in the individual monograph. Where the absorbance is to be measured at a wavelength other than that of maximum absorbance, the abbreviations (min) and (sh) are used to indicate a minimum and shoulder, respectively, in an absorbance spectrum.

SULFUR (by Oxidative Microcoulometry) (Based on ASTM D3120)[10]

[NOTE—All reagents used in this test should be reagent grade; water should be of high purity, and gases must be high-purity grade.]

Apparatus: Use the Dohrmann Microcoulometric Titrating System (MCTS-30), or equivalent (shown in *Figure 20*), unless otherwise specified in an individual monograph. It consists of a constant rate injector, *A*, a pyrolysis furnace, *B*, a quartz pyrolysis tube, *C*, a granular-tin scrubber, *D*, a titration cell, *E*, and a microcoulometer with a digital readout, *F*.

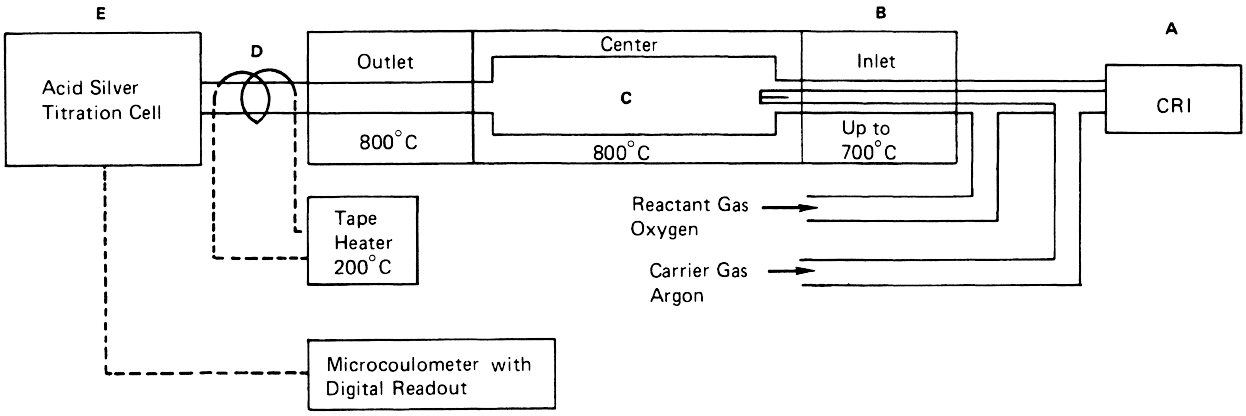


Figure 20. Microcoulometric Titrating System for the Determination of Sulfur in Hexanes

Granular-tin scrubber: Place 5 g of 20- to 30-mesh granular reagent-grade tin between quartz-wool plugs in an elongated 18/9-12/5 standard-taper adaptor that connects the pyrolysis tube and the titration cell.

Microcoulometer: Must have variable attenuation; gain control; and be capable of measuring the potential of the sensing-reference electrode pair, and comparing this potential with a bias potential, amplifying the potential difference, and applying the amplified difference to the working-auxiliary electrode pair to generate a titrant. The microcoulometer output voltage signal must also be proportional to the generating current.

Pyrolysis furnace: The sample should be pyrolyzed in an electric furnace having at least two separate and independently controlled temperature zones, the first being an inlet section that can maintain a temperature sufficient to volatilize all the organic sample. The second zone is a pyrolysis section that can maintain a temperature sufficient to pyrolyze the organic matrix and oxidize all the organically bound sulfur. A third outlet temperature zone is optional.

Pyrolysis tube: Must be fabricated from quartz and constructed in such a way that a sample, which is vaporized completely in the inlet section, is swept into the pyrolysis zone by an inert gas where it mixes with oxygen and is burned. The inlet end of the tube shall hold a septum for syringe entry of the sample and side arms for the introduction of oxygen and inert gases. The center or pyrolysis section should be of sufficient volume to ensure complete pyrolysis of the sample.

Sampling syringe: A microlitre syringe of 10-µL capacity capable of accurately delivering 1–10 µL of sample into the pyrolysis tube. Three-in × 24-gauge needles are recommended to reach the inlet zone of the pyroloysis furnace.

[10] Adapted from ASTM D3120 Standard Test Method for Trace Quantities of Sulfur in Light Liquid Petroleum Hydrocarbons by Oxidative Microcoulometry. The original ASTM method is available in its entirety from ASTM International, 100 Barr Harbor Drive, West Conshohocken, PA 19428. Phone: 610-832-9585, Fax: 610-832-9555, Email: service@astm.org, Website: www.astm.org.

