Status: Currently Official on 17-Feb-2025
Official Date: Official as of 01-Nov-2020
Document Type: Dietary Supplement Monographs
DocId: GUID-DEC96CEA-BD0D-402A-BC13-8762D0532BC9_6_en-US
DOI: https://doi.org/10.31003/USPNF_M88698_06_01
DOI Ref: 0bd9g

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Oil- and Water-Soluble Vitamins with Minerals Tablets

DEFINITION

Change to read:

Oil- and Water-Soluble Vitamins with Minerals Tablets contain one or more of the following oil-soluble vitamins: Vitamin A ▲as retinyl acetate or retinyl palmitate,▲ (USP 1-May-2020) vitamin D as Ergocalciferol (vitamin D_2) or Cholecalciferol (vitamin D_3), Vitamin E ▲as *RRR*- or *all-rac*-alpha-tocopherol, *RRR*- or *all-rac*-alpha-tocopheryl acetate, or *RRR*- or *all-rac*-alpha-tocopheryl acid succinate,▲ (USP 1-May-2020) Phytonadione (vitamin K_1), and Beta Carotene; and 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, pantothenic acid (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. Tablets contain NLT 90.0% and NMT 165.0% of the labeled amount of vitamin A ▲as retinol▲ (USP 1-May-2020) ($C_{20}H_{30}O$); vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$); vitamin E ▲as 2*R*-alpha-tocopherol▲ (USP 1-May-2020) ($C_{29}H_{50}O_2$); phytonadione ($C_{31}H_{46}O_2$); and beta carotene ($C_{40}H_{56}$); and NLT 90.0% and NMT 150.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$); ▲▲ (USP 1-May-2020) 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 ($C_6H_6N_2O$), calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), pyridoxine ▲▲ (USP 1-May-2020) ($C_8H_{11}NO_3$▲▲ (USP 1-May-2020)), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine ▲as thiamine ion ($C_{12}H_{17}N_4OS$)▲ (USP 1-May-2020) and NLT 90.0% and NMT 125.0% of the labeled amount of calcium (Ca), copper (Cu), iron (Fe), manganese (Mn), magnesium (Mg), phosphorus (P), potassium (K), and zinc (Zn); and NLT 90.0% and NMT 160.0% of the labeled amount 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.

IDENTIFICATION

Add the following:

▲• **A.** Characteristic absorption of each mineral as obtained in the tests in *Strength*▲ *(USP 1-May-2020)*

Add the following:

▲• **B.** The retention times of the vitamin peaks of the *Sample solutions* correspond to those of the corresponding vitamin peaks of the *Standard solutions* as obtained in the tests for *Strength*.▲ (USP 1-May-2020)

STRENGTH

[Note—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, with the method used being stated in the labeling only if *Method 1* is not used.]

Change to read:

• **Vitamin A,** *Method 1*

▲Proceed as directed in *Vitamin A Assay ⟨571⟩, Assay, Chromatographic Methods, Procedure 1*, except for the *Sample stock solution* and *Sample solution*.▲ (USP 1-May-2020)

Sample stock solution: Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 5 Tablets, to a container having 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

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volumetric flask. Repeat this extraction with 3 additional 15-mL portions of *n*-hexane. Dilute the extracts in the volumetric flask with *n*-hexane to volume.

Sample solution: Dilute a 10-mL 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 ($C_{20}H_{30}O$).

