



Analysis of Vitamin D₂ and Vitamin D₃ in Infant and Adult Nutritional Formulas by Liquid Chromatography-Tandem Mass Spectrometry: A Multi-Laboratory Testing Study

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Abstract

A multi-laboratory testing study was conducted on AOAC First Action Method 2016.05 “Analysis of Vitamin D₂ and Vitamin D₃ in Fortified Milk Powders, Infant Formulas, and Adult/Pediatric Nutritional Formulas by Liquid Chromatography-Tandem Mass Spectrometry.” Nine laboratories participated in the analysis of duplicate samples of 20 nutritional products. The samples were saponified at high temperature with lipid-soluble components extracted into isooctane; an aliquot was washed and vitamin D derivatized with 4-phenyl-1,2,4-triazoline-3,5-dione to form a high molecular mass, easily ionizable adduct, extracted into acetonitrile and analyzed by reversed-phase LC-tandem MS. Stable isotope labelled internal standards were used for quantitation to correct for losses in extraction and variation in derivatization and ionization efficiencies. Acceptable precision as RSD was demonstrated; repeatability ranged from 1.9 to 5.8% RSD_r and reproducibility values ranged from 6.4 to 12.7% RSD_R, with samples meeting the precision limits specified in the vitamin D Standard Method Performance Requirements and the guidelines recommended for the Horwitz ratio. Method accuracy was assessed using NIST 1849a Standard Reference Material, with a *P*-value of 0.32, indicating an absence of bias against the certified value. As expected, placebo samples not fortified with vitamin D returned negligible results.

Introduction

Vitamin D is not a true vitamin, as individuals with adequate skin exposure to UV radiation produce vitamin D from its precursor, 7-dehydrocholesterol. Vitamin D deficiency is a major public health concern worldwide in all age groups and is a problem even in countries with sun exposure all year round (1). Dietary supplementation of vitamin D is necessary for many, with both infant and adult nutritional formulas typically fortified with vitamin D₃ (cholecalciferol), or less commonly, vitamin D₂ (ergocalciferol). Both vitamin D₂ and vitamin D₃ are metabolized in the liver to their respective 25-hydroxy vitamers, which are the dominant circulating forms in blood. The main biological function of vitamin D is calcium homeostasis, controlling the absorption, transport, and deposition of calcium and phosphorus as part of bone mineralization (2). Recently, vitamin D deficiency has been associated with numerous health issues, including musculoskeletal disorders (falls and fractures), infectious diseases, several types of cancer, autoimmune diseases, cardiovascular disease, type 1 and type 2 diabetes mellitus, neurocognitive dysfunction, and mental illness, as well as infertility, adverse pregnancy, and birth outcomes (3).

Rapid, high-throughput analytical methods for vitamin D are needed for routine testing to meet product specifications, and reference methods utilizing contemporary techniques are needed to demonstrate product compliance with strict global regulations. Given that the internationally accepted multidimensional LC-UV method for vitamin D, AOAC Method 2002.05 (4), has a long total analysis time, an updated reference method for vitamin D was identified by the AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) as a priority. We previously developed an LC-MS/MS method that incorporates saponification, solvent extraction, and 4-phenyl-1,2,4-triazoline-3,5-dione derivatization prior to instrumental analysis (5). The method subsequently underwent a comprehensive single-laboratory validation (SLV) study using the SPIFAN kit (6), a set of infant formula and adult nutritional products selected as a representative subsample of a wide range of commercially available products, and the results were compared with Standard Method Performance Requirements (SMPRs; 7, 8). In March 2016, this LC-MS/MS method was reviewed by the SPIFAN Expert Review Panel (ERP), was approved for First Action Official Method status as AOAC Method 2016.05 (9), and was recommended to advance to a multi-laboratory testing (MLT) study for evaluation of reproducibility.

Collaborative Study

The participating laboratories represented a wide range of food-testing laboratories, including governmental agencies, infant formula manufacturers, and contract analytical services. Prior to commencement of the MLT study, each collaborator received a detailed study protocol to allow familiarization with the technique and an opportunity to communicate any difficulties.

The SPIFAN kit and a candidate Standard Reference Material (SRM), NIST 1869, a soy-based infant/adult nutritional formula fortified with both vitamin D₂ and vitamin D₃, were used in this study. A practice sample, NIST 1849a, was tested by participants and, when acceptable results had been obtained, approval to proceed to the analysis of the SPIFAN kit samples was given. The SPIFAN kit was tested over 2 separate days as blind-coded duplicate pairs.

All data were statistically analyzed using the harmonized guidelines for collaborative studies to establish overall mean, intra-laboratory S_r , RSD_r , inter-laboratory S_R , RSD_R , and Horwitz ratio (HorRat; 10). Cochran's ($P = 0.025$, one-tail) and Grubbs' (single and double, $P = 0.025$, two-tail) tests were used to determine outliers.

Method

The method protocol sent to the collaborating laboratories was as described in AOAC First Action Method 2016.05, with the minor modification of removal of the drying step for acetone.

AOAC Official Method 2016.05

Analysis of Vitamin D₂ and Vitamin D₃ in Fortified Milk

Powders, Infant Formulas, and Adult/Pediatric

Nutritional Formulas by LC-Tandem MS

First Action 2016

Final Action 2017

Applicable to the determination of total vitamin D₂ and total vitamin D₃ in fortified milk powders, infant formulas, and adult/pediatric nutritional formulas.]