Titration cell: Must contain a sensor-reference pair of electrodes to detect changes in triiodide ion concentration and a generator anode–cathode pair of electrodes to maintain constant triiodide ion concentration and an inlet for a gaseous sample from the pyrolysis tube. The sensor electrode shall be platinum foil and the reference electrode platinum wire in saturated triiodide half-cell. The generator anode and cathode half-cell shall also be platinum. The titration cell shall be placed on a suitable magnetic stirrer.

Preparation of apparatus: Carefully insert the quartz pyrolysis tube into the furnace, attach the tin scrubber, and connect the reactant and carrier-gas lines. Add the *Cell electrolyte solution* (see below) to the titration cell, and flush the cell several times. Maintain an electrolyte level of 3.2–6.6 mm above the platinum electrodes. Place the titration cell on a magnetic stirrer, and connect the cell inlet to the tin scrubber outlet. Position the platinum-foil electrodes (mounted on the movable cell head) so that the gas-inlet flow is parallel to the electrodes with the generator anode adjacent to the generator cathode. Assemble and connect the coulometer in accordance with the manufacturer's instructions. Double-wrap the adaptor containing the tin scrubber with heating tape and turn the heating tape on. Adjust the flow of the gases, the pyrolysis furnace temperature, the titration cell, and the coulometer to the desired operating conditions. Typical operating conditions are as follows:

Reactant gas flow (oxygen), cm^3/min	200
Carrier-gas flow (Ar, He), cm^3/min	40
Furnace temperature, °C Inlet zone	700 (maximum)
Pyrolysis zone	800–1000
Outlet zone	800 (maximum)
Tin-scrubber temperature, °C	200
Titration cell	Stirrer speed set to produce slight vortex
Coulometer	
Bias voltage, mV	160
Gain	50
Constant Rate Injector, µL/s	0.25

The tin scrubber must be conditioned to sulfur, nitrogen, and chlorine before quantitative analysis can be achieved. A solution containing 10 mg/kg of butyl sulfide, 100 mg/kg of pyridine, and 200 mg/kg of chlorobenzene in isooctane has proven an effective conditioning agent. With a fresh scrubber installed and heated, two 30-µL samples of this conditioning agent injected at a flow rate of 0.5 µL/s produce a steadily increasing response, with final conditioning indicated by a constant reading from the offset during the second injection.

Reagents

Argon or helium (argon preferred): High-purity grade, used as the carrier gas. Two-stage gas regulators must be used.

Cell electrolyte solution: Dissolve 0.5 g of potassium iodide and 0.6 g of sodium azide in 500 mL of high-purity water, add 5 mL of glacial acetic acid, and dilute to 1 L. Store in a dark bottle or in a dark place and prepare fresh at least every 3 months.

Oxygen: High-purity grade, used as the reactant gas

Iodine: Resublimed, 20-mesh or less, for saturated reference electrode

Sulfur standard (approximately 100 mg/kg): Transfer 0.1569 g of *n*-butyl sulfide, accurately weighed, into a tared 500-mL volumetric flask. Dilute to the mark with isooctane, and reweigh. Calculate the sulfur concentration (S), as a percentage:

$$S = W_b/W_s \times 2.192 \times 10^5$$

in which W_b is the weight of *n*-butyl sulfide and W_s is the weight of the solution.

Calibration: Prepare a calibration standard (approximately 5 mg/kg) by pipetting 5 mL of *Sulfur standard* into a 10-mL volumetric flask and diluting with isooctane to volume. Fill and clamp the syringe onto the constant-rate injector, push the sliding carriage forward to penetrate the septum with the needle, and zero the meter in case of long-term drift in the automatic baseline zero circuitry. Switch S_1 automatically starts the stepper-motor syringe drive and initiates the analysis cycle. At 2.5 min (before the 3-min meter hold point) set the digital meter with the scan potentiometer to correspond to the sulfur content of the known standard to the nearest 0.01 mg/kg. At the 3-min point, the number displayed on the meter stops, the plunger drive block is retracted to its original position, as preset by switch S_2, and a baseline re-equilibration period equal to the injection period elapses before a ready light and a beeper indicate that a new sample may be injected. Repeat the *Calibration* step a total of at least four times.

