Analysis of Vitamin D_2 and Vitamin D_3 in Fortified Milk Powders and Infant and Nutritional Formulas by Liquid Chromatography–Tandem Mass Spectrometry: Single Laboratory Validation, First Action 2016.05

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Abstract

A method for the determination of vitamin D_2 and vitamin D_3 in fortified milk powders and infant and adult nutritional formulas is described. Samples are saponified at high temperature and lipid-soluble components are extracted into isooctane. A portion of the isooctane layer is transferred and washed, and an aliquot of 4-phenyl-1,2,4-triazoline-3,5-dione is added to derivatize the vitamin D to form a high-molecular mass, easily ionizable adduct. The vitamin D adduct is then re-extracted into a small volume of acetonitrile and analyzed by RPLC. Detection is by tandem MS, using multiple reaction monitoring. Stable isotope labeled vitamin D_2 and vitamin D_3 internal standards are used for quantitation to correct for losses in extraction and any variation in derivatization and ionization efficiencies. A single-laboratory validation of the method using AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) kit samples was performed and compared with parameters defined according to the vitamin D Standard Method Performance Requirements (SMPR). Linearity was demonstrated over the range specified in the SMPR, with the LOD being estimated at below that required. Method spike recovery (vitamin D_2, 97.0–99.2%; and vitamin D_3, 96.0–101.0%) and RSD_r

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(vitamin D_3, 1.5–5.2%) were evaluated and compared favorably with limits in the vitamin D SMPR. Acceptable bias for vitamin D_3 was demonstrated against both the certified value for National Institute of Standards and Technology 1849a Standard Reference material ($p_{(\alpha = 0.05)} = 0.25$) and AOAC INTERNATIONAL reference method 2002.05 ($p_{(\alpha = 0.05)} = 0.09$). The method was demonstrated to meet the requirements of the vitamin D SMPR as defined by SPIFAN and was recently approved for Official First Action status by the AOAC Expert Review Panel on SPIFAN nutrient methods.

Introduction

The major biological function of vitamin D is to maintain normal blood levels of calcium and phosphorus. Vitamin D aids in the absorption of calcium, helping to form and maintain strong bones, thereby preventing rickets in children (1). Vitamin D_3 (cholecalciferol) is generated in the skin of animals when a precursor molecule, 7-dehydrocholesterol, absorbs UV light energy. Thus, vitamin D is not a true vitamin because individuals with adequate exposure to sunlight do not require dietary supplementation. Infant formulas are typically fortified with vitamin D_3, and less commonly vitamin D_2, and are subject to strict regulatory control (2).

Accurate, precise, rapid, high-throughput analytical methods for vitamin D are needed for routine testing to ensure that products are manufactured within tight product specifications. Additionally, reference methods utilizing contemporary techniques are needed to guarantee product compliance with global regulations.

The described method was developed to provide an accurate, rapid, and robust technique for the routine compliance testing of vitamin D_3 in infant formulas and adult/pediatric nutritional formulas and was recently reported (3). To meet the requirements specified in the applicability statement of the vitamin D Standard Method Performance Requirements (SMPR; 4), the scope of the analysis was extended to include vitamin D_2. As required by the AOAC Expert Review Panel (ERP) for Nutrient Methods Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) for endorsement as an Official First Action, method performance was evaluated in accordance with single-laboratory validation (SLV) procedures endorsed by the AOAC ERP (5).

In March 2016, this method and associated SLV data were assessed by the ERP and the method approved for Official First Action status. A recommendation by the ERP was added: The effect of temperature-induced inter-conversion of vitamin D and pre-vitamin D, upon final results, should be investigated to provide evidence of the suitability of this method with respect to the applicability statement of the SMPR.

AOAC Official Method 2016.05

Analysis of Vitamin D_2 and Vitamin D_3 in Fortified Milk Powders, Infant Formulas, and Adult/Pediatric Nutritional Formulas

Liquid Chromatography–Tandem Mass Spectrometry

First Action 2016

[Applicable to the determination of vitamin D_2 and vitamin D_3 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; then lipid-soluble components are extracted into isooctane. A portion of the isooctane 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 then re-extracted into a small volume of acetonitrile and analyzed by RPLC. Detection is by MS using multiple reaction monitoring (MRM). Stable isotope-labeled (SIL) *d6*-vitamin D_2 and *d6*-vitamin D_3 internal standards are used for quantitation to correct for losses in extraction and any variation in derivatization and ionization efficiencies.

B. Apparatus

(1) *Ultra-HPLC (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 mass spectrometer.*—Triple Quad 6500 (Sciex, Framingham, MA) or equivalent tandem MS instrument.

(3) *Column.*—Kinetex C_{18} core-shell, 2.6 µm, 2.1 × 50 mm (Phenomenex, Torrance, CA) or equivalent.

(4) *UV 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 centrifuge tubes.

(11) *Pasteur pipette.*—Glass, ~140 mm.

(12) Horizontal shaker.

(13) *Eppendorf vials*.—2 mL.

(14) *Filter membranes.*—0.45 µm nylon.

(15) *Cryogenic vials*.—2 mL.

(16) *Schott bottles*.—1 L, 100 mL.

(17) *HPLC vials, septa, and caps*.

C. Reagents

(1) *Vitamin D_2 (ergocalciferol).*—CAS No. 50-14-6, purity: ≥ 99%.

(2) *Vitamin D_3 (cholecalciferol).*—CAS No. 67-97-0, purity: ≥99%.

(3) d6-*Vitamin D_2.—(*26,26,26,27,27,27-*d6* ergocalciferol), CAS No. 1311259-89-8, enrichment: ≥ 99%, purity: ≥ 99%.

