Key to interpreting proposals

Proposed revisions are shown with language proposed for deletion or replacement crossed off. New text (if any) follows, and is enclosed in symbols as shown in the examples below:

▲new text▲FCC 6

if slated for FCC 6; and

■new text■1S (FCC 6)

if slated for a Supplement to FCC. The same symbols not set off by an extra paragraph break and enclosing text with no increase in type size indicate recent revisions that are already official. Where the symbols appear together with no enclosed text, such as ■ or ▲, it means that text has been deleted and no new text was proposed to replace it. In all revisions, the closing symbol is accompanied by an identifier that indicates the particular Supplement or indicates the FCC as the publication where the revision will appear if approved. For example, ■1S (FCC 6) indicates that the proposed revision is slated for the First Supplement to FCC 6, and ▲FCC 6 indicates that the revisions are proposed for FCC 6.
Alginate-Konjac-Xanthan Polysaccharide Complex. Because there is no existing FCC monograph for this food ingredient, a new monograph is proposed based on comments and data received. This ingredient represents a product which is chemically described as a complex formed from three food ingredients: sodium alginate, konjac flour, and xanthan gum. The proposed monograph is designed to specifically differentiate this ingredient from these three components (either as single products or as simple blends) and from other food ingredients with similar functionalities.

1. **Identification** test A. **Viscometric Characterization** and the **Assay** require viscosity measurements and comparisons to USP Reference Standards, and represent a new use of viscosity determinations for FCC monographs. Comments about the applicability of these determinations are solicited.

2. The **Identification** tests A. Test for Absence of Konjac Flour, B. Test for Absence of Sodium Alginate, and C. Test for Absence of Xanthan Gum are versions of **Identification** tests from the individual FCC monographs for Konjac Flour, Sodium Alginate, and Xanthan Gum, respectively.

3. Comments are specifically requested about the use of largely physical parameters to characterize and assay this ingredient and to ascertain whether or not a chemical means of determining the composition of the ingredient should also be included.

*(Flish: K. Laurvick) C116063*

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**DESCRIPTION**

Alginate-Konjac-Xanthan Polysaccharide Complex occurs as an off-white to light tan granular powder. It is a complex formed by reacting sodium alginate, konjac flour from *Amorphophallus konjac*, and xanthan gum with water at an elevated temperature in a specialized fluidized bed reactor, followed by drying and sieving. The color and granular appearance and rheological properties of the reacted and dried complex are distinctly different from those of the reactants, either separately or mixed.

**Function:** Source of dietary fiber; bulking agent; thickener

**Packaging and Storage:** Store in tight containers in a cool place, protected from moisture and light.

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**IDENTIFICATION**

- **A. TEST FOR ABSENCE OF KONJAC FLOUR**
  - **Sample solution:** 10 mg/mL
  - **Analysis:** At room temperature add 5 mL of a 40-mg/mL sodium borate solution to 5 mL of the **Sample solution** in a test tube, and shake vigorously.
  - **Acceptance criteria:** No gel forms in the test tube. [Note—Konjac flour solutions gel in the presence of sodium borate, as do guar gum and locust bean gum solutions.]

- **B. TEST FOR ABSENCE OF SODIUM ALGINATE**
  - **Sample solution:** 10 mg/mL
  - **Analysis:** To 10 mL of the **Sample solution** add 2 mL of calcium chloride TS. Allow the mixture to stand for
30 min at room temperature.

**Acceptance criteria:** After 30 min there is no observed precipitate.  
[Note—Sodium alginate solutions form a voluminous, gelatinous precipitate upon addition of calcium ions.]

- **C. Test for Absence of Xanthan Gum**
  
  **Sample:** 1.5 g
  
  **Analysis:** Transfer 300 mL of water (at 80°C) to a 400-mL beaker, and stir rapidly with a mechanical stirrer. At the point of maximum agitation, add a dry blend of the Sample and 1.5 g of locust bean gum. Stir until the gum dissolves, and then continue stirring for an additional 30 min. Do not allow the temperature of the solution to drop below 60°C. Discontinue stirring after 30 min, and allow the solution to cool at room temperature for NLT 2 h.

  Similarly conduct a control analysis, replacing the Sample with 1.5 g of xanthan gum and following all subsequent preparation steps. After 2 h, compare the contents of the two beakers.

  **Acceptance criteria:** The beaker containing the xanthan gum (control analysis) forms a firm, rubbery gel after the temperature of the mixture drops below 40°C. The beaker containing the Sample does not form such a gel.

- **D. Viscometric Characterization**

  **Sample:** 7.5 g; weigh material out, and place it in a desiccator until analysis.

  **Standard:** 7.5 g of USP Alginate-Konjac-Xanthan Polysaccharide Complex RS; weigh material out, and place it in a desiccator until analysis.

  **Control:** 7.5 g of USP Blended Polysaccharide for Viscosity Identification RS; weigh material out, and place it in a desiccator until analysis.

  **Analysis of Sample and Standard:** Set up for analysis a Brookfield-type digital viscometer with the appropriate spindle. Use only equipment that has been calibrated according to the manufacturer's instructions. (At a minimum, perform a calibration using ASTM reference oil N4000 on a weekly basis. This reference oil is available from multiple suppliers.) Place the viscometer above a temperature-controlled water bath held at 30°C ± 0.5°C. Position a mechanical (overhead) stirrer with a 4-blade propeller on a stir shaft in such a way that it can vigorously stir reaction mixtures that will be in the water bath. Use a mechanical stirrer capable of maintaining a speed of 1500 rpm. Initialize the viscometer to run with the spindle specified at 1.0 rpm, and autozero the viscometer, if necessary, according to the manufacturer's instructions.

  Weigh 1500 g of water into a 2-L beaker, and place the beaker in the 30°C water bath under the mechanical stirrer (with the propeller in the beaker) until the temperature of the water in the beaker is 30 ± 0.5°C. Set up a laboratory timer for 5 h (300 min). Start the stirrer at 1500 rpm to produce a vortex inside the beaker. Start the laboratory timer, immediately add the Sample to the beaker, and stir until time equals 20 s to ensure dispersion of the Sample. Without stopping the timer, turn off the stirrer, and lower the viscometer spindle into the mixture, ensuring that the viscometer bob is correctly positioned according to the instructions provided by the manufacturer of the viscometer and spindle. Take the first viscosity reading at time equals 1 min, and immediately turn the viscometer off in order for the spindle to return to zero. For the second reading (at time equals 3 min), restart the stirrer 30 s before the desired reading time, continue stirring for exactly 10 s, turn the stirrer off, allow 20 s for the contents of the beaker to settle, then restart the viscometer, and take the viscosity reading at time equals 3 min. Repeat the steps taken for the second reading for subsequent viscosity readings at time equals 4, 5, 10, 15, 20, 25, and 30 min. Record all viscosity readings in cP.

  Immediately after taking the reading at 30 min, turn off and remove the viscometer from the solution in order for the spindle to return to zero for subsequent readings.  
[Note—This step is required due to the
high viscosity and non-Newtonian viscometric behavior exhibited by the solutions containing the Sample and the Standard. Once the viscometer is removed from the solution, blend the mixture by hand, stirring vigorously for 20 s using a wide spatula, because the mixture will be too viscous for the stirrer to fully blend the contents of the beaker. After stirring, immediately lower the cleaned, dried viscometer bob into the solution, and allow the contents of the beaker to settle. At time equals 45 min, restart the viscometer, and record the viscosity of the mixture. Repeat this procedure for subsequent viscosity readings at time equals 60, 120, 180, 240, and 300 min.

Repeat the entire procedure using the Standard in place of the Sample.

Analysis of Control: Use the same type of equipment (prepared as directed) as referenced in the Analysis of Sample and Standard. Weigh 1500 g of water into a 2-L beaker, and place the beaker in the 30°C water bath under the mechanical stirrer (with the propellor in the beaker) until the temperature of the water in the beaker is 30 ± 0.5°C. Set up a laboratory timer for 5 h (300 min). Start the stirrer at 1500 rpm to produce a vortex inside the beaker. Start the laboratory timer, immediately add the Control to the beaker, and stir until time equals 20 s to ensure dispersion of the Control. Without stopping the timer, turn off the stirrer, and lower the viscometer spindle into the mixture, ensuring that the viscometer bob is correctly positioned according to the instructions provided by the manufacturer of the viscometer and spindle. Take the first viscosity reading at time equals 1 min, and immediately turn the viscometer off in order for the spindle to return to zero. For the second reading (at time equals 3 min), restart the stirrer 30 s before the desired reading time, continue stirring for exactly 10 s, turn the stirrer off, allow 20 s for the contents of the beaker to settle, then restart the viscometer, and take the viscosity reading at time equals 3 min. Repeat the steps taken for the second reading for subsequent viscosity readings at time equals 4 and 5 min.

Immediately after taking the reading at 5 min, turn off and remove the viscometer from the solution in order for the spindle to return to zero for subsequent readings. Once the viscometer is removed from the solution, blend the mixture by hand, stirring vigorously for 20 s using a wide spatula, because the mixture will be too viscous for the stirrer to fully blend the contents of the beaker. After stirring, immediately lower the cleaned, dried viscometer bob into the solution, and allow the contents of the beaker to settle. At time equals 10 min, restart the viscometer, and record the viscosity of the mixture. Repeat this procedure for subsequent viscosity readings at time equals 15, 20, 25, 30, 45, 60, 120, 180, 240, and 300 min.

Graph the results of the Sample, Standard, and Control analyses with viscosity, in cP, on the y-axis, and time, in min, on the x-axis.

Acceptance criteria: The viscosity curve of the solution containing the Sample should have slopes, maxima, and minima that are consistent with those from the curve of the solution containing the Standard. The viscosity curve for the solution containing the Control shows a distinctly higher viscosity than the Sample and the Standard from the beginning of the experiment until at least time equals 120 min. The viscosity of all solutions levels off to a constant viscosity after time equals 120 min.

ASSAY

• Viscosity Over Time

Analysis: Use the data obtained from the Sample tested according to Identification test D. Viscometric Characterization.

Acceptance criteria

Viscosity at 60 min: 16,400–21,700 cP
Viscosity at 120 min: 22,700–31,200 cP
IMPURITIES

Inorganic Impurities

- **Arsenic**, *Elemental Impurities by ICP*, Appendix IIIC
  
  **Acceptance criteria**: NMT 3 mg/kg

- **Lead**, *Elemental Impurities by ICP*, Appendix IIIC
  
  **Acceptance criteria**: NMT 5 mg/kg

SPECIFIC TESTS

- **Loss on Drying**, Appendix IIIC: 135° for 6 h
  
  **Acceptance criteria**: NMT 10.0%

1. Use a Brookfield Digital Viscometer Model DV-1+ (RV) with spindle RV3, code 03 (spindle multiplier constant equals 10) from Brookfield Engineering Laboratories, Inc., Middleboro, MA (www.brookfieldengineering.com), or equivalent equipment.


3. Use an IKA model RW20 with stirrer (propellor) model R1342, available from IKA Works, Inc. (www.ika.com), or equivalent equipment.

**Auxiliary Information**— Please check for your question in the FAQs before contacting USP.

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| Monograph      | Kristie Laurvick  
Scientific Liaison  
1-301-816-8356  
(FI2010) Monographs - Food Ingredients |
BRIEFING

Ammonium Salts of Phosphatidic Acid. Because there is no existing FCC monograph for this food ingredient, a new monograph is proposed based on the Ammonium Salts of Phosphatidic Acid monograph from the 55th Session (2000) of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and based on comments and data received. For the Phosphorus Content assay method, it is proposed to slightly modify the JECFA procedure to use a three point standard curve instead of a single point determination. For the Nitrogen Content assay, it is proposed to use the specification from the JECFA monograph, and the test for total nitrogen in FCC’s Appendix IIIIC. It is also proposed to add a limit for arsenic based on the specifications published in the European Union Commission Directive 2008/84/EC and supporting data, and using the existing FCC test procedure for arsenic in Appendix III.

(Fl: J. Moore) C110728

Add the following:

- Ammonium Salts of Phosphatidic Acid

Ammonium phosphatides

Emulsifier YN

Mixed ammonium salts of phosphorylated glycerides

Ammonium salts of phosphorylated glyceride

AMP

![Chemical Structure of AMP](image)

R = H or fatty acid

R’ = H or

INS: 442

DESCRIPTION

Ammonium Salts of Phosphatidic Acid occurs as a brownish, unctuous semisolid to oily liquid. It is soluble in fats, partially soluble in ethanol and acetone, and insoluble in water. The product consists principally of a mixture of the ammonium compounds of phosphatidic acids derived from edible fats and oils. One or two mono- and diglyceride moieties may be attached to phosphorus. Moreover, two phosphorus esters may be linked together as phosphatidyl phosphatides. The product is obtained by glycerolysis of the fat, followed by phosphorylation with phosphorus pentoxide, and neutralization with ammonia. The article of commerce may be further specified as to water content, hexane-insoluble matter, and pH value.

Function: Emulsifier
Packaging and Storage: Keep dry, and store in tight containers at ambient temperature.

IDENTIFICATION

- **Test for Phosphate**
  
  **Sample:** 1 g
  
  **Analysis:** Ignite the *Sample* with 2 g of anhydrous sodium carbonate. Cool, and dissolve the residue in 5 mL of water and 5 mL of nitric acid. Add 5 mL of ammonium molybdate TS, and heat to boiling.
  
  **Acceptance criteria:** A yellow precipitate is formed.

- **Test for Ammonia and Fatty Acid**
  
  **Sample:** 1 g
  
  **Analysis:** Reflux the *Sample* for 1 h with 25 mL of alcoholic potassium hydroxide TS. Cool the residue to 0°.
  
  **Acceptance criteria**
  
  **Acceptance criteria 1:** Ammonia is evolved from the end of the reflux condenser during refluxing, recognizable by its odor and by its reaction on moist, red litmus paper.

  **Acceptance criteria 2:** The cooled residue forms precipitate of potassium soap, indicating the presence of fatty acids.

- **Test for Glycerol**
  
  **Acrolein solution:** 1:1 (v/v) mixture of a 5% solution of disodium pentacyanonitrosylferrate in water and a 20% piperidine solution in water. [Note—Mix the two solutions immediately before use.]

  **Dilute HCl:** 10% (w/v) HCl. Prepare by diluting 266 mL of 36% HCl to 1000 mL.

  **Sample:** 1 g
  
  **Sample preparation:** Reflux the *Sample* with 15 mL of alcoholic potassium hydroxide TS for 1 h. Add 15 mL of water, acidify with about 6 mL of *Dilute HCl*. [Note—Oily drops or a white to yellowish-white solid is produced, which is soluble in hexane.] Add 5 mL of hexane, remove the hexane layer, extract again with 5 mL of hexane, and again remove the hexane layer. Use the resulting aqueous layer as the *Sample preparation*.

  **Analysis:** Transfer 5 mL of the *Sample preparation* (the aqueous layer resulting from the hydrolysis) into a test tube. Add excess calcium hydroxide as a powder, place in boiling water for 5 min, shaking several times, cool, and filter. Transfer one drop of the filtrate into a tube, and add about 50 mg of potassium hydrogen sulfate. Place a filter paper, moistened with *Acrolein solution* on the top of the tube. Heat over a micro flame. Observe the color of the filter paper, add sodium hydroxide TS, and then observe the color again.

  **Acceptance criteria**
  
  **Acceptance criteria 1:** The filter paper is blue after heating the tube over the micro flame.

  **Acceptance criteria 2:** The filter paper color changes to light red after addition of the sodium hydroxide TS.

- **Infrared Absorption, Spectrophotometric Identification Tests, Appendix IIIC**

  **Reference standard:** USP Ammonium Salts of Phosphatidic Acid RS

  **Sample and standard preparation:** A

  [Note—Samples should be homogenized before analysis by stirring at a slow speed to prevent formation of air bubbles.]

  **Acceptance criteria:** The spectrum of the test *Sample* exhibits a strong absorption band at approximately 1100 cm⁻¹ (see example spectra in *Figure 1*) which corresponds in wavelength to those in the spectrum of the *Reference standard*, and corresponds in intensity to the range exhibited by different types of ammonium salts of phosphatidic acid in *Figure 1*. The spectrum of the test *Sample* does not exhibit absorption bands at approximately 1600 cm⁻¹ and 3400 cm⁻¹ as exhibited by soy lecithin in *Figure 2*. 
ASSAY

• **Phosphorus Content**

  **Vanadate-molybdate solution:** Separately dissolve 20 g of ammonium molybdate and 1 g of ammonium vanadate in water. Mix the two solutions in a 1000-mL volumetric flask, add 140 mL of concentrated nitric acid, dilute with water to volume, and mix well.

  **Standard stock solution:** 2.0 mg/mL of P$_2$O$_5$ equivalents. Prepare by diluting 3.8346 g of monobasic potassium phosphate, previously dried at 110°, in water and diluting to 1000 mL.

  **Standard solution:** 0.2 mg/mL of P$_2$O$_5$. Prepare by diluting the **Standard stock solution** with water.

  **Sample:** 1.5–1.6 g

  [Note—Material should be homogenized by slowly stirring before sampling to ensure representative sampling.]

  **Sample preparation:** Weigh the **Sample** into a small glass capsule, and transfer to a 300-mL Kjeldahl flask containing 5 mL of sulfuric acid and 10 mL of nitric acid. Heat the flask, gently at first, with continual swirling, and later more strongly over a bare flame. Add further measured amounts of nitric acid from time to time, cooling the flask prior to addition, and continue the heating until the stage where the digest is clear and assumes a golden color. Cool, add 5 mL of 60% perchloric acid, and continue the oxidation until white acid fumes form in the flask.  

  [Caution—Handle perchloric acid in an appropriate fume hood.]

  Cool again, and add 5 mL of water and continue heating until white fumes are again driven off. Cool, dilute carefully with water, cool again, and transfer quantitatively to a 500-mL volumetric flask. Dilute with water to volume, and mix well.

  **Blank preparation:** Carry out a blank digestion as prescribed above in the **Sample preparation** but omit the sample, and use the same volume of acid as required to wet oxidize the sample.

  **Analysis:** In separate 100-mL volumetric flasks, add the following:

  1. Sample solution: 25.0 mL of **Sample preparation** that corresponds to the equivalent of 5–6 mg of P$_2$O$_5$

  2. Standard solution 1: 25.0 mL of **Standard solution** (equivalent to 5.0 mg P$_2$O$_5$), and 25.0 mL of **Blank preparation**.  

     [Note—The **Blank preparation** is added to compensate for possible traces of phosphorus derived from the acid digest reagents which may be present in the **Sample solution**.]
3. Standard solution 2: 27.5 mL of Standard solution, (equivalent to 5.5 mg P$_2$O$_5$), and 25.0 mL of Blank preparation
4. Standard solution 3: 30.0 mL of Standard solution, (equivalent to 6.0 mg P$_2$O$_5$), and 25.0 mL of Blank preparation

To each flask add 25 mL of Vanadate-molybdate solution, and mix well. Dilute with water to nearly 100 mL, mix well again, adjust the temperature of the solution to 20$^\circ$, dilute with water to volume, and mix again. After 10 min, use an appropriate UV-vis spectrophotometer to measure the absorbance of the Sample solution, Standard solution 1, Standard solution 2, and Standard solution 3 at a wavelength of 420 nm. Prepare a standard curve plotting the mg equivalents of P$_2$O$_5$ versus absorbance, and calculate the mg equivalents of P$_2$O$_5$ in the Sample solution (C$_U$).

Calculate the percentage of phosphorus in the portion of the sample taken:

\[
\text{Result} = C_U \times \frac{F}{W} \times 100
\]

- **Acceptance criteria:** 3.0%–3.4% phosphorus

**Ammonia Nitrogen Content**

**Apparatus for steam distillation:** The apparatus consists of a 2-L flask fitted with a rubber stopper with two holes through which passes an approximately 3-inch length of glass tubing, arranged so that the lower end is near the bottom of the flask, and a shorter L-shaped piece of tubing arranged such that the tube projects about 1/4-inch below the lower surface of the stopper, to act as a steam outlet tube. The flask should be approximately 2/3 filled with water made slightly acidic with dilute sulfuric acid TS and contain a few glass beads to prevent bumping when the contents of the flask are vigorously boiled to act as a steam generator. A tap funnel may be fitted to the flask, if desired, to facilitate replenishing the water in the flask between determinations.

The steam outlet tube is connected via a condensation trap to the inlet of a steam distillation head, fitted to a short necked 1-L round bottomed necked flask. The distillation head should be such that the steam inlet tube reaches almost to the bottom of the 1-L flask, and the outlet should be fitted with two splash traps, one near the top of the 1-L flask and the other near the top of the vertical jointed single-surface condenser to which the distillation head connects. The vertical condenser should be fitted with an extended outlet tube, able to reach to the bottom of a 500-mL conical flask.

**Mixed indicator solution:** Mix 5.0 mL of 0.1% (w/v) alcoholic solution of bromocresol green and 2.0 mL of a 0.1% (w/v) alcoholic solution of methyl red, and dilute with 95% alcohol to 30 mL.

**Sample:** 0.2 g. [Note—Material should be homogenized by slowly stirring before sampling to ensure representative sampling.]

**Analysis:** Assemble, and thoroughly steam out the apparatus. Accurately weigh the Sample into a small glass vial (approx. 3/4 inch diameter, 1/2 inch deep). Transfer the vial and weighed contents to the distillation flask, and add approximately 250 mL of distilled water. Connect the distillation head and splash traps to the distillation flask and vertical condenser, and arrange the condenser such that the outlet dips below the surface of 10 mL of 2% boric acid (w/v) in water and 1 mL of Mixed indicator solution contained in the 500-mL conical flask. Add to the distillation flask, via a funnel attached by means of a short piece of
rubber tubing to the steam inlet tube, 75 mL of 40% aqueous sodium hydroxide (w/v), and wash with water. Detach the funnel, and connect the steam inlet to the steam supply. (Alternatively, the sodium hydroxide may be added to the flask through a tap funnel, fitted to the distillation flask if preferred and washed in with distilled water. If so, a liquid seal should be maintained in the funnel during the addition and distillation). Vigorously steam distill the contents of the distillation flask, and collect 200 mL distillate in the boric acid. During the distillation, gently agitate the distillation flask, if necessary, to avoid the sample being deposited around the upper surfaces of the flask. [Note—During the distillation difficulty may be experienced with frothing of the contents of the distillation flask. If so, two drops of silicone fluid should be added to the distillation flask at the time of adding the sample; and a similar amount included in the blank determination.] When the required amount of distillate has been collected, lower the receiving flask, stop the steam supply, and wash down the inside of the condenser, and the outside of the lower end, with a small quantity of distilled water, collecting the washings in the receiving flask.

Titrate the contents of the receiving flask with 0.02 N hydrochloric acid. Perform a blank determination in exactly the same way, but omit the sample (see General Provisions), and make any necessary correction. Each mL of 0.02 N hydrochloric acid is equivalent to 0.2802 mg of nitrogen.

**Acceptance criteria:** 1.2%–1.5% nitrogen

**IMPURITIES**

Inorganic Impurities

- **Arsenic**, *Arsenic Limit Test*, Appendix IIIB
  - **Sample solution**: Prepare as directed for organic compounds.
  - **Acceptance criterion**: NMT 3 mg/kg
- **Lead**, *Lead Limit Test, Flame Atomic Absorption Spectrophotometric Method*, Appendix IIIB
  - **Sample**: 10 g
  - **Acceptance criterion**: NMT 2 mg/kg

2S (**FCC8**)

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<tr>
<td>Monograph</td>
<td>Jeffrey Moore, Ph.D. Scientific Liaison 1-301-816-8288</td>
<td>(FI2010) Monographs - Food Ingredients</td>
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BRIEFING

Ammonium Polyphosphate. Because there is no existing FCC monograph for this food ingredient, a new monograph is proposed based on the Ammonium Polyphosphate monograph prepared at the 41st Session (1993) of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), the FCC monograph for Ammonium Phosphate Monobasic, and on the basis of comments supplied.

1. The proposed methods of Identification and the Acceptance criteria are consistent with those specified in JECFA. Further comments and data to support more modern methods capable of differentiating this ingredient from similar materials are encouraged.

2. The proposed Assay and its Acceptance criteria are consistent with those specified in JECFA. However, further comments and data are encouraged, especially those related to more modern analytical methods of analysis that support quantitatively the Acceptance criteria of this material.

3. Impurities proposed are consistent with those specified in JECFA. However, the methods and limits proposed for arsenic, fluoride, and lead are based on existing methods in FCC, because the methods referenced by JECFA include insufficient detail. Interested parties are encouraged to submit comments and data pertinent to these methods.

Add the following:

Ammonium Polyphosphate
NH₄(Hₙ₊₂PₙO₃ₙ₊₁)
INS: 452(v)
CAS: [68333-79-9]

DESCRIPTION
Ammonium Polyphosphate occurs as an aqueous solution. It is a heterogeneous mixture of ammonium salts of linearly condensed polyphosphoric acids of general formula Hₙ₊₂PₙO₃ₙ₊₁. The pH of a 1:100 aqueous solution is between 4.0 and 9.0.

Function: Sequestrant, emulsifier

Packaging and Storage: Store in tightly closed containers.

