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Oil- and Water-Soluble Vitamins with Minerals Capsules

DEFINITION

Oil- and Water-Soluble Vitamins with Minerals Capsules contain one or more of the following oil-soluble vitamins: Vitamin A, vitamin D as Ergocalciferol (vitamin D_2) or Cholecalciferol (vitamin D_3), Vitamin E, Phytonadione (vitamin K_1), and Beta Carotene; one or more of the following water-soluble vitamins: Ascorbic Acid or its equivalent as Calcium Ascorbate or Sodium Ascorbate, Biotin, Cyanocobalamin, Folic Acid, Niacin or Niacinamide, Dexpanthenol or Panthenol, vantothenic vcid (as Calcium Pantothenate or Racemic Calcium Pantothenate), Pyridoxine Hydrochloride, Riboflavin, and Thiamine Hydrochloride or Thiamine Mononitrate; and one or more minerals derived from substances generally recognized as safe, furnishing one or more of the following elements in ionizable form: boron, calcium, chromium, copper, fluorine, iodine, iron, magnesium, manganese, molybdenum, nickel, phosphorus, potassium, selenium, tin, vanadium, and zinc. Capsules contain NLT 90.0% and NMT 165.0% of the labeled amounts of vitamin A ($C_{20}H_{30}O$) as retinol or esters of retinol in the form of retinyl acetate ($C_{22}H_{32}O_2$) or retinyl palmitate ($C_{36}H_{60}O_2$); vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$); vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$) or alpha tocopheryl acetate ($C_{31}H_{52}O_3$) or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$); phytonadione ($C_{31}H_{46}O_2$); and beta carotene ($C_{40}H_{56}$); NLT 90.0% and NMT 150.0% of the labeled amounts of ascorbic acid ($C_6H_8O_6$) or its salts as calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$) or sodium ascorbate ($C_6H_7NaO_6$); biotin ($C_{10}H_{16}N_2O_3S$); cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$); folic acid ($C_{19}H_{19}N_7O_6$); niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$); dexpanthenol ($C_9H_{19}NO_4$) or panthenol ($C_9H_{19}NO_4$); calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$); pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$); riboflavin ($C_{17}H_{20}N_4O_6$); and thiamine ($C_{12}H_{17}ClN_4OS$) as thiamine hydrochloride or thiamine mononitrate; and NLT 90.0% and NMT 125.0% of the labeled amount of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), potassium (K), and zinc (Zn); and NLT 90.0% and NMT 160.0% of the labeled amounts of boron (B), chromium (Cr), fluorine (F), iodine (I), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), and vanadium (V).

They may contain other labeled added substances that are generally recognized as safe, in amounts that are unobjectionable.

STRENGTH

[Note—In the following assays, where more than one assay method is given for an individual ingredient, the requirements may be met by following any one of the specified methods, the method used being stated in the labeling only if *Method 1* is not used.]

• **Vitamin A,** *Method 1*

[Note—Where the use of a vitamin A ester (retinyl acetate or retinyl palmitate) is specified in the following procedure, use the chemical form present in the formulation. USP Vitamin A RS is retinyl acetate. It is to be used where USP Vitamin A RS is specified. Use low-actinic glassware throughout this procedure.]

Mobile phase: *n*-Hexane

Standard solution: 15 µg/mL of retinyl acetate from USP Vitamin A RS in *n*-hexane

System suitability stock solution: 15 µg/mL of retinyl palmitate in *n*-hexane

System suitability solution: Mix equal volumes of *System suitability stock solution* and the *Standard solution* to obtain concentrations of 7.5 µg/mL each of retinyl acetate and retinyl palmitate.

Sample stock solution: Transfer the contents of NLT 20 Capsules to a suitable container, mix, and weigh. Transfer a portion of the mixture, equivalent to 5 Capsules, to a container with a polytef-lined screw cap. [Note—For hard gelatin Capsules, remove, as completely as possible, the contents of NLT 20 Capsules by cutting open the Capsule shells, transferring the shells and their contents to a suitable container, and triturating to a homogeneous mass. Transfer a portion of the mass, equivalent to 5 Capsules, to a container with a polytef-lined screw cap.] Add 10 mL of dimethyl sulfoxide and 15 mL of *n*-hexane, and shake for 45 min on a wrist-action shaker in a water bath maintained at 60°. [Note—Set up the wrist-action shaker to ensure that the contents of the container are mixed vigorously and thoroughly.] Centrifuge at 3000 rpm for 10 min, and transfer the hexane layer by means of a pipet to a 100-mL volumetric flask. Add 15 mL of *n*-hexane to the dimethyl sulfoxide layer, shake thoroughly for 5 min, and transfer the hexane layer by means of a pipet to the 100-mL volumetric flask. Repeat this extraction with three additional 15-mL portions of *n*-hexane. Dilute the extracts in the volumetric flask with *n*-hexane to volume.