(4) d6-*Vitamin D_3.—(*26,26,26,27,27,27-*d6* 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 (2,2,4-trimethylpentane).*—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^{-1}).*—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

Because vitamin D is sensitive to light, perform all steps under UV-shielded lighting. If vitamin D_3 is exclusively required for analysis, then standards pertaining to vitamin D_2 need not be used and vice versa.

(1) *Stable isotope-labeled vitamin D_2 or vitamin D_3 stock standard (SILD$_2$SS or SILD3SS; ~10 µg mL^{-1}).—*

(a) Dispense the contents of a 1 mg vial of d6-vitamin D_2 or a 1 mg vial of *d6*-vitamin D_3 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 SILD$_2$SS or SILD$_3$SS at 265 nm. The spectrophotometer should be zeroed against an ethanol blank solution. Calculate and record the concentration.

(d) Immediately dispense aliquots of SILD$_2$SS or SILD$_3$SS (~1.3 mL) into cryogenic vials and freeze at ≤ 15°C.

(2) *Stable isotope-labeled internal standard (SILIS; ~1 µg mL^{-1}).—*

(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 SILD$_2$SS and one vial of SILD$_3$SS from the freezer and allow to warm to room temperature.

(b) Pipette 1.0 mL each of SILD$_2$SS and SILD$_3$SS 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

(3) Non-labeled vitamin D_2 or vitamin D_3 stock standard (NLD$_2$SS or NLD$_3$SS; ~1 mg mL^{-1}).—

(a) Accurately weigh approximately 50 mg vitamin D_2 or vitamin D_3 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 ≤ 15°C for a maximum of 3 months.

(4) Non-labeled vitamin D_2 or vitamin D_3 purity standard (NLD$_2$PS or NLD$_3$PS; ~10 µg mL^{-1}).—

(a) Pipette 1.0 mL NLD$_2$SS or NLD$_3$SS into separate 100 mL volumetric flasks. Dilute to volume with ethanol.

(b) 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.

(c) Make fresh daily.

(5) Non-labeled working standard (NLWS; ~1 µg mL^{-1}).—

(a) Pipette 1.0 mL NLD_2PS and 1.0 mL NLD_3PS into a single 10 mL volumetric flask. Dilute to volume with acetonitrile.

(b) Make fresh daily.

(6) *Calibration standards (CS).*—Make fresh daily. See Table 2016.05A for concentrations of the calibration standard solutions.—

(a) Calibration standard 1.—Pipette 10 µL NLWS and 250 µL SILIS into a 25 mL volumetric flask.

(b) Calibration standard 2.—Pipette 50 µL NLWS and 250 µL SILIS into a 25 mL volumetric flask.

(c) Calibration standard 3.—Pipette 250 µL NLWS and 250 µL SILIS into a 25 mL volumetric flask.

(d) Calibration standard 4.—Pipette 500 µL NLWS and 250 µL SILIS into a 25 mL volumetric flask.

(e) Calibration standard 5.—Pipette 1250 µL NLWS and 250 µL SILIS into a 25 mL volumetric flask.

Table 2016.05A. Nominal concentrations of the 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

Because vitamin D is sensitive to light, perform all steps under UV-shielded lighting.

(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 to mix every 5 min.

(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, then add 0.5 mL SILIS, and then cap and vortex mix.

(2) Add 2 mL potassium hydroxide solution to the boiling tube; cap and vortex mix.

(3) Place the boiling tube in a water bath at 70 °C for 1 h; vortex mix 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 250 × *g* for 15 min.

(7) Transfer a 5 mL aliquot of the upper isooctane layer into a 15 mL centrifuge tube using a Pasteur pipette, taking care not to transfer any of the lower layer.

(8) Add 5 mL water to the centrifuge tube; cap and vortex mix; then place in a centrifuge at 2000 × *g* for 5 min.

(9) Transfer 4–5 mL upper isooctane layer to a new 15 mL disposable centrifuge tube using a disposable pipette, taking care not to transfer any of the lower layer.

(10) Add 75 µL PTAD solution to the centrifuge tube; cap and immediately vortex mix.

(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 vortex mix; then place in a centrifuge at 2000 × *g* for 5 min.

(13) Using a variable volume pipette, transfer 500 µL lower layer into an Eppendorf vial, taking care not to transfer any of the upper layer.

(14) Add 167 µL water to the Eppendorf vial; cap and vortex mix.

(15) Using a syringe filter, transfer an aliquot from the Eppendorf vial to an amber HPLC vial; then 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 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 µL
Initial flow rate	0.6 mL min^{-1}

Table 2016.05C. Gradient procedure for chromatographic separation

Time, min	Flow rate (mL min^{-1})	Phase Composition	
		% A	% B
0 start	0.6	25	75
3.3 pump	0.6	0	100
3.7 pump	1.0	0	100
4.8 pump	1.0	0	100
4.9 pump	0.6	25	75
5.5 stop	0.6	25	75

I. Mass Spectrometry

(1) Set up the mass spectrometer with the instrument settings in Table 2016.05D.

(2) The specific compound parameters to be used are listed in Tables 2016.05E and 2016.05F.