IDENTIFICATION
• AMMONIUM, Appendix IIIA
  Sample solution: 50 mg/mL
  Acceptance criteria: Passes test
• PHOSPHATE, Appendix IIIA
  Sample solution: 50 mg/mL
  Acceptance criteria: Passes tests

ASSAY
• PROCEDURE
  Phenolphthalein indicator solution: In a 100-mL volumetric flask dissolve 0.2 g of phenolphthalein in 60 mL of 90% ethanol, and dilute with water to volume.
  Sample: 200–300 mg
Analysis: Dissolve the Sample in 25 mL of water and 10 mL of nitric acid TS, diluted. Boil the mixture for 30 min. Filter if necessary, and wash any precipitate. Then, dissolve the precipitate by the addition of 1 mL of nitric acid TS, diluted, place in a water bath at about 50°, add 75 mL of ammonium molybdate TS, and incubate at about 50° for 30 min, stirring occasionally. Allow to stand for 16 h or overnight at room temperature. Decant the supernatant through a filter paper, wash the precipitate once or twice with water by decantation using 30–40 mL each time, and pour the washings through the same filter. Transfer the precipitate to the same filter, and wash with a potassium nitrate solution (10 mg/mL) until the filtrate is no longer acid to litmus paper. Transfer the precipitate with filter paper to the original precipitation vessel, add 50.0 mL of 1 N sodium hydroxide, agitate, and stir until the precipitate is dissolved. Add 3 drops of the Phenolphthalein indicator solution, and titrate the excess alkali with 1 N sulfuric acid solution. Each mL of 1 N sodium hydroxide consumed is equivalent to 3.088 mg of P₂O₅.

Acceptance criteria: 55.0%–75.0% on an anhydrous basis, calculated as P₂O₅

IMPURITIES
Inorganic Impurities
- Arsenic, Arsenic Limit Test, Appendix III B
  Sample solution: 1 g in 35 mL of water
  Acceptance criteria: NMT 3 mg/kg
- Fluoride, Fluoride Limit Test, Method IV, Appendix III B
  Buffer solution: [Note—Use the following buffer solution preparation in place of the one specified under Method IV.]  Add two volumes of 6 N acetic acid to 1 volume of water, and adjust the pH to 5.0 with 50% potassium hydroxide solution.
  Acceptance criteria: NMT 10 mg/kg
- Lead, Lead Limit Test, ADPC Extraction Method, Appendix III B
  Acceptance criteria: NMT 4 mg/kg

2S (FCC8)

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<td>Monograph</td>
<td>Carla D Mejia, Ph.D. Scientific Liaison 1-301-816-8571</td>
<td>(Fl2010) Monographs - Food Ingredients</td>
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**BRIEFING**

**o-Anisaldehyde.** Because there is no existing FCC monograph for this food ingredient, a new monograph is proposed based on the o-Anisaldehyde monograph from the 73rd Session (2010) of the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The *Sample and standard preparation* in the *Identification* test is based on information submitted by a stakeholder. Submission of any additional data and information for the sample preparation for the *Identification* test is encouraged.  

(Fl: P. Bhatt)  

Add the following:  

- **o-Anisaldehyde**  
- o-Formylanisole  
- 2-Methoxybenzenecarboxaldehyde  
- 2-Methoxyphenylformaldehyde  
- 2-Methoxybenzaldehyde  
- Salicylaldehyde methyl ether

![Chemical structure of o-Anisaldehyde](image)

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**DESCRIPTION**

- **o-Anisaldehyde** occurs as a light yellow solid.  
- **Odor:** Sweet powdery hawthorn, vanilla and almond aroma  
- **Solubility:** Soluble in water, propylene glycol, and ethanol  
- **Function:** Flavoring agent

**IDENTIFICATION**

---

UNII: 7CP821WF2W [o-anisaldehyde]
INFRARED ABSORPTION, Spectrophotometric Identification Tests, Appendix IIIC

Reference standard: USP o-Anisaldehyde RS

Sample and standard preparation: F

[Note—o-Anisaldehyde is a solid with a low melting point. Solid o-Anisaldehyde is warmed until melted (around 40°), and then a drop of this is placed between two KBr windows.]

Acceptance criteria: The spectrum of the sample exhibits maxima at the same wavelengths as those in the spectrum of the Reference standard.

ASSAY

• Procedure: Proceed as directed under M-1b, Appendix XI.

  Reference standard: USP o-Anisaldehyde RS

  Acceptance criteria: NLT 97%

SPECIFIC TESTS

• Melting Range, Appendix VII

  Acceptance criteria: 34°–40°

2S (FCC8)

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<td>Monograph</td>
<td>Premal Bhatt, M.S. Associate Scientific Liaison 1-301-816-3362</td>
<td>(FI2010) Monographs - Food Ingredients</td>
</tr>
</tbody>
</table>
BRIEFING

Anisyl Butyrate. Because there is no existing FCC monograph for this food ingredient, a new monograph is proposed based on the Anisyl Butyrate monograph from the 57th Session (2001) of the Joint FAO/WHO Expert Committee on Food Additives (JECFA).

Add the following:

- Anisyl Butyrate
- p-Methoxybenzyl Butyrate
- 4-Methoxybenzyl Butanoate

UNII: Z8T0G5UDPH [anisyl butyrate]

DESCRIPTION

Anisyl Butyrate occurs as a colorless liquid.

Odor: Weak, floral, very sweet, plum-like

Solubility: Soluble in organic solvents and oils; insoluble in water; miscible with ethanol at room temperature

Boiling Point: ~270°

Function: Flavoring agent

IDENTIFICATION

- Infrared Absorption, Spectrophotometric Identification Tests, Appendix IIIC
  - Reference standard: USP Anisyl Butyrate 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.

ASSAY

- Procedure: Proceed as directed under M-1b, Appendix XI.
  - Reference standard: USP Anisyl Butyrate RS
  - Acceptance criteria: NLT 97%

SPECIFIC TESTS
- **Acid Value**, M-15, Appendix XI
  - **Acceptance criteria**: NMT 1.0

- **Refractive Index**, Appendix IIB: At 20°
  - **Acceptance criteria**: 1.500–1.505

- **Specific Gravity**: Determine at 25° by any reliable method (see General Provisions).
  - **Acceptance criteria**: 1.047–1.067

**Auxiliary Information**—Please check for your question in the FAQs before contacting USP.

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</table>
| Monograph     | Premal Bhatt, M.S.  
Associate Scientific Liaison  
1-301-816-3362 | (Fi2010) Monographs - Food Ingredients |
**BRIEFING**

**Anisyl Phenylacetate.** Because there is no existing FCC monograph for this food ingredient, a new monograph is proposed based on the Anisyl Phenylacetate monograph from the 57th Session (2001) of the Joint FAO/WHO Expert Committee on Food Additives (JECFA).

(FL: P. Bhatt) C114429

---

**Add the following:**

- Anisyl Phenylacetate
- Anisyl alpha-Toluene
- p-Methoxybenzyl Phenylacetate
- 4-Methoxybenzyl Phenylacetate

![Chemical Structure](image)

\[C_{16}H_{16}O_3\]

*Formula wt 256.3
FEMA: 3740
CAS: [102-17-0]*

**UNII:** 7475820557 [anisyl phenylacetate]

---

**DESCRIPTION**

Anisyl Phenylacetate occurs as a colorless oily liquid.

**Odor:** Faint, honey-like, balsamic-rosy

**Solubility:** Soluble in organic solvents and oils; insoluble in water; miscible with ethanol at room temperature

**Boiling Point:** \(\sim 370^\circ\)

**Function:** Flavoring agent

---

**IDENTIFICATION**

- **Infrared Absorption, Spectrophotometric Identification Tests, Appendix IIIC**
  - **Reference standard:** USP Anisyl Phenylacetate 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.

---

**ASSAY**

- **Procedure:** Proceed as directed under M-1b, Appendix XI.
  - **Reference standard:** USP Anisyl Phenylacetate RS
  - **Acceptance criteria:** NLT 97%
SPECIFIC TESTS

- **Acid Value**, M-15, Appendix XI
  
  **Acceptance criteria**: NMT 1.0

- **Refractive Index**, Appendix II B: At 20°
  
  **Acceptance criteria**: 1.553–1.563

- **Specific Gravity**: Determine at 25° by any reliable method (see *General Provisions*).
  
  **Acceptance criteria**: 1.125–1.133

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</table>
| Monograph     | Premal Bhatt, M.S.  
Associate Scientific Liaison  
1-301-816-3362 | (FI2010) Monographs - Food Ingredients |

Auxiliary Information—Please check for your question in the FAQs before contacting USP.
BRIEFING

Anisyl Propionate. Because there is no existing FCC monograph for this food ingredient, a new monograph is proposed based, in part, on the Anisyl Propionate monograph from the 57th Session (2001) of the Joint FAO/WHO Expert Committee on Food Additives (JECFA).

Add the following:

- Anisyl Propionate
  - Anisyl Propanoate
  - p-Methoxybenzyl Propionate
  - 4-Methoxybenzyl Propanoate

C_{11}H_{14}O_{3}  

Formula wt 194.23
FEMA: 2102
CAS: [7549-33-9]

UNII: IBJ5CR95MK [anisyl propionate]

DESCRIPTION
Anisyl Propionate occurs as a colorless to slightly yellow liquid.

Odor: Sweet, fruity, floral, vanilla–like

Solubility: Soluble in organic solvents, oils; insoluble in water, glycerol, propylene glycol; miscible with ethanol at room temperature

Boiling Point: ~100 to 103° at 0.5 mm Hg

Function: Flavoring agent

IDENTIFICATION
- Infrared Absorption, Spectrophotometric Identification Tests, Appendix III C
  - Reference standard: USP Anisyl Propionate 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.

ASSAY
- Procedure: Proceed as directed under M-1b, Appendix XI.
  - Reference standard: USP Anisyl Propionate RS
Acceptance criteria: NLT 97%

SPECIFIC TESTS

- **Acid Value**, M-15, Appendix XI
  
  Acceptance criteria: NMT 1.0

- **Refractive Index**, Appendix II: At 20°
  
  Acceptance criteria: 1.505–1.510

- **Specific Gravity**: Determine at 25° by any reliable method (see General Provisions).
  
  Acceptance criteria: 1.070–1.086

2S (FCC8)

**Auxiliary Information**—Please check for your question in the FAQs before contacting USP.

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| Monograph      | Liwen Chen  
Assistant Scientific Liaison  
1-301-816-3388 | (FI2010) Monographs - Food Ingredients |
BRIEFING

Benzaldehyde Propylene Glycol Acetal. Because there is no existing FCC monograph for this food ingredient, a new monograph is proposed based, in part, on the Benzaldehyde Propylene Glycol Acetal monograph from the 57th Session (2001) of the Joint FAO/WHO Expert Committee on Food Additives (JECFA).

(Fl: L. Chen) C113919

Add the following:

- Benzaldehyde Propylene Glycol Acetal

4-Methyl-2-phenyl-\textit{m}-dioxolane

4-Methyl-2-phenyl-1,3-dioxolane

\[
\begin{align*}
\text{C}_{10}\text{H}_{12}\text{O}_2
\end{align*}
\]

Formula wt 164.2

FEMA: 2130

CAS: \[2568-25-4\]

UNII: ELQ3FTL5B1 [benzaldehyde propylene glycol acetal]

DESCRIPTION

Benzaldehyde Propylene Glycol Acetal occurs as a colorless liquid.

Odor: Mild, almond-like odor

Solubility: Slightly soluble in water; soluble in oils; miscible in ethanol at room temperature

Boiling Point: \(-83^\circ\text{ to }-85^\circ\) at 4 mm Hg

Function: Flavoring agent

IDENTIFICATION

- \textbf{Infrared Absorption}, \textit{Spectrophotometric Identification Tests}, Appendix IIIC

- \textbf{Reference standard}: USP Benzaldehyde Propylene Glycol Acetal RS

- \textbf{Sample and standard preparation}: F

- \textbf{Acceptance criteria}: The spectrum of the sample exhibits maxima at the same wavelengths as those in the spectrum of the Reference standard.

ASSAY

- \textbf{Procedure}: Proceed as directed under M-1b, Appendix XI.

- \textbf{Reference standard}: USP Benzaldehyde Propylene Glycol Acetal RS

- \textbf{Acceptance criteria}: NLT 95%
SPECIFIC TESTS

• **Acid Value, M-15, Appendix XI**
  **Acceptance criteria:** NMT 1.0

• **Refractive Index, Appendix II: At 20°**
  **Acceptance criteria:** 1.506–1.516

• **Specific Gravity:** Determine at 25° by any reliable method (see General Provisions).
  **Acceptance criteria:** 1.061–1.071

[2S (FCC8)]

**Auxiliary Information**—Please check for your question in the FAQs before contacting USP.

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<tr>
<td>Monograph</td>
<td>Liwen Chen Assistant Scientific Liaison 1-301-816-3388</td>
<td>(FI2010) Monographs - Food Ingredients</td>
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</tbody>
</table>
BRIEFING

Benzenethiol. Because there is no existing FCC monograph for this food ingredient, a new monograph is proposed based, in part, on the Benzenethiol monograph from the 53th Session (1999) of the Joint FAO/WHO Expert Committee on Food Additives (JECFA).

(FI: L. Chen) C113916

Add the following:

- Benzenethiol
- Phenyl Mercaptan
- Thiophenol

\[ C_6H_5S \]

Formula wt 110.17
FEMA: 3616
CAS: [108-98-5]

UNII: 7K011JR4T0 [benzenethiol]

DESCRIPTION

Benzenethiol occurs as a colorless to pale yellow liquid.

Odor: Penetrating, garlic-like

Solubility: Soluble in oils; slightly soluble in alcohol and ether; insoluble in water

Boiling Point: \( \sim 169^\circ \) (46.4° at 10 mm Hg)

Function: Flavoring agent

IDENTIFICATION

- Infrared Absorption, Spectrophotometric Identification Tests, Appendix IIIC
  - Reference standard: USP Benzenethiol 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.

ASSAY

- Procedure: Proceed as directed under M-1b, Appendix XI.
  - Reference standard: USP Benzenethiol RS
Acceptance criteria: NLT 97%

SPECIFIC TESTS

- **Refractive Index**, Appendix II: At 20°
  Acceptance criteria: 1.589–1.593

- **Specific Gravity**: Determine at 25° by any reliable method (see General Provisions).
  Acceptance criteria: 1.073–1.080

2S (FCC8)

**Auxiliary Information**—Please check for your question in the FAQs before contacting USP.

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| Monograph      | Liwen Chen  
  Assistant Scientific Liaison  
  1-301-816-3388 | (FI2010) Monographs - Food Ingredients |
BRIEFING

3-Benzyl-4-Heptanone. Because there is no existing FCC monograph for this food ingredient, a new monograph is proposed based, in part, on the 3-Benzyl-4-Heptanone monograph from the 57th Session (2001) of the Joint FAO/WHO Expert Committee on Food Additives (JECFA).

(Fl: J. Moore) C115539

Add the following:

- 3-Benzyl-4-Heptanone
- Benzyl Dipropyl Ketone
- Morellone
- 1-Phenyl-2-Ethyl-3-Hexanone

![Chemical Structure]

\[ C_{14}H_{20}O \]

Formula wt 204.31
FEMA: 2146
CAS: [7492-37-7]
UNII: 64UYT55005 [3-benzyl-4-heptanone]

DESCRIPTION

3-Benzyl-4-Heptanone occurs as a colorless, oily liquid.

Odor: Fruity, berry, woody, raisin

Solubility: Soluble in organic solvents, oils; miscible with ethanol at room temperature; insoluble in water

Boiling Point: \( \sim 158^\circ - 160^\circ \) (10 mm Hg)

Function: Flavoring agent

IDENTIFICATION

- Infrared Absorption, Spectrophotometric Identification Tests, Appendix III C

Reference standard: USP 3-Benzyl-4-Heptanone 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.

ASSAY
• **PROCEDURE:** Proceed as directed under *M-1b, Appendix XI.*  
  **Reference standard:** USP 3-Benzyl-4-Heptanone RS  
  **Acceptance criteria:** NLT 99% of 3-benzyl-4-heptanone

**SPECIFIC TESTS**

• **Refractive Index,** Appendix IIB: At 20°  
  **Acceptance criteria:** 1.490–1.495

• **Specific Gravity:** Determine at 25° by any reliable method (see General Provisions).  
  **Acceptance criteria:** 0.931–0.937

2S (FCC8)

**Auxiliary Information**—Please check for your question in the FAQs before contacting USP.

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</table>
| Monograph      | Jeffrey Moore, Ph.D.  
Scientific Liaison  
1-301-816-8288   | (FI2010) Monographs - Food Ingredients |
Calcium Phosphate, Monobasic. *FCC 8* page 149. In order to add methods of analysis that reflect what is currently practiced in the industry, and on the basis of comments and data received, it is proposed to revise the current Assay method of this monograph. The proposed method uses ethylenediaminetetraacetate (EDTA) to directly quantify the calcium content in the material. The method is based on the Assay of the monograph for Calcium Dihydrogen Phosphate prepared by Japan’s Specifications and Standards for Food Additives (JSFA) 7th Edition (2000), and is also similar to the Assay currently used in the *FCC* monographs for Calcium Phosphate, Dibasic and Calcium Phosphate, Tribasic. Interested parties are encouraged to submit comments. Comments that provide more modern methods of analysis that could detect adulterated materials are especially encouraged.

(FI: C. Mejia) C115730

**Calcium Phosphate, Monobasic**

Monocalcium Phosphate

Calcium Biphosphate

Acid Calcium Phosphate

\[ Ca(H_2PO_4)_2 \]

\[ Ca(H_2PO_4)_2\cdot H_2O \]

**INS:** 341(i)

**CAS:** anhydrous [7758-23-8]

monohydrate [10031-30-8]

**UNII:** 0N4E6L5449 [calcium phosphate, monobasic, monohydrate]

**UNII:** 701EKV9RMN [calcium phosphate, monobasic, anhydrous]

**DESCRIPTION**

Calcium Phosphate, Monobasic, occurs as white crystals or granules, or as a granular powder. It is anhydrous or contains one molecule of water of hydration, but because of its deliquescent nature, more than the calculated amount of water may be present. It is sparingly soluble in water, and insoluble in alcohol.

**Function:** Buffer; dough conditioner; firming agent; leavening agent; nutrient; yeast food; sequestrant

**Packaging and Storage:** Store in well-closed containers.

**IDENTIFICATION**

1. **PROCEDURE**

   **Sample:** 100 mg

   **Analysis:** Dissolve the Sample by warming it in a mixture of 2 mL of 2.7 N hydrochloric acid and 8 mL of water. Add 5 mL of ammonium oxalate TS.

   **Acceptance criteria:** A white precipitate forms.

2. **PROCEDURE**

   **Analysis:** Add ammonium molybdate TS to a warm solution of sample in a slight excess of nitric acid.
Acceptance criteria: A yellow precipitate of ammonium phosphomolybdate forms.

ASSAY

Change to read:

• CALCIUM

Sample: Amount equivalent to 475 mg of Calcium Phosphate, Monobasic, Anhydrous \( \text{Ca(H}_2\text{PO}_4)\)\(\text{2}\), 450 mg, dried.\(\text{2S (FCC8)}\)

Wash solution: Dilute 10 mL of ammonium oxalate TS to 1000 mL with water.

\(\text{2S (FCC8)}\)

Analysis: Dissolve the Sample in 10 mL of 2.7 N hydrochloric acid; add a few drops of methyl orange TS; and boil for 5 min, keeping the volume and pH of the solution constant during the boiling period by adding hydrochloric acid or water as necessary. Add 2 drops of methyl red TS and 30 mL of ammonium oxalate TS. Then, while constantly stirring, add, dropwise, a mixture of equal volumes of 6 N ammonium hydroxide and water until the pink color of the indicator just disappears. Digest on a steam bath for 30 min, cool to room temperature, allow the precipitate to settle, and filter the supernatant liquid through a sintered-glass crucible, using gentle suction. Wash the precipitate in the beaker with about 30 mL of cold (below 20\(°\)C) Wash solution. Allow the precipitate to settle, and pour the supernatant liquid through the filter. Repeat this washing by decantation three more times. Using the Wash solution, transfer the precipitate as completely as possible to the filter. Finally, wash the beaker and the filter with two 10-mL portions of cold (below 20\(°\)C) water. Place the sintered-glass crucible in the beaker, and add 100 mL of water and 50 mL of cold 1:6 sulfuric acid. Add 35 mL of 0.1 N potassium permanganate from a buret and stir until the color disappears. Heat to about 70\(°\)C, and complete the titration with 0.1 N potassium permanganate. Each mL of 0.1 N potassium permanganate is equivalent to 2.094 mg of Ca.\(\text{1S (FCC8)}\)

In a 250-mL beaker equipped with a magnetic stirrer, dissolve the Sample in a mixture of 5 mL of water and 1 mL of hydrochloric acid, with the aid of gentle heat if necessary, and add 50 mL of water. With constant stirring, add exactly 25 mL of 0.1 M disodium EDTA to the solution. The solution should turn cloudy indicating that enough EDTA has been added. Add to the solution, ammonium hydroxide drop-wise until it becomes clear, then add 20 more drops of ammonium hydroxide and 10 mL ammonia-ammonium chloride buffer TS. Add to the mixture 0.1 mL eriochrome black TS to obtain a clear blue color. Titrate this solution with 0.1 M zinc sulfate until the clear blue color changes to dark red. To obtain the amount of 0.1 M disodium EDTA consumed (mL), subtract the volume (mL) of zinc sulfate used from the initial volume of 0.1 M disodium EDTA that was added (25 mL). Each mL of 0.1 M disodium EDTA consumed is equivalent to 4.008 mg Ca.\(\text{2S (FCC8)}\)

Acceptance criteria

Anhydrous: NLT 16.8% and NMT 18.3% of Ca
Monohydrate: NLT 15.9% and NMT 17.7% of Ca

IMPURITIES

Inorganic Impurities

• ARSENIC, Arsenic Limit Test, Appendix III B

Sample solution: 1 g in 5 mL of 2.7 N hydrochloric acid

Acceptance criteria: NMT 3 mg/kg

• FLUORIDE

[Note—Prepare and store all solutions in plastic containers.]

Anhydrous material: Fluoride Limit Test, Method II, Appendix III B
Monohydrate
Buffer solution: Dissolve 73.5 g of sodium citrate in water, made to 250 mL.

Standard stock solution: 1.1052 mg/mL of USP Sodium Fluoride RS

Standard solution: Transfer 20.0 mL of the Standard stock solution to a 100-mL volumetric flask containing 50 mL of Buffer solution, dilute with water to volume, and mix. (100 µg/mL fluoride ion)

Sample solution: Transfer 2.0 g of sample to a beaker containing a plastic-coated stirring bar, add 20 mL of water and 2.0 mL of hydrochloric acid, and stir until the sample is dissolved. Add 50.0 mL of Buffer solution and sufficient water to make 100 mL.

Electrode system: Use a fluoride-specific, ion-indicating electrode and a silver-silver chloride reference electrode connected to a pH meter capable of measuring potentials with a minimum reproducibility of ±0.2 mV.

Standard response line: Transfer 50.0 mL of Buffer solution and 2.0 mL of hydrochloric acid into a beaker, and add water to make 100 mL. Add a plastic-coated stirring bar, insert the electrodes into the solution, stir for 15 min, and read the potential (mV). Continue stirring, and at 5-min intervals, add 100 µL, 100 µL, 300 µL, and 500 µL of the Standard solution, reading the potential 5 min after each addition. Plot the logarithms of the cumulative fluoride ion concentrations (0.1 µg/mL, 0.2 µg/mL, 0.5 µg/mL, and 1.0 µg/mL) versus potential, in mV.

Analysis: Rinse and dry the electrodes, insert them into the Sample solution, stir for 5 min, and read the potential (mV). From the measured potential and the Standard response line, determine the concentration, C (µg/mL), of fluoride ion in the Sample solution. Calculate the percentage of fluoride in the sample taken by the formula:

\[ \text{Result} = C \times 0.005 \]

Acceptance criteria: NMT 0.005%

• Lead, Lead Limit Test, APDC Extraction Method, Appendix IIIB
  Acceptance criteria: NMT 2 mg/kg

SPECIFIC TESTS

• Loss on Drying, Appendix II C: 60° for 3 h
  [Note—Monohydrate only.]
  Acceptance criteria: NMT 1%

• Loss on Ignition
  [Note—Anhydrous material only.]
  Sample: 3 g
  Analysis: Ignite the Sample, preferably in a muffle furnace, at 800° for 30 min.
  Acceptance criteria: 14.0%–15.5%

OTHER REQUIREMENTS

• Labeling: Indicate the state of hydration.

Auxiliary Information—Please check for your question in the FAQs before contacting USP.
BRIEFING

(+)-Carvone, FCC 8 page 230. On the basis of comments and data received, revisions to the specifications for Specific Gravity and Refractive Index are proposed. Similar revisions, also based on comments and data received, are proposed in the monograph for (-)-Carvone.

(FI: K. Lauvick) C118279

(+)-Carvone

d-Canone

dextro-Canone

d-1-Methyl-4-isopropenyl-6-cyclohexen-2-one

\[
\text{CH}_3
\]

\[
\text{H}_3\text{C} \rightarrow \text{CH}_2
\]

\[
\text{C}_{10}\text{H}_{14}\text{O}
\]

Formula wt 150.22
FEMA: 2249

UNII: 4RWC1CMS3X [carvone, (+)]

DESCRIPTION
(+)-Canone occurs as a colorless to light yellow liquid.

Odor: Caraway

Solubility: Soluble in propylene glycol, most fixed oils; miscible in alcohol; insoluble or practically insoluble in glycerin

Boiling Point: ~230°

Solubility in Alcohol, Appendix VI: One mL dissolves in 5 mL of 60% alcohol.

Function: Flavoring agent

IDENTIFICATION
• Infrared Spectra, Spectrophotometric Identification Tests, Appendix IIIC
Acceptance criteria: The spectrum of the sample exhibits relative maxima at the same wavelengths as those of the spectrum below.