Sample solution: Dilute a volume of the *Sample stock solution* with *n*-hexane to obtain a solution with a concentration of 15 µg/mL of vitamin A as retinol ($C_{20}H_{30}O$).

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Chromatographic system

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

Mode: LC

Detector: UV 325 nm

Column: 4.6-mm × 15-cm; 3-µm packing L8

Flow rate: 1 mL/min

Injection volume: 40 µL

System suitability

Sample: *System suitability solution*

Suitability requirements

Resolution: NLT 10 between all-*trans*-retinyl acetate and all-*trans*-retinyl palmitate

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak area for all-*trans*-retinyl acetate from the *Standard solution* and the peak area for all-*trans*-retinyl acetate or all-*trans*-retinyl palmitate in the chromatogram of the *Sample solution.* For products containing vitamin A acetate or vitamin A palmitate, calculate the percentage of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$), in the portion of Capsules taken:

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

r_U = peak area of the all-*trans*-retinyl ester from the *Sample solution*

r_S = peak area of the all-*trans*-retinyl ester from the *Standard solution*

C_S = concentration of retinyl acetate ($C_{22}H_{32}O_2$) from USP Vitamin A RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of vitamin A, as retinol ($C_{20}H_{30}O$), in the *Sample solution* (µg/mL)

F = factor used to convert retinyl acetate, the ester form present in USP Vitamin A RS, to retinol, 0.872

[Note—The molar responses of retinyl acetate and retinyl palmitate are equivalent.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$)

• **Vitamin A,** *Method 2*

[Note—Where a vitamin A ester (retinyl acetate or retinyl palmitate) is indicated in the following procedure, use the chemical form present in the formulation. USP Vitamin A RS is retinyl acetate. It is to be used where USP Vitamin A RS is specified. Use low-actinic glassware throughout this procedure.]

3 N methanolic sulfuric acid solution: Cautiously add 9 mL of sulfuric acid to 80 mL of methanol in a 100-mL volumetric flask. Cool, and dilute with methanol to volume.

Sodium ascorbate–pyrogallol solution: Transfer 10 g of sodium ascorbate and 5 g of pyrogallol to a 100-mL volumetric flask, and add sufficient water to dissolve. Add 1.7 mL of sulfuric acid, and dilute with water to volume.

Lecithin solution: 5 mg/mL of lecithin in 2,2,4-trimethylpentane

Mobile phase: *n*-Hexane and ethyl acetate (99.7:0.3)

Standard solution: 15 µg/mL of retinyl acetate from USP Vitamin A RS in 2,2,4-trimethylpentane

System suitability stock solution: 15 µg/mL of retinyl palmitate in 2,2,4-trimethylpentane

System suitability solution: Mix equal volumes of the *System suitability stock solution* and the *Standard solution* to obtain concentrations of 7.5 µg/mL each of retinyl acetate and retinyl palmitate.

Sample solution: [Note—This preparation is suitable for the determination of vitamin A, vitamin D, and vitamin E, when present in the formulation.] Weigh NLT 20 Capsules in a tared weighing bottle. Using a sharp blade if necessary, carefully open the Capsules, without loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to 30 µg of the labeled amount of cholecalciferol or ergocalciferol (vitamin D), to a container with a polytef-lined screw cap. If vitamin D is not present in the formulation, use a portion, equivalent to 90 mg of the labeled amount of vitamin E. If vitamin E is not present in the formulation, use a portion, equivalent to 2.5 mg of the labeled amount of vitamin A, as retinol. Add 0.5 g of sodium bicarbonate, 1.5 mL of *Lecithin solution*, and 12.5 mL of 2,2,4-trimethylpentane, and disperse on a vortex mixer. Add 6 mL of *Sodium ascorbate–pyrogallol solution*, shake slowly, and allow the solution to degas. Continue shaking until the evolution of gas has ceased, and then shake for an additional 12 min. Add 6 mL of dimethyl sulfoxide, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 6 mL of *3 N methanolic sulfuric*

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acid solution, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 12.5 mL of 2,2,4-trimethylpentane, mix on a vortex mixer to form a suspension, and shake for 10 min. Centrifuge for 10 min to break up the emulsion and to clarify the supernatant. [NOTE—The supernatant is used for the determination of vitamin A, and also vitamin D and vitamin E, if present in the formulation.] If necessary, quantitatively dilute a volume of the supernatant with 2,2,4-trimethylpentane to obtain a concentration close to that of the *Standard solution*.