Caution: Refer to the material safety data sheets for all chemicals prior to use. Use all appropriate personal protective equipment and follow good laboratory practices.

A. Principle

Samples are saponified at high temperature, and lipid-soluble components are extracted into iso-octane. A portion of the iso-octane layer is transferred and washed, and an aliquot of 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) is added to derivatize vitamin D to form a high-molecular mass, easily ionizable adduct. The vitamin D adduct is subsequently extracted into a small volume of

acetonitrile and analyzed by reversed-phase LC. Detection is by tandem MS (MS/MS) using multiple reaction monitoring. Stable isotope-labeled (SIL) *d6*-vitamin D₂ and *d6*-vitamin D₃ internal standards are used for quantitation to correct for losses in extraction and any variation in derivatization and ionization efficiencies.

B. Apparatus

- 1 Ultra-high performance LC (UHPLC) system.—Nexera (Shimadzu, Kyoto, Japan) or equivalent LC system, consisting of a dual pump system, a sample injector unit, a degasser unit, and a column oven.
- 2 Triple-quadrupole MS.—Triple Quad 6500 (Sciex, Framingham, MA) or equivalent MS/MS instrument.
- 3 Column.—Kinetex C₁₈ core-shell, 2.6 µm, 2.1 × 50 mm (Phenomenex, Torrance, CA) or equivalent.
- 4 Spectrophotometer.—Capable of digital readout to 3 decimal places.
- 5 Centrifuge tubes.—Polypropylene, 15 mL.
- 6 Boiling tubes.—Glass, 60 mL.
- 7 Water baths.—Cold, 20 °C; hot, 70 °C.
- 8 Disposable syringes.—1 mL.
- 9 Syringe filters.—PTFE, 0.2 µm, 13 mm.
- 10 Centrifuge.—Suitable for 60 mL boiling tubes and 15 mL
- 11 Centrifuge tubes.
- 12 Pasteur pipet.—Glass, ~140 mm.
- 13 Horizontal shaker.
- 14 Microcentrifuge vials.—2 mL.
- 15 Filter membranes.—0.45 µm nylon.
- 16 Cryogenic vials.—2 mL.
- 17 Schott bottles.—1 L.
- 18 HPLC vials, septa, and caps.

C. Reagents

- 1 Vitamin D₂ (ergocalciferol).—CAS No. 50-14-6, purity: ≥ 99%.
- 2 Vitamin D₃ (cholecalciferol).—CAS No. 67-97-0, purity: ≥ 99%.
- 3 *d6*-Vitamin D₂ (26,26,26,27,27,27-*d6* ergocalciferol).—CAS No. 1311259-89-8, enrichment: ≥ 99%, purity: ≥ 99%.

- 4 *d*6-Vitamin D₃ (26,26,26,27,27,27-*d*6 cholecalciferol).—CAS No. 118584-54-6, enrichment: ≥ 99%, purity: ≥ 99%.
- 5 PTAD.—Reagent grade (store in desiccator at 2–8 °C).
- 6 Formic acid.—LC-MS grade.
- 7 Potassium hydroxide.—Reagent grade.
- 8 Pyrogallol.—Reagent grade.
- 9 Ethanol.—LC grade.
- 10 Methanol.—LC-MS grade.
- 11 Isooctane.—LC grade.
- 12 Acetone.—LC grade.
- 13 Acetonitrile.—LC-MS grade.
- 14 Water.—Purified, with resistivity ≥ 18 MΩ.

D. Reagent Preparation

- 1 PTAD solution (10 mg/mL).—To a 5 mL volumetric flask, add 50 mg PTAD, then add 4 mL acetone, and dissolve; dilute to volume with acetone. Expiry: 1 day.
- 2 Potassium hydroxide solution (50% w/v).—Dissolve 100 g potassium hydroxide in 200 mL water. Expiry: 1 month.
- 3 Ethanolic pyrogallol solution (1% w/v).—Dissolve 5 g pyrogallol in 500 mL ethanol. Expiry: 1 day.
- 4 Mobile phase A (formic acid; 0.1% v/v).—To 500 mL water, add 0.5 mL formic acid. Expiry: 1 week.
- 5 Mobile phase B (methanol; 100% v/v).—500 mL methanol. Expiry: 1 month.

E. Standard Preparation

Vitamin D is sensitive to light; perform all steps under UV shielded lighting. If vitamin D₃ is exclusively required for analysis, then standards pertaining to vitamin D₂ need not be used and vice-versa.

- 1 SIL vitamin D₂ or vitamin D₃ stock standard (SILD2SS or SILD3SS; ~10 µg/mL).—
 - a. Dispense the contents of a 1 mg vial of *d*6-vitamin D₂ or a 1 mg vial of *d*6-vitamin D₃ into separate 100 mL volumetric flasks.
 - b. Dissolve in ~90 mL ethanol. To promote dissolution, sonicate if necessary. Mix thoroughly; dilute to volume with ethanol.
 - c. Measure the absorbance of an aliquot of SILD2SS or SILD3SS at 265 nm. The spectrophotometer should be zeroed against an ethanol blank solution. Calculate and record the concentration.