(+)–Carvone

ASSAY
• Procedure: Proceed as directed under M-1b, Appendix XI.
  Acceptance criteria: NLT 95.0% of C₁₀H₁₄O

SPECIFIC TESTS
Change to read:
• Refractive Index, Appendix II: At 20°
  Acceptance criteria: Between 1.496 and 1.499

Change to read:
• Specific Gravity: Determine at 25° by any reliable method (see General Provisions).
  Acceptance criteria: Between 0.955 and 0.960

OTHER REQUIREMENTS
• Angular Rotation, Optical (Specific) Rotation, Appendix IIB: Use a 100-mm tube.
  Acceptance criteria: Between +50° and +60°

Auxiliary Information— Please check for your question in the FAQs before contacting USP.

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<td>Liwen Chen Assistant Scientific Liaison 1-301-816-3388</td>
<td>(F12010) Monographs - Food Ingredients</td>
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</table>
BRIEFING

(–)-Carvone, FCC 8 page 231. On the basis of comments and data received, revisions to the specifications for Specific Gravity and Refractive Index are proposed. Similar revisions, also based on comments and data received, are proposed in the monograph for (+)-Carvone.

(FL: K. Laurvick) C118280

(–)-Carvone

l-Carvone

levo-Carvone

1-1-Methyl-4-isopropenyl-6-cyclohexen-2-one

\[
\text{CH}_3
\]

\[
\text{H}_3\text{C} = \text{CH}_2
\]

\[\text{C}_{10}\text{H}_{14}\text{O}\]

Formula wt 150.22

FEMA: 2249

UNII: 5TO7X34D3D [carvone, (–)-]

DESCRIPTION

(–)-Carvone occurs as a colorless to pale strawberry colored liquid.

Odor: Spearminty

Solubility: Soluble in propylene glycol, most fixed oils; miscible in alcohol; insoluble or practically insoluble in glycerin

Boiling Point: \(\sim 231^\circ\)

Solubility in Alcohol, Appendix VI: One mL dissolves in 2 mL of 70% alcohol.

Function: Flavoring agent

IDENTIFICATION

• Infrared Spectra, Spectrophotometric Identification Tests, Appendix IIIC
**Acceptance criteria:** The spectrum of the sample exhibits relative maxima at the same wavelengths as those of the spectrum below.

![Spectrum Image]

**ASSAY**

- **Procedure:** Proceed as directed under M-1b, Appendix XI.
  
  **Acceptance criteria:** NLT 97.0% of C₁₀H₁₄O

**SPECIFIC TESTS**

*Change to read:*

- **Refractive Index,** Appendix II: At 20°
  
  **Acceptance criteria:** Between 1.496 and 1.502 and 1.502 ± 0.002 (FCC8)

*Change to read:*

- **Specific Gravity:** Determine at 25° by any reliable method (see General Provisions).
  
  **Acceptance criteria:** Between 0.956 and 0.960 and 0.956 ± 0.001 and 0.961 ± 0.002 (FCC8)

**OTHER REQUIREMENTS**

- **Angular Rotation,** Optical (Specific) Rotation, Appendix IIB: Use a 100-mm tube.
  
  **Acceptance criteria:** Between -57° and -62°

**Auxiliary Information**— Please check for your question in the FAQs before contacting USP.

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<tr>
<td>Monograph</td>
<td>Carla D Mejia, Ph.D. Scientific Liaison 1-301-816-8571</td>
<td>(F2010) Monographs - Food Ingredients</td>
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*FCC FCC 8 Page 231*
**BRIEFING**

**Cyclamic Acid, FCC 8 page 295.** On the basis of data received, a revision to the Sample and standard preparation in Identification test A. Infrared Absorption is proposed. This revision instructs users to prepare the sample and the Reference Standard as a mineral oil mull and replaces previous instructions to make and use a KBr pellet. Data generated internally indicated that more consistent results are obtained on the mineral oil mull of the material.

(FI: K. Laurvick) C113898

---

**Add the following:**

▲**Cyclamic Acid**

Cyclohexanesulfamic Acid

Cyclohexylsulfamic Acid

![Cyclamic Acid Structure](image)

C₆H₁₃NO₃S

Formula wt 179.24

INS: 952

CAS: [100-88-9]

UNII: HN3OFO5036 [cyclamic acid]

**DESCRIPTION**

Cyclamic Acid occurs as a practically colorless, white crystalline powder. It is soluble in water and in ethanol.

**Function:** Sweetener

**Packaging and Storage:** Store in well-closed containers.

**IDENTIFICATION**

**Change to read:**

• **A. INFRARED ABSORPTION, Spectrophotometric Identification Tests, Appendix III**
  
  **Reference standard:** USP Cyclamic Acid RS
  
  **Sample and standard preparation:** K·M₂S (FCC8)
  
  **Acceptance criteria:** The spectrum of the sample exhibits maxima at the same wavelengths as those in the spectrum of the Reference standard.

• **B. PROCEDURE**
  
  **Sample solution:** 20 mg/mL
  
  **Analysis:** Acidify the Sample solution with hydrochloric acid. Add 1 mL of barium chloride TS to the acidified solution, then filter if any turbidity or precipitate forms. When a clear solution is obtained, add 1 mL of 10%
sodium nitrite solution.

**Acceptance criteria:** A white precipitate forms.

ASSAY

**PROCEDURE**

**Phenolphthalein solution:** Dissolve 0.2 g of phenolphthalein in 60 mL of 90% ethanol, and dilute with water to 100 mL.

**Sample:** 350 mg

**Analysis:** Transfer the **Sample** to a 250-mL flask, and dissolve it in 50 mL of water. Titrate the solution with 0.1 N sodium hydroxide, using **Phenolphthalein solution** as the indicator. Each mL of 0.1 N sodium hydroxide is equivalent to 17.92 mg of C_6H_13NO_3S.

**Acceptance criteria:** 98.0%–102.0% of C_6H_13NO_3S, calculated on the dried basis

IMPURITIES

Inorganic Impurities

**Lead, Lead Limit Test, Flame Atomic Absorption Spectrophotometric Method, Appendix IIIB**

**Sample:** 5 g

**Acceptance criteria:** NMT 1.0 mg/kg

Organic Impurities

**Cyclohexanamine, Aniline, and N-Cyclohexylcyclohexanamine**

**Internal standard solution:** 0.02 µL/mL of tetradecane in methylene chloride

**Solution A:** Dissolve 10 mg of cyclohexanamine, 1 mg of N-cyclohexylcyclohexanamine, and 1 mg of aniline in water, then dilute with the same solvent to 1000 mL. Dilute 10 mL of this solution with water to 100 mL.

**Solution B:** 42% (w/v) sodium hydroxide solution

**Standard solution:** To 20 mL of **Solution A** add 0.5 mL of **Solution B**, and extract with 30 mL of toluene. Shake 20 mL of the upper layer with 4 mL of a mixture of equal volumes of water and an acetic acid solution (12% w/v). Separate the lower layer, add 0.5 mL of **Solution B** and 0.5 mL of the **Internal standard solution**, and shake. Use the lower layer immediately after separation.

**Sample solution:** Dissolve 2 g of sample in 20 mL of water, add 0.5 mL of **Solution B**, and shake with 30 mL of toluene. Shake 20 mL of the upper layer with 4 mL of a mixture of equal volumes of an acetic acid solution (12% w/v) and water. Separate the lower layer, add 0.5 mL of **Solution B** and 0.5 mL of the **Internal standard solution**, and shake. Use the lower layer immediately after separation.

**Chromatographic system, Appendix IIA**

**Mode:** Gas chromatography

**Detector:** Flame ionization

**Column:** 25-cm × 0.32-mm (i.d.) fused-silica column with poly(dimethyl)(diphenyl)siloxane containing 95% of methyl groups and 5% of phenyl groups¹ as stationary phase (film thickness 0.51 µm)

**Carrier gas:** Helium

**Flow rate:** 1.8 mL/min

**Temperatures**

**Injection port:** 250°

**Detector:** 270°

**Column:** See the temperature program in the table below.
**Injection volume:** 1.5 µL. Use a split vent at a flow rate of 20 mL/min.

**Analysis:** Separately inject equal volumes of the *Standard solution* and *Sample solution* into the chromatograph, record the chromatograms, and measure the responses. [Note—The approximate retention times (relative to cyclohexanamine, which has a retention time of about 2.3 min) for aniline, tetradecane, and *N*-cyclohexylcyclohexanamine are about 1.4, 4.3, and 4.5 min, respectively.]

**Acceptance criteria**
- *Cyclohexanamine*: NMT 10.0 mg/kg
- *Aniline*: NMT 1.0 mg/kg
- *N*-Cyclohexylcyclohexanamine: NMT 1.0 mg/kg

**SPECIFIC TESTS**
- **Loss on Drying,** Appendix IIIC: 105°C for 1 h
  - **Acceptance criteria:** NMT 1%

1 DB-5 available from J&W Scientific, SE-52 available from Restek Corp., or equivalent.

**Auxiliary Information**— Please check for your question in the FAQs before contacting USP.

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<tbody>
<tr>
<td>Monograph</td>
<td>Carla D Mejia, Ph.D. Scientific Liaison 1-301-816-8571</td>
<td>(FI2010) Monographs - Food Ingredients</td>
</tr>
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</table>

*FCC Forum - June 2012*
BRIEFING

Cyclohexanecarboxylic Acid. Because there is no existing FCC monograph for this food ingredient, a new monograph is proposed based, in part, on the Cyclohexanecarboxylic Acid monograph from the 59th Session (2002) of the Joint FAO/WHO Expert Committee on Food Additives (JECFA).

(FI: L. Chen) C117065

Add the following:

- Cyclohexanecarboxylic Acid

Benzoic Acid, Hexahydropyridine
Carboxycyclohexane
Cyclohexanoic Acid
Cyclohexylcarboxylic Acid
Cyclohexylmethanoic Acid
Hexahydrobenzoic Acid
Cyclohexanecarboxylic Acid

\[
\text{C}_7\text{H}_{12}\text{O}_2
\]

Formula wt 128.17
FEMA: 3531
CAS: [98-89-5]

UNII: H9VKD9VL18 [cyclohexanecarboxylic acid]

DESCRIPTION

Cyclohexanecarboxylic Acid occurs as a white solid under ambient temperature. At higher temperatures, it melts and occurs as a colorless, viscous liquid.

Odor: Rum, raisins, fruity, fatty sweet

Solubility: Slightly soluble in water; miscible in fat; miscible in ethanol at room temperature

Boiling Point: \(-232^\circ\text{C} - 233^\circ\text{C}\)
Function: Flavoring agent

IDENTIFICATION
- **INFRARED ABSORPTION**, *Spectrophotometric Identification Tests*, Appendix IIIC
  - **Reference standard**: USP Cyclohexanecarboxylic Acid 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.

ASSAY
- **Procedure**: Proceed as directed under *M-1b*, Appendix XI.
  - **Reference standard**: USP Cyclohexanecarboxylic Acid RS
  - **Acceptance criteria**: NLT 98%

SPECIFIC TESTS
- **Refractive Index**, Appendix II: At 20°
  - **Acceptance criteria**: 1.516–1.520

- **Specific Gravity**: Determine at 25° by any reliable method (see *General Provisions*).
  - **Acceptance criteria**: 1.029–1.037

- **Melting Range or Temperature Determination**, Appendix IIB
  - **Acceptance criteria**: 28°–32°

2S (FCC8)

**Auxiliary Information**—Please check for your question in the FAQs before contacting USP.

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<tr>
<td>Monograph</td>
<td>Liwen Chen Assistant Scientific Liaison 1-301-816-3388</td>
<td>(FI2010) Monographs - Food Ingredients</td>
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</table>
BRIEFING

High-Fructose Corn Syrup, FCC 8 page 545.

1. On the basis of comments received, it is proposed to revise the test for Lead to add an alternate matrix modifier solution which can be used in procedure without air ashing step in the furnace program. Interested parties are encouraged to submit comments.

2. It is also proposed to add clarification to the sample preparation for the Lead test. Interested parties are encouraged to submit comments.

(FI: P. Bhatt) C118011 C118012

High-Fructose Corn Syrup
UNII: XY6UN3QB6S [high fructose corn syrup]

DESCRIPTION
High-Fructose Corn Syrup (HFS) occurs as a water, white to light yellow, somewhat viscous liquid that darkens at high temperatures. It is a saccharine mixture prepared as a clear, aqueous solution from high-dextrose-equivalent corn starch hydrolyzed by the partial enzymatic conversion of glucose (dextrose) to fructose, using an insoluble glucose isomerism preparation that complies with 21CFR 184.1372 and that has been obtained from a pure culture fermentation that produces no antibiotics. It is miscible in all proportions with water.

Function: Nutritive sweetener
Packaging and Storage: Store in tight containers.

IDENTIFICATION

• PROCEDURE
  Sample solution: 100 mg/mL
  Analysis: Add a few drops of the Sample solution to 5 mL of hot alkaline cupric tartrate TS.
  Acceptance criteria: A copious red precipitate of cuprous oxide forms.

ASSAY

• PROCEDURE
  Mobile phase: Degassed, purified water passed through a 0.22-µm filter before use. [Note—Maintain the water at 85° during operation of the chromatograph.]
  Standard solution: Prepare a solution containing a total of about 10% solids, using sugars of known purity (e.g., USP Fructose RS; USP Dextrose RS or NEST Standard Reference Material; maltose, Aldrich Chemical Company; or equivalent) that approximates, on the dry basis, the composition of the sample to be analyzed. Dissolve each standard sugar, in 20 mL of purified water contained in a 50-mL beaker. Heat on a steam bath until all sugars are dissolved, then cool, and transfer to a 100-mL volumetric flask. Dilute with water to volume, and mix. [Note—Freeze the solution if it is to be reused.]
  Sample solution: Dilute to approximately 10% solids using the result from the test for Total Solids (below).
  Chromatographic system, Appendix III
  [Note—Use a suitable high-performance liquid chromatography system such as the one described in Standard Analytical Methods of the Corn Refiners Association.]
  Mode: High-performance liquid chromatography
  Detector: Differential refractometer
  Column: 22- to 31-cm stainless steel column, or equivalent with a stationary phase of prepacked macro reticular polystyrene sulfonated divinyl benzene cation-exchange resin (2% to 8% cross-linked, 8- to 25-
µm particle size), preferably in the calcium or silver form. Examples of acceptable resins are Bio-Rad Amines HDX-87C, or equivalent, for separating DP₁-DP₄ saccharine, and Amines HDX-42C and HDX-42A, or equivalent, for separating DP₁-DP₇ saccharine.

Column temperature: 85°C
Detector temperature: 45°C ± 0.005°C
Injection volume
  Standard solution: 10–20 µL
  Sample solution: 10–50 µL, appropriate for the specific solids content

Standardization: If a corn syrup or maltodextrin is used to supply a DP₄⁺ fraction, take care to include all saccharides in the standard composition calculation. Calculate the dry-basis concentration, in percent, of each individual component in the Standard solution by the formula:

\[
\text{Result} = \frac{W_C}{\sum W_l} \times 100
\]

\[ W_C \] = weight of the sugar of interest
\[ \sum W_l \] = sum of the weights of all sugar components

Standardize by injecting 10–20 µL (about 1.0–2.0 mg of solids) of the Standard solution into the chromatograph. Integrate the peaks and normalize. Sum the individual DP₄⁺ responses from the normalized printout to obtain the total DP₄⁺ normalized response. Calculate the response factors as follows:

\[ R_l = \frac{\text{(known concentration, dry basis %)}}{\text{(measured concentration, normalized %)}} \]

\[ R_l \] = response factor for component l

Compute the response factor for each component relative to glucose (\( R'_l \)) using the following equation:

\[ R'_l = \frac{R_l}{R_G} \]

\[ R_G \] = response factor for glucose
\[ R_l \] = response factor for component l
\[ R'_l \] = response factor relative to glucose for component l

The \( R'_l \) for DP₄⁺ should be programmed as a default value (if automated equipment is used) and used to compute the concentration of higher saccharides.

Analysis: Inject a volume of the Sample solution (sized appropriately for the specific solids content) into the chromatograph, and record the resulting chromatogram. Calculate the concentration of each component as follows:

\[ C_l = \frac{A_l \times R_l \times 100}{\sum A_l R_l} \]

\[ C_l \] = concentration of component l
\[ A_l \] = peak area recorded for component l
\[ R_l \] = response factor for component l

\[ \sum A_l R_l \] sum of the product of the areas (A) and response factors (R) for all components detected

Acceptance criteria
42% HFCS: NLT 97.0% total saccharides, expressed as a percent of solids, of which:

- NLT 42.0% consists of fructose
- NLT 92.0% consists of monosaccharides
- NMT 8.0% consists of other saccharides

55% HFCS: NLT 95.0% total saccharides, expressed as a percent of solids, of which:

- NLT 55.0% consists of fructose
- NLT 95.0% consists of monosaccharides
- NMT 5.0% consists of other saccharides

IMPURITIES

Change to read:

Inorganic Impurities

- **Arsenic, Arsenic Limit Test, Appendix IIIB**
  
  **Sample solution**: Prepare as directed for organic compounds.
  
  **Control**: 1 µg As (1 mL of Standard Arsenic Solution)
  
  **Acceptance criteria**: NMT 1 mg/kg

- **Lead, Lead Limit Test, Atomic Absorption Spectrophotometric Graphite Furnace Method, Method I, Appendix IIIB**
  
  **Sample**: 5 g. [Note—To ensure that a representative subsample of test sample is used for analysis, ultrasonic and/or vortex mixing of the test sample prior to weighing is recommended.]  
  
  **Analysis**: An acceptable alternative method is to omit air ashing step in furnace program and use matrix modifier. If matrix modifier is used instead of air ashing, prepare Modifier solution as directed below.
  
  **Modifier Stock Solutions**: Palladium, 10,000 ppm$^1$ and Magnesium nitrate, 10,000 ppm$^2$
  
  **Modifier Solution**: [Note—Prepare this solution fresh just before use.] Add 0.3 mL of Palladium, 10,000 ppm and 5 mL of Magnesium nitrate, 10,000 ppm to 9.7 mL of 5% nitric acid and mix well.
  
  **Acceptance criteria**: NMT 0.1 mg/kg

- **Sulfur Dioxide, Sulfur Dioxide Determination, Appendix X**
  
  **Sample**: 50 g
  
  **Acceptance criteria**: NMT 0.003%

SPECIFIC TESTS

- **Color**
  
  **Standard solution**: 100 µg/mL of reagent-grade potassium dichromate
  
  **Analysis**: Use a suitable variable-wavelength spectrophotometer capable of measuring percent transmittance throughout the visible spectrum and designed to permit the use of sample and reference cells with path lengths of 2–4 cm. The transmittance of all paired cells should agree within 0.5%.
  
  Using water in the sample and reference cells of 2-cm pathlength, normalize the percent transmittance scale of the spectrophotometer to 100%. Leave the reference cell in place, and replace the water in the sample cell with the Standard solution. Determine the wavelength at which the solution exhibits exactly 54.5% transmittance. This wavelength is defined as $\lambda_c$, the corrected 450-nm wavelength.
  
  Remove the 2-cm cells from the spectrophotometer and, with water in the sample and reference cells of 4-cm pathlength, adjust the percent transmittance scale to 100% with the spectrophotometer set at $\lambda_c$. 
Leave the reference cell in place, and replace the water in the sample cell with sample. Measure the percent transmittance \( T_{450} \). Remove the sample cell, set the wavelength at 600 nm, replace the sample with water, and adjust the percent transmittance scale to 100%. Determine the percent transmittance at 600 nm \( T_{600} \) with the same sample in the sample cell.

Calculate the color of the Sample taken using the formula:

\[
\text{Result} = \frac{\log T_{600} - \log T_{450}}{4}
\]

\( T_{600} \) = percent transmittance at 600 nm
\( T_{450} \) = percent transmittance at \( \lambda_c \) nm

**Acceptance criteria:** Within the range specified by the vendor

- **Residue on Ignition (Sulfated Ash), Appendix IIC**
  
  **Sample:** 10 g
  
  **Acceptance criteria:** NMT 0.05%

- **Total Solids, High Fructose Corn Syrup Solids, Appendix X**
  
  **Analysis:** Determine the refractive index of a sample at 20° or 45°, and use the tables in the test referenced to obtain the percent Total Solids.

  **Acceptance criteria**
  
  - 42% HFCS: NLT 70.5%
  - 55% HFCS: NLT 76.5%

**OTHER REQUIREMENTS**

- **Labeling:** Indicate the color range and presence of sulfur dioxide if the residual concentration is greater than 10 mg/kg.

1 NIST traceable.
2 NIST traceable.

**Auxiliary Information**— Please check for your question in the FAQs before contacting USP.

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<tr>
<td>Monograph</td>
<td>Jeffrey Moore, Ph.D. Scientific Liaison 1-301-816-8288</td>
<td>(FI2010) Monographs - Food Ingredients</td>
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</table>

FCC FCC 8 Page 545
BRIEFING

Pectins, FCC 8 page 851. In an effort to modernize test procedures in the FCC, it is proposed to revise the method for Methanol, Ethanol, and Isopropanol determination in the Organic Impurities section. The revision replaces the existing packed-column gas chromatography method with a capillary column gas chromatography method, and it is based on the equivalent method present in the monograph for Pectin published in USP 35–NF 30. Interested parties are encouraged to submit comments.

(FI: C. Mejia) C115732

Pectins
INS: 440
CAS: [9000-69-5]
UNII: 89NA02M4RX [pectin]

DESCRIPTION

Pectins occur as white, yellow, light gray, or light brown powders. They consist mainly of the partial methyl esters of polygalacturonic acid and their sodium, potassium, calcium, and ammonium salts. They are obtained by extraction in an aqueous medium of appropriate edible plant material, usually citrus fruits or apples. No organic precipitants shall be used other than methanol, ethanol, and isopropanol. In some types of Pectins, a portion of the methyl esters may have been converted to primary amides by treatment with ammonia under alkaline conditions. Pectins dissolve in water, forming an opalescent, colloidal dispersion. They are practically insoluble in ethanol. The commercial product is normally diluted with sugars for standardization purposes. In addition to sugars, Pectins may be mixed with suitable food-grade salts required for pH control and desirable setting characteristics.

Function: Gelling agent; thickener; stabilizer

Packaging and Storage: Store in well-closed containers.

[Note—The following tests and procedures apply to the Pectins as supplied, whether standardized or not, except for specifications covering amide substitution and the weight percent of total galacturonic acid in the Pectin component, in which cases the test procedures include provisions for removing the sugars and soluble salts before analysis of the Pectin component.]

IDENTIFICATION

• Procedure

Sample stock solution: Transfer 0.05 g of sample into a 100-mL volumetric flask, and moisten with pure isopropanol. Add 50 mL of water, and mix with a magnetic stirrer. Using 0.5 M sodium hydroxide, adjust to a pH of 12, stop the stirrer, and leave the solution undisturbed and at room temperature for 15 min. Reduce the pH to 7.0 with 0.5 M hydrochloric acid. Dilute with water to 100.0 mL.

Tris buffer solution: Dissolve 6.055 g of Tris (hydroxylmethyl) aminomethane and 0.147 g of calcium chloride dihydrate in 1 L of water. Adjust to a pH of 7.0 with 1 M hydrochloric acid.

Enzyme solution: 10 mg/mL of pectate lyase in Tris buffer solution

[Note—Solutions to which this solution is added should be analyzed immediately (see Analysis.)]

Sample blank: In a quartz cuvette, mix thoroughly 0.5 mL of Tris buffer solution, 1.0 mL of Sample solution, and 1.0 mL of water.

Enzyme Blank: In a quartz cuvette, mix thoroughly 0.5 mL of Tris buffer solution, 1.5 mL of water, and 0.5 mL of Enzyme solution.

Sample solution: In a quartz cuvette, mix thoroughly 0.5 mL of Tris buffer solution, 1.0 mL of Sample solution, and 1.0 mL of water.
stock solution, 0.5 mL of water, and 0.5 mL of Enzyme solution.

**Analysis:** Using a suitable UV spectrophotometer, measure the absorbance of the solutions at 235 nm immediately following addition of the Enzyme solution (time 0) and at 10 min after. Using the absorbance values measured for each solution at the specified times, calculate the corrected absorbance, $A_t$, at $t = 0$ min and $t = 10$ min with the following equation:

$$A_t = A_S - (A_{EB} + A_{SB})$$

$A_S$ = absorbance of the Sample solution at time $t$

$A_{EB}$ = absorbance of the Enzyme blank at time $t$

$A_{SB}$ = absorbance of the Sample blank at time $t$

Calculate the amount of unsaturated product (U) produced as follows:

$$U = (A_{t=10} - A_{t=0})/(4600 \times l)$$

$I$ = thickness (cm) of the cuvette

Report the value of $U$ as $U \times 10^5$

**Acceptance criteria:** $U$ is greater than 0.5, whereas other gums show essentially no change

**IMPURITIES**

**Inorganic Impurities**

- **Lead**

  [Note—Use deionized water throughout this procedure.]

  **Standard stock solution:** 1000 µg/mL of Pb.  [Note—Use a commercially available certified solution.]

  **Standard solution:** 2 µg/mL Pb, prepared immediately before use by pipetting 0.10 mL of Standard stock solution into a 50-mL volumetric flask containing 30 mL of water, 4 mL of 20% (v/v) hydrochloric acid, and 4 mL of 0.1 M EDTA. Dilute with water to volume, and mix.

  **Control solution:** 0.4 µg/mL Pb, prepared by pipetting 5.0 mL of the Standard solution into a 25-mL volumetric flask containing 10 mL of water, 2 mL of 20% (v/v) hydrochloric acid, and 2 mL of 0.1 M EDTA. Dilute with water to volume, and mix.

  **Blank solution:** Transfer 30 mL of water, 4 mL of 20% (v/v) hydrochloric acid, and 4 mL of 0.1 M EDTA into a 50-mL volumetric flask. Dilute with water to volume, and mix.