Chromatographic system

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

Mode: LC

Detector: UV 325 nm

Column: 4.6-mm × 25-cm; 5-µm packing L24

Flow rate: 1.5 mL/min

Injection volume: 40 µL

System suitability

Sample: *System suitability solution*

Suitability requirements

Resolution: NLT 8.0 between all-*trans*-retinyl acetate and all-*trans*-retinyl palmitate

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak area for all-*trans*-retinyl acetate from the *Standard solution* and the peak area of all-*trans*-retinyl acetate or all-*trans*-retinyl palmitate from the *Sample solution.*

Calculate the percentage of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$), in the portion of Capsules taken:

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

r_U = peak area of the all-*trans*-retinyl ester from the *Sample solution*

r_S = peak area of the all-*trans*-retinyl ester from the *Standard solution*

C_S = concentration of retinyl acetate ($C_{22}H_{32}O_2$) from USP Vitamin A RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of vitamin A, as retinol ($C_{20}H_{30}O$), in the *Sample solution* (µg/mL)

F = factor used to convert retinyl acetate, the ester form present in USP Vitamin A RS, to retinol, 0.872

[NOTE—Account for the initial extraction volume of 26.5 mL of 2,2,4-trimethylpentane to calculate the nominal concentration. The molar responses of retinyl acetate and retinyl palmitate are equivalent.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$)

Change to read:

• **VITAMIN A,** *Method 3*

[NOTE—Where a vitamin A ester (retinyl acetate or retinyl palmitate) is indicated in the following procedure, use the chemical form present in the formulation. USP Vitamin A RS is retinyl acetate. It is to be used where USP Vitamin A RS is specified. Use low-actinic glassware throughout this procedure.]

Extraction solvent: *n*-Hexane and methylene chloride (3:1)

Potassium hydroxide solution: 800 mg/mL of potassium hydroxide in water. [NOTE—Cautiously add potassium hydroxide in water. Mix, and cool.]

Diluent: 10 mg/mL of pyrogallol in alcohol

Mobile phase: *n*-Hexane and isopropyl alcohol (92:8)

Standard stock solution: 30 µg/mL of retinyl acetate from USP Vitamin A RS in *Diluent*. [NOTE—This solution may be stored in a refrigerator for 1 week.]

Standard solution: Dilute a volume of *Standard stock solution* with *Diluent* to obtain a concentration of 1 µg/mL of retinyl acetate from USP Vitamin A RS. Transfer 10.0 mL of this solution to a stoppered 125-mL flask, and add 5 mL of water, 5 mL of *Diluent*, and 3 mL of *Potassium hydroxide solution*. Insert the stopper tightly, shake for 15 min over a water bath maintained at 60 ± 5°, and cool to room temperature. Add 7 mL of water and 25.0 mL of *Extraction solvent*. Insert the stopper tightly, and shake vigorously for 60 s. Rinse the sides of the flask with 60 mL of water, and allow to stand for 10 min until the layers separate. Withdraw a portion of the organic layer for injection into the chromatograph. This *Standard solution* contains 0.34 µg/mL of retinol.

Sample solution: Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing with several portions of ether. Discard the

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washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to 1.5 mg of retinyl acetate, to a stoppered 125-mL flask. Add 5 mL of water, 15 mL of *Diluent*, and 3 mL of *Potassium hydroxide solution*. Insert the stopper tightly, shake for 15 min over a water bath maintained at 60 ± 5°, and cool to room temperature. Add 7 mL of water and 25.0 mL of *Extraction solvent*. Insert the stopper tightly, and shake vigorously for 60 s or longer, if necessary, for complete extraction. Rinse the sides of the flask with 60 mL of water, and allow to stand for 10 min until the layers separate. [NOTE—Do not shake, because an emulsion may form.] Withdraw a portion of the organic layer, and dilute quantitatively, and stepwise if necessary, with *Extraction solvent* to obtain a concentration of 0.34 µg/mL of retinol.

Chromatographic system

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

Mode: LC

Detector: UV 335 nm

Column: 6.2-mm × 8-cm; packing L3

Column temperature: 40°

Flow rate: 4 mL/min

Injection volume: 50 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for 13-*cis*-retinol and all-*trans*-retinol are about 0.92 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for all-*trans*-retinol and 13-*cis*-retinol.

Calculate the percentage of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$), in the portion of Capsules taken:

$$\text{Result} = (r_{T1}/r_{T2}) \times (C_S/C_U) \times F \times 100$$

r_{T1} = sum of the areas of the all-*trans*-retinol and 13-*cis*-retinol peaks from the *Sample solution*

r_{T2} = sum of the areas of all-*trans*-retinol and 13-*cis*-retinol peaks from the *Standard solution*

C_S = concentration of retinyl acetate (▲$C_{22}H_{32}O_2$▲ (ERR 1-Nov-2023)) from USP Vitamin A RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of vitamin A, as retinol ($C_{20}H_{30}O$), in the *Sample solution* (µg/mL)

F = factor used to convert retinyl acetate, the ester form present in USP Vitamin A RS, to retinol, 0.872

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$)

• **CHOLECALCIFEROL OR ERGOCALCIFEROL (VITAMIN D),** *Method 1*

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Mobile phase: *n*-Hexane and isopropyl alcohol (99:1)

Standard solution: 2 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in *n*-hexane

System suitability solution: Heat a volume of the *Standard solution* at 60° for 1 h to partially isomerize vitamin D (cholecalciferol or ergocalciferol) to its corresponding precursor.