- d. Immediately dispense aliquots of SILD2SS or SILD3SS (~1.3 mL) into cryogenic vials and freeze at $\leq 15^{\circ}\text{C}$.
- 1 SIL internal standard (SILIS; ~1 $\mu\text{g/mL}$).—
 - a. Prepare an adequate volume of SILIS for the daily sample numbers. For every 15 samples (or part thereof) in an analytical run, remove one vial of SILD₂SS and one vial of SILD₃SS from the freezer and allow to warm to room temperature.
 - b. Pipette 1.0 mL each of SILD₂SS and SILD₃SS into the same 10 mL volumetric flask (use a separate 10 mL volumetric flask for each set of 15 samples). Dilute to volume with acetonitrile and mix thoroughly.
 - c. Pool all 10 mL volumetric flasks together and mix thoroughly.
 - d. Make fresh daily.
- 2 Non-labeled vitamin D₂ or vitamin D₃ stock standard (NLD₂SS or NLD₃SS; ~1 mg/mL).—
 - a. Accurately weigh approximately 50 mg vitamin D₂ or vitamin D₃ into separate 50 mL volumetric flasks.
 - b. Dissolve in ~40 mL ethanol. To promote dissolution, sonicate if necessary. Mix thoroughly; dilute to volume with ethanol. Store in a freezer at $\leq 15^{\circ}\text{C}$ for a maximum of 3 months.
 - c. Non-labelled vitamin D₂ or vitamin D₃ purity standard (NLD₂PS or NLD₃PS; ~10 $\mu\text{g/mL}$).—
 - d. Pipette 1.0 mL NLD₂SS or NLD₃SS into separate 100 mL volumetric flasks. Dilute to volume with ethanol.
 - e. Measure the absorbance of an aliquot of each solution at 265 nm. The spectrophotometer should be zeroed against an ethanol blank solution. Record the absorbance and calculate the concentration.
 - f. Make fresh daily.
- 3 Non-labeled working standard (NLWS; ~1 $\mu\text{g/mL}$).—
 - a. Pipette 1.0 mL NLD₂PS and 1.0 mL NLD₃PS into a single 10 mL volumetric flask. Dilute to volume with acetonitrile.
 - b. Make fresh daily.
- 4 Calibration standard solutions (CS).—See Table 2016.05A for concentrations of the CS solutions.—
 - a. CS1.—Pipette 10 μL NLWS and 250 μL SILIS into a 25 mL volumetric flask.
 - b. CS2.—Pipette 50 μL NLWS and 250 μL SILIS into a 25 mL volumetric flask.
 - c. CS3.—Pipette 250 μL NLWS and 250 μL SILIS into a 25 mL volumetric flask.
 - d. CS4.—Pipette 500 μL NLWS and 250 μL SILIS into a 25 mL volumetric flask.
 - e. CS5.—Pipette 1250 μL NLWS and 250 μL SILIS into a 25 mL volumetric flask.
 - f. To each CS, add 5 mL acetonitrile and 75 μL PTAD solution; shake to mix.

- g. Leave the CS in the dark for 5 min.
- h. Add 6.25 mL water to each CS and dilute to volume with acetonitrile; shake to mix.
- i. Transfer ~1 mL of each CS to an HPLC vial ready for analysis.

Table 2016.05A. Nominal concentrations of calibration standards

Calibration standard	Concentration, ng mL ⁻¹	
	Vitamin D	SIL d6-vitamin D
CS1	0.4	10
CS2	2.0	10
CS3	10	10
CS4	20	10
CS5	50	10

F. Sample Preparation

Vitamin D is sensitive to light; perform all steps under UV shielded lighting. Sample preparation step

- 1 Powder sample preparation.—Accurately weigh 1.8–2.2 g powder sample into a boiling tube. Record the weight.
- 2 Slurry sample preparation.—
 - a. Accurately weigh 19.0–21.0 g powder into a disposable slurry container. Record the weight.
 - b. Accurately weigh ~80 mL water into the container. Record the weight.
 - c. Shake thoroughly until mixed. Place in the dark at room temperature for 15 min and shake every 5 min to mix.
 - d. Accurately weigh 9.5–10.5 g slurry or reconstituted powder sample into a boiling tube. Record the weight.
- 3 Liquid sample preparation.—Accurately weigh 10.0 mL liquid milk into a boiling tube. Record the weight.

G. Extraction and Derivatization

- 1 To a powder, slurry, or liquid sample in a boiling tube, add 10 mL ethanolic pyrogallol solution; add 0.5 mL SILIS, cap, and mix on a vortex mixer.
- 2 Add 2 mL potassium hydroxide solution to the boiling tube; cap and mix on the vortex mixer.
- 3 Place the boiling tube in a water bath at 70 °C for 1 h; mix on the vortex mixer every 15 min.
- 4 Place the boiling tube in a water bath at room temperature until cool.