  **Sample:** 2.0 g

  **Sample solution:** Transfer the Sample into a clean, 100-mL glass beaker, add 25 mL of 70% (v/v) nitric acid, cover with a watch glass, and heat at low to moderate heat on a hot plate in a fume hood for 2 h. Remove the watch glass, and continue to heat until the sample is dry with no visible fumes. Add 0.5 mL of 70% (v/v) nitric acid, and heat to dryness. Cool to room temperature, and add 2 mL of 20% (v/v) hydrochloric acid and 2 mL of 0.1 M EDTA. Quantitatively transfer the solution to a 25-mL volumetric flask, dilute with water to volume, and mix.

  **Analysis:** Set up an inductively coupled plasma emission spectrometer according to manufacturer's instructions. Using the lead emission line of 220.35 nm, calibrate the instrument with the Blank solution and the Standard solution. Then analyze the Sample solution and the Control solution.

  **Acceptance criteria:** The concentration in the Sample solution is not more than that in the Control solution. (NMT 5 mg/kg)

- **Sulfur Dioxide, Sulfur Dioxide Determination, Appendix X**

  **Analysis:** Proceed as directed, but use the following modification for the Sample Introduction and Distillation. Transfer 20 g of sample into the round-bottom flask, C, and add 20 mL of ethanol to moisten
the sample. Add 400 mL of water, swirling vigorously to disperse the sample. Reassemble the apparatus, making sure that the tapered joints are clean and greased with stopcock grease, and continue as directed beginning with, "the nitrogen flow through the 3% Hydrogen Peroxide Solution..." in the first paragraph.

Acceptance criteria: NMT 0.005%

Change to read:

Organic Impurities

- Methanol, Ethanol, and Isopropanol

[Note—Internal standard solution, Standard stock solution, and Standard solution are equilibrated and brought to volume at 20°. Keep solutions in a dry place.]

Internal standard solution: 0.05 mg/mL of n-propanol 5000 µg/mL of USP 2-Butanol RS. [Note—This solution can be stored at 5°–8° for 3 months.]

Standard stock solution: 5000 µg/mL each of USP Methyl Alcohol RS, USP Alcohol Determination-Alcohol RS, and USP 2-Propanol RS

Standard solution: To a 250-mL volumetric flask add 2.5 mL of Internal standard solution and 2.5 mL of Standard stock solution. Dilute with water to volume, and mix. Each mL of this solution contains 50 µg each of USP Methyl Alcohol RS, USP Alcohol Determination-Alcohol RS, USP 2-Propanol RS, and USP 2-Butanol RS. Transfer 1.0 g of this solution to a 10-mL headspace vial. [Note—This solution can be stored at 5°–8° for 3 months.]

Sample: 1.0 g of pectin

Sample solution: 100 mg of sample dissolved in 10 mL of water. [Note—Use sodium chloride as a dispersing agent, if necessary.] Transfer the Sample and 5 g of sucrose to a stoppered 100-mL Erlenmeyer flask containing 90 mL of water, add 1.0 mL of Internal standard solution, and dilute with water to 100 mL. Mix the solution using a magnetic stirrer. Continue stirring until all the pectin has been completely dissolved; typically it takes about 1–2 h. This solution contains 50 µg/mL of USP 2-Butanol RS. Transfer 1.0 g of this solution to a 10-mL headspace vial.

Standard alcohol solution: Using a micropipet, transfer 50 mg each of methanol (63.21 µL); ethanol (63.35 µL); and isopropanol (63.65 µL) into a 1000-mL volumetric flask, dilute to volume, and mix.

Sodium nitrite solution: 250 mg/mL sodium nitrite

Chromatographic system, Appendix IIA

Mode: Gas chromatography; chromatograph equipped with a balanced-pressure headspace autosampler

Detector: Flame ionization

Column: 90-cm x 4-mm (id) glass column, or equivalent, with the first 15 cm packed with Chrompack (or equivalent) and the remainder packed with 120– to 150-mesh Porapak R (or equivalent) 30-m x 0.32-mm (i.d.) capillary column with a 6% cyanopropylphenyl–94% dimethylpolysiloxane stationary phase and a 1.8-µm film thickness. [Note—An alternative column, such as a 25-m x 0.32-mm (i.d.) capillary column of a highly cross-linked copolymer of ethylvinylbenzene and divinylbenzene and a 5-µm film thickness, can be used as long as the system suitability requirements are met.]

Column temperature: Isothermal at 150°

Injection port temperature: 250°

Temperature
Column: 70°
Injection port: 200°
Detector: 280°

Carrier gas: Nitrogen
Flow rate: 80 mL/min

Split flow rate: 30 mL/min
Injection size: 2 mL
Split ratio: 20:1

Sampler parameters
Equilibration time: 10 min
Equilibration temperature: 70°
Agitation speed: 500 rpm
Agitation-on time: 5 s
Agitation-off time: 90 s
Syringe temperature: 80°
Syringe size: 2.5 mL
Fill speed: 100 µL/s
Pull-up delay: 2.0 s

[Note—A GC run time of 10.5 min should be sufficient to complete the analysis. However, these conditions should be optimized according to the instruments used.]

System suitability
Sample: Standard solution.  [Note—The relative retention times for methanol, ethanol, 2-propanol, and 2-butanol are 0.5, 0.6, 0.7 and 1.0, respectively.]

Suitability requirements
Suitability requirement 1: The resolution (R) is NLT 1.5, between each pair of analytes.
Suitability requirement 2: The relative standard deviation is NMT 10% for each analyte.

Analysis: Weigh 200 mg of urea, and place it in a 25-mL amber-glass vial. Purge the urea with nitrogen for 5 min, add 1 mL of saturated oxalic acid solution, close the vial with a rubber stopper, and swirl. Add 1 mL of Sample solution and 1 mL of Internal standard solution; simultaneously start a stopwatch (t = 0). Swirl the vial and recap it with an open screw cap fitted with a silicone rubber septum. Swirl the vial until t = 30 s. At t = 45 s, inject 0.6 mL of Sodium nitrite solution through the septum. Swirl until t = 70 s, and at t = 150 s, use a pressure lock syringe to withdraw 1.0 mL of the headspace through the septum. Inject the headspace sample into the injection port of the gas chromatograph. Repeat this procedure, but use 1 mL of the Standard alcohol solution instead of the Sample solution. Separately inject equal volumes of the Standard solution and Sample solution into the chromatograph, record the chromatograms, and measure the responses.

Calculate the total amount (mg) percentage of methanol, ethanol, and isopropanol (T) present in 1 mL of the Sample solution in the sample:

\[ T = \left[ V_{MS} \times (R_{MU} / R_{MS}) \times d_M \right] + \left[ V_{ES} \times (R_{EU} / R_{ES}) \times d_E \right] + \left[ V_{IS} \times (R_{IU} / R_{IS}) \times d_I \right] \]
V_{MS} = volume of methanol in the Standard alcohol solution (mL)
R_{MU} = ratio of the peak area of methanol in the Sample solution to that of n-propanol in the Internal standard solution
R_{MS} = ratio of the peak area of methanol in the Standard alcohol solution to that of n-propanol in the Internal Standard solution
d_{M} = density of methanol, 0.791 g/mL
V_{ES} = volume of ethanol in the Standard alcohol solution (mL)
R_{EU} = ratio of the peak area of ethanol in the Sample solution to that of n-propanol in the Internal standard solution
R_{ES} = ratio of the peak area of ethanol in the Standard alcohol solution to that of n-propanol in the Internal Standard solution
d_{E} = density of ethanol, 0.7893 g/mL
V_{IS} = volume of isopropanol in the Standard alcohol solution (mL)
R_{IU} = ratio of the peak area of isopropanol in the Sample solution to that of n-propanol in the Internal standard solution
R_{IS} = ratio of the peak area of isopropanol in the Standard alcohol solution to that of n-propanol in the Internal Standard solution
d_{I} = density of isopropanol, 0.7855 g/mL

\[\text{Result} = \left(\frac{R_{U}}{R_{S}}\right) \times \left(\frac{C_{S}}{C_{U}}\right) \times F \times 100\]

R_{U} = internal standard ratio (peak response of the respective alcohol to the peak response of the internal standard) from the Sample solution
R_{S} = internal standard ratio (peak response of the respective alcohol to the peak response of the internal standard) from the Standard solution
C_{S} = concentration of the respective residual alcohol (methanol, or ethanol, or 2-propanol) in the Standard solution (µg/mL)
C_{U} = concentration of the respective residual alcohol (methanol, or ethanol, or 2-propanol) in the Sample solution (g/mL)
F = conversion factor (10^{-6} g/µg)

\[\text{Result} = \left(\frac{1000T}{W}\right)\]

W = weight of the sample taken to prepare the Sample solution (mg)

\[\text{Acceptance criteria: NMT 1.0% total methanol, ethanol, and isopropanol}\]

\[\text{Sodium Methyl Sulfate}\]

\[\text{Mobile phase: Prepare a 0.04 M potassium hydrogen phthalate solution by transferring 16.4 g of potassium hydrogen phthalate into a 2-L volumetric flask, diluting with water to volume, and mixing. Then, filter the}\]
solution through a 0.45-µm pore size filter.

**Standard solution:** 0.1 mg/mL of anhydrous sodium methyl sulfate in *Mobile phase*

**Sample solution:** Suspend 1 g of the sample in 10.0 mL of 50% (v/v) ethanol solution. Stir for 30 min using a Teflon-coated stirring bar. Allow the suspension to precipitate and filter. Evaporate a 1.0-mL aliquot to dryness under reduced pressure (10 mm Hg), and heat at 60°. Redissolve the residue in 1.0 mL of the *Mobile phase*.

**Chromatographic system,** Appendix IIA

- **Mode:** Liquid chromatography
- **Detector:** Refractive index
- **Column:** 25-cm × 4.6-mm (id) column packed with Nucleosil 10SB (or equivalent)
- **Column temperature:** 40°
- **Flow rate:** 1 mL/min
- **Injection volume:** 20 µL

**System suitability**

- **Sample:** Standard solution
- **Suitability requirement:** The relative standard deviation for three replicate injections is NMT 4.0% for the response factor of the sodium methyl sulfate peak obtained using the formula:

\[
\text{Result} = \left( \frac{A_S}{C_S} \right)
\]

- \(A_S\) = peak area of the *Standard solution*
- \(C_S\) = concentration (mg/mL) of sodium methyl sulfate in the *Standard solution*

**Analysis:** Separately inject the *Standard solution* and *Sample solution* into the chromatograph and record the peak areas. Calculate the percentage of methyl sulfate in the sample:

\[
\text{Result} = \left( \frac{C_S A_U}{A_S W} \right)
\]

- \(C_S\) = concentration (mg/mL) of sodium methyl sulfate in the *Standard solution*
- \(A_U\) = peak area obtained from the *Sample solution*
- \(A_S\) = peak area obtained from the *Standard solution*
- \(W\) = weight of the sample taken to prepare the *Sample solution* (g)

**Acceptance criteria:** NMT 0.1%

### SPECIFIC TESTS

- **ASH (ACID-INSOLUBLE),** Appendix IIC
  - **Acceptance criteria** NMT 1.0%

- **Degree of Amide Substitution and Total Galacturonic Acid in the Pectin Component**

  **Clark’s solution:** Mix 100 g of magnesium sulfate heptahydrate with 0.3 mL of sulfuric acid and sufficient water to make 180 mL of solution.

  - **Sample:** 5 g
  - **Sample solution:** Transfer the *Sample* to a suitable beaker, add a mixture of 5 mL of 2.7 N hydrochloric acid and 100 mL of 60% ethanol, and stir for 10 min.

  **Analysis:**

  1. **Step 1:** Transfer the *Sample solution* to a fritted-glass filter tube (30–60-mL capacity), and wash the filtrate with six 15-mL portions of the same hydrochloric acid–60% ethanol mixture, followed by 60% ethanol,
until the filtrate is free of chlorides. Finally, wash with 20 mL of ethanol, dry for 2.5 h in an oven at 105°C, cool, and weigh. Transfer exactly one-tenth of the total net weight of the now ash-free, dried sample (representing 0.5 g of the original, unwashed sample) to a 250-mL conical flask, and moisten the sample with 2 mL of ethanol. Add 100 mL of recently boiled and cooled water, stopper the flask, and swirl occasionally until a complete solution is formed. Add 5 drops of phenolphthalein TS, titrate with 0.1 N sodium hydroxide, and record the results as the initial titer ($V_1$).

**Step 2:** Add exactly 20 mL of 0.5 N sodium hydroxide to the flask, stopper, shake the flask vigorously, and let it stand for 15 min. Add exactly 20 mL of 0.5 N hydrochloric acid, and shake the flask until the pink color disappears. Titrate with 0.1 N sodium hydroxide to a faint pink color that persists after vigorous shaking; record this value as the saponification titer ($V_2$).

**Step 3:** Quantitatively transfer the contents of the conical flask into a 500-mL distillation flask fitted with a Kjeldahl trap and a water-cooled condenser, the delivery tube of which extends well beneath the surface of a mixture of 150 mL of carbon dioxide-free water and 20.0 mL of 0.1 N hydrochloric acid in a receiving flask. Add 20 mL of a 1:10 sodium hydroxide solution to the distillation flask, seal the connections, and begin heating carefully to avoid excessive foaming. Continue heating until 80–120 mL of distillate has been collected. Add a few drops of methyl red TS to the receiving flask, titrate the excess acid with 0.1 N sodium hydroxide, and record the volume required (mL) as $S$. Perform a blank determination (see General Provisions) on 20.0 mL of 0.1 N hydrochloric acid, and record the volume of 0.1 N sodium hydroxide required (mL) as $B$. Record the amide titer ($B - S$) as $V_3$.

**Step 4:** Transfer exactly one-tenth of the total net weight of the dried sample (representing 0.5 g of the original, unwashed sample) prepared in Step 1 to a 50-mL beaker and wet with about 2 mL of ethanol. Dissolve the sample in 25 mL of 0.125 M sodium hydroxide. Agitate the solution for 1 h at room temperature. Quantitatively transfer the saponified solution to a 50-mL volumetric flask, and dilute with water to volume. Transfer 25.0 mL of this solution to the round-bottom flask of the distillation apparatus, and add 20 mL of *Clark's solution*. Start the distillation by heating the round-bottom flask. Collect the first 15 mL of distillate separately in a graduated cylinder. Then start the steam supply, and continue distillation until 150 mL of distillate has been collected in a 200-mL beaker. Quantitatively combine the distillates, titrate with 0.05 M sodium hydroxide to a pH of 8.5, and record the volume of titrant required (mL) as $S$. Perform a blank determination (see General Provisions) using 20 mL of water. Record the required volume of 0.05 M sodium hydroxide (mL) as $B$. Record the acetate ester titer ($S - B$) as $V_4$.

**Calculations:** Calculate the degree of amidation (as the percentage of total carboxyl groups):

\[\text{Result} = 100\left[\frac{V_3}{V_1 + V_2 + V_3 - V_4}\right]\]

Calculate the weight of galacturonic acid (mg):

\[\text{Result} = 19.41(V_1 + V_2 + V_3 - V_4)\]

The amount (mg) of galacturonic acid obtained in this way is the content of one-tenth of the weight of the washed and dried sample. To calculate the percentage of galacturonic acid on a moisture- and ash-free basis, multiply the number of mg obtained by 1000/x, in which x is the weight, (mg), of the washed and dried sample. If the sample is known to be of the non-amidated type, only $V_1$ and $V_2$ need to be determined; $V_3$ may be regarded as zero in the formula for calculating mg of galacturonic acid.

**Acceptance criteria**

**Degree of amide substitution in the pectin component:** NMT 25% of total carboxylic groups

**Total galacturonic acid in the pectin component:** NLT 65.0%, calculated on the ash-free, dried basis
• **Loss on Drying,** Appendix IIC: 105° for 2 h

  **Acceptance criteria:** NMT 12.0%

• **Total Insoluble Substances**

  [Note—Use deionized water free from dust on insoluble particles throughout this procedure.]

  **Sample:** 1 g

  **Analysis:** Dry 70-mm glass fiber filter paper\(^4\) for 1 h in an oven equipped with a fan and set to 105°. Transfer the paper to a desiccator containing silica gel, allow it to cool, and weigh.

  Transfer the Sample into a 250-mL beaker, add 5 mL of isopropanol to the beaker and, while stirring, add 100 mL of a previously mixed and filtered solution of 0.03 M sodium hydroxide containing 0.1% (w/w) tetrasodium ethylenediamine tetraacetic acid (EDTA). Stir the mixture for 30 min at room temperature, then heat to boiling. **[Caution—Some Pectins foam when heated.]** Filter the hot solution through the previously dried filter paper under a vacuum. Rinse the beaker and filter five times with 100 mL of warm (approximately 50°) water. Dry the filter paper in the oven at 105° for 1 h. Transfer it to the desiccator and allow it to cool. Weigh the paper. Calculate the percentage of insoluble material:

  \[
  \text{Result} = \left(\frac{M_2 - M_1}{S}\right) \times 100
  \]

  \(S\) = sample weight (g)

  \(M_1\) = weight of the filter paper before filtration step (g)

  \(M_2\) = weight of the filter paper after filtration and drying steps (g)

  **Acceptance criteria:** NMT 3.0%

**OTHER REQUIREMENTS**

• **Labeling:** Indicate the presence of sulfur dioxide if the residual concentration is greater than 10 mg/kg.

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\(^1\) Zebron ZB-624 (Phenomenex, Torrance, CA), or equivalent. \(^2\) Reacti-Flasks, or equivalent

\(^3\) Precision Sampling Corporation, or equivalent

\(^4\) GF/B (Whatman 1821 070, or equivalent)

**Auxiliary Information**— Please check for your question in the FAQs before contacting USP.

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<td>Carla D Mejia, Ph.D. Scientific Liaison 1-301-816-8571</td>
<td>(FI2010) Monographs - Food Ingredients</td>
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FCC FCC 8 Page 851
Polydextrose, *FCC 8* page 899. On the basis of comments received, it is proposed to revise the text under *Description*. Comments received indicate that the material can comprise concentrations of less than 1% of citric acid or less than 0.1% of phosphoric acid. Comments are encouraged.

(FI: P. Bhatt) C115831

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**Polydextrose**

**INS:** 1200

**CAS:** [68424-04-4]

**UNII:** VH2XOU12IE [polydextrose]

### DESCRIPTION

**Change to read:**

Polydextrose occurs as an off-white to light tan solid. It is a randomly bonded polymer prepared by the condensation of a melt that consists of approximately 90% d-glucose, 10% sorbitol, and 1% citric acid or 0.1% phosphoric acid on a weight basis. The 1,6-glycosidic linkage predominates in the polymer, but other possible bonds are present. The product contains small quantities of free glucose, sorbitol, and d-anhydroglucoses (levoglucosan), with traces of citric acid or phosphoric acid. It may be partially reduced by transition metal catalytic hydrogenation in an aqueous solution. It may be neutralized with any food-grade base and decolorized and deionized for further purification. It is very soluble in water.

**Function:** Bulking agent; humectant; texturizer

**Packaging and Storage:** Store in tight, light-proof containers.

### IDENTIFICATION

- **A. PROCEDURE**
  - **Sample solution:** 100 mg/mL
  - **Analysis:** Add 4 drops of 5% aqueous phenol solution to 1 drop of the *Sample solution*, then rapidly add 15 drops of sulfuric acid.
  - **Acceptance criteria:** A deep yellow to orange color appears.

- **B. PROCEDURE**
  - **Sample solution:** 100 mg/mL
  - **Analysis:** While vigorously swirling (vortex mixer), add 1.0 mL of acetone to 1.0 mL of the *Sample solution*.
    - [Note—Retain this solution for *Identification* test C (below).]
  - **Acceptance criteria:** The solution remains clear.

- **C. PROCEDURE**
  - **Analysis:** While vigorously swirling, add 2.0 mL of acetone to the retained solution from *Identification* test B.
  - **Acceptance criteria:** A heavy, milky turbidity develops immediately.

- **D. PROCEDURE**
  - **Sample solution:** 20 mg/mL
  - **Sample:** Add 4 mL of alkaline cupric citrate TS to 1 mL of the *Sample solution*. Boil vigorously for 2–4 min.
    - Remove from heat, and allow the precipitate (if any) to settle.
  - **Acceptance criteria:** The supernatant is blue or blue-green.
ASSAY

**PROCEDURE**

**Standard stock solution:** 0.2 mg/mL of α-d-glucose

**Standard solutions:** 50 µg/mL, 40 µg/mL, 20 µg/mL, 10 µg/mL, and 5 µg/mL of α-d-glucose made from the standard stock solution

**Sample stock solution:** 1.0 mg/mL

**Sample solution:** 40 µg/mL made from the sample stock solution

**Phenol solution:** Add 20 mL of water to 80 g of phenol.

**Analysis:** On a daily basis, pipet 2.0 mL of each standard solution and the sample solution into separate, acetone-free, 15-mL screw-cap vials. Add 0.12 mL of the phenol solution, and mix gently. Uncap each vial and rapidly add 5.0 mL of sulfuric acid. Immediately recap each vial, and shake vigorously. [Caution—Wear rubber gloves and a safety shield while adding sulfuric acid.]

Let the vials stand at room temperature for 45 min, then determine the absorbance of each sample at 490 nm in a suitable spectrophotometer, using a phenol solution–sulfuric acid reagent blank in the reference cell. Repeat the procedure three times and obtain the mean absorbance value. For the standard curve, plot mean absorbance values versus concentrations, in µg/mL, obtained from triplicate standard solutions.

Calculate the percentage of polymer by the formula:

$$\text{Result} = 1.05 \left( \frac{100(A - Y)}{(S \times C) - P_G - 1.11P_L} \right)$$

1.05 = an experimentally derived correction factor to account for the polymer (which also contains a small amount of sorbitol) not giving the exact amount of color given by an equivalent amount of glucose monomers

A = sample absorbance

Y = the y-intercept of the standard curve

S = slope (approximately 0.02) of absorbance versus glucose concentration, in g/mL, obtained from the standard curve

C = concentration of the sample stock solution, adjusted for ash and moisture (g/mL)

P_G = percentage of glucose determined under the test for monomers (below)

P_L = percentage of levoglucosan determined under the test for monomers (below)

1.11 = conversion factor from levoglucosan, which gives an equivalent amount of color to an equivalent weight of glucose

**Acceptance criteria:** NLT 90.0% polymer, calculated on the anhydrous, ash-free basis

**IMPURITIES**

**Inorganic Impurities**

**Lead**

[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.]
**Apparatus:** Use a suitable spectrophotometer (Perkin-Elmer Model 6000, or equivalent), a graphite furnace containing a L’vov platform (Perkin-Elmer Model HGA-500, or equivalent), and an autosampler (Perkin-Elmer Model AS-40, or equivalent). Use a lead hollow-cathode lamp (lamp current of 10 mA), a slit width of 0.7 mm (set low), the wavelength set at 283.3 nm, and a deuterium arc lamp for background correction.

**Lead nitrate solution:** 100 µg of lead (Pb) ion/mL prepared as follows. Dissolve 159.8 mg of lead nitrate 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 stock solution:** 10 µg of lead (Pb) ion/mL from the Lead nitrate solution. [Note—Prepare on the day of use.]

[Note—As an alternative to preparing the Lead nitrate solution and Standard stock solution, NIST Standard Reference Material containing 10 mg of lead/kg, or equivalent may be used.]

**Standard solutions:** 0.02 µg, 0.05 µg, 0.1 µg, and 0.2 µg of lead (Pb) ion/mL from the Standard stock solution

**Matrix modifier solution:** 10 mg/mL of dibasic ammonium phosphate

**Sample solution:** Transfer 1 g of sample into a 10-mL volumetric flask, add 5 mL of water, and mix. Dilute to volume, and mix.

**Spiked sample solution:** Prepare a solution as directed under Sample solution, but add 100 µL of the Standard stock solution, dilute to volume, and mix. This solution contains 0.1 µg of lead/mL.

**Analysis:** With the use of an autosampler, atomize 10-µL aliquots of the four Standard solutions, using the following sequence of conditions:

1. Dry at 130° with a 20-s ramp period, a 40-s hold time, and a 300-mL/min argon flow rate;
2. Char at 800° with a 20-s ramp period, a 40-s hold time, and a 300-mL/min argon flow rate;
3. Atomize at 2400° for 6 s with a 50-mL/min argon flow rate;
4. Clean at 2600° with a 1-s ramp period, a 5-s hold time, and a 300-mL/min argon flow rate; and
5. Recharge at 20° with a 2-s ramp period, a 20-s hold time, and a 300-mL/min argon flow rate.

Atomize 10 µL of the Matrix modifier solution in combination with either 10 mL of the Sample solution or 10 µL of the Spiked sample solution under identical conditions used for the Standard solutions.

Plot a standard curve using the concentration, in µg/mL, of each Standard solution versus its maximum absorbance value compensated for background correction, and draw the best straight line. From the standard curve, determine the concentrations, $C_S$ and $C_A$, in µg/mL, of the Sample solution and the Spiked sample solution, respectively.

Calculate the quantity, in mg/kg, of lead in the sample taken by the formula:

\[
\text{Result} = 10C_S/W
\]

\[W = \text{weight of the sample taken (g)}\]

Calculate the recovery by the formula:

\[
\text{Result} = 100[(C_A - C_S)/0.1]
\]

\[0.1 = \text{amount of lead added to the Spiked sample solution (µg/mL)}\]

**Acceptance criteria:** NMT 0.5 mg/kg

**Nickel Limit Test, Method II, Appendix IIIIB:** For Hydrogenated Polydextrose

**Acceptance criteria:** NMT 2 mg/kg

**Organic Impurities**
• **5-HYDROXYMETHYLFURFURAL AND RELATED COMPOUNDS**

  **Sample solution:** 10 mg/mL

  **Analysis:** Read the absorbance of the *Sample solution* against a water blank at 283 nm in a 1-cm quartz cell in a spectrophotometer.