Procedure: Rinse the syringe several times with sample; then fill it, clamp it onto the constant-rate injector, push the sliding carriage forward to penetrate the septum with the needle, and zero the meter. Turn on switch S_1 to start the stepper-motor syringe drive automatically and initiate the analysis cycle. After the 3-min hold point, the number displayed on the meter corresponds to the sulfur content of the injected sample.

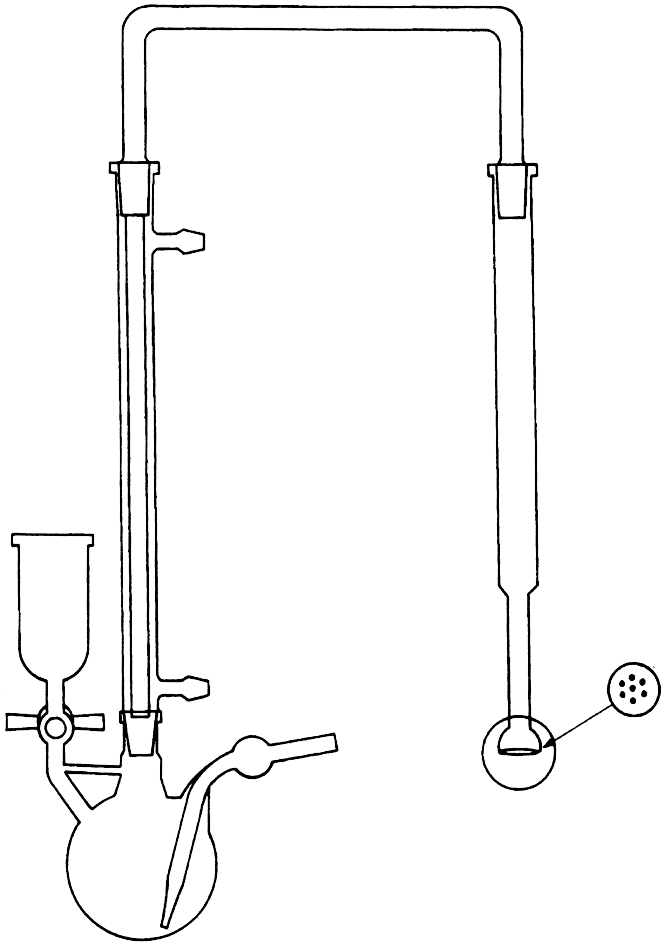


Figure 21. Raney Nickel Reduction Apparatus

APPENDIX XIII: ADULTERANTS AND CONTAMINANTS IN FOOD INGREDIENTS

Tests contained within this Appendix are not monograph requirements, but rather are provided as informational methods for *FCC* users.

DIETHYLENE GLYCOL AND ETHYLENE GLYCOL IN GLYCERIN[1]

This method was developed for the identification and quantification of low levels of ethylene glycol and diethylene glycol in glycerin.

Standard solution: 0.025 mg/mL of USP Ethylene Glycol RS, 0.025 mg/mL of USP Diethylene Glycol RS, 50 mg/mL of USP Glycerin RS, and 0.05 mg/mL of 2,2,2-trichloroethanol (internal standard) in methanol

Sample solution: 50 mg/mL of sample and 0.05 mg/mL of 2,2,2-trichloroethanol (internal standard) in methanol

Chromatographic system, Appendix IIA

Mode: GC

Detector: Flame ionization

Column: 0.53-mm × 30-m fused-silica analytical; coated with 3.0-µm 6% cyanopropylphenyl–94% dimethylpolysiloxane stationary phase[2]

Temperatures

Injector: 220°

Detector: 250°

Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
100	—	100	4
100	50	120	10
120	50	220	6

Carrier gas: Helium

Injection volume: 1.0 µL

Flow rate: 4.5 mL/min

Injection type: Split flow ratio is about 10:1.