- 5 Add 10 mL isooctane to the boiling tube; cap the boiling tube tightly and place on a horizontal shaker for 10 min.
- 6 Add 20 mL water to the boiling tube and invert the tube 10 times; place in a centrifuge at $\geq 250 \times g$ for 15 min.
- 7 Transfer a 5 mL aliquot of the upper isooctane layer into a 15 mL centrifuge tube using a Pasteur pipet, taking care not to transfer any of the lower layer.
- 8 Add 5 mL water to the centrifuge tube; cap and mix on the vortex mixer; place in a centrifuge at $2000 \times g$ for 5 min.
- 9 Transfer 4–5 mL upper isooctane layer to a new 15 mL disposable centrifuge tube using a disposable pipet, taking care not to transfer any of the lower layer.
- 10 Add 75 μ L PTAD solution to the centrifuge tube; cap and immediately mix on the vortex mixer.
- 11 Allow to stand in the dark for 5 min to allow the derivatization reaction to complete.
- 12 Add 1 mL acetonitrile to the centrifuge tube; cap and mix on the vortex mixer, then place in a centrifuge at $2000 \times g$ for 5 min.
- 13 Using a variable volume pipet, transfer 500 μ L lower layer into a microcentrifuge vial, taking care not to transfer any of the upper layer.
- 14 Add 167 μ L water to the microcentrifuge vial; cap and mix on the vortex mixer.
- 15 Using a syringe filter, transfer an aliquot from the microcentrifuge vial to an amber HPLC vial; cap.

H. Chromatography

- 1 Set up the UHPLC system with the configuration shown in Table 2016.05B.
- 2 Form gradients by high-pressure mixing of the two mobile phases, A and B, using the procedure shown in Table 2016.05C.

Table 2016.05B Chromatographic instrument settings

Instrument parameter	Value
Mobile phase A	Formic acid, 0.1%
Mobile phase B	Methanol, 100%
Column	Kinetex C ₁₈
Oven temperature	40 °C
Chiller temperature	15 °C
Injection volume	3 μ L
Initial flow rate	0.6 mL min ⁻¹

Table 2016.05C Gradient procedure for chromatographic separation

Time,	Flow rate,	Mobile phase composition

min	mL min ⁻¹	% A	% B
0	0.6	25	75
3.3	0.6	0	100
3.7	1.0	0	100
4.8	1.0	0	100
4.9	0.6	25	75
5.5	0.6	25	75

I. Mass Spectrometry

- 1 Set up the MS with the instrument settings shown in Table 2016.05D.

Table 2016.05D Mass spectrometer instrument settings^a

Instrument parameter	Value
Ionization mode	ESI+
Curtain gas	30 psi
Nebulizer gas GS1	40 psi
Heater gas GS2	40 psi
Collision gas	N ₂
Source temperature	300°C
Ion spray voltage	5500 V

^a These settings are suitable for the 6500 triple-quadrupole mass spectrometer (Sciex). Optimal settings on alternative instruments may differ.

- 2 The specific compound parameters to be used are shown in Tables 2016.05E and 2016.05F.

Table 2016.05E Compound parameters (vitamin D₂ instrument method only)

Vitamin D ₂ ion ^a	Precursor ion, m/z	Product ion, m/z	DP ^b , V	EP ^c , V	CE ^d , V	CXP ^e , V	Dwell time, ms
Analyte quantifier	572.2	298.0			23	22	120
Analyte qualifier	572.2	280.0	81	10	39	16	80
Internal standard quantifier	578.2	298.0			23	22	120
Internal standard qualifier	578.2	280.0			39	16	80

^a Analyte = vitamin D₂-PTAD adduct, Internal standard ion = *d6*-vitamin D₂-PTAD adduct

^b DP = declustering potential

^c EP = entrance potential

^d CE = collision energy

^e CXP = collision cell exit potential

Table 2016.05F Compound parameters (vitamin D₃ instrument method only)

Vitamin D ₃ ion ^a	Precursor ion, m/z	Product ion, m/z	DP ^b , V	EP ^c , V	CE ^d , V	CXP ^e , V	Dwell time, ms
Analyte quantifier	560.2	298.0	151	10	21	18	120
Analyte qualifier	560.2	280.0			37	18	80

Internal standard quantifier	566.2	298.0	21	18	120
Internal standard qualifier	566.2	280.0	37	18	80

^a Analyte = vitamin D₂–PTAD adduct, Internal standard ion = *d6*-vitamin D₂–PTAD adduct

^b DP = declustering potential

^c EP = entrance potential

^d CE = collision energy

^e CXP = collision cell exit potential

J. Calculations

- 1 Concentration of stable isotope-labeled vitamin D₂ in the stock standard, SILD₂SS.—

$$\text{SILD}_{2\text{SS}}_{\text{D2conc}} = \frac{\text{SILD}_{2\text{SS}}_{\text{abs}(\lambda_{\text{max}})}}{E_{1\text{cm}}^{1\%}} \times 10000$$

where:

SILD₂SS_{D2conc} = concentration of *d6*-vitamin D₂ in stock standard (μg mL⁻¹);

SILD₂SS_{abs(λ_{max})} = UV absorbance of stock standard at 265 nm (cm⁻¹);

E_{1cm}^{1%} = extinction coefficient for vitamin D₂ in ethanol (461 dL g.cm⁻¹);

10000 = concentration conversion factor (g dL⁻¹ to μg mL⁻¹).