  Calculate the percentage of 5-hydroxymethylfurfural and related compounds by the formula:

  \[
  \text{Result} = \left( \frac{0.749 \times A}{C} \right)
  \]

  0.749 = a composite proportionality constant that includes the extinction coefficient and other molecular weight, unit, and volume conversions

  \[
  A = \text{absorbance of the Sample solution}
  \]

  \[
  C = \text{concentration of the Sample solution, corrected for ash and moisture (mg/mL)}
  \]

  **Acceptance criteria:** NMT 0.1%, calculated on the anhydrous, ash-free basis

• **MONOMERS**

  **Octadecane solution:** 0.5 mg/mL of *n*-octadecane in pyridine

  **Standard solution:** Transfer 50 mg of α-glucose, 40 mg of anhydrous d-sorbitol, and 35 mg of d-anhydroglucoses, all accurately weighed, into a 100-mL volumetric flask; dissolve in and dilute with pyridine to volume.

  **Silylated standard solution:** Transfer 1.0 mL of the *Standard solution* to a screw-cap vial, and add 1.0 mL of *Octadecane solution* and 0.5 mL of *N*-trimethylsilylimidazole. Cap the vial, and immerse it in an ultrasonic bath at 70° for 60 min.

  **Sample solution:** Transfer 20 mg of sample into a screw-cap vial, and add 1.0 mL of *Octadecane solution*, 1 mL of pyridine, and 0.5 mL of *N*-trimethylsilylimidazole. Cap the vial, and immerse it in an ultrasonic bath at 70° for 60 min.

  **Chromatographic system, Appendix IIA**

  **Mode:** Gas chromatography

  **Detector:** Flame-ionization detector

  **Column:** 250-cm × 2-mm (id) glass column, or equivalent, packed with 3% OV-1 stationary phase on 100- to 120-mesh Gas Chrom Q, or equivalent

  **Temperatures**

  **Column:** 175°

  **Injection port:** 210°

  **Detector:** 230°

  **Injection volume:** About 3 µL

  **Analysis:** Initially, inject the *Silylated standard solution* into the gas chromatograph. Repeat twice, then inject duplicate portions of the *Sample solution.* [Note—The relative retention times for d-anhydroglucoses (levoglucosan), pyranose form; furanose form (not present in standard); *n*-octadecane; α-glucose; d-sorbitol; and β-glucose are 3.7, 4.3, 5.1, 8.7, 11.3, and 13.3 min.]

  Calculate the percentage of each monomer by the formula:

  \[
  \text{Result} = \left( \frac{R \times W_S}{R_S \times W} \right)
  \]
\[ R = \frac{\text{ratio of the area of the monomer peak to the area of the octadecane peak in the sample injection}}{\text{octadecane peak}} \]
\[ W_S = \text{weight of the respective monomer in the Silylated standard solution (mg)} \]
\[ R_S = \frac{\text{mean ratio of the area of the monomer peak to the area of the octadecane peak in the standard injections}}{\text{octadecane peak}} \]
\[ W = \text{weight of the sample taken, adjusted for residue on ignition and moisture (mg)} \]

**Acceptance criteria**
- **\( d^- \)Anhydroglucoses:** NMT 4.0%, calculated on the anhydrous, ash-free basis
- **Glucose and sorbitol:** NMT 6.0%, calculated on the anhydrous, ash-free basis

**SPECIFIC TESTS**
- **MOLECULAR WEIGHT LIMIT**
  - **Mobile phase:** Dissolve 35.0 g of sodium nitrate and 1.0 g of sodium azide in 100 mL of HPLC-grade water. Filter through a 0.45-µm filter into a 4-L flask. Dilute with HPLC-grade water to volume. Degas by applying an aspirator vacuum for 30 min. The resulting eluent is 0.1 N sodium nitrate containing 0.025% sodium azide.
  - **Standard solution:** Transfer 20 mg each of dextrose\(^2\); stachyose\(^2\); and 5800, 23,700, and 100,000 molecular weight (MW) pullulan standards\(^2\) into a 10-mL volumetric flask. Dissolve in and dilute with Mobile phase to volume. Filter through a 0.45-µm syringe filter.
  - **Sample solution:** 5 mg/mL in Mobile phase, and filtered through a 0.45-µm filter
  - **Chromatographic system,** Appendix IIA
    - **Mode:** High-performance liquid chromatography
    - **Detector:** Differential refractometer
    - **Column:** Waters Ultrahydrogel 250 A size-exclusion column, or equivalent
    - **Column temperature:** 45\(^\circ\)
    - **Detector cell temperature:** 35\(^\circ\) ± 0.1\(^\circ\)
    - **Flow rate:** 0.8 mL/min, reproducible to 0.5%
    - **Injection volume:** 50 µL
  - **Setup:** Use either a loop injector or suitable autosampler, a column heating block or oven and a computing integrator, or a computer data handling system with molecular weight determination capabilities. Set the differential refractometer at a sensitivity of \(4 \times 10^{-6}\) refractive index units full scale, and set the plotter of the integrator to 64 mV full scale. Noise attributable to the detector and electronics should be less than 0.1% full scale.
  - **Column equilibration:** After installing a new column in the HPLC, pump Eluent through it overnight at 0.3 mL/min. Before calibration or analysis, increase the flow slowly to 0.8 mL/min over a 1-min period, then pump at 0.8 mL/min for at least 1 h before the first injection. Check the flow gravimetrically, and adjust it if necessary. Reduce the flow to 0.1 mL/min when the system is not in use.
  - **Data system setup:** Set the integrator or computerized data-handling system as its respective manual instructs for normal gel permeation chromatographic determinations. Set the integration time to 15 min.
  - **Column standardization:** After equilibrating the HPLC system at a flow rate of 0.8 mL/min for at least 1 h, inject 50 µL of the Standard solution five times, allowing 15 min between injections. Record the retention times of the various components in the Standard solution. Retention times for each component should agree within ±1 s. Insert the average retention time along with the molecular weight of each component into the calibration table of the molecular weight distribution software.
**System suitability:** Check the regression results for a cubic fit of the calibration points. They should have an $R^2$ value of 0.9999+. Dextrose and stachyose should be baseline resolved from one another and from the 5800 MW pullulan standard. Elevated valleys are usually observed between the 5800, the 23,700, and the 100,000 MW pullulan standards.

**Analysis:** Inject 50 µL of the Sample solution, following the same conditions and procedure as described under Column standardization. Using the Molecular Weight Distribution software of the data-reduction system, generate a molecular weight distribution curve of the sample.

**Acceptance criteria:** There is no measurable peak above a molecular weight of 22,000.

- **pH, pH Determination, Appendix IIB**
  - **Sample solution:** 100 mg/mL
  - **Acceptance criteria**
    - Untreated polydextrose: 2.5–7.0
    - Neutralized or decolorized polydextrose: 5.0–6.0

- **Residue on Ignition (Sulfated Ash), Method I, Appendix IIC**
  - **Acceptance criteria**
    - Untreated polydextrose: NMT 0.3%
    - Neutralized or decolorized polydextrose: NMT 2.0%

**Change to read:**

- **Water, Water Determination, Appendix IIB**
  - **Analysis:** Determine as directed, but using pyridine—a mixture of hydral solvent and hydral formamide dry (2:1) as solvent—instead of methanol in the titration vessel.
  - **Acceptance criteria:** NMT 4.0%

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1 Available from NIST.

2 Available from Polymer Laboratories, Inc., Technical Center, Amherst Fields Research Park, 160 Old Farm Road, Amherst, MA 01002.

**Auxiliary Information—** Please check for your question in the FAQs before contacting USP.

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<td>(Fl2010) Monographs - Food Ingredients</td>
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FCC FCC 8 Page 899
BRIEFING

Rebaudioside A, **FCC 8 page 975.** On the basis of comments and data received, it is proposed to revise the HPLC Assay and Related Steviol Glycosides procedures based on the new C18 HPLC procedure proposed for the Steviol Glycosides monograph in the December 2011 FCC Forum. The proposed new HPLC method harmonizes the HPLC testing approaches for stevia-based ingredients in FCC. It also addresses the potential co-elution of rebaudioside A with “iso-rebA”, when running the current monograph’s amine-column separation which could result in an over estimation of rebaudioside A contents. The major differences between the method proposed in this monograph and that proposed for the Steviol Glycosides monograph in the December 2011 FCC Forum include:

1. This proposed monograph requires use of only one external quantitative reference standard (USP Rebaudioside A) to quantify all nine steviol glycosides using relative response factors as opposed to the use of multiple external reference standards proposed in the Steviol Glycosides monograph.
2. This proposed monograph uses a different concentration of rebaudioside A in the Standard solutions for the Assay and Related Steviol Glycosides procedures compared to that proposed in the Steviol Glycosides proposal. This change is based on the expected concentration range of steviol glycosides in high-purity rebaudioside A ingredients, and is supported by validation data.
3. The calculation for the Related Steviol Glycosides test in this proposal corrects an error in the proposed calculation in the Steviol Glycosides monograph proposal.
4. The relative response factors for the HPLC procedure in this proposal are different from those proposed in the Steviol Glycosides monograph. This is based on new data submitted.
5. The resolution requirement for system suitability is tightened to NLT 1.7 based on data received.

(FIEC: J. Moore) C117313

Rebaudioside A

Reb A

Rebiana

Kaur-16-en-18-oic acid, 13-[(O-β-d-glucopyranosyl-(1→2)-O-β-d-glucopyranosyl-(1→3)]-β-d-glucopyranosyl]oxy]-, β-d-glucopyranosyl ester, (4α)

13-{(2-O-β-d-glucopyranosyl-3-O-β-d-glucopyranosyl-β-d-glucopyranosyl]oxy] kaur-16-en-18-oic acid β-d-glucopyranosyl ester

\[\text{C}_{44}\text{H}_{70}\text{O}_{23}\]

Formula wt 967.01
UNII: B3FUD0528F [rebaudioside a]

DESCRIPTION
Rebaudioside A is a white to off-white, hygroscopic fine crystal, granule, or powder having a sweet taste. It is freely soluble in ethanol:water 50/50 (v/v), sparingly soluble in water, and sparingly soluble in ethanol. It is obtained from the leaves of the *Stevia rebaudiana* (Bertoni) plant in a multi-step separation and purification process. Principle steps include extraction of steviol glycosides from the leaves using an aqueous or aqueous alcoholic (ethanol or methanol) solvent and purification of rebaudioside A from the resulting mixture of steviol glycosides by resin absorption followed by recrystallization from an aqueous or aqueous alcoholic (ethanol or methanol) solvent. It is composed predominantly of rebaudioside A, a glycoside of the ent-kaurenoid diterpenoid aglycone known as steviol.

Function: Nonnutritive sweetener; sugar substitute

Packaging and Storage: Keep dry, and store in tight containers at ambient temperature.

IDENTIFICATION

- **A. INFRARED ABSORPTION, Spectrophotometric Identification Tests, Appendix IIIC**
  - **Reference standard:** USP Rebaudioside A RS
  - **Sample and standard preparation:** A
  - **Acceptance criteria:** The spectrum of the sample exhibits maxima at the same wavelengths as those in the spectrum of the *Reference standard*.

**Change to read:**

- **B. PROCEDURE**
  - **Acceptance criteria:** The retention time of the major peak (excluding the solvent peak) in the chromatogram of the *Sample solution* is the same as that of the 4750 mg/L *Rebaudioside A standard solution* corresponds to that of *Standard solution 1* (FCC8) in the Assay.

ASSAY

**Change to read:**

- **PROCEDURE**

  [Note—Rebaudioside A is hygroscopic, and accurate quantitative analysis requires moisture equilibration before analysis. Equilibrate sample and rebaudioside A standard specimens in the lab NLT 24 h before weighing. Intermittent stirring will ensure uniform moisture absorption. The Water content used in the calculation should be determined at the time of weighing and after equilibration.]

  **Acetate buffer:** Dissolve 0.125 g of ammonium acetate in 900 mL of water, adjust to a pH of 4.3 with glacial acetic acid solution, and dilute to 1 L.  [Note—It may be necessary to adjust the ratio of ammonium acetate to acetic acid. Changing the pH adjusts the retention time of rebaudioside A and related glycosides. Decreasing the pH of the buffer will decrease the retention time of rebaudioside A.]

  **Mobile phase:** 13% (v/v) Acetate buffer in acetonitrile

  **Diluent:** 25% (v/v) Acetate buffer in acetonitrile. [Note—Allow Diluent to come to room temperature before use.]

  **Rebaudioside A standard solutions:** 250, 1000, 2500, and 5000 mg/L of USP Rebaudioside A RS in Diluent

  **Stevioside standard stock solution:** 250 mg/L of USP Stevioside RS in Diluent
Stevioside standard solutions: 0.5, 5.0, 25, and 250 mg/L of USP Stevioside RS in Diluent: from
Stevioside standard stock solution
Sample solution: 5000 mg/L in Diluent
Chromatographic system, Appendix IIA
Mode: High-performance liquid chromatography
Detector: UV 210 nm
Column: 15-cm × 4.6-mm, packed with a propyl amino silane phase bonded to silica gel (5-µm particle
diameter)¹
Column temperature: 30°C
Flow rate: 1.5 mL/min
Injection size: 15 µL
System suitability
Samples: 5000 mg/L Rebaudioside A standard solution and 0.5 mg/L Stevioside standard solution
Suitability requirements
Detector response: Peak-to-noise ratio (peak height/baseline noise) is NLT 3 for the stevioside peak
from the 0.5 mg/L Stevioside standard solution, where peak height is expressed in mAU, and
baseline noise is the maximum deflection of the baseline (mAU) in a blank at the retention time of
stevioside over the same baseline peak width in min:
Relative standard deviation: NMT 2.0% for rebaudioside A peak area and retention time from the
5000 mg/L Rebaudioside A standard solution
Retention time: The retention time for the rebaudioside A peak from the 5000 mg/L Rebaudioside A
standard solution is less than 15.0 min:
Tailing factor: NMT 2.0 for the rebaudioside A peak from the 5000 mg/L Rebaudioside A standard
solution
Analysis: Separately inject equal volumes of the Rebaudioside A standard solutions, Stevioside standard
solutions, and Sample solution into the chromatograph, and measure the responses for the major peaks
on the resulting chromatograms. [Note—The approximate retention times for rebaudioside A and its
related steviol-glycosides are listed in Chromatographic Profile Table 1. If the retention time for
rebaudioside A is below 11 min, adjust the ratio of ammonium acetate to acetic acid.]

Chromatographic Profile Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Approx.-Retention Time (min)</th>
<th>Molecular Weight (g/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rubusoside</td>
<td>2.6</td>
<td>642.73</td>
</tr>
<tr>
<td>Dulcoside A</td>
<td>4.3</td>
<td>788.87</td>
</tr>
<tr>
<td>Stevioside</td>
<td>6.6</td>
<td>804.88</td>
</tr>
<tr>
<td>Rebaudioside C</td>
<td>8.5</td>
<td>951.01</td>
</tr>
<tr>
<td>Rebaudioside F</td>
<td>9.6</td>
<td>936.99</td>
</tr>
<tr>
<td>Rebaudioside A</td>
<td>14</td>
<td>967.01</td>
</tr>
<tr>
<td>Steviolbioside</td>
<td>29</td>
<td>642.73</td>
</tr>
<tr>
<td>Rebaudioside D</td>
<td>41</td>
<td>1429.15</td>
</tr>
<tr>
<td>Rebaudioside B</td>
<td>66</td>
<td>804.88</td>
</tr>
</tbody>
</table>

Prepare a standard curve for rebaudioside A by plotting rebaudioside A peak areas versus concentrations
in mg/L, corrected for purity, based on the USP Reference Standard label claim. [Note—Peak
responses for all other steviol-glycosides besides rebaudioside A are used in the Related-Steviol
Glycosides impurities test procedure. From the standard curve, calculate the concentration \( C_U \) of rebaudioside A in the Sample solution in mg/L. Calculate the percentage of rebaudioside A in the portion of the sample taken:

\[
\text{Result} = \frac{C_U}{C_{SMP}} \times 100
\]

\( C_U \) = concentration of rebaudioside A in the Sample solution determined from the standard curve (mg/L)

\( C_{SMP} \) = concentration of the sample in the Sample solution (mg/L)

**Solution A:** 5 mM potassium phosphate buffer, pH 3.0. Prepare by dissolving 1.36 g of potassium dihydrogen phosphate (KH$_2$PO$_4$) in water in a 2-L volumetric flask, adjusting with phosphoric acid to a pH of 3.0, diluting to volume with water, and passing through a 0.45-µm filter.

**Solution B:** Acetonitrile

**Mobile phase:** See Table 1.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Solution A (%)</th>
<th>Solution B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>65</td>
<td>35</td>
</tr>
<tr>
<td>25</td>
<td>65</td>
<td>35</td>
</tr>
<tr>
<td>35</td>
<td>25</td>
<td>75</td>
</tr>
<tr>
<td>50</td>
<td>25</td>
<td>75</td>
</tr>
<tr>
<td>52</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>67</td>
<td>90</td>
<td>10</td>
</tr>
</tbody>
</table>

**Diluent:** Solution A and Solution B (65:35 v/v)

**Standard solution 1:** 1.2 mg/mL of USP Rebaudioside A RS in Diluent. Prepare by dissolving in 80% volume of Diluent, sonicating until dissolved (2–5 min), and diluting to volume.

**Standard solution 2:** 0.03 mg/mL of USP Rebaudioside A RS in Diluent. Prepare by dissolving in 80% volume of Diluent, sonicating until dissolved (2–5 min), and diluting to volume.

**System suitability solution:** 1.2 mg/mL of USP Steviol Glycosides System Suitability RS in Diluent. Prepare by dissolving in 80% volume of Diluent, sonicating until dissolved (2–5 min), and diluting to volume.

**Sample:** Rebaudioside A is hygroscopic, and accurate quantitative analysis requires moisture equilibration before analysis. Equilibrate sample specimens in the lab NLT 24 h before weighing by spreading into a thin layer NMT 1/4 inch. Intermittent stirring will ensure uniform moisture absorption. The water content of the equilibrated samples should be determined at the time of weighing using the Karl Fischer method (Water, Water Determination, Method I, Appendix IIB).

**Sample solution:** 1.2 mg/mL of the Sample in Diluent. Prepare by dissolving in 80% volume of Diluent, sonicating until dissolved (2–5 min), and diluting to volume.

**Chromatographic system,** Appendix IIA

**Mode:** High-performance liquid chromatography

**Detector:** UV 210 nm

**Column:** 25-cm × 4.6-mm, packed with 5-µm reversed phase C18 silica stationary phase

**Column temperature:** 32°C

**Flow rate:** 0.5 mL/min

**Injection volume:** 15 µL
System suitability

Sample: System suitability solution

Suitability requirements

Suitability requirement 1: The relative standard deviation of the rubusoside peak area is NMT 2.5% for five replicate injections.

Suitability requirement 2: The resolution, R, between rebaudioside A and stevioside is NLT 1.7.

Suitability requirement 3: The relative standard deviation of the rebaudioside A peak area is NMT 1.5% for five replicate injections.

Analysis: Separately inject equal volumes of the System suitability solution, Standard solution 1, and Sample solution into the chromatograph. Use the chromatogram of the Standard solution to identify the rebaudioside A peak, and the chromatogram of the System suitability solution to identify the peaks corresponding to the other eight steviol glycosides listed in Table 2.

Table 2: Chromatographic Profile

<table>
<thead>
<tr>
<th>Compound</th>
<th>Relative Retention Time</th>
<th>Relative Response Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rebaudioside D</td>
<td>0.80</td>
<td>0.83</td>
</tr>
<tr>
<td>Rebaudioside A</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Stevioside</td>
<td>1.03</td>
<td>1.22</td>
</tr>
<tr>
<td>Rebaudioside F</td>
<td>1.10</td>
<td>1.02</td>
</tr>
<tr>
<td>Rebaudioside C</td>
<td>1.15</td>
<td>0.98</td>
</tr>
<tr>
<td>Dulcoside A</td>
<td>1.21</td>
<td>1.16</td>
</tr>
<tr>
<td>Rubusoside</td>
<td>1.44</td>
<td>1.06</td>
</tr>
<tr>
<td>Rebaudioside B</td>
<td>1.59</td>
<td>1.07</td>
</tr>
<tr>
<td>Steviolbioside</td>
<td>1.61</td>
<td>1.38</td>
</tr>
</tbody>
</table>

Calculate the percentage of rebaudioside A in the portion of the sample taken:

\[
\text{Result} = \left( \frac{r_U}{r_S} \right) \times \left( \frac{C_S}{C_U} \right) \times 100
\]

\[r_U\] = peak area for the analyte in the Sample solution

\[r_S\] = peak area for rebaudioside A in Standard solution 1

\[C_S\] = concentration of rebaudioside A in Standard solution 1, corrected for purity and water content based on the USP RS label information (mg/mL)

\[C_U\] = concentration of the sample in the Sample solution corrected for water content (as prescribed in the Sample) and solvents (as prescribed under Ethanol and Methanol, below) (mg/mL)

Acceptance criteria: NLT 95.0%, calculated on the anhydrous and solvent-free basis

IMPURITIES

Inorganic Impurities

- Arsenic, Arsenic Limit Test, Appendix IIIB

[Note—Alternatively, the arsenic content may be determined by the following method.]

[Note—When water is specified as a diluent, use deionized ultra-filtered water. When nitric acid is specified, use nitric acid of a grade suitable for trace element analysis with as low a content of arsenic as practical.]
Dilute nitric acid: Dilute 2.0 mL of nitric acid with water to 100 mL.

Yttrium internal standard solution: Use a commercially available 1000 µg/kg yttrium ICP standard solution.
[Note—The internal standard should be 20 µg/kg in all blanks, standards, and samples.]

Standard stock solution: Dilute a 1000 mg/kg commercially available arsenic ICP standard solution to 1000 µg/kg with Dilute nitric acid, transfer 10.0 mL of this solution to a 100-mL volumetric flask, add 2.0 mL of Dilute nitric acid, and dilute with water to volume (100 µg/kg).
[Note—Prepare this solution fresh every 2 weeks.]

Standard solution: 10 µg/kg arsenic prepared as follows. Transfer 5.0 mL of the Standard stock solution to a 50-mL volumetric flask, add 3.0 mL of Dilute nitric acid, add 1.0 mL of Yttrium internal standard solution, and dilute with water to volume (10 µg/kg).
[Note—Prepare this solution fresh weekly.]

Standard blank solution: Transfer 1.0 mL of the Yttrium internal standard solution to a 50-mL volumetric flask, add 3.0 mL of Dilute nitric acid, and dilute with water to volume.

Sample solution
[Caution—Wear proper eye protection, protective clothing, and gloves during sample preparation. Closely follow the manufacturer’s safety instructions for use of the microwave digestion apparatus.]
Transfer 500 mg of sample into a Teflon digestion vessel liner. Prepare samples in duplicate. Add 10 mL of nitric acid, and swirl gently. Cover the vessels with lids, leaving the vent fitting off. Predigest for at least 1 h under a hood. Place the rupture membrane in the vent fitting, and tighten the lid. Place all vessels on the turntable of a microwave oven with a magnetron frequency of about 2455 MHz and a selectable output power of 0–950 watts in 1% increments, equipped with advanced composite vessels with 100-mL polytef liners\(^2\). Connect the vent tubes to the vent trap, and connect the pressure-sensing line to the appropriate vessel. Initiate a two-stage digestion procedure by heating the microwave at 15% power for 10 min, followed by 25% power for 10 min. Remove the turntable of vessels from the oven, and allow the vessels to cool to room temperature (a cool water bath may be used to speed the cooling process). Vent the vessels when they reach room temperature. Transfer the cooled digests into 50-mL volumetric flasks, add 1.0 mL of the Yttrium internal standard solution, and dilute with deionized water to volume.

Spectrophotometric system, Plasma Spectrochemistry, Appendix IIC

Mode: Inductively coupled plasma-mass spectrometer (ICP-MS)

ICP-MS: Use a system equipped with a quadrupole mass spectrometer and an ion detector maintained under vacuum; the system may include a suppression system to mitigate interference from the \(^{40}\text{Ar}^{35}\text{Cl}^+\) ion. If not, correction for this interference must be determined by a suitable method, such as that recommended by the instrument manufacturer. The isotope ratio of \(^{40}\text{Ar}^{35}\text{Cl}^+\)/\(^{40}\text{Ar}^{37}\text{Cl}^+\) in the Standard blank solution may be used to correct this interference.

Analysis
[Note—Instrument performance must be verified to conform to the manufacturer’s specifications for resolution and sensitivity. Before analyzing samples, the instrument must pass a suitable performance check.]
Aspirate the Standard blank solution, Standard solution, and Sample solution, at least in duplicate. The Standard blank solution should not yield a significant intensity for arsenic. Calculate the internal standard ratios for the Sample solution and Standard solution as ratio of the arsenic to the yttrium intensities. Calculate the concentration (mg/kg) of arsenic in the sample taken:

\[
\text{Result} = \left(\frac{R_U}{R_S}\right) \times C_S \times \left(50/S\right)
\]
RU = internal standard ratio (arsenic response/yttrium response) from the Sample solution
RS = internal standard ratio (arsenic response/yttrium response) from the Standard solution
CS = concentration of arsenic in the Standard solution (µg/kg)
50 = sample dilution factor
S = weight of sample used to prepare the Sample solution (mg)

Acceptance criteria: NMT 1 mg/kg, calculated on the anhydrous basis

- Lead, Lead Limit Test, Atomic Absorption Spectrophotometric Graphite Furnace Method, Method I, Appendix IIIB

  [Note—Alternatively, the lead content may be determined by the following method.]
  [Note—When water is specified as a diluent, use deionized ultra-filtered water. When nitric acid is specified, use nitric acid of a grade suitable for trace element analysis with as low a content of lead as practical.]