Table 2016.05D. Mass spectrometer instrument settings[a]

Instrument parameter	Value
Ionization mode	ESI^+
Curtain gas	30
Nebulizer gas GS1	40
Heater gas GS2	40
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

Table 2016.05E. Compound parameters (vitamin D_2 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	81	10	23	22	120
Analyte qualifier	572.2	280.0			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 D_2–PTAD adduct, Internal standard ion = *d6*-vitamin D_2–PTAD adduct.
[b] DP = declustering potential
[c] EP = entrance potential
[d] CE = collision energy
[e] CXP = collision cell exit potential

Table 2016.05E. Compound parameters (vitamin D_3 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 D_3–PTAD adduct, Internal standard ion = *d6*-vitamin D_3–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 D_2 in the stock standard, $SILD_2SS$.—

$$SILD_2SS_{D2conc} = \frac{SILD_2SS_{abs(\lambda max)}}{E^{1\%}_{1cm}} \times 10000$$

where:

$SILD_2SS_{D2conc}$ = concentration of *d6*-vitamin D_2 in stock standard (µg mL^{-1});

$SILD_2SS_{abs(\lambda max)}$ = UV absorbance of stock standard at 265 nm (cm^{-1});

$E^{1\%}_{1cm}$ = extinction coefficient for vitamin D_2 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 D_3 in stock standard $SILD_3SS$.—

$$SILD_3SS_{D3conc} = \frac{SILD_3SS_{abs(\lambda max)}}{E^{1\%}_{1cm}} \times 10000$$

where:

$SILD_3SS_{D3conc}$ = concentration of *d6*-vitamin D_3 in stock standard (µg mL^{-1});

$SILD3SS_{abs(\lambda max)}$ = UV absorbance of stock standard at 265 nm (cm^{-1});

$E^{1\%}_{1cm}$ = extinction coefficient for vitamin D_3 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 D_2 in internal standard SILIS.—

$$SILIS_{D2conc} = SILD_2SS_{D2conc} \times \frac{1.0}{10} \times 1000$$

where:

$SILIS_{D2conc}$ = concentration of *d6*-vitamin D_2 in internal standard (ng mL^{-1});

$SILD2SS_{D2conc}$ = concentration of *d6*-vitamin D_2 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.—

$$SILIS_{D3conc} = SILD_3SS_{D3conc} \times \frac{1.0}{10} \times 1000$$

where: $SILIS_{D3conc}$ = concentration of *d6*-vitamin D_3 in internal standard (ng mL^{-1});

$SILD2SS_{D3conc}$ = concentration of *d6*-vitamin D_3 in stock standard (µg mL^{-1});

1000 = concentration conversion factor (µg mL^{-1} to ng mL^{-1}).

(5) *Concentration of non-labelled vitamin D_2 in purity standard NLD_2PS.—*

$$NLD_2PS_{D2conc} = \frac{NLD_2PS_{abs(\lambda max)}}{E^{1\%}_{1cm}} \times 10000$$

where:

NLD_2PS_{D2conc} = concentration of vitamin D_2 in purity standard (µg mL^{-1});

$NLD_2PS_{abs(\lambda max)}$ = UV absorbance of purity standard at 265 nm (cm^{-1});

$E^{1\%}_{1cm}$ = extinction coefficient for vitamin D_2 in ethanol (461 dL g.cm^{-1});

10000 = concentration conversion factor (g dL^{-1} to µg mL^{-1}).

(6) *Concentration of non-labeled vitamin D_3 in purity standard NLD_3PS.—*

$$NLD_3PS_{D3conc} = \frac{NLD_3PS_{abs(\lambda max)}}{E^{1\%}_{1cm}} \times 10000$$

where:

NLD_3PS_{D3conc} = concentration of vitamin D_3 in purity standard (µg mL^{-1});

$NLD_3PS_{abs(\lambda max)}$ = UV absorbance of purity standard at 265 nm (cm^{-1});

$E^{1\%}_{1cm}$ = extinction coefficient for vitamin D_3 in ethanol (485 dL g.cm^{-1});

10000 = concentration conversion factor (g dL^{-1} to µg mL^{-1}).

(7) *Concentration of non-labeled vitamin D_2 in working standard NLWS.—*

$$NLWS_{D2conc} = NLD_2PS_{D2conc} \times \frac{1.0}{10} \times 1000$$

where: $NLWS_{D2conc}$ = concentration of vitamin D_2 in working standard (ng mL^{-1});

NLD_2PS_{D2conc} = concentration of vitamin D_2 in purity standard (µg mL^{-1});

1000 = concentration conversion factor (µg mL^{-1} to ng mL^{-1}).

(8) Concentration of non-labeled vitamin D_3 in working standard NLWS.—

$$NLWS_{D3conc} = NLD_3PS_{D3conc} \times \frac{1.0}{10} \times 1000$$

where:

$NLWS_{D3conc}$ = concentration of vitamin D_3 in working standard (ng mL^{-1});

NLD_3PS_{D3conc} = concentration of vitamin D_3 in purity standard (µg mL^{-1});

1000 = concentration conversion factor (µg mL^{-1} to ng mL^{-1}).

(9) Concentrations of vitamin D_2 and vitamin D_3 in calibration standards CS1–CS5.—

$$CS1_{Dconc} = NLWS_{Dconc} \times \frac{0.01}{25}$$

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

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

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

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

where:

$CS1–CS5_{Dconc}$ = concentration of vitamin D_2 or vitamin D_3 in calibration standards (ng mL^{-1});

$NLWS_{Dconc}$ = concentration of vitamin D_2 or vitamin D_3 in working standard (ng mL^{-1}).

(10) Concentrations of stable isotope-labeled d6-*vitamin D_2 and* d6-*vitamin D_3 in calibration standards CS1–CS5.—*

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

where:

$CS1–CS5_{Dconc}$ = concentration of *d6*-vitamin D_2 or *d6*-vitamin D_3 in calibration standards (ng mL^{-1});

$SILIS_{Dconc}$ = concentration of *d6*-vitamin D_2 or *d6*-vitamin D_3 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).

(12) 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.