- 2 Concentration of stable isotope-labeled vitamin D₃ in stock standard SILD₃SS.—

$$\text{SILD}_{3\text{SS}}_{\text{D3conc}} = \frac{\text{SILD}_{3\text{SS}}_{\text{abs}(\lambda_{\text{max}})}}{E_{1\text{cm}}^{1\%}} \times 10000$$

where:

SILD₃SS_{D3conc} = concentration of *d6*-vitamin D₃ in stock standard (μg mL⁻¹);

SILD₃SS_{abs(λ_{max})} = UV absorbance of stock standard at 265 nm (cm⁻¹);

E_{1cm}^{1%} = extinction coefficient for vitamin D₃ in ethanol (485 dL g.cm⁻¹);

10000 = concentration conversion factor (g dL⁻¹ to μg mL⁻¹).

- 3 Concentration of stable isotope-labeled vitamin D₂ in internal standard SILIS.—

$$\text{SILIS}_{\text{D2conc}} = \text{SILD}_{2\text{SS}}_{\text{D2conc}} \times \frac{1.0}{10} \times 1000$$

where:

SILIS_{D2conc} = concentration of *d6*-vitamin D₂ in internal standard (ng mL⁻¹);

SILD₂SS_{D2conc} = concentration of *d6*-vitamin D₂ in stock standard (μg mL⁻¹);

1000 = concentration conversion factor (μg mL⁻¹ to ng mL⁻¹).

- 4 Concentration of stable isotope-labeled vitamin D₃ in internal standard SILIS.—

$$\text{SILIS}_{\text{D3conc}} = \text{SILD}_{3\text{SS}}_{\text{D3conc}} \times \frac{1.0}{10} \times 1000$$

where:

SILIS_{D3conc} = concentration of d6-vitamin D₃ in internal standard (ng mL⁻¹);

SILD2SS_{D3conc} = concentration of d6-vitamin D₃ in stock standard (µg mL⁻¹);

1000 = concentration conversion factor (µg mL⁻¹ to ng mL⁻¹).

- 5 Concentration of non-labelled vitamin D₂ in purity standard NLD₂PS.—

$$\text{NLD}_2\text{PS}_{\text{D2conc}} = \frac{\text{NLD}_2\text{PS}_{\text{abs}(\lambda_{\text{max}})}}{E_{1\text{cm}}^{1\%}} \times 10000$$

where:

NLD₂PS_{D2conc} = concentration of vitamin D₂ in purity standard (µg mL⁻¹);

NLD₂PS_{abs(λ_{max})} = UV absorbance of purity standard at 265 nm (cm⁻¹);

E_{1cm}^{1%} = extinction coefficient for vitamin D₂ in ethanol (461 dL g.cm⁻¹);

10000 = concentration conversion factor (g dL⁻¹ to µg mL⁻¹).

- 6 Concentration of non-labeled vitamin D₃ in purity standard NLD₃PS.—

$$\text{NLD}_3\text{PS}_{\text{D3conc}} = \frac{\text{NLD}_3\text{PS}_{\text{abs}(\lambda_{\text{max}})}}{E_{1\text{cm}}^{1\%}} \times 10000$$

where:

NLD₃PS_{D3conc} = concentration of vitamin D₃ in purity standard (µg mL⁻¹);

NLD₃PS_{abs(λ_{max})} = UV absorbance of purity standard at 265 nm (cm⁻¹);

E_{1cm}^{1%} = extinction coefficient for vitamin D₃ in ethanol (485 dL g.cm⁻¹);

10000 = concentration conversion factor (g dL⁻¹ to µg mL⁻¹).

- 7 Concentration of non-labeled vitamin D₂ in working standard NLWS.—

$$\text{NLWS}_{\text{D2conc}} = \text{NLD}_2\text{PS}_{\text{D2conc}} \times \frac{1.0}{10} \times 1000$$

where:

NLWS_{D2conc} = concentration of vitamin D₂ in working standard (ng mL⁻¹);

NLD₂PS_{D2conc} = concentration of vitamin D₂ in purity standard (µg mL⁻¹);

1000 = concentration conversion factor (µg mL⁻¹ to ng mL⁻¹).

- 8 Concentration of non-labeled vitamin D₃ in working standard NLWS.—

$$\text{NLWS}_{\text{D3conc}} = \text{NLD}_3\text{PS}_{\text{D3conc}} \times \frac{1.0}{10} \times 1000$$

where:

NLWS_{D3conc} = concentration of vitamin D₃ in working standard (ng mL⁻¹);

NLD₃PS_{D3conc} = concentration of vitamin D₃ in purity standard (µg mL⁻¹);

1000 = concentration conversion factor (µg mL⁻¹ to ng mL⁻¹).

- 9 Concentrations of vitamin D₂ and vitamin D₃ in calibration standards CS1–CS5.—

$$\text{CS1}_{\text{Dconc}} = \text{NLWS}_{\text{Dconc}} \times \frac{0.01}{25}$$

$$CS2_{Dconc} = NLWS_{Dconc} \times \frac{0.05}{25}$$

$$CS3_{Dconc} = NLWS_{Dconc} \times \frac{0.25}{25}$$

$$CS4_{Dconc} = NLWS_{Dconc} \times \frac{0.5}{25}$$

$$CS5_{Dconc} = NLWS_{Dconc} \times \frac{1.25}{25}$$

where:

CS1–CS5_{Dconc} = concentration of vitamin D₂ or vitamin D₃ in calibration standards (ng mL⁻¹);

NLWS_{Dconc} = concentration of vitamin D₂ or vitamin D₃ in working standard (ng mL⁻¹).