  **Dilute nitric acid:** Dilute 2.0 mL of nitric acid with water to 100 mL.

  **Thallium internal standard solution:** Commercially available 1000 µg/kg thallium ICP standard solution.

  [Note—The internal standard should be 20 µg/kg in all blanks, standards, and samples.]

  **Standard stock solution:** Dilute a 1000 mg/kg commercially available lead ICP standard solution to 1000 µg/kg with Dilute nitric acid, transfer 10.0 mL of this solution to a 100-mL volumetric flask, add 2.0 mL of Dilute nitric acid, and dilute with water to volume (100 µg/kg).

  [Note—Prepare this solution fresh every 2 weeks.]

  **Standard solution:** 10 µg/kg of lead prepared as follows: transfer 5.0 mL of the Standard stock solution to a 50-mL volumetric flask, add 3.0 mL of Dilute nitric acid, add 1.0 mL of Thallium internal standard solution, and dilute with water to volume (10 µg/kg).

  [Note—Prepare this solution fresh weekly.]

  **Standard blank solution:** Transfer 1.0 mL of the Thallium internal standard solution to a 50-mL volumetric flask, add 3.0 mL of Dilute nitric acid, and dilute with water to volume.

  **Sample solution**

    [Caution—Wear proper eye protection, protective clothing, and gloves during sample preparation. Closely follow the manufacturer's safety instructions for use of the microwave digestion apparatus.]

    Transfer 500 mg of sample into a Teflon digestion vessel liner. Prepare samples in duplicate. Add 10 mL of nitric acid, and swirl gently. Cover the vessels with lids, leaving the vent fitting off. Predigest for at least 1 h under a hood. Place the rupture membrane in the vent fitting, and tighten the lid. Place all vessels on the turntable of a microwave oven with a magnetron frequency of about 2455 MHz and a selectable output power of 0–950 watts in 1% increments, equipped with advanced composite vessels with 100-mL polytef liners². Connect the vent tubes to the vent trap, and connect the pressure-sensing line to the appropriate vessel. Initiate a two-stage digestion procedure by heating the microwave at 15% power for 10 min, followed by 25% power for 10 min. Remove the turntable of vessels from the oven, and allow the vessels to cool to room temperature (a cool water bath may be used to speed the cooling process). Vent the vessels when they reach room temperature. Transfer the cooled digests into 50-mL volumetric flasks, add 1.0 mL of the Thallium internal standard solution, and dilute with deionized water to volume.

  **Spectrophotometric system, Plasma Spectrochemistry, Appendix IIC**

  **Mode:** Inductively coupled plasma-mass spectrometer (ICP-MS)

  **ICP-MS:** Use a system equipped with a quadrupole mass spectrometer and an ion detector maintained under vacuum. The instrument should read all isotopes for lead (206 amu, 207 amu, and 208 amu) and the thallium internal standard (205 amu), and should report the total lead content using the most naturally abundant isotope at 208 amu.

  **Analysis**
[Note—Instrument performance must be verified to conform to the manufacturer's specifications for resolution and sensitivity. Before analyzing samples, the instrument must pass a suitable performance check.]

Aspirate the Standard blank solution, Standard solution, and Sample solution, at least in duplicate. The Standard blank solution should not yield a significant intensity for lead. Calculate the internal standard ratios for the Sample solution and Standard solution as ratio of the lead to the thallium intensities. Calculate the concentration (mg/kg) of lead in the sample taken:

\[
\text{Result} = \left( \frac{R_U}{R_S} \right) \times C_S \times \frac{50}{S}
\]

- \( R_U \) = internal standard ratio (lead response/thallium response) from the Sample solution
- \( R_S \) = internal standard ratio (lead response/thallium response) from the Standard solution
- \( C_S \) = concentration of lead in the Standard solution (µg/kg)
- 50 = sample dilution factor
- \( S \) = weight of sample used to prepare the Sample solution (mg)

Acceptance criteria: NMT 1 mg/kg, calculated on the anhydrous basis

Change to read:

Organic Impurities

- **Ethanol and Methanol**
  
  **Internal standard solution**: 10 µg/mL of 1-butanol
  
  **Standard stock solution**: 12.5 mg/mL of ethanol and 12.5 mg/mL of methanol. [Note—Use water free of organics. Prepare fresh daily.]

  **Standard solutions**: By serial dilution of the Standard stock solution, prepare solutions with ethanol and methanol concentrations of 1250 µg/mL, 625 µg/mL, 125 µg/mL, 62.5 µg/mL, 12.5 µg/mL, and 1.25 µg/mL. Separately add 4.0 mL each of these ethanol–methanol solutions and 1.0 mL of Internal standard solution to headspace vials, and cap tightly.

  **Sample**: 100 mg
  
  **Sample solution**: Transfer the Sample into a headspace vial, add 4.0 mL of water, add 1 mL of Internal standard solution, and cap tightly.

  **Blank**: Transfer 4.0 mL of water into a headspace vial, and cap tightly.

Chromatographic system, Appendix IIA

**Mode**: Gas chromatography equipped with a headspace analyzer

**Detector**: Flame ionization

**Column**: 30-m × 0.32-mm (id) high polarity capillary column with a crosslinked and bonded poly(ethylene glycol) stationary phase and a 1-µm film thickness\(^3\)

**Column temperature**: 3 min at 35°; increase to 180° at 10°/min; maintain at 180° for 1 min

**Injection port temperature**: 250°

**Detector temperature**: 250°

**Carrier gas**: Helium

**Flow rate**: 35 cm/s linear velocity

**Incubation**: 80° for 20 min

**Injection syringe**: Heated, gas-tight, 85°

**Injection size**: 1 mL of headspace
System suitability

Sample: Standard solution

Suitability requirement 1: The resolution, R, between any two components is NLT 3.

Suitability requirement 2: The relative standard deviation of the individual peak responses from replicate injections is NMT 15%.

Analysis: Separately inject equal volumes of the Standard solutions and Sample solution into the chromatograph, record the chromatograms, and measure the peak responses. [Note—The approximate retention times for ethanol and methanol are 8.1 min and 7.4 min, respectively.]

Prepare standard curves for ethanol and methanol by plotting on the y-axis the ratios of analyte peak area to internal standard peak area and on the x-axis the concentration of analyte (µg/mL). [Note—The coefficient of determination for each standard curve should be NLT 0.995.]

Determine the concentration (C_U), in µg/mL, of each analyte in the Sample solution using the appropriate standard curve and the ratio of each analyte peak area to the internal standard peak area from the Sample solution chromatogram. Determine the percentage of each analyte (ethanol and methanol) in the portion of the Sample taken:

\[
\text{Result} = C_U \times \frac{4}{S} \times 0.1
\]

- \( C_U \) = concentration of analyte in the Sample solution determined from the standard curve (µg/mL)
- 4 = total volume of water used dissolve the Sample for the Sample solution (mL)
- \( S \) = Sample weight (mg)
- 0.1 = correction factor, taking into account unit conversion from µg/mg to µg/µg and conversion to percentage

Acceptance criteria

Ethanol: NMT 0.50%
Methanol: NMT 0.020%

• Related Steviol Glycosides

Acetate buffer, Mobile phase, Diluent, Rebaudioside A standard solutions, Stevioside standard stock solution, Stevioside standard solutions, Sample solution, Chromatographic system, and System suitability: Prepare as directed in the Assay.

Analysis: Proceed as directed in the Assay, but with the following modifications for the standard curve and calculations.

Using the peak area responses from the Stevioside standard solutions, prepare a standard curve for stevioside by plotting stevioside peak areas versus concentrations, in mg/L, corrected for purity, based on the USP Reference Standard label claim. From this standard curve, determine the concentration (mg/L) of stevioside in the Sample solution. Calculate the percentage of stevioside in the sample taken:

\[
\text{Result} = \frac{C_U}{C_{SMP}} \times 100
\]

- \( C_U \) = concentration of stevioside in the Sample solution determined from the standard curve (mg/L)
- \( C_{SMP} \) = concentration of the sample in the Sample solution (mg/L)

For the seven other steviol glycoside impurities (rubusoside, dulcoside A, rebaudioside C, rebaudioside F, rebaudioside D, steviolbioside, and rebaudioside B) use the stevioside standard curve prepared above to calculate the mg/L stevioside equivalents for each. Separately calculate the percentage of each analyte (rubusoside, dulcoside A, rebaudioside C, rebaudioside F, rebaudioside D, steviolbioside, and
rebaudioside B) in the sample taken, which takes into account the differences in molecular weights between the analytes and stevioside:

\[
\text{Result} = \left( \frac{C_U \times M_r1}{M_r2} \right) \times \frac{C_{SMP}}{100}
\]

- \(C_U\) = concentration of stevioside equivalents in the Sample solution determined from the standard curve (mg/L)
- \(M_r1\) = molecular weight of the analyte (see Chromatographic Profile Table 1 in the Assay)
- \(M_r2\) = molecular weight of stevioside, 804.88
- \(C_{SMP}\) = concentration of the sample in the Sample solution (mg/L)

**Solution A, Solution B, Mobile Phase, Diluent, System suitability solution, Sample, Sample solution, Standard solution 2, Chromatographic system, and System suitability:** Prepare as directed in the Assay.

**Analysis:** Separately inject equal volumes of the System suitability solution, Standard solution 2, and Sample solution into the chromatograph. Calculate the percentages of rebaudioside D, stevioside, rebaudioside F, rebaudioside C, dulcoside A, rubusoside, rebaudioside B, and steviolbioside in the portion of the sample taken as follows, which takes into account the UV response factors between the analytes and rebaudioside A:

\[
\text{Result} = \left( \frac{r_U}{r_S} \right) \times \left( \frac{C_S}{C_U} \right) \times \left( \frac{1}{F} \right) \times 100
\]

- \(r_U\) = peak area for the analyte in the Sample solution
- \(r_S\) = peak area for rebaudioside A in Standard solution
- \(C_S\) = concentration of USP Rebaudioside A RS in Standard solution 2, corrected for purity and water content based on the USP RS label information (mg/mL)
- \(C_U\) = concentration of the sample in the Sample solution corrected for water content (as prescribed in the Sample) and solvents (as prescribed under Ethanol and Methanol, below) (mg/mL)
- \(F\) = relative response factor (see Table 2)

Add together the individual percentages of the eight measured steviol glycoside impurities corrected for water (as prescribed in the Sample section of the Assay) and solvents (as prescribed under Ethanol and Methanol, below). 12S (FCC8)

**Acceptance criteria:** The sum of the percentages for all eight steviol glycoside impurities is NMT 5%, calculated on the anhydrous and solvent-free basis.

**SPECIFIC TESTS**

- **pH, pH Determination, Appendix IIB**
  - **Sample:** 10 mg/mL
  - **Acceptance criteria:** Between 4.5 and 7.0
- **Residue on Ignition (Sulfated Ash), Appendix IIC**
  - **Sample:** 1 g
  - **Acceptance criteria:** NMT 1%, calculated on the anhydrous basis
- **Water, Water Determination, Method I, Appendix IIB**
  - **Acceptance criteria:** NMT 6%

1-Cosmosil Sugar-D (Nacalai Tesque), or equivalent.
1 YMC-Pack ODS-AQ (YMC America Inc., http://www.ymcamerica.com), or equivalent.

2 MDS 2100 (CEM Corporation, Matthews, NC, USA) or equivalent.

3 DB-WAXetr (Agilent Technologies), or equivalent.

**Auxiliary Information**— Please check for your question in the FAQs before contacting USP.

<table>
<thead>
<tr>
<th>Topic/Question</th>
<th>Contact</th>
<th>Expert Committee</th>
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<tbody>
<tr>
<td>Monograph</td>
<td>Jeffrey Moore, Ph.D.</td>
<td>(FL2010) Monographs - Food Ingredients</td>
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<tr>
<td></td>
<td>Scientific Liaison</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1-301-816-8288</td>
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*FCC FCC 8 Page 975*
BRIEFING

**Rosemary Extract.** On the basis of comments and data received, a new monograph for Rosemary Extract is proposed.

1. The Identification section contains two thin-layer chromatographic tests which are designed to separate and develop polar and nonpolar components of the sample separately. These tests are based on high-performance thin-layer chromatographic procedures, which are new to FCC. Revisions to *Thin-Layer Chromatography, Appendix IIA* are also proposed in this *FCC Forum*. See briefing under *Thin-Layer Chromatography, Appendix IIA.*

2. This monograph is intended to address different formulations of Rosemary Extract which are approved for uses in foods, therefore Assay specifications for *Carnosic Acid and Carnosol Content* are based on the content claimed by the manufacturer of the ingredient being tested.

(FI: K. Laurvick) C113721

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*Add the following:*

- **Rosemary Extract**

**Powdered Rosemary Extract**

**INS:** 392

**CAS:** [84604-14-8]

**DESCRIPTION**

Rosemary Extract may occur as a powder or a liquid. It is prepared by extraction from the leaves of *Rosmarinus officinalis*, using food grade ethanol or acetone as extraction solvents. It may also be extracted using supercritical carbon dioxide. Subsequent production steps may include filtration, purification, solvent evaporation, drying and sieving. Rosemary Extract may be deodorized, decolorized, and standardized using diluents and carriers permitted for use in foods. It is composed primarily of phenolic acids, flavonoids, diterpenes, diterpenoids, and triterpenes, and is characterized by the content of carnosic acid and carnosol. Rosemary Extract is insoluble in water.

**Function:** Flavoring agent; antioxidant

**Packaging and Storage:** Store in tight, light-resistant containers, protected from heat, light, moisture, and oxygen.

**IDENTIFICATION**

- **A. Procedure**
  - **Acceptance criteria:** The chromatogram obtained from the Sample solution displays major peaks that exhibit the same relative retention times as the major peaks observed in the chromatogram of Standard solution A, as obtained in the Assay for *Carnosic Acid and Carnosol Content*.

- **B. Thin-Layer Chromatography (Polar Compounds), Thin-Layer Chromatography, Appendix IIA**
  - **Sample solution:** 20 mg/mL in methanol
  - **Standard solution:** 20 mg/mL of USP Powdered Rosemary Extract RS in methanol
  - **System suitability solution:** 0.5 mg/mL of USP Rosmarinic Acid RS and 0.6 mg/mL of USP Caffeic Acid RS in methanol
  - **Adsorbent:** 0.25-mm layer of chromatographic silica gel with a pore size of 60 Å, loaded with a fluorescent
indicator with an excitation wavelength of 254 nm

**Derivatization reagent A**: 5 mg/mL of 2-aminoethyl diphenylborinate in ethyl acetate

**Derivatization reagent B**: 50 mg/mL of polyethylene glycol 400 in dichloromethane

**Developing solvent system**: Ethyl acetate, formic acid, and water [15:1:1] (v/v/v)

**Application volume**: 8 µL for the Sample solution, and 2 µL for the Standard solution and the System suitability solution

**Analysis**: The suitability of this chromatographic system is demonstrated by the separation of the System suitability solution into two distinct bands with approximate R\(_F\) values of 0.73 (light blue fluorescent band: rosmarinic acid) and 0.76 (light blue fluorescent band: caffeic acid).

Using a suitable high-performance thin-layer chromatographic system, separately apply the Sample solution, Standard solution, and System suitability solution to an appropriate high-performance thin-layer chromatographic plate. Develop the plate in a saturated solvent chamber (allow 15 min for chamber saturation prior to development; use plates with a controlled humidity of 33%) to a distance of 70 mm from the lower edge of the plate. Remove the plate from the developing chamber, and dry for 3 min at 100 °. Apply Derivatization reagent A then Derivatization reagent B (allowing the plate to dry between applications of the reagents), and examine the plate under UV light at 366 nm.

**Acceptance criteria**: The principal bands obtained from the Sample solution correspond in color, size, and R\(_F\) value to those obtained from the Standard solution. Bands for rosmarinic acid and caffeic acid should be present (as described in Analysis) as well as the following bands: a red band directly below the solvent front; a green band with an approximate R\(_F\) value of 0.50; an intense orange band with an approximate R\(_F\) value of 0.21; and a characteristic pattern of lightly-colored bands with approximate R\(_F\) values of 0.1–0.5.

- **C. Thin-Layer Chromatography (Nonpolar Compounds)**, Thin-Layer Chromatography, Appendix IIA

  **Sample solution**: 20 mg/mL in methanol
  **Standard solution A**: 20 mg/mL of USP Powdered Rosemary Extract RS in methanol
  **Standard solution B**: 0.5 mg/mL of USP Carnosic Acid RS in methanol

  **Adsorbent**: 0.25-mm layer of chromatographic silica gel with a pore size of 60 Å, loaded with a fluorescent indicator with an excitation wavelength of 254 nm

  **Derivatization reagent**: Carefully add 1 mL of anisaldehyde, 20 mL of glacial acetic acid, 170 mL of methanol, and 10 mL of sulfuric acid (in the exact order written) to a beaker of appropriate size. [Caution—Heat is generated; prepare with caution.]

  **Developing solvent system**: Toluene and ethyl acetate [70:30] (v/v)

  **Application volume**: 1 µL

  **Analysis**: Using a suitable high-performance thin-layer chromatographic system, separately apply the Sample solution, Standard solution A, and Standard solution B to an appropriate high-performance thin-layer chromatographic plate. Develop the plate in a saturated solvent chamber (allow 15 min for chamber saturation prior to development; use plates with a controlled humidity of 33%) to a distance of 60 mm from the lower edge of the plate. Dry the plate in a stream of cool air for 10 min, then apply the Derivatization reagent to the plate. Heat the plate at 100 ° for 10 min, then examine the plate under UV light at 366 nm.

  **Acceptance criteria**: The principal bands obtained from the Sample solution correspond in color, size, and R\(_F\) value to those obtained from Standard solution A. The Sample solution exhibits a band corresponding in color and R\(_F\) value to that obtained from Standard solution B (presence of carnosic acid).

**ASSAY**

- **Carnosic Acid and Carnosol Content**

  **Diluent**: Add 0.5 mL of phosphoric acid to 100 mL of methanol.
Solution A: 0.5% phosphoric acid in water (v/v)

Mobile phase: Acetonitrile and Solution A [65:35]

Standard solution A: 200–500 µg/mL of USP Powdered Rosemary Extract RS in Diluent. Sonicate for 5 min, then filter through a 0.45-µm filter.

Standard solution B: 100 µg/mL of USP Carnosic Acid RS in Diluent. Sonicate for 5 min, then filter through a 0.45-µm filter.

Sample solution: 500 µg/mL in Diluent. Sonicate for 5 min, then filter through a 0.45-µm filter.

Chromatographic system, Appendix II

Mode: High-performance liquid chromatography

Detector: UV 230 nm

Column: 4.6-mm × 250-mm column that contains 5-µm porous silica microparticles chemically bonded to octadecylsilane

Flow rate: 1.5 mL/min

Temperature: 25°

Injection size: 5 µL

System suitability

Sample: Standard solution B

Suitability requirement 1: The tailing factor for the carnosic acid peak is 0.90–1.30.

Suitability requirement 2: The relative standard deviation for the carnosic acid peak response (replicate injections) is NMT 2%.

Analysis: Separately inject the Standard solutions and the Sample solution into the chromatograph, and record the resulting chromatograms. Identify the peaks present in the chromatograms by comparison to the reference chromatograms supplied with the USP Reference Standards.

Calculate (separately) the percentages of carnosic acid and carnosol in the portion of the sample taken:

\[
\text{Result} = \left( \frac{r_U}{r_S} \right) \times \left( \frac{C_S}{C_U} \right) \times F \times \left( \frac{M_{W1}}{M_{W2}} \right) \times 100
\]

\(r_U\) = peak area of the analyte of interest obtained from the chromatogram of the Sample solution

\(r_S\) = peak area of carnosic acid obtained from the chromatogram of Standard solution B

\(C_S\) = concentration of carnosic acid in Standard solution B (µg/mL)

\(C_U\) = concentration of the Sample solution (µg/mL)

\(F\) = relative response factor of the analyte (1.00 for carnosic acid; 0.92 for carnosol)

\(M_{W1}\) = molar weight of carnosic acid (332.4 g/mol)

\(M_{W2}\) = molar weight of carnosol (330.4 g/mol)

[Note—Add the individual percentages of carnosic acid and carnosol calculated, and report the result as the total content of carnosic acid and carnosol in the sample taken.]

Acceptance criteria: The total content of carnosic acid and carnosol is 95%–105% of the content claimed by the manufacturer. The ratio of carnosic acid to carnosol is NLT 7%.

Impurities

• Inorganic Impurities

Arsenic, Elemental Impurities by ICP, Method I, Appendix IIIC

Acceptance criteria: NMT 3 mg/kg
Lead, *Elemental Impurities by ICP, Method I, Appendix IIIC*

Acceptance criteria: NMT 2 mg/kg

- **Organic Impurities**

Residual Solvents

**Solution A:** 10% (w/w) solution of sodium chloride

**Internal standard solution:** 160 µg/g of 1-propanol

**Standard stock solution:** 260 µg/g each of ethanol and acetone

**Standard solution 1:** Weigh 250 mg of sunflower oil, 50 mg of the *Standard stock solution*, 2700 mg of *Solution A*, and 1000 mg of the *Internal standard solution* in a 20-mL glass autosampler vial.

**Standard solution 2:** Weigh 250 mg of sunflower oil, 100 mg of the *Standard stock solution*, 2650 mg of *Solution A*, and 1000 mg of the *Internal standard solution* in a 20-mL glass autosampler vial.

**Standard solution 3:** Weigh 250 mg of sunflower oil, 200 mg of the *Standard stock solution*, 2550 mg of *Solution A*, and 1000 mg of the *Internal standard solution* in a 20-mL glass autosampler vial.

**Standard solution 4:** Weigh 250 mg of sunflower oil, 500 mg of the *Standard stock solution*, 2250 mg of *Solution A*, and 1000 mg of the *Internal standard solution* in a 20-mL glass autosampler vial.

**Standard solution 5:** Weigh 250 mg of sunflower oil, 1000 mg of the *Standard stock solution*, 1750 mg of *Solution A*, and 1000 mg of the *Internal standard solution* in a 20-mL glass headspace vial.

**Sample solution:** Weigh 250 mg of rosemary extract, 2750 mg of *Solution A*, and 1000 mg of the *Internal standard solution* in a 20-mL glass headspace vial.

**Chromatographic system, Appendix IIA**

- **Mode:** Headspace gas chromatography
- **Detector:** Flame ionization
- **Column:** 15-m × 0.15-µm column with a 0.84-µm film of 6% cyanopropylphenyl/94% dimethylpolysiloxane
- **Carrier gas:** Helium
- **Flow rate:** 0.8 mL/min
- **Temperatures**
  - **Injector:** 250°C
  - **Syringe headspace:** 120°C
  - **Detector:** 300°C
  - **Oven:** Hold at 40°C for 5 min; ramp to 250°C at 25°C/min. [Note—The total run time is 13.4 min.]

**Headspace sampler**

- **Sample heating temperature:** 90°C
- **Sample heating time:** 10 min
- **Sample agitation speed:** 400 rpm
- **Injection size:** 1000 µL
- **Injection type:** Split (1:50)
- **Injection speed:** 1 mL/s
- **Injection liner:** 78.5-mm × 6.3-mm (o.d.) split/splitless liner with a recessed gooseneck and a quartz wool plug; 4.0-mm (i.d.)

**Analysis:** Place the *Sample solution* and the five *Standard solutions* into the sample tray of the headspace gas chromatograph. Record the resulting chromatograms and, using the results from the *Standard solutions*, calculate the response factors, F, for the two analytes of interest (ethanol and acetone). Separately calculate the response factors for each analyte in each of the five *Standard solutions* (five values for F will result for each of the two analytes of interest):
\[ F = \left( \frac{C_S}{C_{IS}} \right) \times \left( \frac{R_{IS}}{R_S} \right) \]

- \( C_S \) = concentration of the analyte of interest in the relevant Standard solution analyzed (µg/g)
- \( C_{IS} \) = concentration of 1-propanol in the relevant Standard solution analyzed (µg/g)
- \( R_{IS} \) = peak area response for 1-propanol obtained from the chromatogram of the relevant Standard solution
- \( R_S \) = peak area response for the analyte of interest obtained from the chromatogram of the relevant Standard solution

Calculate the average response factor, \( F_X \), for each of the two analytes:

\[ F_X = \frac{\Sigma(F)}{5} \]

[Note—Add the five values \( F \) obtained above for each analyte separately, then divide each sum by the number of Standard solutions analyzed to generate the values for \( F \) (5) to obtain an average response factor for acetone and an average response factor for ethanol.]

Finally, separately calculate the concentrations of acetone and ethanol in the sample taken:

\[ \text{Result} = F_X \times \left( \frac{R_U}{R_{IS}} \right) \times \left( \frac{C_{IS}}{C_U} \right) \times 1000 \]

- \( F_X \) = average response factor for the analyte of interest
- \( R_U \) = peak area response for the analyte of interest obtained from the chromatogram of the Sample solution
- \( R_{IS} \) = peak area response for 1-propanol obtained from the chromatogram of the Sample solution
- \( C_{IS} \) = concentration of 1-propanol in the Sample solution (µg/g)
- \( C_U \) = concentration of rosemary extract in the Sample solution (mg/g)

**Acceptance criteria**
- **Acetone:** NMT 100 ppm
- **Ethanol:** NMT 500 ppm

**SPECIFIC TESTS**

- **Loss on Drying**
  
  **Analysis:** Weigh 0.8–1.0 g of the sample into an aluminum dish in a thin layer. Load the dish into an infrared moisture analyzer, and set the instrument to run at temperature of 105\(^\circ\)C. At the endpoint of the analysis record the results, and calculate the percentage of the original sample weight lost in the drying process.
  