Sample solution: Transfer NLT 20 mL of the solution prepared as directed for the *Sample stock solution* in *Vitamin A, Method 1* to a suitable container, and evaporate, if necessary, under vacuum at room temperature to obtain a solution with an expected concentration of 2 µg/mL of cholecalciferol or ergocalciferol.

Chromatographic system

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

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 15-cm; 3-µm packing L8

Flow rate: 1 mL/min

Injection volume: 100 µL

System suitability

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

Suitability requirements

Resolution: NLT 10 between the vitamin D form present and its corresponding precursor, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for vitamin D. Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of Capsules taken:

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

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*

r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*

C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

F = correction factor to account for the average amount of previtamin D present in the *Sample solution*, 1.09

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

• **Cholecalciferol or Ergocalciferol (Vitamin D),** *Method 2*

[Note—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

3 N methanolic sulfuric acid solution, Sodium ascorbate–pyrogallol solution, Lecithin solution, and **Sample solution:** Proceed as directed in *Vitamin A, Method 2*.

Mobile phase: *n*-Hexane and tertiary butyl alcohol (98.75:1.25)

Standard solution: 1 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in 2,2,4-trimethylpentane

System suitability solution: Heat a volume of the *Standard solution* at 60° for 1 h to partially isomerize vitamin D (cholecalciferol or ergocalciferol) to its corresponding precursor.

Chromatographic system

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

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 25-cm; 5-µm packing L24

Flow rate: 1 mL/min

Injection volume: 40 µL

System suitability

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

Suitability requirements

Resolution: NLT 4.0 between the vitamin D form present and its corresponding precursor, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for vitamin D. Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of Capsules taken:

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

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*

r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*

C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

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Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

• **Cholecalciferol or Ergocalciferol (Vitamin D),** *Method 3*

[Note—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Diluted acetic acid: Glacial acetic acid solution (1 in 10) in water

Phenolphthalein solution: 10 mg/mL of phenolphthalein in alcohol

Potassium hydroxide solution: Slowly dissolve 14 g of potassium hydroxide in a mixture of 31 mL of dehydrated alcohol and 5 mL of water. Prepare fresh daily.

Extraction solvent: Methylene chloride and isopropyl alcohol (99.8:0.2)

Mobile phase: Acetonitrile and methanol (91:9)

Standard stock solution: 0.2 mg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in dehydrated alcohol. [Note—Prepare fresh every 4 weeks. Store in a freezer.]

Standard solution: [Note—Condition the solid-phase extraction column specified for use in the *Standard solution* and the *Sample solution* by initially washing the column with 4.0 mL of a mixture of methylene chloride and isopropyl alcohol (4:1), followed by 5.0 mL of *Extraction solvent*. Do not allow the column to dry.] Dilute a volume of *Standard stock solution* with dehydrated alcohol to obtain a concentration of 5 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS. Prepare this solution fresh daily. Transfer 2.0 mL of this solution to a stoppered 125-mL flask. Add 15.0 mL of water and 15.0 mL of *Potassium hydroxide solution*, insert the stopper, and shake for 30 min in a water bath maintained at 60°. Allow to cool to room temperature, and transfer the contents of the flask to a 250-mL separatory funnel. Add 15.0 mL of water to the flask, insert the stopper, shake vigorously, and transfer this solution to the separatory funnel. Rinse the flask with 60 mL of *n*-hexane, and transfer the rinsing to the separatory funnel. Insert the stopper, shake vigorously for 90 s, and allow to stand for 15 min until the layers separate. Drain, and discard the aqueous layer. Add 15.0 mL of water to the hexane layer in the separatory funnel, insert the stopper, and shake vigorously. Allow to stand for 10 min until the layers separate, and discard the aqueous layer. Add 1 drop of *Phenolphthalein solution* and 15.0 mL of water to the separatory funnel. Add *Diluted acetic acid* dropwise, with shaking, until the washing is neutral. Allow to stand for 10 min until the layers separate. Drain, and discard the aqueous layer. Filter the hexane layer through anhydrous sodium sulfate supported by a small pledget of cotton into a 100-mL round-bottom flask. Rinse the funnel and sodium sulfate with a few mL of *n*-hexane, and collect the rinsings in the same flask. Evaporate the hexane in the flask on a rotary evaporator at 50° to dryness. Immediately add 2.0 mL of *Extraction solvent* to dissolve the residue. Transfer this solution to a freshly conditioned solid-phase extraction column containing silica packing with a sorbent mass-to-column volume ratio of 500 mg to 2.8 mL or equivalent, rinse the round-bottom flask with 1.0 mL of *Extraction solvent*, and transfer to the column. Elute the column with 2.0 mL of *Extraction solvent*, and discard this fraction. Elute the column with 7.0 mL of *Extraction solvent*, and collect the eluate in a suitable flask. Place the flask in a warm water bath maintained at 42°, and evaporate the solvent with the aid of a stream of nitrogen. Immediately add 2.0 mL of acetonitrile to the residue, and use the solution for injection into the chromatograph.