▲▲ (USP 1-May-2020)

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 2*

▲Proceed as directed in *Vitamin A Assay ⟨571⟩, Assay, Chromatographic Methods, Procedure 2*.▲ (USP 1-May-2020)

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*

▲Proceed as directed in *Vitamin A Assay ⟨571⟩, Assay, Chromatographic Methods, Procedure 3*.▲ (USP 1-May-2020)

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

Change to read:

• **Vitamin D (Cholecalciferol or Ergocalciferol),** *Method 1*

▲Proceed as directed in *Vitamin D Assay ⟨581⟩, Assay, Chromatographic Methods, Procedure 1*, except for the *Sample solution*.▲ (USP 1-May-2020)

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

▲▲ (USP 1-May-2020)

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

Change to read:

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

▲Proceed as directed in *Vitamin D Assay ⟨581⟩, Assay, Chromatographic Methods, Procedure 2*.▲ (USP 1-May-2020)

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

Change to read:

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

▲Proceed as directed in *Vitamin D Assay ⟨581⟩, Assay, Chromatographic Methods, Procedure 3*, except for the *Analysis*.▲ (USP 1-May-2020)

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 Tablets 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)

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

Change to read:

• **Vitamin E,** *Method 1*

▲Proceed as directed in *Vitamin E Assay ⟨551⟩, Assay, Procedure 1*, except for the *Sample solution* and *Analysis*.▲ (USP 1-May-2020)

Sample solution: Prepare as directed for the *Sample stock solution* in *Vitamin A, Method 1*. Transfer NLT 20 mL of this solution to a suitable container, and evaporate under vacuum at room temperature to dryness. Transfer the residue with the aid of methanol to a suitable

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volumetric flask, and dilute with methanol to volume to obtain a concentration of 2 mg/mL of alpha-tocopherol, alpha-tocopheryl acetate, or alpha-tocopheryl acid succinate.

▲▲ (USP 1-May-2020)

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 ($C_{33}H_{54}O_5$) ▲as 2*R*-alpha-tocopherol ($C_{29}H_{50}O_2$)▲ (USP 1-May-2020) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \text{ ▲}\times F\text{▲ (USP 1-May-2020)} \times 100$$

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 ▲*all-rac*-alpha-tocopherol from either USP Alpha Tocopherol RS or USP Alpha Tocopheryl Acetate RS or *RRR*-alpha-tocopherol from USP Alpha Tocopheryl Acid Succinate RS▲ (USP 1-May-2020) in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding form of vitamin E ▲as *2R*-alpha-tocopherol▲ (USP 1-May-2020) in the *Sample solution* (mg/mL)

▲F = conversion factor for the content of *all-rac*-alpha-tocopherol to 2*R*-alpha-tocopherol, 1/2 (for products labeled to contain *all-rac* vitamin E sources) and 1 (for products labeled to contain *RRR* vitamin E sources)▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–165.0% 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 ($C_{33}H_{54}O_5$) as ▲2*R*-alpha-tocopherol▲ (USP 1-May-2020)

Change to read:

• **Vitamin E,** *Method 2*

▲Proceed as directed in *Vitamin E Assay ⟨551⟩, Assay, Procedure 2*, except for the *Analysis*.▲ (USP 1-May-2020)

Analysis

Samples: *Standard solution* and *Sample solution*

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 ($C_{33}H_{54}O_5$) as 2R-alpha-tocopherol ($C_{29}H_{50}O_2$) in the portion of Tablets taken as follows:

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

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 *all-rac*-alpha-tocopherol from either USP Alpha Tocopherol RS or USP Alpha Tocopheryl Acetate RS or *RRR*-alpha-tocopherol from USP Alpha Tocopheryl Acid Succinate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding form of vitamin E as *2R*-alpha-tocopherol in the *Sample solution* (mg/mL)

F = conversion factor for the content of *all-rac*-alpha-tocopherol to 2*R*-alpha-tocopherol, 1/2 (for products labeled to contain *all-rac* vitamin E sources) and 1 (for products labeled to contain *RRR* vitamin E sources)

Acceptance criteria: 90.0%–165.0% 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 ($C_{33}H_{54}O_5$) as ▲2*R*-alpha-tocopherol▲ (USP 1-May-2020)

Change to read:

• **Vitamin E,** *Method 3*

▲Proceed as directed in *Vitamin E Assay ⟨551⟩, Assay, Procedure 3*, except for the *Analysis*.