  [Note—Some instruments may report the result as the percentage of moisture lost. Alternatively, the Loss on Drying may be determined according to Appendix IIC at 105\(^\circ\)C until constant weight is achieved.]

  **Acceptance criteria:** NMT 8.0%

1 EMD Merck HPTLC silica gel 60 F254 plates (available at www.emdchemicals.com), or equivalent.
2 ZORBAX SB-C18 (Agilent Technologies), or equivalent.
3 Agilent VF-624 MS (available from www.agilent.com), or equivalent.
4 Item number 092010 from SGE Analytical Science (available at www.sge.com), or equivalent.
**Auxiliary Information**— Please check for your question in the FAQs before contacting USP.

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BRIEFING

Sodium Hydrogen dl-Malate. Because there is no existing FCC monograph for this food ingredient, a new monograph is proposed, based on comments and data received and based on the Sodium Hydrogen dl-Malate monograph from the 67th Session (2006) of the Joint Expert Committee on Food Additives (JECFA). Data and comments are requested to support the Acceptance criteria for the Assay, or to propose a more modern assay method that is consistent with other protease assay methods commonly used in industry and which includes use of a more specific substrate material. Data and comments are also requested regarding the applicability of the proposed method for Loss on Drying versus a Karl Fischer titration method, which is used in a current proposal for a new FCC monograph for a similar ingredient (see briefing under Sodium dl-Malate).

(Fl: K. Laurvick) C114724

Add the following:

- Sodium Hydrogen dl-Malate
  2-Hydroxybutanedioic Acid Monosodium Salt
  Malic Acid Monosodium Salt
  Monosodium dl-Malate
  Monosodium 2-dl-hydroxy Succinate

\[
\text{Na}^+ \quad \text{C}_4\text{H}_5\text{NaO}_5
\]

DESCRIPTION
Sodium Hydrogen dl-Malate occurs as an odorless white powder.

Function: Buffer; humectant

Packaging and Storage: Store in well-closed containers.

IDENTIFICATION
- A. Sodium, Appendix IIIA
  Acceptance criteria: Passes tests
- B. Procedure
  Sample solution: 50 mg/mL
  Analysis: Transfer 5 mL of the Sample solution to a porcelain dish, and add 10 mg of sulfanilic acid. Place the dish in a shallow water bath, and heat for a few min, then add 5 mL of a 200-mg/mL sodium nitrite solution, and heat slightly. Add sodium hydroxide TS until the solution is alkaline.

INS: 350(i)
CAS: [58214-38-3]
**Acceptance criteria:** A red color is produced (passes test).

**ASSAY**

**PROCEDURE**

**Sample:** 1.5 g, previously dried

**Analysis:** Weigh the Sample into a platinum or porcelain crucible 20–30 mm in diameter. Heat the Sample very gently, gradually raising the temperature. Continue heating for 2 h, and carbonize the material thoroughly. The heating temperature is 300°–400°, at which the crucible shows a dull red color. [Note—If a gas burner is used, do not allow the flame to come in contact with the carbonized mass.] After allowing the carbonized mass in the crucible to cool, disintegrate the mass with a glass rod, and transfer the mass and the crucible to a beaker. Add 50 mL of water and 50 mL of 0.5 N sulfuric acid to the beaker, then cover the beaker with a watch glass, and heat the contents on a boiling water bath for 1 h. Filter the contents of the beaker through filter paper. [Note—if the filter paper is colored, weigh out another Sample and repeat the carbonization, boiling, and filtering steps.] Wash the beaker, the crucible, and the residue on the filter paper with hot water until the washings become neutral to blue litmus paper. Combine the washings with the filtrate. Titrate the excess sulfuric acid in the combined solution with 0.5 N sodium hydroxide, using 3 drops of methyl red TS as the indicator. Each mL of 0.5 N sulfuric acid is equivalent to 78.04 mg of C₄H₅NaO₅.

**Acceptance criteria:** NLT 99.0% of C₄H₅NaO₅, on the dried basis

**IMPURITIES**

**Inorganic Impurities**

- **Lead,** *Elemental Impurities by ICP, Method II, Appendix IIIC*  [Note—Alternately, the lead content may be determined on a 5-g sample using the method: Lead Limit Test, *Flame Atomic Absorption Spectrophotometric Method, Appendix IIIB.*]

**Acceptance criteria:** NMT 2 mg/kg

**Organic Impurities**

- **Fumaric and Maleic Acids**

  [Note—For all reference standards, do not dry before use, and keep the containers tightly closed and protected from light. Determine the water content of the USP Fumaric Acid RS titrimetrically before use, and make the necessary correction in preparing the *Standard solution.*]

**Mobile phase:** 0.01 N sulfuric acid, filtered and degassed

**System suitability solution:** 1 mg/mL of sample, 10 µg/mL of USP Fumaric Acid RS, and 4 µg/mL of USP Maleic Acid RS in *Mobile phase*

**Standard solution:** 5 µg/mL of USP Fumaric Acid RS and 2 µg/mL of USP Maleic Acid RS in *Mobile phase*

**Sample:** 100 mg

**Sample solution:** Transfer the Sample into a 100-mL volumetric flask, dilute with *Mobile phase* to volume, and mix.

**Chromatographic system,** Appendix IIA

- **Mode:** High-performance liquid chromatography
- **Detector:** UV 210 nm
- **Column:** 30-cm × 6.5-mm (id) packed with a strong cation exchange resin consisting of sulfonated crosslinked styrene-divinylbenzene copolymer in the hydrogen form (Polypore H from Brownlee Laboratories, Inc., or equivalent)
- **Column temperature:** 37 ± 1°
- **Flow rate:** About 0.6 mL/min
Injection volume: About 20 µL

System suitability

Sample: System suitability solution

Suitability requirement 1: From the System suitability solution, the resolution, R, between maleic acid and sample peaks is NLT 2.5.

Suitability requirement 2: From the System suitability solution, the resolution, R, between fumaric acid and sample peaks is NLT 7.0.

Suitability requirement 3: From the System suitability solution, the maleic acid peak area response relative standard deviation for replicate injections is NMT 2.0%.

Analysis: Separately inject the Standard solution and the Sample solution into the chromatograph, record the chromatograms, and measure the peak area responses. [Note—The relative retention times are approximately 0.6 for maleic acid, 1.0 for malic acid, and 1.5 for fumaric acid.]

Calculate (separately) the percentages of maleic acid and fumaric acid in the portion of the Sample taken:

\[
\text{Result} = \left(\frac{r_U}{r_S}\right) \times \left(\frac{C_S}{C_U}\right) \times 100
\]

- \(r_U\) = peak area response of the analyte of interest obtained from the chromatogram of the Sample solution
- \(r_S\) = peak area response of the analyte of interest obtained from the chromatogram of the Standard solution
- \(C_S\) = concentration of the analyte of interest in the Standard solution (mg/mL)
- \(C_U\) = concentration of the Sample solution (mg/mL)

Acceptance criteria

- Fumaric acid: NMT 1.0%
- Maleic acid: NMT 0.05%

SPECIFIC TESTS

- Loss on Drying, Appendix II C: 110° for 3 h
  Acceptance criteria NMT 2%

Auxiliary Information—Please check for your question in the FAQs before contacting USP.

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| Monograph      | Kristie Laurvick  
Scientific Liaison  
1-301-816-8356 | (FI2010) Monographs - Food Ingredients |
**BRIEFING**

Sodium dl-Malate. Because there is no existing FCC monograph for this food ingredient, a new monograph is proposed, based on comments and data received and on the Sodium dl-Malate monograph from the 67th Session (2006) of the Joint Expert Committee on Food Additives (JECFA). The method for *Water Determination* is proposed based on comments and data received indicating that a simple oven drying technique, as is specified in the JECFA monograph, is insufficient for complete drying, particularly of the trihydrated form of this ingredient.

(FI: K. Laurvick) C111738

*Add the following:*

- Sodium DL-Malate
  - dl-Disodium Malate
  - Disodium dl-Malate
  - Hydroxybutanedioic Acid Disodium Salt
  - Malic Acid Disodium Salt
  - Malic Acid Sodium Salt
  - Sodium Malate

![Chemical structure of Sodium dl-Malate](image)

\[ C_4H_4Na_2O_5 \cdot 3H_2O \]

\[ C_4H_4Na_2O_5 \cdot \frac{1}{2}H_2O \]

Formula wt, hemihydrate 187.1

Formula wt, trihydrate 232.1

INS: 350(ii)

CAS: [676-46-0]

**DESCRIPTION**

Sodium dl-Malate occurs as a white to off-white odorless crystalline powder (with or without lumps). It is manufactured by reacting malic acid with sodium hydroxide and purifying the reaction product. Sodium dl-Malate is freely soluble in water.

**Function:** Buffer; neutralizing agent

**Packaging and Storage:** Store in well-closed containers.

**IDENTIFICATION**

- **A. Sodium**, Appendix IIIA
  - Acceptance criteria: Passes tests
- **B. Procedure**
  - Sample solution: 50 mg/mL
Analysis: Transfer 5 mL of the Sample solution to a porcelain dish, and add 10 mg of sulfanilic acid. Place the dish in a shallow water bath, heat for a few min, then add 5 mL of a 200-mg/mL sodium nitrite solution, and heat slightly. Add sodium hydroxide TS until the solution is alkaline.

Acceptance criteria: A red color is produced (passes test).

ASSAY

• PROCEDURE

Sample: 200 mg, previously dried

Analysis: Dissolve the Sample in 30 mL of glacial acetic acid, and titrate with 0.1 M perchloric acid. Determine the endpoint potentiometrically or using crystal violet TS as the indicator. [Note—The crystal violet indicator will cause the solution to change color from purple to blue to green. The green color indicates the endpoint of the titration.] Perform a blank determination (see General Provisions). Each mL of 0.1 M perchloric acid is equivalent to 8.903 mg of C₄H₄Na₂O₅.

Acceptance criteria: 98.0%–102.0% of C₄H₄Na₂O₅, on the dried basis

IMPURITIES

Inorganic Impurities

• ARSENIC, Arsenic Limit Test, Appendix IIIB

Sample solution: Dissolve 1.0 g of the sample in 10 mL of water. Cautiously neutralize the solution to litmus paper using sulfuric acid. Cool the solution before use.

Acceptance criteria: NMT 3 mg/kg

• LEAD, Elemental Impurities by ICP, Method II, Appendix IIIC

[Note—Alternatively, the lead content may be determined on a 5-g sample using the method: Lead Limit Test, Flame Atomic Absorption Spectrophotometric Method, Appendix IIIB.]

Acceptance criteria: NMT 2 mg/kg

Organic Impurities

• FUMARIC AND MALEIC ACIDS

[Note—For all reference standards, do not dry before use, and keep the containers tightly closed and protected from light. Determine the water content of the USP Fumaric Acid RS titrimetrically before use, and make the necessary correction in preparing the Standard solution.]

Mobile phase: 0.01 N sulfuric acid, filtered and degassed

System suitability solution: 1.0 mg/mL of USP Sodium d,l-Malate RS, 10 µg/mL of USP Fumaric Acid RS, and 4 µg/mL of USP Maleic Acid RS in Mobile phase

Standard solution: 5 µg/mL of USP Fumaric Acid RS and 2 µg/mL of USP Maleic Acid RS in Mobile phase

Sample solution: 1.0 mg/mL in Mobile phase

Chromatographic system, Appendix II A

Mode: High-performance liquid chromatography
Detector: UV 210 nm
Column: 30-cm × 6.5-mm (id) column packed with a strong cation exchange resin consisting of sulfonated crosslinked styrene-divinylbenzene copolymer in the hydrogen form (Polypore H from Brownlee Laboratories, Inc., or equivalent)

Column temperature: 37 ± 1 °C
Flow rate: About 0.6 mL/min
Injection volume: About 20 µL
System suitability

Suitability requirement 1: From the System suitability solution, the resolution, R, between the maleic acid and sodium d,l-malate peaks is NLT 2.5.
Suitability requirement 2: From the System suitability solution, the resolution, R, between the fumaric acid and sodium d,l-malate peaks is NLT 7.0.

Suitability requirement 3: From the System suitability solution, the maleic acid peak relative standard deviation for replicate injections is NMT 2.0%.

Analysis: Separately inject the Standard solution and the Sample solution into the chromatograph, record the chromatograms, and measure the peak responses. [Note—The relative retention times are approximately 0.6 for maleic acid, 1.0 for malic acid, and 1.5 for fumaric acid.] Calculate (separately) the percentages of maleic acid and fumaric acid in the portion of the sample taken:

\[
\text{Result} = \left( \frac{r_U}{r_S} \right) \times \left( \frac{C_S}{C_U} \right) \times 100
\]

- \(r_U\) = peak response of the analyte of interest obtained from the chromatogram of the Sample solution
- \(r_S\) = peak response of the analyte of interest obtained from the chromatogram of the Standard solution
- \(C_S\) = concentration of the analyte of interest in the Standard solution (mg/mL)
- \(C_U\) = concentration of the Sample solution (mg/mL)

Acceptance criteria
- Fumaric acid: NMT 1.0%
- Maleic acid: NMT 0.05%

SPECIFIC TESTS

- **Alkalinity (as Na\(_2\)CO\(_3\))**
  - **Sample**: 1.0 g, previously dried
  - **Indicator solution**: Dissolve 200 mg of phenolphthalein in 60 mL of 90% ethanol, and then dilute with water to 100 mL.
  - **Analysis**: Dissolve the Sample in 20 mL of fresh, carbon dioxide-free water, and add 2 drops of the Indicator solution.
  - **Acceptance criteria**: Any pink color that is formed upon addition of the Indicator solution is completely discharged by adding NMT 0.4 mL of 0.1 N sulfuric acid (NMT 0.2%, as Na\(_2\)CO\(_3\), on the dried basis).

- **Water Determination, Method I (Karl Fischer Titrimetric Method), Appendix IIB**
  - **Acceptance criteria**
    - Hemihydrate: NMT 7.0%
    - Trihydrate: 20.5%–23.5%

**Auxiliary Information**— Please check for your question in the FAQs before contacting USP.
BRIEFING

Sodium Potassium Polyphosphates, Glassy. Because there is no existing FCC monograph for this food ingredient, a new monograph is proposed based on the Sodium Polyphosphates, Glassy FCC monograph and on comments and data received. Interested parties are encouraged to submit comments, including new methods, particularly in reference to the proposed Assay.

(FI: C. Mejia) C112491

Add the following:

- Sodium Potassium Polyphosphates, Glassy

Glassy Sodium Potassium Polyphosphates

Metaphosphoric Acid, Sodium Potassium Salt

Sodium Potassium Hexametaphosphate

Sodium Potassium Phosphate, Glassy

\((\text{Na}(1-x)\text{K}_x\text{PO}_3), 0<x<1\)

CAS: [67183-30-6]

DESCRIPTION

Sodium Potassium Polyphosphates, Glassy occurs as colorless or white, transparent platelets, granules, or powder. It consists of several amorphous, water-soluble polyphosphates composed of linear chains of metaphosphate units \((\text{MPO}_3)_n\) for which \(M = \text{Na} \) and \(K\) and average chain length, \(n\), is NLT 4. The material is usually identified by the \(\text{M}_2\text{O}/\text{P}_2\text{O}_5\) ratio or the \(\text{P}_2\text{O}_5\) content. The \(\text{M}_2\text{O}/\text{P}_2\text{O}_5\) ratios vary from about 1.0 to 1.3. Its manufacture is based on an aqueous solution of phosphoric acid, potassium hydroxide, and sodium hydroxide or sodium carbonate that is heated to evaporate water and forms a high-temperature melt. This melt is then chilled to form a solid (amorphous glass), which is then milled and screened to obtain the appropriate granule size for use as a final product. Sodium Potassium Polyphosphates, Glassy is very soluble to freely soluble in water. The pH of a 1% aqueous solution ranges from 6.0 to 8.0

Function: Emulsifier; sequestrant; texturizer

Packaging and Storage: Store in tightly closed containers.

IDENTIFICATION

- A. Sodium, Appendix IIIA
  
  Sample solution: 50 mg/mL

  Acceptance criteria: Passes tests

- B. Potassium, Appendix IIIA
  
  Sample solution: 50 mg/mL

  Acceptance criteria: Passes tests

- C. Phosphate, Appendix IIIA
  
  Sample: 100 mg

  Analysis: Dissolve the Sample in 5 mL of hot 1.7 N nitric acid, warm on a steam bath for 10 min, and cool.
Neutralize to litmus paper with 1 N sodium hydroxide, and add silver nitrate TS.

**Acceptance criteria:** A yellow precipitate that is soluble in 1.7 N nitric acid forms.

**ASSAY**

- **Procedure**
  - **Sample:** 800 mg
  - **Sample solution:** Transfer the Sample into a 400-mL beaker, add 100 mL of water and 25 mL of nitric acid, cover with a watch glass, and boil for 10 min on a hot plate. Rinse any condensate from the watch glass into the beaker, cool the solution to room temperature, transfer it quantitatively into a 500-mL volumetric flask, dilute with water to volume, and mix thoroughly.
  - **Analysis:** Pipet 20.0 mL of Sample solution into a 500-mL Erlenmeyer flask, add 100 mL of water, and heat just to boiling. While stirring, add 50 mL of quimociac TS, cover with a watch glass, and boil for 1 min in a well-ventilated hood. Cool to room temperature, swirling occasionally while cooling, then pass through a tared, sintered-glass filter crucible of medium porosity, and wash the precipitate with five 25-mL portions of water. Dry the precipitate at about 225°C for 30 min, cool, and weigh. Each mg of precipitate obtained is equivalent to 32.074 µg of P$_2$O$_5$.
  - **Acceptance criteria:** 59.0%–63.0% of P$_2$O$_5$

**IMPURITIES**

Inorganic Impurities

- **Arsenic, Arsenic Limit Test, Appendix IIIB**
  - **Sample solution:** 1 g of sample in 35 mL of water
  - **Acceptance criteria:** NMT 3 mg/kg
- **Fluoride, Fluoride Limit Test, Method III, Appendix IIIB**
  - **Sample:** 2 g
  - **Acceptance criteria:** NMT 50 mg/kg
- **Lead, Lead Limit Test, APDC Extraction Method, Appendix IIIB**
  - **Sample:** 5 g
  - **Acceptance criteria:** NMT 2 mg/kg

**SPECIFIC TESTS**

- **Insoluble Substances**
  - **Sample:** 10 g
  - **Analysis:** Dissolve the Sample in 100 mL of hot water, and filter through a tared filtering crucible. Wash the insoluble residue with hot water, dry at 105°C for 2 h, cool, and weigh.
  - **Acceptance criteria:** NMT 2.0%

**Auxiliary Information—** Please check for your question in the FAQs before contacting USP.

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<tr>
<td>Monograph</td>
<td>Carla D Mejia, Ph.D. Scientific Liaison 1-301-816-8571</td>
<td>(FL2010) Monographs - Food Ingredients</td>
</tr>
</tbody>
</table>
BRIEFING

**Taurine.** Because there is no existing FCC monograph for this food ingredient, a new monograph is proposed based on the monograph for Taurine published by the Japanese Pharmacopoeia 15th ed., the Taurine monograph in USP 35–NF 30, and on the basis of comments received. Limited data have been received to support these limits. Interested parties are encouraged to submit comments including new methods, particularly in reference to the proposed Assay.

1. The impurities proposed are consistent with those specified in the Japanese Pharmacopoeia, and on comments received. Comments and data pertaining to **Acceptance criteria** are solicited to support the limits stated in these documents for these impurities.

2. The method and **Acceptance criteria** used in the test for **Lead** are based on the existing methods and criteria used in FCC for various amino acids, because the method referenced by other sources includes insufficient detail. Interested parties are encouraged to submit comments and data pertinent to these methods.

(FL: C. Mejia) C112925

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**Add the following:**

- **Taurine**
  - 2–Aminoethanesulfonic Acid
  - 2-Aminoethylsulfonic Acid
  - 2-Sulfoethylamine

```
  \[
    \text{C}_2\text{H}_7\text{NO}_3\text{S} \\
    \begin{array}{c}
      \text{O} \\
      \text{S} \\
      \text{OH} \\
    \end{array}
  \]

  \text{Formula wt 125.15} \\
  \text{CAS: [107-35-7]}
```

**DESCRIPTION**

Taurine occurs as white or colorless crystals, or as a white crystalline powder. It is soluble in water and practically insoluble in ethanol.

**Function:** Nutrient

**Packaging and Storage:** Store in tight containers in a cool, dry place.

**IDENTIFICATION**

- **Infrared Absorption,** *Spectrophotometric Identification Tests, Appendix IIIC*
  - **Reference standard:** USP Taurine RS
  - **Sample and standard preparation:** K
  - **Acceptance criteria:** The spectrum of the sample exhibits maxima at the same wavelengths as those in
the spectrum of the Reference standard.

- Thin Layer Chromatography, Appendix IIA
  - Standard solution: 10 mg/mL of USP Taurine RS in water
  - Sample solution: 10 mg/mL of Taurine in water
  - Adsorbent: 0.25-mm layer of chromatographic silica gel mixture
  - Developing system: A mixture of butyl alcohol, glacial acetic acid, and water (60:20:20, v/v)
  - Spray reagent: Dissolve 0.2 g of ninhydrin in 100 mL of a mixture of butyl alcohol and 2 N acetic acid (95:5, v/v).
  - Application volume: 5 µL
  - Analysis: After developing the plate in the Developing system, remove the plate from the developing chamber, and dry the plate at 80°C for 30 min. Spray the plate with the Spray reagent, and heat at 80°C for about 10 min. Examine the plate under white light.
  - Acceptance criteria: The Rf value of the principal spot obtained from the chromatogram of the Sample solution corresponds to that obtained from the chromatogram of the Standard solution. [Note—The Rf value for taurine spots is about 0.2.]

Assay

- Total Nitrogen, Nitrogen Determination, Appendix III C
  - Analysis: Each mL of 0.01 N sulfuric acid is equivalent to 1.25 mg of C₂H₇NO₃S.
  - Acceptance criteria: 98.5%–101.5% of C₂H₇NO₃S, calculated on the dried basis

Impurities

Inorganic impurities

- Ammonium
  - Phenol–sodium nitroferricyanide solution: In a 500-mL volumetric flask, dissolve 5 g of phenol and 0.025 g of sodium nitroferricyanide (III) in water, and dilute to volume. [Note—Preserve in a dark, cold place.]
  - Sodium hypochlorite solution: Pass chlorine into sodium hydroxide TS while cooling with ice, to obtain NLT 4.2% (w/v) as sodium hypochlorite (NaClO: Formula wt., 74.44). Alternatively, the solution may be prepared using a commercially available stock solution. Test the content periodically by performing the following assay.
    - Pipet 10 mL of Sodium hypochlorite solution into a 100-mL volumetric flask, and dilute with water to volume. Transfer exactly 10.0 mL of this solution to a glass-stoppered flask, add 90 mL of water, 2 g of potassium iodide, and 6 mL of diluted acetic acid (1:2). Tightly stopper the flask, shake well, and allow to stand for 5 min in a dark place. Titrate the liberated iodine with standardized 0.1 mol/L sodium thiosulfate solution (indicator: 3 mL starch TS). Perform a blank determination, and make any necessary correction. Each mL of standardized 0.1 mol/L sodium thiosulfate solution is equivalent to 3.722 mg of sodium hypochlorite.
    - Sodium hypochlorite–sodium hydroxide solution: Transfer a volume of Sodium hypochlorite solution equivalent to 1.05 g of sodium hypochlorite to a 1000-mL volumetric flask, add 15 g of sodium hydroxide, and dilute with water to volume. [Note—Prepare this solution just before use.]
  - Ammonium standard solution: Weigh exactly 2.97 g of ammonium chloride, dissolve in a 1000-mL volumetric flask, and dilute with water to volume. Transfer 10 mL of this solution into another 1000-mL volumetric flask, and dilute with water to volume. Each mL of this solution contains 0.01 mg of ammonium (NH₄⁺).
  - Sample: 0.25 g
  - Sample solution: [Note—Boil all rubber parts used in the vacuum distillation apparatus (see Figure 1]
below) for 10–30 min in sodium hydroxide TS, then for 30–60 min in water, and finally wash thoroughly with water before use. Transfer the Sample into a vacuum distillation flask (L), and add 70 mL of water and 1 g of magnesium oxide. Connect the apparatus, and to the receiver (M) add 20 mL of boric acid solution (1:200) as absorbing liquid. Put the end of the branch of the distillation flask (L) in the absorbing liquid, and keep the distillation flask (L) at 60° using a water bath or alternative. Cool the receiver (M) with running water during the distillation process. Adjust the pressure to get the distillate at a rate of 1–2 mL/min, and continue the distillation until 30 mL of distillate are obtained. Transfer the distillate to a 100-mL volumetric flask, and dilute with water to volume.

**Standard solution:** Transfer 5.0 mL of Ammonium standard solution into the distillation flask, and proceed as directed for the Sample solution.

Figure 1. Vacuum distilling apparatus for ammonium limit test

**Analysis:** Place 30 mL of the Sample solution distillate and 30 mL of the Standard solution distillate into separate Nessler tubes, add 6.0 mL of Phenol–sodium nitroferricyanide solution to each solution, and mix. Then, add 4 mL of Sodium hypochlorite–sodium hydroxide solution and water to make 50 mL, mix, and allow to stand for 60 min. Compare the color of both solutions by viewing through the Nessler tubes downward and transversely.

**Acceptance criteria:** The color developed by the Sample solution is not more intense than that developed by the Standard solution (NMT 200 mg/kg).