Sample solution: Proceed as directed in *Vitamin A, Method 3*, through "calculate the net weight of the Capsule contents." Transfer a portion of the Capsule contents, equivalent to 10 µg of cholecalciferol or ergocalciferol, to a stoppered 125-mL flask, and proceed as directed for the *Standard solution*, beginning with "Add 15.0 mL of water and 15.0 mL of *Potassium hydroxide solution*".

Chromatographic system

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

Mode: LC

Detector: UV 265 nm

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

Column temperature: 27°

Flow rate: 0.7 mL/min

Injection volume: 15 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 4.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for vitamin D. Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of Capsules taken:

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

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*

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r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*

C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

• **Vitamin E,** *Method 1*

[Note—Where vitamin E (alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Solution A: Phosphoric acid solution (1 in 100) in water

Mobile phase: Methanol and *Solution A* (19:1)

System suitability solution: Prepare a 0.65-mg/mL solution of USP Ergocalciferol RS in methanol. Transfer 1.0 mL of this solution to a 100-mL volumetric flask containing 100 mg of USP Alpha Tocopheryl Acetate RS. Dissolve in 30 mL of methanol, with the aid of sonication if necessary, and dilute with methanol to volume. Store this solution in a refrigerator.

Standard solution: 2 mg/mL of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, or USP Alpha Tocopheryl Acid Succinate RS in methanol

Sample solution: Transfer NLT 20 mL of the solution prepared as directed for the *Sample stock solution* in *Vitamin A, Method 1* to a suitable container, and evaporate, under vacuum at room temperature to dryness. Transfer the contents of the flask to a suitable volumetric flask with the aid of methanol, and dilute with methanol to volume, to obtain a solution with an expected concentration of 2 mg/mL of alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate.

Chromatographic system

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

Mode: LC

Detector: UV 254 nm

Column: 8-mm × 10-cm; 5-µm packing L1

Flow rate: 2 mL/min

Injection volume: 100 µL

System suitability

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

[Note—The relative retention times for ergocalciferol and alpha tocopheryl acetate are about 0.5 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 12 between ergocalciferol and alpha tocopheryl acetate, *System suitability solution*

Tailing factor: Between 0.8 and 1.2, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas. Calculate the percentage of the labeled amount of alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$) in the portion of Capsules taken:

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

r_U = peak area of the relevant vitamin E form from the *Sample solution*

r_S = peak area of the relevant vitamin E form from the *Standard solution*

C_S = concentration of the corresponding USP Reference Standard in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding form of vitamin E in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$)

• **Vitamin E,** *Method 2*

[Note—Where vitamin E (alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

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Mobile phase: Mix 240 mL of methanol with 10 mL of water, followed by 0.5 mL of 50% phosphoric acid, and dilute with acetonitrile to 1000 mL.

System suitability solution: 2 mg/mL each of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, and USP Alpha Tocopheryl Acid Succinate RS in methanol

Standard solution: 2 mg/mL of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, or USP Alpha Tocopheryl Acid Succinate RS in methanol

3 N methanolic sulfuric acid solution: Cautiously mix sulfuric acid and methanol (9 in 100) in a 100-mL volumetric flask. [NOTE—Dissolve in a portion of methanol, cool, and then dilute to final volume.]

Sodium ascorbate–pyrogallol solution: Transfer 10 g of sodium ascorbate and 5 g of pyrogallol to a 100-mL volumetric flask. Add sufficient water to dissolve. Add 1.7 mL of sulfuric acid, and dilute with water to volume.