System suitability

Sample: *Standard solution*

Suitability requirements

Resolution: NLT 1.5 between diethylene glycol and glycerin

Analysis: Separately inject equal volumes of the *Standard solution* and *Sample solution* into the chromatograph, and measure the responses for the major peaks on the resulting chromatograms. Diethylene glycol and ethylene glycol can be identified in the *Sample solution* on the basis of peak retention times compared to those in the *Standard solution*. [NOTE—The relative retention times for ethylene glycol, 2,2,2-trichloroethanol, diethylene glycol, and glycerin are about 0.3, 0.6, 0.8, and 1.0, respectively. See *Figure 1* for example chromatograms.] The percentages of diethylene glycol and ethylene glycol in the portion of sample taken are calculated using the following formula:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = analyte relative response (analyte peak response/2,2,2-trichloroethanol peak response) from the *Sample solution*

R_S = analyte relative response (analyte peak response/2,2,2-trichloroethanol peak response) from the *Standard solution*

C_S = concentration of analyte in the *Standard solution* (mg/mL)

C_U = concentration of sample in the *Sample solution* (mg/mL)

Performance characteristics

Limit of quantitation: 0.025% (w/w) for ethylene glycol and diethylene glycol

Range: 0.013–0.031 mg/mL for ethylene glycol and 0.012–0.030 mg/mL for diethylene glycol. [NOTE—Wider linear ranges may be achievable but were not investigated when developing this method.]

Accuracy: 99%–107% recovery from samples spiked with diethylene glycol and ethylene glycol at levels of 50%–120%

Precision: Instrument precision: Less than 3.0% RSD; repeatability less than 4.0% RSD for analysis in the 50%–120% range

[1] Based on the following published method: Holloway G., Maheswaran R., Bradby, S., and Wahab, S. 2010. Screening method for ethylene glycol and diethylene glycol in glycerin-containing products. *J Pharm Biomed Anal.* 51:507-511.

[2] DB-624 (J & W Scientific), or equivalent.

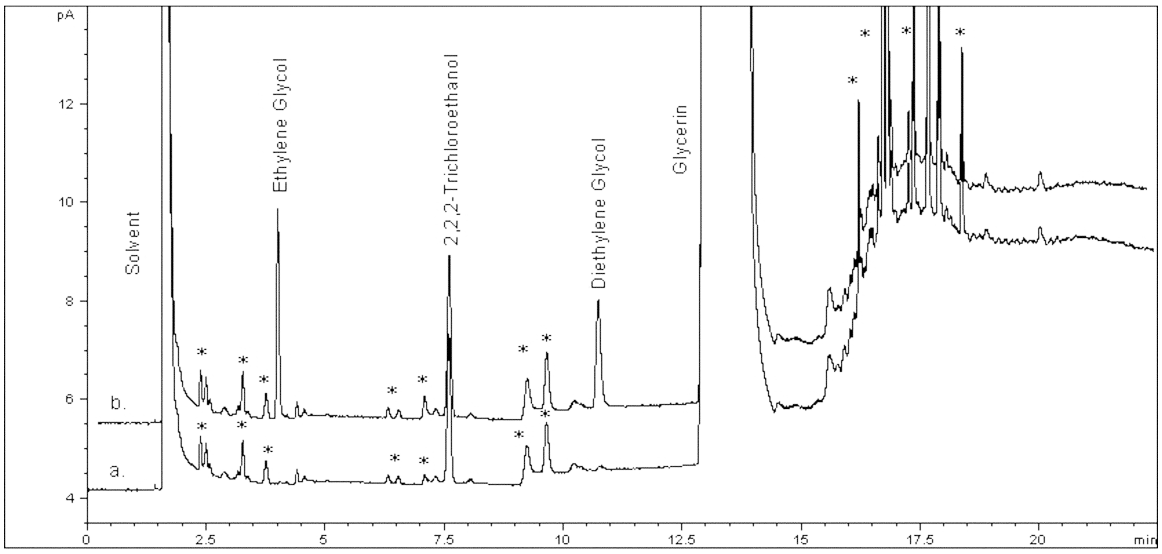


Figure 1. Overlay chromatogram of (a) *Sample solution* and (b) *Standard solution*. *Peaks from solvent and glycerin.

PESTICIDE RESIDUES

The methods and information contained in this section are designed to measure pesticide residues in food ingredients of botanical origin.

• General Method for Pesticide Residues Analysis

Definition

Where used in this compendium, the designation pesticide applies to any substance or mixture of substances intended to prevent, destroy, or control any unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage, transport, or marketing of pure articles. The designation includes substances intended for use as growth regulators, defoliants, or desiccants, and any substance applied to crops before or after harvest to protect the product from deterioration during storage and transport.

Limits

Limits for pesticides for foods are determined by the Environmental Protection Agency (EPA), and where no limit is set, the limit is zero. The limits contained in *Table 1*, therefore, may not be applicable in the United States and are provided for guidance purposes only, and not for the purpose of meeting a regulatory requirement in the United States. The limits may be applicable in other countries where the presence of pesticide residues is permitted. Unless otherwise specified in the individual monograph, the article under test contains NMT the amount of any pesticide indicated in *Table 1*.