(13) The concentration (*w/w*) of vitamin D_2 or vitamin D_3 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 D_2 or vitamin D_3 concentration in sample (µg hg^{-1});

PA_{NLD} = peak area of vitamin D_2 or vitamin D_3 in sample;

PA_{SILD} = peak area of d6- vitamin D_2 or *d6*-vitamin D_3 in sample;

$SILIS_{Dconc}$ = concentration of *d6*-vitamin D_2 or *d6*-vitamin D_3 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}).

(14) The concentration (*w/v*) of vitamin D_2 or vitamin D_3 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 D_2 or vitamin D_3 concentration in sample (µg hg^{-1});

PA_{NLD} = peak area of vitamin D_2 or vitamin D_3 in sample;

PA_{SILD} = peak area of *d6*-vitamin D_2 or *d6*-vitamin D_3 in sample;

$SILIS_{Dconc}$ = concentration of *d6*-vitamin D_2 or *d6*-vitamin D_3 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}).

(15) The concentration of vitamin D_2 or vitamin D_3 as IU hg^{-1} in the sample is calculated as:

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

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.

Results and Discussion

Method Optimization

The advantages of using the described derivatization strategy for the analysis of vitamin D are that many compounds (such as plant sterols) that are isobaric with vitamin D_2 and vitamin D_3 are excluded from detection because they lack the conjugated diene structure, and therefore do not form adducts. The derivatization of vitamin D with PTAD produces two epimers, 6*S* and 6*R*, because PTAD reacts with the *cis*-diene moiety from both the α-side and the β-side, with the ratio of 6*S*:6*R* being approximately 4:1 (6). The 6*S*/6*R* epimers co-elute using the described chromatographic conditions, and the typical MRM chromatograms for a sample are shown in Figures 1 and 2.

Product ion scans of the fragmentation of authentic vitamin D_3–PTAD $[M+H]^+$ and vitamin D_2–PTAD $[M+H]^+$ ions were performed (Figures 3 and 4). Product ions (298.0 and 280.0 *m/z*) were identified as being suitable quantifier and qualifier ion candidates for both vitamin D_2 and vitamin D_3. The method was optimized to enhance the signal of the transitions 572.2→298.0 and 572.2→280.0 for vitamin D_2 and the transitions 560.2→298.0 and 560.2→280.0 for vitamin D_3.

Single-Laboratory Validation

A wide range of infant formula and adult nutritional products that are available in the SPIFAN kit, plus an in-house vitamin D_3 QC milk powder sample, were used for the validation of this method (Table 1).

Linearity was evaluated by the analysis of six-level calibration standards on three different days. Visual inspection of the linear regression lines and residuals plots, back-calculation of standard concentrations (data not shown), and regression equations and correlation coefficients (Table 2) were used to demonstrate a linear relationship between instrument response and analyte concentration over the working range specified in the SMPR. The linear ranges for vitamin D_2 and vitamin D_3 extended beyond both the lower limit and the upper limit of the range specified in the vitamin D SMPR.

Precision was assessed for all of the fortified samples by testing duplicate samples on six separate days by two different analysts on a single instrument, with fresh calibration standards and reagents being made each day (Table 3). The repeatability of the method for the SPIFAN kit samples ranged between 1.5 and 5.2%, which complied with the ≤ 11.0% limit set in the SMPR. The HorRat values were within acceptability criteria for repeatability of 0.3–1.3 (7). Intermediate precision ranged between 3.1 and 7.9%, with a mean value of 5.5%, less than the 15% limit for reproducibility defined in the SMPR.

The LOD and LOQ were initially estimated by evaluating multiple whole-milk powder samples spiked at a range of concentrations and by determining the spike concentration that gave an S/N of

approximately 10. This was determined to be a concentration of 2 ng of vitamin D spiked into a 2 g sample. The LOD and the LOQ were then determined from 10 independent analyses. The LOD and the LOQ for vitamin D_2 were determined to be 0.12 and 0.15 μg hg^{-1}, which were equivalent to 0.013 and 0.016 μg hg^{-1} as RTF, specified in the SMPR. The LOD and the LOQ for vitamin D_3 were determined to be 0.16 and 0.25 μg hg^{-1}, equivalent to 0.018 and 0.028 μg hg^{-1} as RTF. The LOD and the LOQ for both vitamin D_2 and vitamin D_3 were lower than those defined in the vitamin D SMPR.

Recovery was evaluated using unfortified samples within the SPIFAN kit. Each matrix was spiked at two levels: 50% (5 μg hg^{-1} ≈ 0.6 μg hg^{-1} RTF) and 100% (10 μg hg^{-1} ≈ 1.1 μg hg^{-1} 1 RTF) of typical infant formula concentrations. Spike samples were analyzed on three separate days. The mean recoveries measured were between 97.0 and 99.2% for vitamin D_2 and between 96.0 and 101.0% for vitamin D_3 (Table 4), within the limits set in the SMPR of 90–110%. Bias was evaluated by replicate analyses of the National Institute of Standards and Technology (NIST) 1849a Standard Reference Material (SRM). Differences between the measured value and the certified value were determined with the mean and SD of the differences, and the test statistic was calculated. A $P_{(\alpha = 0.05)}$ of 0.25 indicates that there was no bias between the measured results and the certified value (Table 5). As part of initial method validation, the LC-MS/MS was evaluated for bias against an HPLC-UV method based on AOAC 2002.05 (8, 9). A $P_{(\alpha = 0.05)}$ of 0.09 indicates that there was no bias between the methods (Table 6). Bias against a certified reference material or a reference method is not a defined parameter within the SMPR.