Analysis

Samples: *Standard solution* and *Sample solution*

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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 ($C_{33}H_{54}O_5$) as 2*R*-alpha-tocopherol ($C_{29}H_{50}O_2$) in the portion of Tablets taken as follows:

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

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 *all-rac*-alpha-tocopherol from either USP Alpha Tocopherol RS and USP Alpha Tocopheryl Acetate RS or *RRR*-alpha-tocopherol from USP Alpha Tocopheryl Acid Succinate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding form of vitamin E as *2R*-alpha-tocopherol in the *Sample solution* (mg/mL)

F = conversion factor for the content of *all-rac*-alpha-tocopherol to 2*R*-alpha-tocopherol, 1/2 (for products labeled to contain *all-rac* vitamin E sources) and 1 (for products labeled to contain *RRR* vitamin E sources)

▲ (USP 1-May-2020)

Acceptance criteria: 90.0%–165.0% 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 ($C_{33}H_{54}O_5$) as ▲2*R*-alpha-tocopherol▲ (USP 1-May-2020)

• **Phytonadione,** *Method 1*

[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 the *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 the *Standard stock solution* diluted with methanol

Sample solution: Prepared as directed for the *Sample stock solution* in *Vitamin A, Method 1*. Transfer NLT 20 mL of the solution to a suitable container, and, if necessary, evaporate under vacuum at room temperature to dryness. Transfer the residue to a suitable volumetric flask with the aid of methanol, and dilute with methanol to volume to obtain a 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 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 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 Tablets taken:

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

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

r_S = peak area of phytonadione 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)

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

• **Phytonadione,** *Method 2*

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

Solvent: Methanol and isopropyl alcohol (19:1)

Mobile phase: Mix 800 mL of methanol, 200 mL of methylene chloride, 0.1 mL of glacial acetic acid, 1.36 g of zinc chloride, and 0.41 g of sodium acetate.

Internal standard solution: 5 µg/mL of menaquinone 4 (vitamin K_2) in the *Solvent*. [Note—A concentrated stock solution of menaquinone 4 (100 µg/mL) can be stored for 2 months in a refrigerator.]

Standard stock solution: 5 µg/mL of USP Phytonadione RS, prepared by dissolving in methylene chloride with the aid of sonication and diluting with *Solvent* to final volume

Standard solution: Transfer 1.0 mL of the *Standard stock solution* and 1.0 mL of the *Internal standard solution* to a suitable flask, and dilute with *Solvent* to 5 mL. Pass through a membrane filter of 0.45-µm or finer pore size.

Sample solution: Finely powder NLT 20 Tablets. To a centrifuge tube fitted with a cap transfer an amount of powder not exceeding 800 mg and equivalent to an amount of phytonadione not exceeding 50 µg. Add 4 mL of water. Insert the stopper, and mix using a vortex mixer until the sample is dispersed. Place the tube in a water bath at 60° for 5 min. Remove from the bath, and again shake or mix using a vortex mixer for 1 min while the preparation is still hot. Add 8 mL of alcohol, and swirl the contents to mix. Place the tube in a water bath at 60° for 5 min. Remove from the bath, and again shake or mix using a vortex mixer for 2 min while the preparation is still hot. Cool to room temperature. Add a volume of the *Internal standard solution*, equivalent to 1.0 mL per each 5 µg of the expected amount of phytonadione in the aliquot taken. Add 20.0 mL of petroleum ether, and cap the tube tightly. Shake or mix using a vortex mixer for 15 min to thoroughly mix the contents. Centrifuge to separate the 2 layers. Transfer a volume of the top layer of petroleum ether, equivalent to 5–50 µg of the nominal amount of phytonadione, to an appropriate flask. Place the flask in a water bath at 35°–45°, and evaporate the solvent under a stream of nitrogen until an oily residue is left. Dissolve the residue in a volume of the *Solvent* to obtain a concentration of 1 µg/mL of phytonadione.