Table 1

Substance	Limit (mg/kg)
Alachlor	0.02
Aldrin and Dieldrin (sum of)	0.05
Azinphos-methyl	1.0
Bromopropylate	3.0
Chlordane (sum of cis- and trans- isomers and oxychlordane)	0.05
Chlorfenvinphos	0.5
Chlorpyrifos	0.2
Chlorpyrifos-methyl	0.1
Cypermethrin (and isomers)	1.0
DDT (sum of *p,p′*-DDT, *o,p′*-DDT, *p,p′*-DDE, and *p,p′*-TDE)	1.0
Deltamethrin	0.5
Diazinon	0.5
Dichlorvos	1.0

Table 1 *(continued)*

Substance	Limit (mg/kg)
Dithiocarbamates (as CS_2)	2.0
Endosulfan (sum of endosulfan isomers and endosulfan sulfate)	3.0
Endrin	0.05
Ethion	2.0
Fenitrothion	0.5
Fenvalerate	1.5
Fonofos	0.05
Heptachlor (sum of heptachlor and heptachlor epoxide)	0.05
Hexachlorobenzene	0.1
Hexachlorocyclohexane isomers (other than γ)	0.3
Lindane (γ-hexachlorocyclohexane)	0.6
Malathion	1.0
Methidathion	0.2
Parathion	0.5
Parathion-methyl	0.2
Permethrin	1.0
Phosalone	0.1
Piperonyl butoxide	3.0
Pirimiphos-methyl	4.0
Pyrethrins (sum of)	3.0
Quintozene (sum of quintozene, pentachloroaniline and methyl pentachlorophenyl sulfide)	1.0

Sampling

For articles in containers holding less than 1 kg, mix the contents, and withdraw a quantity sufficient for the tests. For articles in containers holding between 1 and 5 kg, withdraw equal portions from the upper, middle, and lower parts of the container, each of the samples being sufficient to carry out the tests. Thoroughly mix the samples, and withdraw an amount sufficient to carry out the tests. For containers holding more than 5 kg, withdraw three samples, each weighing NLT 250 g, from the upper, middle, and lower parts of the container. Thoroughly mix the samples, and withdraw a portion sufficient to carry out the tests.

If the number of the containers, n, is three or fewer, withdraw samples from each container as indicated above. If the number of containers is more than three, take samples from $\sqrt{n} + 1$ containers, rounding up to the nearest whole number if necessary.

[NOTE—Conduct tests without delay to avoid possible degradation of the residues. If this is not possible, store the samples in hermetic containers suitable for food contact, at a temperature below 0, and protected from light.]

Reagents

Use reagents and solvents that are free from any contaminants, especially pesticides, that might interfere with the analysis. It is often necessary to use special grade solvents suitable for pesticide residue analysis or solvents that have recently been redistilled in an apparatus made entirely of glass. In any case, suitable blank tests must be performed.

Preparation of Apparatus

Clean all equipment, especially glassware, to ensure that it is free from pesticides. Soak all glassware for a minimum of 16 h in a solution of phosphate-free detergent, rinse with copious quantities of distilled water, and then wash with acetone, followed by hexane or heptane.

Qualitative and Quantitative Analysis of Pesticide Residues

Use validated analytical procedures that satisfy the following criteria. The method, especially with respect to its purification steps, is suitable for the combination of pesticide residue and substance under test, and is not susceptible to interference from co-extractives. Measure the limits of detection and quantification for each pesticide matrix combination to be analyzed: the method is shown to recover between 70% and 110% of each pesticide; the repeatability and reproducibility of the method are NLT the appropriate values indicated in *Table 2*; and the concentrations of test and reference solutions and the setting of the apparatus are such that a linear response is obtained from the analytical detector.

Table 2

Concentration of Pesticide (mg/kg)	Repeatability (difference, ± mg/kg)	Reproducibility (difference, ± mg/kg)
0.010	0.005	0.01
0.100	0.025	0.05
1.000	0.125	0.25

• TEST FOR PESTICIDES

Depending on the substance being examined, it may be necessary to modify, sometimes extensively, the procedure described hereafter. Additionally, it may be necessary to perform another method with another column having a different polarity, another detection method (e.g., mass spectrometry), or a different method (e.g., immunochemical method) to confirm the results.