Vitamin D–Previtamin D Interconversion

Although the described method specifically detects vitamin D and not the pre-vitamin D isomer, the method quantifies an aggregate result for both pre-vitamin D and vitamin D. This satisfies the requirement of the applicability statement of the SMPR, which specifies total vitamin D_2 or vitamin D_3, including their pre-vitamin isomers. It was assumed in this analysis, as with all analytical methods for vitamin D that use calciferol internal standards, that the pre-vitamin D: vitamin D ratio was equivalent for the sample analyte and the internal standard. For deuterated internal standards, the labelled site must be remote from the triene center because of the difference in inter-conversion behaviour between the analyte and the internal standard (10). To confirm this assumption, the effect of temperature on the final results was evaluated. Experiments were performed with saponification assessed in three different ways: (1) at 70 °C for 1 h, according to the described method protocol; (2) at 20 °C for 7.5 h; and (3) at 70 °C for 7.5 h. A 7.5 h saponification was chosen because this is the time needed, as previously reported, for a pure solution of vitamin D to reach equilibrium with pre-vitamin D at 70 °C (11). Samples 1–6 and 13–18, which were saponified at 70 °C, showed significantly lower absolute peak areas for the vitamin D–PTAD quantifier ion than samples 7–12, which were saponified at 20 °C. This was as expected because a higher proportion of vitamin D is converted to

pre-vitamin D at the elevated temperature. This effect was seen for both the analyte vitamin D in the sample and the SIL *d6*-vitamin D internal standard, illustrating the appropriateness of the internal standard to account for any temperature-induced inter-conversion between pre-vitamin D and vitamin D (Figure 5). The final results obtained showed that, within sample error, there was no difference between the three experiments, which was consistent with the premise that the described method measures an aggregate result for both pre-vitamin D and vitamin D forms (Figure 6).

The separate measurement of pre-vitamin D was investigated as part of an independent initial method proof of concept and in which a number of practical reasons for not quantifying pre-vitamin D separately were discussed (12). Its inclusion as part of the analysis would add complexity, with no material improvement to the estimation of vitamin D because (i) the relative ionization and fragmentation efficiencies of vitamin D–PTAD and pre-vitamin D–PTAD are not known; (ii) the pre-vitamin D–PTAD peak has a different retention time from the vitamin D–PTAD peak and may be subject to different ion suppression, thereby making accurate quantitation of this form difficult; and (iii) a pure standard for pre-vitamin D is not available (12).

It has been demonstrated that separate detection and measurement of pre-vitamin D in this method was not necessary and that the results obtained would be consistent with the requirements of the SMPR.

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Table 1. Samples used during method validation

Sample description	Type	Code	Fortified
Child formula	Powder	00847RF00	No
Infant elemental	Powder	00796RF	No
Adult nutritional, high-protein	RTF	00821RF00	No
Adult nutritional, high-fat	RTF	00820RF00	No
Infant formula, milk-based	RTF	EV4H2Q	No
Infant formula, partially hydrolyzed, milk-based	Powder	410057652Z	Vitamin D_3
Infant formula, partially hydrolyzed, soy-based	Powder	410457651Z	Vitamin D_3
Toddler formula, milk-based	Powder	4052755861	Vitamin D_3
Infant formula, milk-based	Powder	4044755861	Vitamin D_3
Adult nutritional, low-fat	Powder	00859RF00	Vitamin D_3
Child formula	Powder	00866RF00	Vitamin D_3
Infant elemental	Powder	00795RF	Vitamin D_3
Infant formula FOS/GOS-based[a,b]	Powder	50350017W1	Vitamin D_3
Infant formula, milk-based	Powder	K16NTAV	Vitamin D_3
Infant formula, soy-based	Powder	E10NWZC	Vitamin D_3
Infant formula, milk-based	RTF[c]	EV4H2R	Vitamin D_3
Adult nutritional, high-protein	RTF	00730RF00	Vitamin D_3
Adult nutritional, high-fat	RTF	00729RF00	Vitamin D_3
NIST 1849a SRM[d]	Powder	CLC10-b	Vitamin D_3
In-house QC infant formula	Powder	—	Vitamin D_3

[a] FOS = Fructooligosaccharide
[b] GOS = Galactooligosaccharide
[c] RTF = Ready-to-feed
[d] SRM = Standard Reference Material

Table 2. Linearity and range for vitamin D_2 and vitamin D_3

Analyte	Linear regression	Correlation coefficient	Range, ng mL^{-1}	Range RTF[a], (µg hg^{-1})	SMPR (µg hg^{-1})
Vitamin D_2	$y = 0.87x + 0.015$	1.0000	0.3–59.1	0.04–7.3	0.12–5.1
Vitamin D_3	$y = 0.87x + 0.015$	0.9999	0.5–92.8	0.06–11.3	

[a] RTF = Ready-to-feed at a concentration of 25 g dissolved in 200 mL

Table 3. Repeatability and intermediate precision of the method for vitamin D

Sample	Repeatability RSD, % (HorRat)	Intermediate precision RSD, %
Infant formula, partially hydrolyzed, milk-based	4.4 (0.2)	7.4
Infant formula, partially hydrolyzed, soy-based	1.8 (0.1)	5.0
Toddler formula, milk-based	2.2 (0.1)	4.4
Infant formula, milk-based	2.1 (0.1)	4.4
Adult nutritional, low-fat	3.7 (0.1)	6.3
Child formula	3.3 (0.1)	5.8
Infant elemental	3.5 (0.1)	3.1
Infant formula, FOS/GOS-based[a,b]	1.5 (0.1)	4.7
Infant formula, milk-based	3.3 (0.1)	6.4
Infant formula, soy-based	2.6 (0.1)	3.6
Infant formula, milk-based	2.3 (0.1)	7.8
Adult nutritional, high-protein	1.6 (0.1)	5.2
Adult nutritional, high-fat	4.9 (0.2)	7.9
NIST 1849a SRM[c]	2.8 (0.1)	5.4
In-house QC infant formula	5.2 (0.2)	5.4