Lecithin solution: 5 mg/mL of lecithin in 2,2,4-trimethylpentane

Sample solution: Proceed as directed in *Vitamin A, Method 2*, through "calculate the net weight of the Capsule contents." Transfer a portion of the Capsule contents, equivalent to 55 mg of vitamin E, to a container with a polytef-lined screw cap. Add 0.5 g of sodium bicarbonate, 1.5 mL of *Lecithin solution*, and 12.5 mL of 2,2,4-trimethylpentane, and disperse on a vortex mixer. Add 6 mL of *Sodium ascorbate–pyrogallol solution*, shake slowly, and allow the solution to degas. Continue shaking until the evolution of gas has ceased, and then shake for an additional 12 min. Add 6 mL of dimethyl sulfoxide, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 6 mL of *3 N methanolic sulfuric acid solution*, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 12.5 mL of 2,2,4-trimethylpentane, mix on a vortex mixer to form a suspension, and shake for 10 min. Centrifuge for 10 min to break up the emulsion and to clarify the supernatant layer. Transfer a volume of the supernatant 2,2,4-trimethylpentane layer to a suitable volumetric flask, the volume of the specimen withdrawn from the 2,2,4-trimethylpentane layer and the size of the volumetric flask being such that the final concentration of the *Sample solution* is equivalent to that of the *Standard solution*. Evaporate nearly to dryness, add several mL of methanol, and evaporate the remaining 2,2,4-trimethylpentane. Dilute with methanol to volume.

Chromatographic system

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

Mode: LC

Detector: UV 280 nm

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

Flow rate: 1.5 mL/min

Injection volume: 25 µL

System suitability

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

[NOTE—The relative retention times for alpha tocopheryl acid succinate, alpha tocopherol, and alpha tocopheryl acetate are about 0.6, 0.8, and 1.0, respectively.]

Suitability requirements

Resolution: NLT 4.0 between alpha tocopheryl acid succinate and alpha tocopherol and NLT 3.0 between alpha tocopherol and alpha tocopheryl acetate, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas. Calculate the percentage of the labeled amount of alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$) in the portion of Capsules taken:

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

r_U = peak area of the relevant vitamin E form from the *Sample solution*

r_S = peak area of the relevant vitamin E form from the *Standard solution*

C_S = concentration of the corresponding USP Reference Standard in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding form of vitamin E in the *Sample solution* (mg/mL)

[NOTE—Account for the initial extraction volume of 26.5 mL of 2,2,4-trimethylpentane and the dilution factor to exchange the solvent from 2,2,4-trimethylpentane to methanol to calculate the nominal concentration.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$)

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• **Vitamin E,** *Method 3*

Diluent: Acetonitrile and ethyl acetate (1:1)

Mobile phase: Methanol, acetonitrile, and *n*-hexane (46.5:46.5:7.0)

Standard solution: 0.3 mg/mL of USP Alpha Tocopherol RS in methanol

Sample solution: Proceed as directed in *Vitamin A, Method 3*, through "calculate the net weight of the Capsule contents." Transfer a portion of the Capsule contents, equivalent to 8.0 mg of alpha tocopherol, to a glass-stoppered conical flask. Add 25.0 mL of water, 25.0 mL of dehydrated alcohol, and 3.5 g of potassium hydroxide pellets. Shake for 1 h in a water bath maintained at 55°. Cool, and transfer with the aid of a minimum volume of water to a 125-mL separatory funnel. Rinse the flask with 50 mL of *n*-hexane, and add the rinsing to the separatory funnel. Insert the stopper, shake vigorously for 60 s, and allow the layers to separate. Drain the aqueous layer into a second 250-mL separatory funnel, and repeat the extraction with 50 mL of *n*-hexane. Discard the aqueous layer, and combine the hexane extracts. Wash the combined extracts with 25 mL of water, allow the layers to separate, and discard the aqueous layer. Add 3 drops of glacial acetic acid, and repeat the washing procedure two more times. Filter the washed hexane layer through anhydrous sodium sulfate into a 250-mL round-bottom flask. Rinse the funnel and sodium sulfate with a few mL of *n*-hexane, and add the rinsing to the hexane solution in the flask. Place the flask in a water bath maintained at 50°, and evaporate the hexane solution with the aid of a rotary evaporator to dryness. Immediately add 25.0 mL of *Diluent*, and swirl to dissolve the residue.

Chromatographic system

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

Mode: LC

Detector: UV 291 nm

Column: 4.6-mm × 25-cm; packing L1

Column temperature: 40°

Flow rate: 3 mL/min

Injection volume: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas. Calculate the percentage of the labeled amount of vitamin E, as alpha tocopherol ($C_{29}H_{50}O_2$), in the portion of Capsules taken:

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

r_U = peak area of alpha tocopherol from the *Sample solution*

r_S = peak area of alpha tocopherol from the *Standard solution*

C_S = concentration of alpha tocopherol in the *Standard solution* (mg/mL)

C_U = nominal concentration of vitamin E, as alpha tocopherol, in the *Sample solution* (mg/mL)

[Note—Calculate the alpha tocopherol equivalent ($C_{29}H_{50}O_2$) of alpha tocopheryl acetate ($C_{31}H_{52}O_3$) or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$) by multiplying their contents by the factors 0.91 or 0.81, respectively.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin E

• **Phytonadione**

[Note—Use low-actinic glassware throughout this procedure.]

Mobile phase: Methanol and water (19:1)

Standard stock solution: 200 µg/mL of USP Phytonadione RS in methanol. Dissolve with the aid of sonication if necessary.