Chromatographic system

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

Mode: LC

Detector: Fluorometric detector

Excitation: 320 nm

Emission: 420 nm

Column: 4.6-mm × 25-cm; 5-µm, end-capped packing L1, and a postcolumn reactor constituted with a 4.6-mm × 3-cm PEEK column tightly packed with zinc powder. [Note—Prepare the postcolumn reactor daily, or as necessary, to meet the *System suitability* requirements.]

Flow rate: 1 mL/min

Injection volume: 25 µL

System suitability

Sample: *Standard solution*

[Note—The relative retention times for the internal standard and phytonadione are 1.0 and 1.4, respectively.]

Suitability requirements

Column efficiency: NLT 2500 theoretical plates for the phytonadione peak

Tailing factor: NMT 1.5 for the phytonadione peak

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of phytonadione ($C_{31}H_{46}O_2$) in the portion of Tablets taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of phytonadione to the internal standard from the *Sample solution*

R_S = peak response ratio of phytonadione to the internal standard 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 labeled amount of phytonadione ($C_{31}H_{46}O_2$)

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▲• **Vitamin A, Vitamin D (Cholecalciferol or Ergocalciferol),** and **Vitamin E,** *Method 4*; **Phytonadione,** *Method 3*

[Note—Where vitamin A (retinyl acetate or retinyl palmitate), vitamin D (cholecalciferol or ergocalciferol), or vitamin E (alpha-tocopherol, alpha-tocopheryl acetate, or alpha-tocopheryl acid succinate) are specified in the following procedure, use the chemical form present in the formulation and the relevant Reference Standard. Use low-actinic glassware.]

Diluent: Methanol and water (90:10, v/v)

Solution A: 0.1% (v/v) trifluoroacetic acid in *Diluent*

Solution B: 0.1% (v/v) trifluoroacetic acid in methanol

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
19	0	100
27	0	100
28	100	0
30	100	0

Vitamin A standard stock solution: A solution containing the equivalent of 0.2 mg/mL of retinol from USP Retinyl Acetate RS or USP Retinyl Palmitate RS in isopropyl alcohol. Dissolve with the aid of sonication if necessary.

Vitamin D standard stock solution: 0.05 mg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in isopropyl alcohol. Dissolve with the aid of sonication if necessary.

Vitamin E standard stock solution: 3.0 mg/mL of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, or USP Alpha Tocopheryl Acid Succinate RS in isopropyl alcohol. Dissolve with the aid of sonication if necessary.

Phytonadione standard stock solution: 0.04 mg/mL of USP Phytonadione RS in isopropyl alcohol. Dissolve with the aid of sonication if necessary.

Sample solution 1: Finely powder NLT 20 Tablets. Transfer an accurately weighed portion of the powder into a 50-mL volumetric flask to obtain a solution containing known nominal concentrations in the ranges of 0.05–0.1 mg/mL of vitamin A and 0.5–0.15 mg/mL of vitamin E. Add 10 mL of dimethyl sulfoxide and sonicate for 30 min with vigorous intermittent shaking. Cool to room temperature, dilute with isopropyl alcohol to volume, and mix well. Pass a portion of the solution through a nylon filter of 0.45-µm pore size, and discard the first milliliter of the filtrate.

Sample solution 2: Transfer an accurately weighed portion of the powder from NLT 20 finely powdered Tablets into a 50-mL volumetric flask to obtain a solution containing known nominal concentrations in the ranges of 1.0–3.5 µg/mL of vitamin D and 1.0–3.5 µg/mL of phytonadione. Add 5 mL of water and sonicate for 10 min with intermittent shaking. Add 10 mL of dimethyl sulfoxide and sonicate for 30 min with vigorous intermittent shaking. Cool to room temperature, dilute with isopropyl alcohol to volume, and mix well. Pass a portion of the solution through a nylon filter of 0.45-µm pore size, and discard the first milliliter of the filtrate.