[a] FOS = Fructooligosaccharide

[b] GOS = Galactooligosaccharide

[c] SRM = Standard Reference Material

Table 4. Recoveries for vitamin D_2 and vitamin D_3

Sample	Recovery, % (RSD, %)	
	Vitamin D_2	Vitamin D_3
Child formula powder	99.2 (3.7)	100.5 (2.2)
Infant elemental powder	97.6 (1.5)	97.0 (1.4)
Adult nutritional RTF, high-proteina	98.5 (1.1)	97.7 (0.9)
Adult nutritional RTF, high-fat	98.3 (2.3)	101.0 (2.7)
Infant formula RTF, milk-based	97.0 (3.1)	96.0 (2.0)

[a] RTF = Ready-to-feed

Table 5. Results for the bias experiment against NIST 1849a SRM[a]

Parameter	Value
Certified value (μg hg^{-1})	11.1
Uncertainty (μg hg^{-1})	1.7
Certified range (μg hg^{-1})	9.4–12.8
Coverage factor (k)	2
Degrees of freedom (DF_{CRV})	60
Mean, $\bar{x}$ (μg hg^{-1})	10.1
SD	0.53
Number of replicates (n)	13
95% Confidence interval (μg hg^{-1})	9.8–10.4
T_{stat}	1.165
Degrees of freedom	63.92
$p_{(\alpha = 0.05)}$	0.25

[a] SRM = Standard Reference Material

Table 6. Results for the bias experiment against AOAC 2002.05

Parameter	Reference method	LC-MS/MS method
Mean, $\bar{x}$ (μg hg^{-1})	10.5	10.8
SD	3.18	3.66
Number of replicates, n	40	40
95% Confidence interval (μg hg^{-1})	10.0–11.0	10.2–11.4
Mean of paired differences	–0.3	
SD of paired differences	1.27	
T_{stat}	1.73	
Degrees of freedom	38	
$P_{(\alpha = 0.05)}$	0.09	