- 10 Concentrations of stable isotope-labeled d6-vitamin D₂ and d6-vitamin D₃ in calibration standards CS1–CS5.—

$$CS1-5_{Dconc} = SILIS_{Dconc} \times \frac{0.25}{25}$$

where:

CS#_{Dconc} = concentration of d6-vitamin D₂ or d6-vitamin D₃ in calibration standards (ng mL⁻¹);

SILIS_{Dconc} = concentration of d6-vitamin D₂ or d6-vitamin D₃ in internal standard (ng mL⁻¹).

- 11 Mass of powder in slurried sample.—

$$S_{mass} = \frac{D_{mass}}{(D_{mass} + W_{mass})} \times A_{mass}$$

where:

S_{mass} = the mass of sample (g);

D_{mass} = the mass of dry powder used to make the slurry (g);

W_{mass} = the mass of water used to make the slurry (g);

A_{mass} = the mass of the aliquot of slurried sample used in the analysis (g).

Determine the linear regression curve $y = mx + c$ (using the "least squares" method) for the ratio of peak areas (non-labeled vitamin D/stable isotope-labeled d6-vitamin D) vs. the ratio of concentrations (non-labeled vitamin D/stable isotope-labeled d6-vitamin D) for five calibration standards with the y-intercept forced through zero. Interpolate the nucleotide contents in unknown samples from this calibration curve.

- 12 The concentration (w/w) of vitamin D₂ or vitamin D₃ in dry powders is calculated as:

$$\text{Result D} = \frac{PA_{NLD}}{PA_{SILD}} \times \frac{SILIS_{Dconc}}{L} \times \frac{SILIS_{alqt}}{S_{mass}} \times \frac{100}{1000}$$

where:

Result D = vitamin D₂ or vitamin D₃ concentration in sample (μg hg⁻¹);

PA_{NLD} = peak area of vitamin D₂ or vitamin D₃ in sample;

PA_{SILD} = peak area of d6- vitamin D₂ or d6- vitamin D₃ in sample;

$SILIS_{Dconc}$ = concentration of d6-vitamin D₂ or d6-vitamin D₃ in SILIS (ng mL⁻¹);

L = slope of calibration curve

$SILIS_{alqt}$ = volume of SILIS aliquot spiked into sample (0.5 mL);

S_{mass} = mass of sample (g); 1000 = concentration conversion factor (ng g⁻¹ to µg g⁻¹);

100 = concentration conversion factor (µg g⁻¹ to µg hg⁻¹).

- 13 The concentration (w/v) of vitamin D₂ or vitamin D₃ in ready-to-feed (RTF) liquids is calculated as:

$$\text{Result D} = \frac{PA_{NLD}}{PA_{SILD}} \times \frac{SILIS_{Dconc}}{L} \times \frac{SILIS_{alqt}}{S_{vol}} \times \frac{100}{1000}$$

where: Result D = vitamin D₂ or vitamin D₃ concentration in sample (µg hg⁻¹);

PA_{NLD} = peak area of vitamin D₂ or vitamin D₃ in sample;

PA_{SILD} = peak area of d6- vitamin D₂ or d6- vitamin D₃ in sample;

$SILIS_{Dconc}$ = concentration of d6-vitamin D₂ or d6-vitamin D₃ in SILIS (ng mL⁻¹);

L = slope of calibration curve;

$SILIS_{alqt}$ = volume of SILIS aliquot spiked into sample (0.5 mL);

S_{vol} = volume of sample (mL);

1000 = concentration conversion factor (ng mL⁻¹ to µg mL⁻¹);

100 = concentration conversion factor (µg mL⁻¹ to µg dL⁻¹).

- 14 The concentration of vitamin D₂ or vitamin D₃ as IU hg⁻¹ in the sample is calculated as:

$$\text{Result (IU hg}^{-1}\text{)} = \text{Result (}\mu\text{g hg}^{-1}\text{)} \times 40$$

where:

40 = dietary conversion factor (µg hg⁻¹ to IU hg⁻¹).

K. Data Handling

Report result as µg hg⁻¹ to one decimal place or as IU hg⁻¹ to zero decimal places.

J. Data Handling

Report results in mg/hg or mg/dL to 1 decimal place.

Results and Discussion

The initial phase of method evaluation within the participating laboratories involved the analysis of a practice sample. NIST 1849a SRM was selected as the practice sample as it provided confidence that

the method was implemented appropriately within each laboratory and that accurate results could be obtained.

A total of 12 laboratories agreed to participate as part of this study; however, only 9 laboratories were able to submit data for evaluation prior to the submission deadline, with 2 laboratories not reporting any data and 1 laboratory unable to achieve acceptable results for the practice sample as defined by the ERP (6).

Upon completion of the analysis of all samples, each participating laboratory reported measured results as well as additional information, such as sample identification, weights, volumes, UV absorbances, and peak areas. Participants were also asked to document any deviation from the method and any other pertinent comments based on their experiences in adapting the method into their laboratory. The results received from participants were tabulated and are summarized in Table 1. All nine collaborating laboratories returned acceptable standard calibration parameters based on linear regression correlation coefficients ($r^2 \geq 0.998$).

Only a single pair of results for vitamin D₂ from Laboratory 6 was excluded as Cochran outliers; no other outliers were identified, and all other results were used in the generation of precision values. Repeatability ranged from 1.9 to 5.8% RSD_r and reproducibility values ranged from 6.4 to 12.7% RSD_R (Table 2), with HorRat_R values for the method ranging from 0.2 to 0.6 (expected range 0.5–2.0; 10).