System suitability solution: 0.65 mg/mL of USP Alpha Tocopheryl Acetate RS and 20 µg/mL of USP Phytonadione RS from *Standard stock solution* diluted with methanol. [Note—Dissolve USP Alpha Tocopheryl Acetate RS in a portion of methanol, add the *Standard stock solution*, and then dilute with methanol to volume.]

Standard solution: 20 µg/mL of USP Phytonadione RS from *Standard stock solution* diluted with methanol

Sample solution: Transfer NLT 20 mL of the solution prepared as directed for the *Sample stock solution* in *Vitamin A, Method 1* to a suitable container, and evaporate, if necessary, under vacuum at room temperature to dryness. Transfer the contents of the flask to a suitable

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volumetric flask with the aid of methanol, and dilute with methanol to volume to obtain a solution with an expected concentration of 20 µg/mL of phytonadione.

Chromatographic system

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

Mode: LC

Detector: UV 254 nm

Column: 8-mm × 10-cm; 5-µm packing L1

Flow rate: 2.0 mL/min

Injection volume: 100 µL

System suitability

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

[NOTE—The relative retention times for alpha tocopheryl acetate and phytonadione are about 0.68 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 5 between alpha tocopheryl acetate and phytonadione, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas. Calculate the percentage of the labeled amount of phytonadione ($C_{31}H_{46}O_2$) in the portion of Capsules taken:

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

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Phytonadione RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of phytonadione in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of phytonadione ($C_{31}H_{46}O_2$)

• **BETA CAROTENE**

[NOTE—Use low-actinic glassware throughout this procedure.]

Potassium hydroxide solution: Dissolve 58.8 g of potassium hydroxide in 50 mL of water.

Iodine solution: Transfer 10 mg of iodine to a 100-mL volumetric flask. Dissolve in cyclohexane, and dilute with cyclohexane to volume. Dilute 10 mL of this solution with cyclohexane to 100 mL. [NOTE—Prepare this solution fresh daily.]

Sample solution A (for preparations containing beta carotene in oil solutions)**:** Proceed as directed in *Vitamin A, Method 1*, except use cyclohexane instead of *n*-hexane as the extraction solvent, and dilute the filtered extracts with cyclohexane, to obtain a concentration of 2 µg/mL of beta carotene.

Sample solution B (for preparations containing beta carotene in dry powder)**:** Remove the contents of NLT 20 Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the Capsule contents, equivalent to 2 mg of beta carotene, to a 500-mL saponification flask. Add 100 mL of alcohol, 6 mL of *Potassium hydroxide solution*, and a magnetic stirring bar. Attach an air condenser to the flask, and heat under reflux for 45 min with constant stirring. Cool to room temperature. Add 170 mL of solvent hexane, and stir for 30 min. Quantitatively transfer the contents of the flask to a 500-mL separatory funnel with portions of solvent hexane. Allow the layers to separate for 5–10 min, and transfer the upper organic layer to a 500-mL volumetric flask. Transfer the lower aqueous layer into the saponification flask. Add 170 mL of solvent hexane, and stir for an additional 20 min. Quantitatively transfer the contents of the saponification flask to the separatory funnel with the aid of portions of solvent hexane. Allow the layers to separate for 10 min. Drain the lower aqueous layer, and discard. Transfer the organic layer to the volumetric flask containing the previously collected organic layer. Rinse the separatory funnel with small portions of solvent hexane, and transfer the washings to the volumetric flask. Dilute the hexane extracts with solvent hexane to volume. Add 3 g of anhydrous sodium sulfate, shake, and allow to settle. Quantitatively transfer a volume of this solution, equivalent to 100 µg of beta carotene, to a 50-mL volumetric flask. Evaporate under a stream of nitrogen to dryness, and immediately add cyclohexane. Add 2 mL of *Iodine solution*, and heat for 15 min in a water bath maintained at 65°. Cool rapidly, and dilute with cyclohexane to volume.

Instrumental conditions

(See *Ultraviolet-Visible Spectroscopy (857)*.)

Mode: Vis

Analytical wavelength: 452 nm

Blank: Cyclohexane

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Analysis

Sample: *Sample solution*

Determine the absorbance against the *Blank*. Calculate the percentage of the labeled amount of beta carotene ($C_{40}H_{56}$) in the portion of Capsules taken:

$$\text{Result} = (A_U/F) \times (100/C_U)$$

A_U = absorbance of the *Sample solution*

F = absorptivity of beta carotene at 452 nm, 223

C_U = nominal concentration of beta carotene in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of beta carotene ($C_{40}H_{56}$)

• **Ascorbic Acid, Calcium Ascorbate,** and **Sodium Ascorbate**

(See *Vitamin C Assay ⟨580⟩*.)