[a] SRM = Standard Reference Material

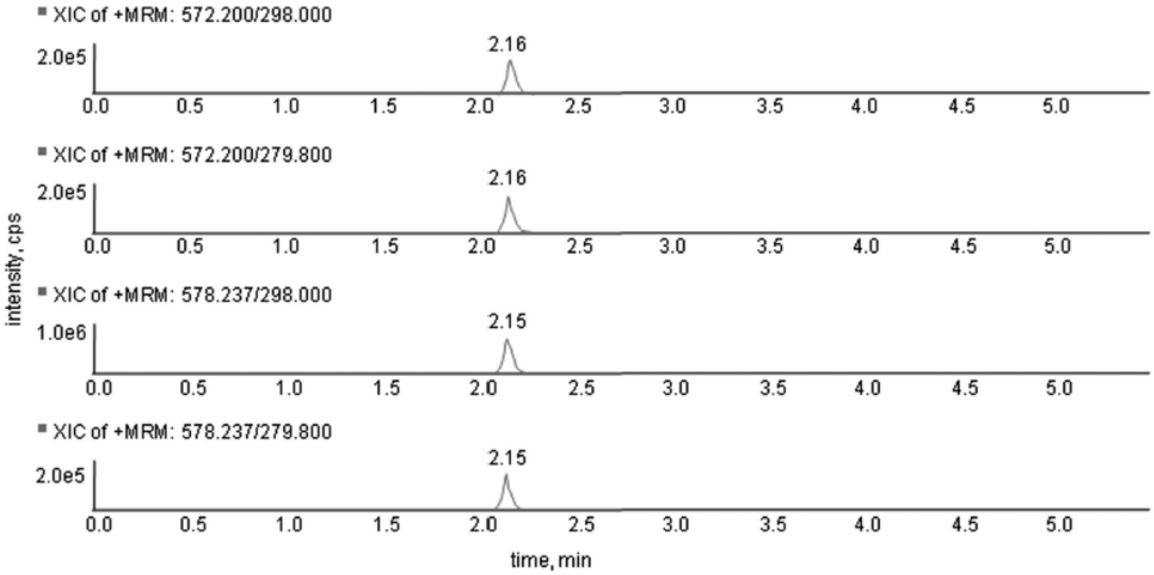

Figure 1. MRM chromatogram for vitamin D_2

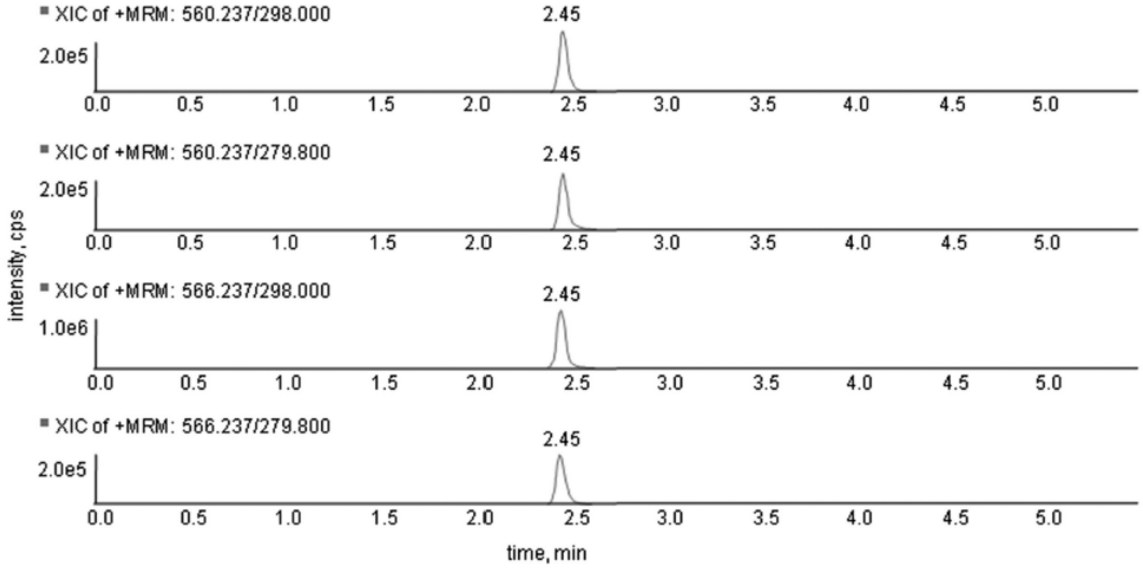

Figure 2. MRM chromatogram for vitamin D_3

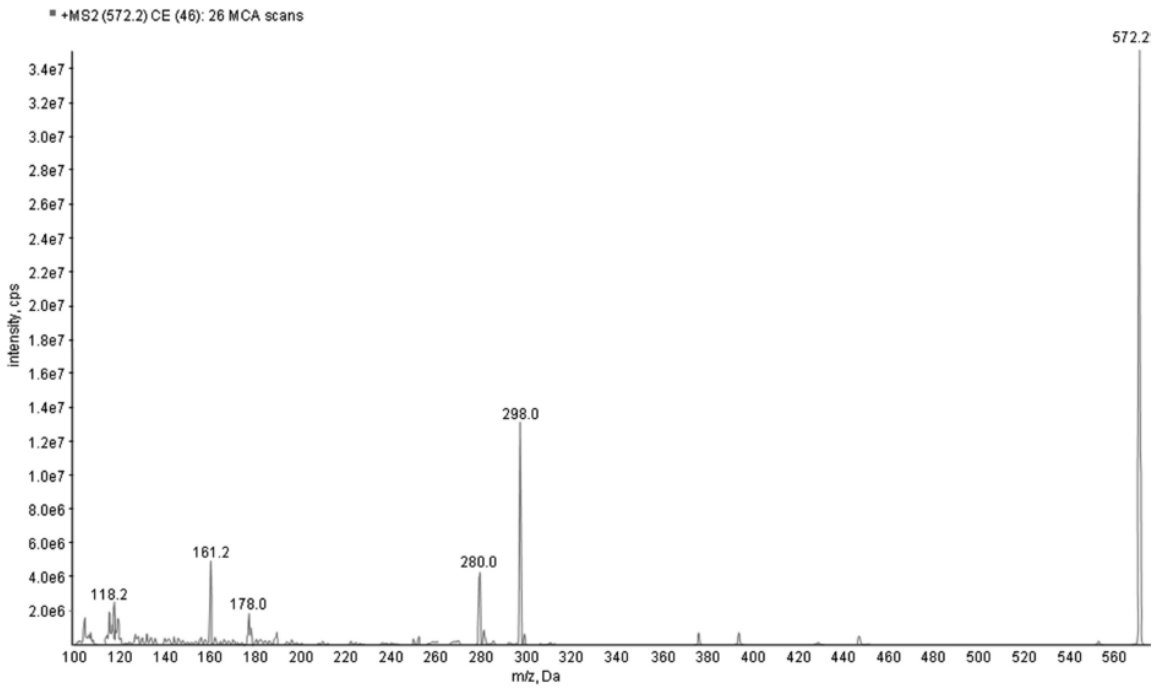

Figure 3. Product ion spectrum of vitamin D_2

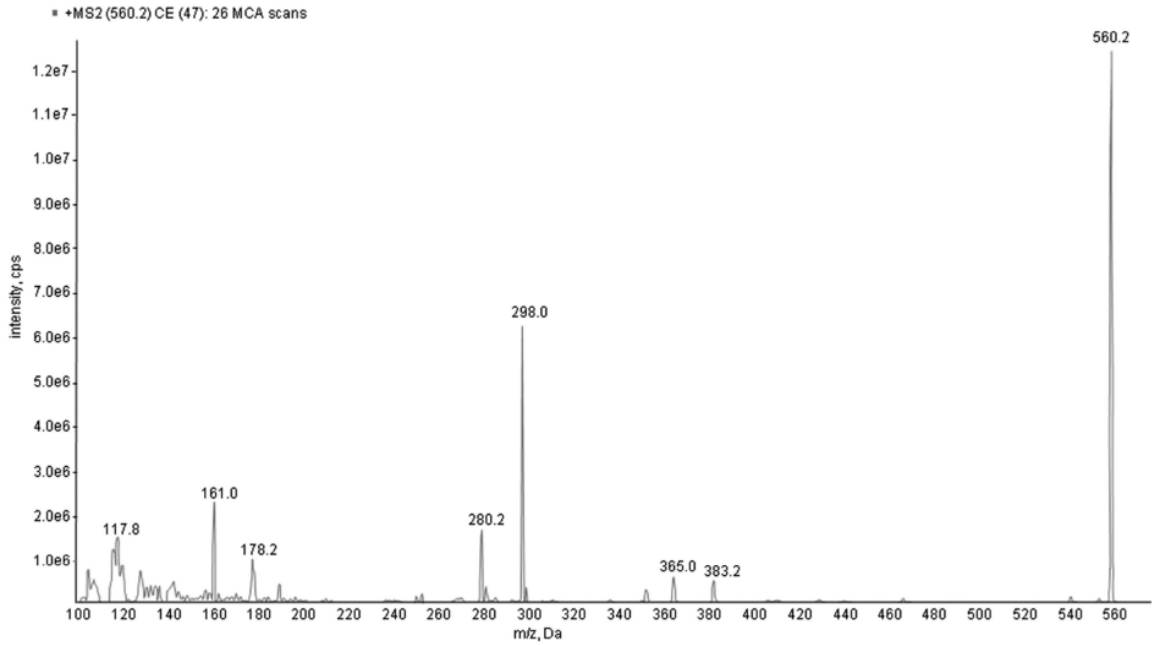