Extraction: [NOTE—Use the following procedure for the analysis of samples and articles having a water content of less than 15%. Samples having a higher water content may be dried, provided that the drying procedure does not significantly affect the pesticide content.]

To 10 g of the coarsely powdered substance under test, add 100 mL of acetone, and allow to stand for 20 min. Add 1 mL of a solution in toluene containing 1.8 µg/mL of carbophenothion. Mix in a high-speed blender for 3 min. Filter this solution, and wash the residue with two 25-mL portions of acetone. Combine the filtrate and the washings, and heat, in a rotary evaporator, maintaining the temperature of the bath below 40° until the solvent has almost completely evaporated. To the residue add a few mL of toluene, and heat again until the acetone is completely removed. Dissolve the residue in 8 mL of toluene. Pass through a membrane filter having a 45-µm porosity, rinse the flask and the filter with toluene, and dilute with toluene to 10.0 mL. This is *Solution A*.

Purification

Organochlorine, organophosphorus, and pyrethroid insecticides: The size-exclusion chromatograph is equipped with a 7.8-mm × 30-cm stainless steel column containing 5-µm packing of styrene-divinylbenzene copolymer. Toluene is used as the mobile phase at a flow rate of 1 mL/min.

Performance of the column: Inject 100 µL of a solution in toluene containing, in each mL, 0.5 mg of methyl red and 0.5 mg of oracet blue. The column is not suitable unless the color of the eluate changes from orange to blue at an elution volume of about 10.3 mL. If necessary, calibrate the column, using a solution in toluene containing suitable concentrations of the pesticide of interest having the lowest molecular weight (for example, dichlorvos) and that having the highest molecular weight (for example, deltamethrin). Determine which fraction of the eluate contains both pesticides.

Purification of the sample solution: Inject a suitable volume (100–500 µL) of *Solution A* into the chromatograph. Collect the fraction (*Solution B*) as determined above under *Performance of the column*. Organophosphorus pesticides elute between 8.8 and 10.9 mL. Organochlorine and pyrethroid pesticides elute between 8.5 and 10.3 mL.

Organochlorine and pyrethroid insecticides: Into a 5-mm × 10-cm chromatographic column, introduce a piece of fat-free cotton and 0.5 g of silica gel treated as follows. Heat chromatographic silica gel in an oven at 150° for NLT 4 h. Allow to cool, and add dropwise a quantity of water corresponding to 1.5% of the weight of the silica gel used. Shake vigorously until agglomerates have disappeared, and continue shaking by mechanical means for 2 h. Condition the column with 1.5 mL of hexane.

[NOTE—Prepacked columns containing about 0.50 g of a suitable silica gel may also be used, provided they have been previously validated.]

Concentrate *Solution B* almost to dryness, with the aid of a stream of helium or oxygen-free nitrogen, and dilute with toluene to a suitable volume (200 µL to 1 mL, according to the volume injected in the preparation of *Solution B*). Quantitatively transfer this solution to the column, and proceed with the chromatography, using 1.8 mL of toluene as the mobile phase. Collect the eluate (*Solution C*).

Quantitative Analysis of Organophosphorus Insecticides

Sample solution: Concentrate *Solution B* almost to dryness, with the aid of a stream of helium, and dilute with toluene to 100 µL.

Standard solution: Prepare at least three solutions in toluene containing each of the pesticides of interest and carbophenothion at concentrations suitable for plotting a calibration curve.

Chromatographic system, Appendix IIA

Mode: GC

Detector: Flame-ionization (alkali) or flame-photometric

Column: 0.32-mm × 30-m fused silica; coated with a 0.25-µm layer of dimethylpolysiloxane

Carrier gas: Hydrogen (may also use helium or nitrogen)

Temperatures

Injector: 250°

Detector: 275°

Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
80	—	80	1
80	30	150	3

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
150	4	280	1

Analysis: Use carbophenothion as the internal standard. [NOTE—If necessary, use a second internal standard to identify any possible interference with the peak corresponding to carbophenothion.] Inject the chosen volume of each solution, record the chromatograms, and measure the peak responses. [NOTE—The approximate relative retention times are listed in *Table 3*.] Calculate the content of each pesticide from the peak areas and the concentrations of the solution.