Standard solution: Transfer calculated volumes of *Vitamin A standard stock solution*, *Vitamin D standard stock solution*, *Vitamin E standard stock solution*, and *Phytonadione standard stock solution* into a suitable volumetric flask, and dilute with isopropyl alcohol to volume to obtain a solution with final vitamin concentrations similar to those obtained for the corresponding vitamins in *Sample solution 1* and *Sample solution 2*.

Chromatographic system

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

Mode: LC

Detectors

For *Sample solution 1* containing retinyl acetate: UV 325 nm for 7 min, then switch to 265 nm

For *Sample solution 1* containing retinyl palmitate: UV 265 nm for 18 min, then switch to 325 nm

For *Sample solution 2*: UV 265 nm

Column: 4.6-mm × 10-cm; 3-µm packing L1

Temperatures

Sample: 4°

Column: 35°

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Flow rate: 1.5 mL/min

Injection volume: 20 µL

System suitability

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

Suitability requirements

Relative standard deviation: NMT 2.0% for each individual peak, *Standard solution*

Peak purity of vitamin D and vitamin A (as retinyl palmitate): Monitor the chromatograms of *Sample solution 1* and *Sample solution 2* at 450 nm. The area of any peak at the retention time of retinyl palmitate from *Sample solution 1* and any peak at the retention time of vitamin D (as cholecalciferol or ergocalciferol) from *Sample solution 2* should be NMT 5% of the corresponding peak areas detected at 325 nm and 265 nm, respectively. [Note—This test is required because of possible interference with carotenoids (cryptoxanthin and lycopene) that may be present in the formulation. Use another procedure in the monograph for analysis of vitamin D and vitamin A (as retinyl palmitate) if the system is not suitable]

Analysis

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

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

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

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 retinol in the *Standard solution* (mg/mL)

C_U = nominal concentration of vitamin A, as retinol, in the *Sample solution* (mg/mL)

Calculate the percentage of the labeled amount of vitamin E, as 2*R*-alpha-tocopherol ($C_{29}H_{50}O_2$), in the portion of Tablets taken:

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

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 *all-rac*-alpha-tocopherol from either USP Alpha Tocopherol RS or USP Alpha Tocopheryl Acetate RS or *RRR*-alpha-tocopherol from USP Alpha Tocopheryl Acid Succinate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of vitamin E, as 2*R*-alpha-tocopherol, in the *Sample solution* (mg/mL)

F = conversion factor for the content of *all-rac*-alpha-tocopherol to *RRR*-alpha-tocopherol equivalent, 1/2 (for products labeled to contain *all-rac* vitamin E sources) and 1 (for products labeled to contain *RRR* vitamin E sources)

Calculate the percentage of the labeled amount of vitamin D, as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$), and phytonadione ($C_{31}H_{46}O_2$) in the portion of Tablets taken:

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

r_U = peak area of the related vitamin from the *Sample solution*

r_S = peak area of the related vitamin from the *Standard solution*

C_S = concentration of related vitamin in the *Standard solution* (µg/mL)

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

Acceptance criteria: 90.0%–165.0% of the labeled amount of each individual vitamin▲ (USP 1-May-2020)

• **Beta Carotene**

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

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

Iodine solution: 0.01 mg/mL of iodine in cyclohexane. [Note—Prepare this solution fresh daily.]

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Sample solution: Weigh NLT 20 Tablets. Grind the Tablets to a fine powder, and transfer a quantity of the powder, 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

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 Tablets 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 ($C_{40}H_{56}$)

Change to read:

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

(See *Vitamin C Assay ⟨580⟩*.)