[Note—For labeling purposes, consider *Method I—Titrimetric Method* as *Method 1*.]

Acceptance criteria: 90.0%–150.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$) or its salts as calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$) or sodium ascorbate ($C_6H_7NaO_6$)

• **Biotin,** *Method 1*

[Note—Use low-actinic glassware throughout this procedure.]

Mobile phase: Mix 85 mL of acetonitrile, 1 g of sodium perchlorate, and 1 mL of phosphoric acid, and dilute with water to 1000 mL.

Standard stock solution: 0.333 mg/mL of USP Biotin RS in dimethyl sulfoxide

Standard solution: 5 µg/mL of USP Biotin RS prepared by diluting the *Standard stock solution* in water

Sample solution: Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without the loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing, if necessary, with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the average net weight per Capsule. Transfer a portion of the Capsule contents, equivalent to 1 mg of biotin, to a 200-mL volumetric flask. Add 3 mL of dimethyl sulfoxide, and swirl to wet the contents. Place the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with water to volume, and filter.

Chromatographic system

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

Mode: LC

Detector: UV 200 nm

Column: 4.6-mm × 15-cm; 3-µm packing L7

Flow rate: 1.2 mL/min

Injection volume: 100 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of biotin. Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Capsules taken:

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

r_U = peak area of biotin from the *Sample solution*

r_S = peak area of biotin from the *Standard solution*

C_S = concentration of USP Biotin RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of biotin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

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• **Biotin,** *Method 2*

[Note—Use low-actinic glassware throughout this procedure.]

Dehydrated mixtures yielding formulations similar to the media described herein may be used provided that, when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described herein.

Standard stock solution: 50 µg/mL of USP Biotin RS in 50% alcohol. Store this solution in a refrigerator.

Standard solution: 0.1 ng/mL of USP Biotin RS in water, prepared by dilution of the *Standard stock solution* with water on the day of the assay

Sample solution: Proceed as directed in *Biotin, Method 1* through "calculate the average net weight per Capsule." Transfer a portion of the Capsule contents, equivalent to 100 µg of biotin, to a 200-mL volumetric flask. Add 3 mL of 50% alcohol, and swirl to wet the contents. Heat the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with 50% alcohol to volume, and filter. Dilute a volume of the filtrate quantitatively, and stepwise if necessary, with water to obtain a solution with a concentration of 0.1 ng/mL.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and dilute with water to 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature NLT 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water. Heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and dilute with water to 1000 mL. Store under toluene in a cool place at a temperature NLT 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and dilute with water to 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Calcium pantothenate solution: 10 µg/mL of calcium pantothenate in 50% alcohol. Store in a refrigerator.

Riboflavin–thiamine hydrochloride solution: 20 µg/mL of riboflavin and 10 µg/mL of thiamine hydrochloride in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

***p*-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution:** 10 µg/mL of *p*-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid, and mix. Store under toluene.

Basal medium stock solution: Dissolve the *anhydrous Dextrose* and *anhydrous Sodium acetate* in the solutions previously mixed according to *Table 1*, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Table 1

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Calcium pantothenate solution	5 mL
Riboflavin–thiamine hydrochloride solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

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Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water. Add 500 mg of *anhydrous Dextrose*, 500 mg of *anhydrous Sodium acetate*, and 1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to test tubes, close or cover the tubes, sterilize in an autoclave at 121° for 15 min, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*,[1] incubating for 16–24 h at a temperature of between 30° and 37° held constant to within ±0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution* add 5.0 mL of water containing 0.5 ng of biotin. Plug the tubes with cotton, sterilize in an autoclave at 121° for 15 min, and cool.

Inoculum: [Note—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Transfer cells from the *Stock culture of Lactobacillus plantarum* to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature of between 30° and 37° held constant to within ±0.5°. The cell suspension so obtained is the *Inoculum*.

Analysis

Samples: *Standard solution* and *Sample solution*

To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool. Add 1 drop of *Inoculum* to each tube, except two of the four tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature of between 30° and 37° held constant to within ±0.5° until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of Standard during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to a spectrophotometer cell. Place the cell in a spectrophotometer that has been set at a specific wavelength of 540–660 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittance (Σ_S) as the difference, $y = 2.00 - \Sigma_S$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_U$, adding together the two transmittances (Σ_U) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of *y* that falls within the range of lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, *X*, for each dosage level. Average the values of *X* for each of three or more dosage levels to obtain $\overline{X}$, which equals the log-relative potency, *M*', of the *Sample solution*. Determine the quantity, in µg, of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Capsules taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of µg of biotin assumed to be present in the portion of the Capsules taken

Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of the Capsules taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of biotin in the portion of Capsules taken (µg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies *M* is NMT 0.08, their mean, $\overline{M}$, is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* ⟨111⟩, *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional

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