Method accuracy was assessed in accordance with SPIFAN procedures (6) based on results from NIST 1849a SRM (Table 3). A *P*-value of 0.32 indicated that no bias against the certified value was found. As expected, placebo samples not fortified with vitamin D returned negligible results.

The method demonstrated its compliance with the applicability statement of vitamin D SMPR 2011.004 (7) and has been demonstrated to be suitable for the analysis of total vitamin D (previtamin D and vitamin D) in a wide range of infant formulas and nutritional products, as illustrated with the range of different matrixes used in the SLV study (8) and this MLT study.

A summary of each laboratory's performance was sent to participants, along with an invitation to make comments on the performance of the method in their laboratory. In general, comments were positive with respect to the ease of use of the method. Laboratories 3 and 9 found that centrifugation of the samples at 250 × *g* did not give a good separation between the two layers and recommended that a higher centrifuge speed be used. It was noted by Laboratory 4 that the drying step for acetone was not necessary and they did not use it. We also noted this and all participants were advised not to dry acetone during the MLT study. Laboratory 6 used vials in a heating block rather than boiling tubes

in a water bath because of limitations of equipment, and the results did not appear to have been compromised.

Safety concerns with this method were evaluated, and there were no major hazards beyond those typically found in chemistry laboratories. Users of the method are directed to use appropriate safety equipment when handling acids, bases, and solvents, and to refer to Material Safety Data Sheets for detailed safety instructions for each chemical used.

The described protocol provides an accurate, precise, rapid, and robust method for the analysis of vitamin D that is suitable both for routine compliance testing and as a reference method to demonstrate product compliance against global regulations.

Conclusions

An MLT study of AOAC First Action Method 2016.05, an LC-MS/MS method for the analysis of vitamin D₂ and vitamin D₃ in infant formulas and nutritional products, was undertaken. The method was implemented in nine laboratories and demonstrated acceptable precision and accuracy.

Recommendation

A study report summarizing the outcomes of this multi-laboratory collaborative study was submitted with the recommendation that AOAC First Action Method 2016.05 be accepted as a SPIFAN-endorsed AOAC Final Action method. The ERP evaluated the study data in March 2017 and endorsed the recommendation, which was subsequently approved by the AOAC Official Methods Board.

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Table 1. Tabulated raw data for replicate analyses of SPIFAN kit

Lab ^a	Sample Vitamin D ₃ , µg/hg																					
	A		B		C		D		E		F		G		H		I		J			
3	10.78	10.19	10.52	10.05	7.89	8.93	9.21	8.96	0.97	0.98	11.54	10.36	0.00	0.00	9.10	9.00	0.00	0.00	0.70	0.72	1.30	1.34
4	9.92	10.55	10.61	10.51	8.69	9.11	8.02	7.95	0.97	0.94	10.28	10.45	0.02	0.01	8.61	8.86	0.10	0.16	0.68	0.69	1.38	1.24
6	10.69	10.94	10.48	10.85	9.13	9.53	8.61	8.29	1.10	1.08	10.62	10.33	0.00	0.00	8.70	9.00	0.00	0.00	0.72	0.71	1.39	1.46
7	10.22	10.58	10.13	10.28	9.07	8.83	8.33	8.53	0.98	0.92	9.81	10.02	0.01	0.00	8.59	8.71	0.01	0.02	0.67	0.66	1.26	1.28
8	10.04	10.13	10.09	9.87	8.11	8.26	8.04	8.14	0.93	0.92	9.76	9.64	0.00	0.00	7.82	7.93	0.00	0.00	0.67	0.65	1.19	1.16
9	11.11	11.73	12.18	11.34	9.22	9.74	9.07	9.00	1.21	1.11	11.43	10.77	0.00	0.00	8.44	8.87	0.08	0.05	0.81	0.73	1.30	1.24
10	9.83	9.39	9.44	9.46	8.26	8.38	7.50	7.52	0.85	0.94	9.38	9.45	0.00	0.00	7.95	7.92	0.02	0.02	0.59	0.59	1.23	1.25
11	9.58	9.12	9.27	9.61	8.49	8.21	8.03	7.64	1.02	0.89	8.78	9.77	0.00	0.00	8.57	7.44	0.00	0.00	0.63	0.68	1.23	1.27
12	9.60	8.92	10.20	10.14	7.34	6.90	7.40	6.87	0.77	0.74	7.82	7.54	0.00	0.00	7.66	6.84	0.00	0.00	0.51	0.52	1.18	1.13