[Note—For labeling purposes, consider *⟨580⟩, Method I—Titrimetric Method* as *Method 1*; ▲*⟨580⟩, Method II—Chromatographic Method, Procedure 1* as *Method 2*; and *⟨580⟩, Method II—Chromatographic Method, Procedure 2* as *Method 3*.▲ (USP 1-May-2020)]

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, 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: Finely powder NLT 20 Tablets. Transfer a portion of the powder, 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*

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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 Tablets 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 ($C_{10}H_{16}N_2O_3S$)

Change to read:

• **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 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: Finely powder NLT 30 Tablets. Transfer a portion of the powder, 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 make 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 not below 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 add water to make 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 add water to make 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 2*,▲ (USP 1-May-2020) and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

▲**Table 2**▲ (USP 1-May-2020)

Acid-hydrolyzed casein solution	25 mL

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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

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 3 or more of the tubes, using a pure culture of *Lactobacillus plantarum*,[1] incubating for 16–24 h at a temperature 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 between 30° and 37° held constant to within ±0.5°. The cell suspension 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 4 similar but 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 3 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 2 of the 4 tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature 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 milliliter of the *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.

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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 3 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 Tablets taken:

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

R = amount of biotin assumed to be present in the portion of the Tablets taken (µg)

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

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

N = nominal amount of biotin in the portion of the Tablets 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 determinations. From the mean of two or more values of *M* that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

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

• **Biotin,** *Method 3*

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

Solution A: Transfer 800 mL of water and 100 mL of triethylamine to a 1000-mL volumetric flask. Add 80 mL of 85% phosphoric acid, and dilute with water to volume.

Mobile phase: Transfer 80 mL of acetonitrile and 10 mL of *Solution A* to a 1000-mL volumetric flask. Dilute with water to volume.

Standard solution: 0.6 µg/mL of USP Biotin RS in water. [Note—A portion of the *Standard solution* will be used to determine the percent recovery of biotin from the *Solid-phase extraction* procedure.]

Sample solution: Finely powder NLT 20 Tablets. Transfer an amount of powdered Tablets to a volumetric flask to obtain a nominal concentration of 0.6 µg/mL of biotin. Add water up to 80% of the flask capacity, and sonicate for 30–40 min, with occasional mixing, to dissolve. Dilute with water to volume, and filter. Adjust the pH of the solution with either dilute acetic acid or 0.1 N sodium hydroxide to a pH of 6.0–7.0.

Solid-phase extraction: [Note—Condition the extraction column specified in this procedure in the following manner. Wash the column with a 2-mL portion of methanol. Equilibrate with a 2-mL portion of water.] Separately pipet 5.0 mL each of the *Sample solution* and *Standard solution* into freshly conditioned solid-phase extraction columns consisting of a mixed-mode packing with a sorbent-mass of 60 mg. [Note—The mixed-mode packing consists of anion-exchange and reversed-phase sorbents. The reverse-phase component is a polymer of copolymer *N*-vinylpyrrolidone and divinylbenzene. The anion exchange moiety is a trialkylamino group.[2]]

Wash the column with 10 mL of 30% (v/v) methanol in water. Apply an appropriate volume (4.9 mL) of 30% (v/v) methanol in 0.1 N hydrochloric acid to the column. Collect the eluate in a 5-mL volumetric flask, containing 100 µL of 40% (w/v) sodium acetate in water, and dilute with 30% (v/v) methanol in 0.1 N hydrochloric acid to volume.

Chromatographic system

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

Mode: LC

Detector: UV 200 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 2 mL/min

Injection volume: 100 µL

System suitability

Samples: *Standard solution* and portion of the *Standard solution* that has undergone *Solid-phase extraction*

Suitability requirements

Tailing factor: NMT 1.5, *Standard solution*

Relative standard deviation: NMT 2.0%, *Standard solution* and *Standard solution* that has undergone *Solid-phase extraction*

Recovery: 95%–100%, *Standard solution* that has undergone *Solid-phase extraction*

Analysis

Samples: *Standard solution* and the *Sample solution* that have both undergone *Solid-phase extraction*

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