Figure 4. Product ion spectrum of vitamin D_3

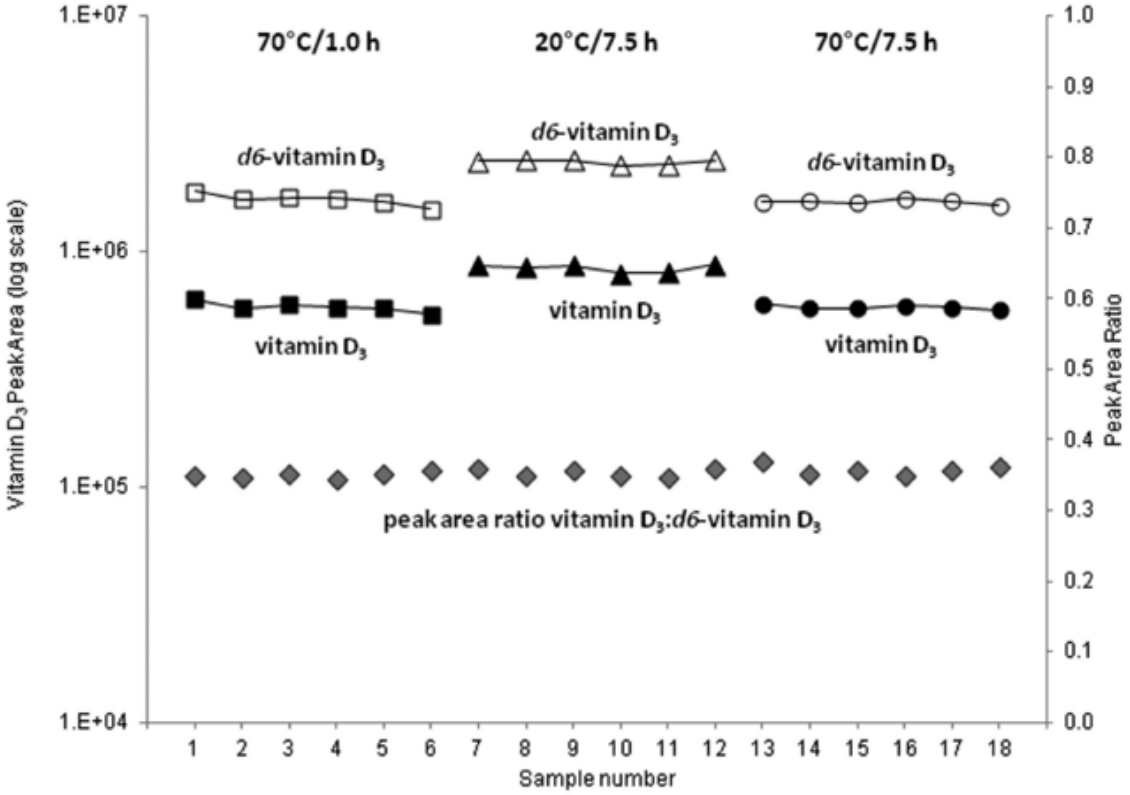

Figure 5. Effect of saponification time/temperature on vitamin D and *d6*-vitamin D

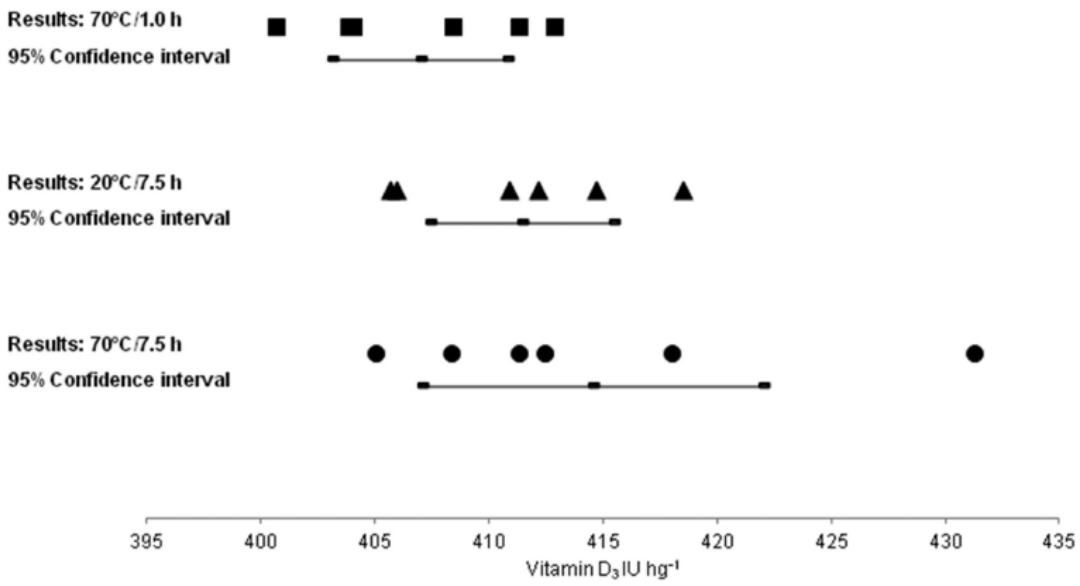

Figure 6. Effect of saponification time/temperature on the measured results for vitamin D