- **Arsenic, Appendix IIIIB**
  - Sample: 1 g
  - Control: 2 µg of As (2.0 mL of Standard Arsenic Solution)
  - Acceptance criteria: NMT 2 mg/kg.

- **Chloride, Chloride and Sulfate Limit Tests, Chloride Limit Test, Appendix IIIIB**
  - Sample: 0.5 g
  - Control: 50 µg of chloride (5 mL of Standard Chloride Solution)
  - Acceptance criteria: Any turbidity produced by the Sample solution does not exceed that produced by the Control (NMT 100 mg/kg).

- **Iron, Elemental Impurities by ICP, Appendix IIIIC**
  - Acceptance criteria: NMT 10 mg/kg

- **Lead, Elemental Impurities by ICP, Appendix IIIIC**
  - Acceptance criteria: NMT 5 mg/kg

- **Sulfate, Chloride and Sulfate Limit Tests, Sulfate Limit Test, Appendix IIIIB**
  - Sample: 0.5 g
  - Control: 50 µg of sulfate (5 mL of Standard Sulfate Solution)
  - Acceptance criteria: Any turbidity produced by the Sample solution does not exceed that produced by the
SPECIFIC TESTS

- **Loss on Drying**, Appendix IIC: 105°, 3h
  
  Acceptance criteria: NMT 0.3%

- **Residue on Ignition (Sulfated Ash)**, Appendix IIC
  
  Sample: 1 g
  
  Acceptance criteria: NMT 0.1%

- **Related Substances**, Thin-Layer Chromatography, Appendix IIA
  
  **Standard solution**: 0.05 mg/mL of USP Taurine RS in water
  
  **Sample solution**: 10 mg/mL of sample in water
  
  **Analysis**: Proceed as directed in the *Identification, Thin-Layer Chromatography* test above.
  
  **Acceptance criteria**: No spot, other than the principal spot, in the chromatogram of the Sample solution is larger or more intense than the principal spot obtained from the Standard solution (NMT 0.5% approx.).

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L-Tryptophan, FCC 8 page 1164. On the basis of comments and data received, it is proposed to add a new HPLC related compounds test and limit to the monograph based on *Organic Impurities, Procedure 1* in the USP 35 monograph for Tryptophan, similar to the *Impurity A and Other Related Substances* limit and test in the European Pharmacopeia 7.0 monograph for tryptophan. It is also proposed to use the HPLC test as an *Identification* test in the monograph, complimentary to the existing IR method.

The rationale for this proposed test is two-fold:

1. To update and harmonize the FCC monograph to reflect the latest impurity testing approaches established in USP–NF and EP. These standards were developed in an effort to minimize the exposure to L-tryptophan contaminants because of their potential link to eosinophilia myalgia cases in the 1980’s and recently in 2011.
2. To modernize the FCC monograph to include a chromatographic approach for better characterization of the identity and impurities of this food ingredient.

The proposed total impurities limit for FCC is consistent to that established in USP–NF and EP, but does not include the 10 ppm limit for 1,1’-ethylidenebis-(L-Tryptophan) (EBT) in USP–NF and EP, since this specific impurity was never conclusively linked as the sole contaminant responsible for reported adverse events.

(Ft: J. Moore) C117185

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**L-Tryptophan**

L-α-Amino-3-indolepropionic Acid

![Chemical structure](image)

C₁₁H₁₂N₂O₂

Formula wt 204.22
CAS: [73-22-3]

UNII: 8DUH1N11BX

**DESCRIPTION**

L-Tryptophan occurs as white to yellow-white crystals, or as a crystalline powder. One g dissolves in about 100 mL of water. It is soluble in hot alcohol, in dilute hydrochloric acid, and in alkali hydroxide solutions.

**Function:** Nutrient

**Packaging and Storage:** Store in well-closed, light-resistant containers.

**IDENTIFICATION**

*Change to read:*

- **A₂S (FCC)** *Infrared Absorption, Spectrophotometric Identification Tests, Appendix IIIC*

  **Reference standard:** USP L-Tryptophan RS
Sample and standard preparation: *M*

Acceptance criteria: The spectrum of the sample exhibits maxima at the same wavelengths as those in the spectrum of the Reference standard.

**Add the following:**

- **B. Procedure**
  - **Analysis:** Proceed as directed in the test for Related Compounds, except use the following Standard solution and Sample solution.
  - **Standard solution:** 10 µg/mL of USP L-Tryptophan RS
  - **Sample solution:** 10 µg/mL
  - **Acceptance criteria:** The major peak in the chromatogram of the Sample solution (representing NLT 98.5% of the total area of all peaks excluding those due to solvents) corresponds to that of L-Tryptophan in the Standard solution. [2S (FCC8)]

**ASSAY**

- **Procedure**
  - **Sample:** 300 mg
  - **Analysis:** Dissolve the Sample in 3 mL of formic acid and 50 mL of glacial acetic acid. Add 2 drops of crystal violet TS, and titrate with 0.1 N perchloric acid to a green endpoint or until the blue color disappears completely. Perform a blank determination (see General Provisions), and make any necessary correction. Each mL of 0.1 N perchloric acid is equivalent to 20.42 mg of C_{11}H_{12}N_{2}O_{2}.  
  - **Acceptance criteria:** 98.5%–101.5% of C_{11}H_{12}N_{2}O_{2}, calculated on the dried basis

**IMPURITIES**

Inorganic Impurities

- **Lead,** Lead Limit Test, Appendix IIB
  - **Sample solution:** Prepare as directed for organic compounds.
  - **Control:** 5 µg Pb (5 mL of Diluted Standard Lead Solution)
  - **Acceptance criteria:** NMT 5 mg/kg

**Add the following:**

- **Organic Impurities**
  - **Related Compounds**
    - **Solution A:** 1 mL/L of trifluoroacetic acid in water
    - **Solution B:** 1 mL/L of trifluoroacetic acid in a mixture of acetonitrile and water (80:20, v/v).
    - **Mobile phase:** See the gradient table below.
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<th>Solution B (%)</th>
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<td>5</td>
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<tr>
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**Standard solution:** 1.0 µg/mL of USP Tryptophan Related Compound B RS in water

**Sample solution:** 10.0 mg/mL in water

**Chromatographic system**, Appendix IIA
- **Mode:** High-performance liquid chromatography
- **Detector:** UV 220 nm
- **Column:** 25 cm × 4.6-mm column packed with 5-µm reversed phase C18 silica stationary phase
- **Column temperature:** 30°C
- **Flow rate:** 1.0 mL/min
- **Injection volume:** 20 µL

**System suitability**
- **Sample:** Standard solution

**Suitability requirements**
- **Suitability requirement 1:** The relative standard deviation for peak area response for related compound B from replicate injections is NMT 5%.

**Analysis:** Separately inject equal volumes of the Standard solution and Sample solution into the chromatograph, and measure the responses for all peaks, except l-Tryptophan, on the resulting chromatograms.

Calculate the percentage of each unspecified impurity eluting before and after l-Tryptophan (except l-Tryptophan related compound B) in the sample taken:

\[
\text{Result} = \left( \frac{r_U}{r_S} \right) \times \left( \frac{C_S}{C_U} \right) \times \left( \frac{1}{F} \right) \times 100
\]

- \( r_U \) = peak area for analyte in the Sample solution
- \( r_S \) = peak area for related compound B in the Standard solution
- \( C_S \) = concentration of related compound B in the Standard solution (µg/mL)
- \( C_U \) = concentration of the sample in the Sample solution (mg/mL)
- \( F \) = mg-to-µg conversion factor, 1000

**Acceptance criteria**
- **Total impurities 1:** The sum of all impurities eluting prior to the l-Tryptophan peak is NMT 0.01%
- **Total impurities 2:** The sum of all impurities eluting after to the l-Tryptophan peak (excluding tryptophan related compound B) is NMT 0.03%

SPECIFIC TESTS
- **Loss on Drying**, Appendix IIIC: 105°C for 3 h
Acceptance criteria: NMT 0.3%

- **Optical (Specific) Rotation**, Appendix IIIB
  
  Sample solution: 1 g of previously dried sample in sufficient water to make 100 mL
  
  Acceptance criteria
  
  \([\alpha]_D^{20}\) between \(-30.0^\circ\) and \(-33.0^\circ\), on the dried basis; or
  
  \([\alpha]_D^{25}\) between \(-29.7^\circ\) and \(-32.7^\circ\), on the dried basis

- **Residue on Ignition (Sulfated Ash)**, Appendix IIC
  
  Sample: 1 g
  
  Acceptance criteria: NMT 0.1%

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1 Ultrasphere ODS (Hichrom), or equivalent.

**Auxiliary Information**— Please check for your question in the FAQs before contacting USP.

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FCC FCC 8 Page 1164
BRIEFING

Appendix II: Physical Tests and Determinations, A. Chromatography, Thin-Layer Chromatography. FCC 8 page 1223. In order to support a new proposed FCC monograph for Rosemary Extract in this Forum, a revision to include reference to high-performance thin-layer chromatography (HPTLC) in the existing section on Thin-Layer Chromatography is proposed. The added information is consistent with the method described in the Thin-Layer Chromatography section of Chromatography <621> in USP 35–NF 30.

(F: K. Laurvick)  C118088

THIN-LAYER CHROMATOGRAPHY

Change to read:

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 predesorbent zone can be used if they are specified in a monograph. Sample applied to the predesorbent region develops into sharp, narrow bands at the predesorbent-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.

Change to read:

Apparatus Acceptable apparatus and materials for thin-layer chromatography consist of the following:

Glass Plates: Flat glass plates of uniform thickness throughout their areas. The most common sizes are 20 cm, 10 cm, and 5 cm × 20 cm. (Aluminum plates also are commonly used.)

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.]

Change to read:

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.

2S (FCC8)

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.

Apply the Sample Solution and the Standard Solution at points about 1.5 cm apart and about 2 cm from the lower edge of the plate (the lower edge is the first part over which the spreader moves in the application of the adsorbent layer), and allow to dry. A template will aid in determining the spot points and the 10 cm to 15 cm distance through which the solvent front should move.

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; this usually requires 15 min to 1 hr.

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.

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BRIEFING

Appendix III B: Fluoride Limit Test, FCC 8 page 1269. On the basis of comments received, and in an effort to modernize test procedures in the FCC by incorporating the use of digital scale currently present in most pH/mV meters in the market, a revision to Method II in the Fluoride Limit Test is proposed. A revision of the formula for calculating the fluoride content in the sample is also proposed to allow a routine standard addition measurement as described in Fluoride Limit Test, Method II. Comments by interested parties are encouraged.

(FI: C. Mejia) C115731

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 having 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 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 F/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.

Change to read:

**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 mg of fluoride per mL.

**Fluoride Standard Solution** Transfer 1 mL of the Fluoride Standard to a 1000-mL volumetric flask, and dilute with water to volume. Take 1 mL of this solution and transfer 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. Adjust the calibration control until the indicator needle points to the center on the logarithmic concentration scale, allowing sufficient time for equilibration (about 20 min), and stirring constantly during the equilibration period and throughout the remainder of the procedure. Allow the initial equilibrated reading in mV, Pipet 1.0 mL of a solution containing 100 µg of fluoride (F) ion per mL (prepared by dissolving 22.2 mg of sodium fluoride, previously dried at 200 ° for 4 h, in sufficient water to make 100.0 mL) into the beaker, allow the electrode to come to equilibrium, and record the final reading on the logarithmic concentration scale 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:

\[
\text{Result} = \frac{IA}{(R - I)} \times 100 \times \frac{200}{25W}
\]

in which \(I\) is the initial scale reading before the addition of the sodium fluoride solution; \(A\) is the concentration, in µg/mL, of fluoride in the sodium fluoride solution added to the sample solution; \(R\) is the final scale reading after addition of the sodium fluoride solution; \(W\) is the original weight, in g, of the sample; \(E_1\) is the initial equilibrated reading, in mV; \(E_2\) is the final equilibrated reading in mV; \(S\) is the electrode slope; 800 is a factor that corrects for the sample dilutions; and 1.01 corresponds to the correction factor that is the relationship between the volume of standard used for standard addition and the volume of the sample dilution on which standard addition is performed, \((V_s/V)\).

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 mL, 2.0 mL, 3.0 mL, 5.0 mL, 10.0 mL, 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 F per 100 mL 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:

\[
\text{Result} = \left( \frac{C}{W_S} \right) \times 0.000001 \times 100\%
\]

in which C is the content of fluoride, in µg, in the sample, determined from the Calibration Curve; \(W_S\) is the sample weight, in g; and 0.000001 is a factor converting 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°C for Buffer Solution A and in the range of 54–60 mV at 25°C 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°C. 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 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°C, then maintain at 160°C–165°C 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°C, 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°C...".

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BRIEFING

Appendix IIIB: Lead Limit Test, ADPC Extraction Method and Atomic Absorption Spectrophotometric
Graphite Furnace Method, FCC 8 page 1270.

1. On the basis of comments received, and in order to clarify the use of a reagent blank in the analysis, it
is proposed to add a section that specifically describes the reagent blank and its use in the
Procedure. Interested parties are encouraged to submit comments to Carla Mejia at cdm@usp.org.
2. On the basis of comments received, and to clarify the Graphite Furnace Atomic Absorption (GFAA)
method, it is proposed to emphasize the importance of the air ashing step during the furnace program
under Apparatus and to clarify the addition of matrix modifier as well as the sample digestion
procedure. Interested parties are encouraged to submit comments to Premal Bhatt at pyb@usp.org.

(FL: C. Mejia; P. Bhatt)   C116065   C118010

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 \[\text{Pb(NO}_3\text{)}_2\] 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 \([\text{Pb(NO}_3\text{)}_2]\) 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:

\[
\text{Result} = 10C/W_S
\]
in which $C$ is the concentration, in µg/mL, of lead from the standard curve; and $W_S$ is the weight, in grams, of the sample taken.

**Change to read:**

**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 °C. 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.

- **2S (FCC8)**

**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 2S (FCC8) 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.] 2S (FCC8) (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.

Use the autosampler to inject 20 µL each of blanks, calibration standards, and sample solutions and 5 µL of *Modifier Working Solution*. 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. 2S (FCC8) Inject each respective solution in triplicate, and average 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. 2S Calculate the characteristic mass ($m_o$) (mass of Pb pg necessary to produce an integrated absorbance of 0.0044 abs-sec) as follows:

$$m_o = \frac{(0.0044 \text{ abs-sec})(25 \text{ pg/µL})(20 \text{ µL})}{(\text{measured 25 pg/µL abs-sec})}$$

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. 2S 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:

\[
DL = (3) \times (\text{s.d. blank abs-sec})(10 \text{ pg}/\mu\text{L})(20 \mu\text{L}) / (\text{abs-sec 10 ng/mL std})
\]

\[
QL = (10) \times (\text{s.d. blank abs-sec})(10 \text{ pg}/\mu\text{L})(20 \mu\text{L}) / (\text{abs-sec 10 ng/mL std})
\]

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:

\[
Pb \text{ (ng/g)} = (\text{blank-corrected Pb ng/mL})(\text{DF})(\text{sample vol (10 mL)}) / (\text{sample wt (approx. 1.5 g)})^2
\]

**Quality Assurance** To ensure analytical accuracy, National Institute of Standards and Technology (NIST) SRM 1643c acidified water or a similar material should be analyzed before the unknown samples are. The certified content of SRM 1643c is 35.3 ± 0.9 ng/mL. If the concentration determined is not within 10% of the mean reference value (31.8–38.8 ng/mL), 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 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$_2$WO$_4$) 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 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°C for 20 s, char at 700°C–900°C for 20 s, and with the argon flow stopped, atomize at 2700°C 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°C for 30 s, with a 20-s ramp period and a 10-s hold time; then char at 700°C 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 \([\text{Pb(NO}_3\text{)}_2]\) 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, and 10 mL of water to a second 150-mL beaker to serve as the blank. Add to each 2S (FCC8) 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** Concomitantly determine the absorbances of the appropriate Standard Lead Solution and the
Sample Preparation against the blank at the lead emission line of 283.3 nm, using a slit-width of 0.7 nm. 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 electrode-less discharge lamp (EDL), or equivalent; an air-acetylene flame; and a 4-in burner head. Use water as the blank. 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.

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).

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BRIEFING

Pepsin Activity, FCC 8 page 1327. On the basis of comments and data received, major revisions to this method of measuring Pepsin Activity are proposed. Revisions proposed are as follows.

1. A new USP Pepsin Unit is proposed, based on the change in the assay substrate and mechanism. The new unit is based on the amount of tyrosine that is produced per minute under standardized conditions and will be calculated from the activity of the USP Pepsin for Assay RS used in the analysis.

2. The proposed assay utilizes USP Hemoglobin Protease Substrate RS in the Substrate solution in order to ensure interlaboratory consistency of substrates.

3. The proposed assay requires pH adjustment of the Substrate solution as pH is recognized as a critical consideration in enzyme analysis. There is no solution given for adjustment to a higher pH because data indicates that the pH of the substrate, when prepared exactly as directed in the procedure, is reliably 1.6 or higher (not lower).

4. Users of this method must independently determine the variability of the products they are testing because product variability was not addressed in the validation of this protocol. Users must always perform in-house testing to transfer analytical procedures developed and validated elsewhere.

5. The variability of this method was determined to be 95%–105% when users collect data from a minimum of two independent assays (or until the coefficient of variation is \( \leq 5.1 \)).

(FL: K. Laurvick)  C114884

PEPSIN ACTIVITY

Change to read:

Application This procedure is to be applied to preparations derived from porcine or other animal stomachs. This procedure is used to determine the protease activity of pepsin preparations derived from porcine or other animal stomach tissue.

Delete the following:

Apparatus

Measuring Vessels Use 100-mL conically shaped measuring vessels complying with the following descriptions: (1) diameters not exceeding 1 cm at the bottom; (2) comply in other respects with the water and sediment tube ASTM Standard Method D96-68; (3) graduated from 0 to 0.5 mL in 0.05-mL graduations, from 2 to 3 mL in 0.1-mL graduations, from 3 to 5 mL in 0.2-mL graduations, from 5 to 10 mL in 1-mL graduations, from 10 to 25 mL in 5-mL graduations, and with graduation marks at 50, 75, and 100 mL.

[Note—Measuring vessels other than the type described herein may be used if they are of such design and graduation to permit measurement of the residue with equivalent accuracy.]

(FL: FCC)

Change to read:
Reagents and Solutions

*Hydrochloric Acid Solution*— Mix 35 mL of 1.0 N hydrochloric acid with 385 mL of water.

*Substrate*— Boil one or more hen eggs for 15 min to provide coagulated albumen (Miles, Inc.), and cool rapidly by immersion in cold water. Remove the shell and pellicle and all of the yolk, and at once rub the albumen through a clean, dry No. 40 sieve, rejecting the first portion that passes through the sieve.

*Substrate Preparation*— Place 10 g of the Substrate in each of as many 100-mL wide-mouth bottles as needed for the test, and immediately add 35 mL of Hydrochloric Acid Solution (all at one time or in portions). By suitable means, thoroughly disintegrate the particles of albumen. Equilibrate to 52° before use in the Procedure, below.

*Standard Preparation*— Dissolve 100 mg of USP Pepsin Reference Standard in 150 mL of Hydrochloric Acid Solution. Use this solution within 1 h.

*Sample Preparation*— Dissolve 100 mg of the pepsin sample, or an amount of the enzyme preparation that will provide a solution similar to or slightly stronger than the Standard Preparation, in 150 mL of Hydrochloric Acid Solution. Use this solution within 1 h.

* Dilute Hydrochloric Acid Solution * Dilute 30 mL of 1.0 N hydrochloric acid with water to 1000 mL, then adjust to a pH of 1.6 ± 0.1 with 1.0 N hydrochloric acid, if necessary.

*TCA Solution*— Prepare a 4.0% (w/v) solution of trichloroacetic acid in water.

*Substrate Solution*— Weigh 5.0 g of USP Hemoglobin Protease Substrate RS into a large beaker. Add 100 mL of Dilute Hydrochloric Acid Solution to the beaker, and stir using a magnetic stirrer. Once the hemoglobin is fully dissolved, adjust the pH of the solution to 1.6 ± 0.1 (use 1.0 N hydrochloric acid, dropwise, to adjust the pH as needed). Quantitatively transfer the solution to a 250-mL volumetric flask, then dilute with Dilute Hydrochloric Acid Solution to volume. [Note—Prepare fresh daily.]

*Standard Solutions*— Quantitatively prepare four serial dilutions of USP Pepsin for Assay RS in Dilute Hydrochloric Acid Solution covering the activity range of 0.45–0.90 USP Pepsin U/mL. [Note—Prepare immediately before use.]

*Sample Solution*— Quantitatively prepare a solution of pepsin in Dilute Hydrochloric Acid Solution containing approximately 0.60–0.70 USP Pepsin U/mL. [Note—Prepare immediately before use.]

*Change to read:*

*Procedure*— Pipet 5.0 mL of the Standard Preparation into each of two bottles containing the Substrate Preparation. To two or more additional substrate bottles add graduated aliquots of the Sample Preparation so that one bottle will contain approximately the same amount, and the others will contain successively lesser amounts, of pepsin as is contained in the 5.0 mL of the Standard Preparation, using, for example, 5.0, 4.9, and 4.8 mL. When less than 5.0 mL of the Sample Preparation is used, add sufficient Hydrochloric Acid Solution to make 5.0 mL of combined Sample Preparation plus acid added. At once stopper the bottles securely, invert them three times, and heat in a water bath, maintained at 52° ± 0.5°, for 2.5 h, agitating the contents equally every 10 min by inverting the bottles once. Remove the bottles from the bath, and pour the contents of each into separate measuring vessels.

Transfer the undigested albumen that adheres to the sides of the bottles into the respective measuring vessel with the aid of small portions of water until 50 mL has been used for each. Mix the contents of each vessel, allow them to stand for 30 min, and then read for each the volume of undigested albumen. Average the
sediment volumes in the two standard vessels, and note which of the sample vessels contains undigested albumen closest to the average for the standards. Finally, record as V the volume, in mL, of Sample Preparation that produced the undigested albumen closest to the average produced by the Standard Preparations.

For the standard curve, prepare test tubes as follows (use tubes that can accommodate and allow vortex mixing of a volume of not less than 16 mL). Separately transfer 1.0-mL aliquots of each of the Standard Solutions into four test tubes—two tubes for the analysis of each Standard Solution and two blank tubes for each Standard Solution. Prepare a substrate blank by transferring 1.0 mL of Dilute Hydrochloric Acid Solution into a single test tube. A full set of tubes for creation of the standard curve will require seventeen test tubes.

For each pepsin sample being tested, prepare test tubes as follows. Separately transfer 1.0-mL aliquots of the Sample Solution into four test tubes—two tubes for the analysis of each sample and two blank tubes for each sample.

To each of the blank tubes (the substrate blank, the Standard Solution blanks, and the Sample Solution blanks), add 10.0 mL of TCA Solution, and mix by vortexing. Place all of the test tubes into a water bath maintained at 25°C ± 0.1°C. Add 5.0 mL of the Substrate Solution (previously equilibrated to 25°C ± 0.1°C) to each of the blank tubes, and mix by vortexing. Using a stopwatch and starting at time equals zero, rapidly pipet 5.0 mL of the Substrate Solution (previously equilibrated to 25°C ± 0.1°C) successively and at intervals of exactly 30 s into each of the tubes containing the Sample Solution and Standard Solutions for analysis. Vortex each tube immediately after adding the Substrate Solution, then return the tube to the water bath. Exactly 10.0 min after the addition of the Substrate Solution, stop the reaction by rapidly pipetting 10.0 mL of the TCA Solution into each analysis tube at intervals of exactly 30 s, immediately vortexing each tube after addition of the TCA Solution. Remove all tubes from the water bath.

Allow all of the tubes (including blanks) to sit at room temperature for 25 min. Prepare clean test tubes for filtering the solutions, allowing two tubes for each solution to be filtered. Place funnels in each tube, and line the funnels with fluted or folded filter paper (use Whatman No. 41 ashless filter circles with a minimum diameter of 125 mm, or equivalent). After 25 min, vortex each tube, then transfer the contents of each tube into a clean test tube. Re-filter each of the filtrates through the same paper into a second clean test tube. Measure the absorbance of each of the filtrates at 280 nm using a suitable UV-visible spectrophotometer that has previously been zeroed with the substrate blank in a 1-cm cell. Record the absorbance of each filtrate, and calculate the average absorbance reading for each Standard Solution, Sample Solution, and their respective blanks. Determine the net absorbance for each of the Standard Solutions by subtracting the average absorbance of the Standard Solution blank from the average absorbance of the corresponding Standard Solution. Plot a standard curve of the net absorbance of each Standard Solution versus its concentration, in mg/mL. Determine the slope (m) and y-intercept (b) of the resulting curve. □

**Change to read:**

**Calculation** One pepsin unit is defined as that quantity of enzyme that digests 3000 times its weight of coagulated egg albumen produces the equivalent of 1 µmol of tyrosine per min under the conditions of the assay.

Calculate the activity of the enzyme preparation by the equation

\[
\text{Pepsin units/mg} = 3000 \times \frac{(S/u)}{(5.0/V)}
\]

in which S is the weight, in mg, of USP Pepsin Reference Standard used to make the Standard Preparation; u
is the weight, in mg, of enzyme preparation taken for analysis; and \( v \) is as defined in the Procedure.

\[
Pepsin \text{ Units/mg} = \frac{[(A_S - b) \times P]}{(m \times C)}
\]

in which \( A_S \) is the average absorbance for the sample corrected for the average absorbance of the sample blanks; \( b \) is the y-intercept of the standard curve; \( P \) is the activity of the USP Pepsin for Assay RS used to prepare the Standard Solutions (U/mg); \( m \) is the slope of the standard curve; and \( C \) is the concentration of the Sample Solution (mg/mL). 2S (FCC8)

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