Lab ^a	Sample Vitamin D ₃ , µg/hg																					
	K		L		M		N		O		P		Q		R		S		T			
3	0.00	0.00	7.93	7.75	9.05	8.86	9.60	9.36	10.35	10.63	7.16	7.44	0.00	0.00	0.00	0.00	3.20	3.02	15.07	14.76	13.14	14.05
4	0.02	0.01	8.32	8.09	9.29	9.27	8.94	9.28	10.36	10.25	7.17	7.00	0.50	0.30	0.06	0.04	3.74	3.55	13.62	14.49	13.75	14.44
6	0.00	0.00	8.21	8.65	9.25	9.35	9.36	9.15	10.58	10.67	7.01	6.83	0.00	0.00	0.00	0.00	3.28	3.56	21.1 ^b	18.0 ^b	14.24	14.10
7	0.00	0.00	7.36	7.37	9.04	8.76	9.06	9.24	10.11	10.23	6.95	6.66	0.01	0.04	0.01	0.02	3.35	3.45	12.79	12.80	13.19	12.72
8	0.00	0.00	7.78	7.78	8.32	8.41	8.72	8.44	9.77	10.03	6.70	6.77	0.00	0.00	0.00	0.00	2.64	2.83	13.20	13.34	12.60	12.64
9	0.01	0.01	7.26	7.52	9.90	9.63	9.87	9.67	11.86	10.78	8.09	7.93	0.03	0.03	0.03	0.04	4.06	3.81	16.75	17.54	17.61	17.34
10	0.00	0.00	7.50	7.50	8.47	8.39	8.40	8.32	9.48	9.61	6.57	6.49	0.01	0.00	0.00	0.01	3.15	3.13	14.30	13.30	13.30	13.40
11	0.00	0.00	6.82	6.77	8.85	7.75	7.02	7.45	8.78	9.79	5.75	6.23	0.00	0.00	0.00	0.00	2.98	2.83	15.44	15.49	13.06	11.93
12	0.00	0.00	7.43	7.64	8.32	8.29	8.09	7.41	8.10	9.66	7.04	5.80	0.00	0.00	0.00	0.00	2.89	3.25	10.49	11.14	12.21	11.99

^a Data for Labs 1, 2, and 5 not submitted prior to MLT submission deadline.

^b * Results removed as Cochran outlier prior to precision calculation.

Sample: A = SRM NIST 1849a, B = Partially hydrolyzed soy-based infant formula powder, C = Infant elemental powder, D = High-protein adult nutritional ready-to-feed, E = Soy-based infant formula, F = High-protein adult nutritional ready-to-feed (unfortified), G = Infant formula powder, H = High-fat adult nutritional ready-to-feed (unfortified), I = Milk-based infant formula ready-to-feed, J = High-fat adult nutritional ready-to-feed, K = Milk-based infant formula ready-to-feed (unfortified), L = Milk-based child formula powder, M = Partially hydrolyzed milk-based infant formula powder, N = Child elemental powder, O = Milk-based infant formula powder, P = Fructooligosaccharide, galactooligosaccharide-based infant formula powder, Q = Infant elemental powder (unfortified), R = Milk-based child formula powder (unfortified), S = Low-fat adult nutritional powder, T = Candidate SRM NIST 1869

Table 2. Method precision for vitamin D in fortified SPIFAN kit samples

Precision parameters	Symbol	Sample														
		A	B	C	D	E	G	I	J	L	M	N	O	P	S	T
Total number of laboratories	p	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
Total number of replicates	n	36	18	18	18	18	18	18	18	18	18	18	18	18	18	34
Number of outliers	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Overall mean (µg/hg)	\bar{x}	10.2	8.6	8.2	1.0	9.9	8.3	0.7	1.3	7.6	8.8	8.7	10.1	6.9	3.3	13.8
Repeatability SD (µg/hg)	SD _r	0.3	0.3	0.2	0.0	0.4	0.4	0.0	0.0	0.1	0.3	0.2	0.5	0.3	0.2	0.8
Reproducibility SD (µg/hg)	SD _R	0.7	0.8	0.7	0.1	1.1	0.6	0.1	0.1	0.5	0.6	0.9	0.8	0.6	0.4	1.8
Repeatability RSD (%)	RSD _r	3.2	3.9	2.3	5.1	4.2	4.3	3.6	3.4	1.9	3.2	2.7	5.1	4.9	4.6	5.8
SMPR repeatability limit	-	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0
Reproducibility RSD (%)	RSD _R	7.2	8.8	8.2	12.4	10.9	7.7	11.4	6.9	6.5	6.4	9.8	8.3	9.1	12.0	12.7
SMPR reproducibility limit	-	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0
Horwitz ratio	HorRat _R	0.3	0.4	0.3	0.4	0.5	0.3	0.3	0.2	0.3	0.3	0.4	0.4	0.4	0.4	0.6

Sample: A = SRM NIST 1849a, B= Partially hydrolyzed soy-based infant formula powder, C = Infant elemental powder, D = High-protein adult nutritional ready-to-feed, E = Soy-based infant formula, G = Infant formula powder, I = Milk-based infant formula ready-to-feed, J = High-fat adult nutritional ready-to-feed, L = Milk-based child formula powder, M = Partially hydrolyzed milk-based infant formula powder, N = Child elemental powder, O = Milk-based infant formula powder, P = Fructooligosaccharide, galactooligosaccharide-based infant formula powder, S = Low-fat adult nutritional powder, T = Candidate SRM NIST 1869

Table 3. Method accuracy for vitamin D in SRM NIST 1849a

Bias parameters	Symbol	Value
Overall mean (µg/hg)	\bar{x}	10.2
Total number of replicates	n	36
Certified value (µg/hg)	μ	11.1
Uncertainty(µg/hg)	U_{CRV}	1.7
Coverage factor	k	2.0
Student's test-statistic	t_{STAT}	1.0
Degrees of freedom	DF	63.0
p-value		0.32