Table 3

Substance	Relative Retention Time
Dichlorvos	0.20
Fonofos	0.50
Diazinon	0.52
Parathion-methyl	0.59
Chlorpyrifos-methyl	0.60
Pirimiphos-methyl	0.66
Malathion	0.67
Parathion	0.69
Chlorpyrifos	0.70
Methidathion	0.78
Ethion	0.96
Carbophenothion	1.00
Azinphos-methyl	1.17
Phosalone	1.18

Quantitative Analysis of Organochlorine and Pyrethroid Insecticides

Sample solution: Concentrate *Solution C* almost to dryness, with the aid of a stream of helium or oxygen-free nitrogen, and dilute with toluene to 500 µL.

Standard solution: Prepare at least three solutions in toluene containing each of the pesticides of interest and carbophenothion at concentrations suitable for plotting a calibration curve.

Chromatographic system, Appendix IIA

Mode: GC

Detector: Electron capture

Column: 0.32-mm × 30-m fused silica; coated with a 0.25-µm layer of dimethylpolysiloxane

Carrier gas: Hydrogen (may also use helium or nitrogen)

Temperatures

Injector: 275°

Detector: 300°

Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
80	—	80	1
80	30	150	3
150	4	280	1

Analysis: Use carbophenothion as the internal standard. [NOTE—If necessary, use a second internal standard to identify any possible interference with the peak corresponding to carbophenothion.] Inject the chosen volume of each solution, record the chromatograms, and measure the peak responses. [NOTE—The approximate relative retention times are listed in *Table 4*.] Calculate the content of each pesticide from the peak areas and the concentrations of the solution.

Table 4

Substance	Relative Retention Time
α-Hexachlorocyclohexane	0.44

Table 4 *(continued)*

Substance	Relative Retention Time
Hexachlorobenzene	0.45
β-Hexachlorocyclohexane	0.49
Lindane	0.49
δ-Hexachlorocyclohexane	0.54
ε-Hexachlorocyclohexane	0.56
Heptachlor	0.61
Aldrin	0.68
cis-Heptachlor epoxide	0.76
o,p′-DDE	0.81
α-Endosulfan	0.82
Dieldrin	0.87
p,p′-DDE	0.87
o,p′-DDD	0.89
Endrin	0.91
β-Endosulfan	0.92
o,p′-DDT	0.95
Carbophenothion	1.00
p,p′-DDT	1.02
cis-Permethrin	1.29
trans-Permethrin	1.31
Cypermethrin[a]	1.40
Fenvalerate[a]	1.47
	1.49
Deltamethrin	1.54

[a] The substance shows several peaks.

Add the following:

▲ADDED COLORS IN SPICES

The methods and information contained in this section are designed to detect the presence of potentially undeclared colors in spices and spice mixes.

• NON-TARGETED SCREENING FOR SYNTHETIC COLORS IN SPICES USING THIN-LAYER CHROMATOGRAPHY (TLC)

Principle: Thin-layer chromatography and paper chromatography are two commonly used methods to detect colors in foods. To protect food integrity and public health, a non-targeted screening method for colors added to spices or spice blends using thin-layer chromatography is introduced. This method examines the chromatogram of authentic spices and determines whether there are abnormal color peaks in the samples present under visual light. For separation and identification of different colors, three different procedures were developed and validated. Users should test a sufficient number of samples to establish chromatograms of authentic spices or spice blends. Users may choose to run one or more of the following procedures depending on the specific colors suspected as adulterants. If no information is available on specific added colors, users may wish to perform all three procedures. Chromatograms of spices or spice blends that do not match the established sample results may warrant further examination using more specific techniques. The spots on the chromatogram can be compared to the spots from the colors listed in *Table 5* to identify colors. The detection limit for the colors listed is 1000 ppm. The detection limit can be extended to lower levels with supportive experimental data.

Scope: This method is applicable to synthetic colors in paprika, chili powder, turmeric, curry, and sumac.

Sample solution: Transfer 2 g of the powdered spice sample into a 50-mL amber conical centrifuge tube, add 10 mL of water and 10 mL acetonitrile, then add 4 g of magnesium sulfate, 1 g of sodium chloride, and 1.5 g of sodium citrate dihydrate. Shake the tube at 400 rpm for 15 min, centrifuge at 1789 × g for 10 min, and collect the supernatant as the *Sample solution*.

Diluent: Acetonitrile and water (8:2, v/v)

Color stock solutions: Prepare individual solutions of each of the following colors. Transfer 2 mg of the color into a 20-mL volumetric flask and add 5 mL of water to dissolve with mixing and sonication. Dilute with acetonitrile to volume. For Sudan III, Sudan IV, and Para red, use acetone in place of water and acetonitrile to dissolve and dilute the colors.

Table 5. Colors

Colors	CAS
Acid black 1	1064-48-8
Orange II (Acid orange 7)	633-96-5
Ponceau 4R	2611-82-7
Yellow 5 (Tartrazine)	1934-21-0
Allura red AC (Food red 17)	25956-17-6
Carmoisine (Azorubine)	3567-69-9
Brilliant black BN	2519-30-4
Yellow 6 (Food yellow 3)	2783-94-0
Metanil yellow (Acid yellow 36)	587-98-4
Sudan I	842-07-9
Sudan II	3118-97-6
Sudan III	85-86-9
Sudan IV	85-83-6
Para red	6410-10-2
Rhodamine B	81-88-9
Auramine O	2465-27-2
Amaranth (Acid red 27)	915-67-3

Procedure 1

Thin-Layer Chromatography, Appendix IIA

Adsorbent: 0.2-mm layer of reverse-phase modified silica[3]

Mixed color solution A: Transfer 1.0 mL of each of the *Color stock solutions* for yellow 5, pouceau 4R, carmoisine, and para red into a 10-mL volumetric flask, and dilute with *Diluent* to volume.

Mixed color solution B: Transfer 1.0 mL of each of the above *Color stock solutions* for brilliant black BN, rhodamine B, and allura red AC into a 10-mL volumetric flask and dilute with *Diluent* to volume.

Application volume: 10 µL

Developing solvent system: 1-Butanol, methyl ethyl ketone, ammonia, and water (125:75:25:50, v/v/v/v)

Analysis: Following development, remove the plate from the chamber, dry thoroughly in air, and visualize under white light. [NOTE—See *Table 6* for the approximate R_F values of the colors.]

Table 6

Color	R_F Value
Yellow 5 (Tartrazine)	0.42
Brilliant black BN	0.44
Ponceau 4R	0.55
Carmoisine (Azorubine)	0.64
Rhodamine B	0.66
Para red	0.72
Allura red AC (Food red 17)	0.76

Acceptance criteria: The spots from *Mixed color solution A* and *Mixed color solution B* are absent from the chromatogram of the *Sample solution*.

Procedure 2

Thin-Layer Chromatography, Appendix IIA

Adsorbent: 0.2-mm layer of silica[4]

Mixed color solution C: Transfer 1.0 mL of each of the *Color stock solutions* for Sudan I, Sudan II, Sudan III, and Sudan IV into a 10-mL volumetric flask and dilute with *Diluent* to volume.

Application volume: 10 µL

Developing solvent system: *n*-Hexane and ethyl acetate (85:15, v/v)

[3] ▲Merck TLC Silica gel 60 RP-18 F_{254}s, available at Millipore Sigma (www.emdmillipore.com), catalog #115685, or equivalent.▲ *(FCC 12)*

[4] ▲Merck TLC Silica gel 60 F_{254}, available at Millipore Sigma (www.emdmillipore.com), catalog #105554, or equivalent.▲ *(FCC 12)*

Analysis: Following development, remove the plate from the chamber, dry thoroughly in air, and visualize under white light. [NOTE—See *Table 7* for the approximate R_F values of the colors.]

Table 7

Color	R_F Value
Sudan III	0.27
Sudan IV	0.31
Sudan I	0.35
Sudan II	0.39

Acceptance criteria: The spots from the *Mixed color solution C* are absent from the chromatogram of the *Sample solution*.

Procedure 3

Thin-Layer Chromatography, Appendix IIA

Adsorbent: 0.2-mm layer of silica[4]

Mixed color solution D: Transfer 1.0 mL of each of the *Color stock solution* for amaranth, yellow 6, acid black, orange II, metanil yellow, and auramine O into a 10-mL volumetric flask and dilute with *Diluent* to volume.

Application volume: 10 µL

Developing solvent system: 1-Butanol, water, and formic acid (40:15:10, v/v/v)

Analysis: Following development, remove the plate from the chamber, dry thoroughly in air, and visualize under white light. [NOTE—See *Table 8* for the approximate R_F values of the colors.]

Table 8

Color	R_F Value
Amaranth (Acid red 27)	0.08
Yellow 6 (Food yellow 3)	0.24
Acid black 1	0.38
Orange II (Acid orange 7)	0.52
Metanil yellow (Acid yellow 36)	0.58
Auramine O	0.67

Acceptance criteria: The spots from *Mixed color solution D* are absent from the chromatogram of the *Sample solution*.▲ *(FCC 12)*