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Analysis of Vitamin D_2 and Vitamin D_3 by LC-MS/MS in Milk Powders, Infant Formulas, and Adult Nutritionals

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AOAC Official Method 2016.05

Analysis of Vitamin D2 and Vitamin D3 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 supplemented milk powders, infant formula and adult/pediatric nutritional formula).

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, washed, and an aliquot of 4-phenyl-1,2,4-triazoline-3,5-dione is added to derivatize vitamin D to form a high molecular mass, easily ionisable adduct. The vitamin D-adduct is then re extracted into a small volume of acetonitrile and analysed by reverse-phase liquid chromatography. Detection is by triple quadrupole mass spectrometer using multiple reaction monitoring (MRM). Stable isotope labelled (SIL) vitamin D2 and vitamin D3 internal standards are used for quantitation to correct for losses in extraction and any variation in derivatisation and ionisation efficiencies.

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2. Apparatus

(a) UHPLC system, consisting of dual pump system, a sample injector unit, a degasser unit, and a column oven (Shimadzu Nexura or equivalent).

(b) Triple quadrupole mass spectrometer (Sciex 6500 QTrap or equivalent)

(c) Column, Kinetex C_{18} core-shell, 2.6 µm, 2.1 mm × 50 mm, (Phenomenex or equivalent)

(d) Spectrophotometer.—Capable of digital readout to 3 decimal places.

(e) Centrifuge tubes, polypropylene, 15 mL.

(f) Boiling tubes, glass, 60 mL.

(g) Water baths, cold 20 °C, hot 70 °C.

(h) Disposable syringes, capacity 1 mL.

(i) Syringe filters, PTFE, 0.2 µm, 13 mm.

(j) Centrifuges, suitable for 60 mL boiling tubes and 15 mL centrifuge tubes.

(k) Pasteur pipettes, glass, ~140 mm.

(l) Horizontal shaker.

(m) Eppendorf vials, 2 mL.

(n) Filter membranes, 0.2 µm × 47 mm, nylon.

(o) Cryogenic vials, 2 mL.

(p) Schott bottles, 1 L, 100 mL.

2.2. Reagents

(a) Standards.—Should be ≥99% pure.

(1) Vitamin D_2, ergocalciferol.

(2) Vitamin D_3, cholecalciferol.

(3) *d6*-Vitamin D_2, 26,26,26,27,27,27-*d6* ergocalciferol.

(4) *d6*-Vitamin D_3, 26,26,26,27,27,27-*d6* cholecalciferol.

(b) PTAD (4-phenyl-1,2,4-triazoline-3,5-dione).

(c) Formic acid (HCO_2H), LC-MS grade.

(d) Potassium hydroxide (KOH).

(e) Pyrogallol ($C_6H_3(OH)_3$).

(f) Ethanol (C_2H_5OH).

(g) Methanol (CH_3OH), LC-MS grade

(h) Isooctane ($(CH_3)_3CCH_2CH(CH_3)_2$).

(i) Acetone (CH_3COCH_3).

(j) Acetonitrile (CH_3CN). LC-MS grade

(k) Water.—Purified with resistivity ≥18 MΩ.

2.3. Standards

(a) PTAD Solution (4-phenyl-1,2,4-triazoline-3,5-dione, 10 mg/mL). Dissolve 50 mg PTAD in 5.0 mL acetone.

(b) Potassium Hydroxide Solution (KOH, 50% w/v). Dissolve 100 g potassium hydroxide in 200 mL water.

(c) Ethanolic Pyrogallol Solution (C6H3(OH)3, 1% w/v). Dissolve 5 g pyrogallol in 500 mL of ethanol.

(d) Mobile Phase A (HCO_2H, 0.1% v/v). To 500 mL of water, add 0.5 mL formic acid

(e) Mobile Phase B (CH_3OH, 100% v/v). Methanol, 500 mL

2.4. Sample collection

Vitamin D is sensitive to light; perform all steps under low-level incandescent lighting. If exclusively vitamin D_3 is required for analysis, then standards pertaining to vitamin D_2 need not be used and vice versa. Calibration standards should be bracketed at the beginning and at the end of an analytical run.

(a) Vitamin D_2 Stable Isotope Labelled Stock Standard Solution (~10 µg/mL)—Dispense the contents of a 1 mg vial of d6 vitamin D_2 into a 100 mL volumetric flask. Dissolve in 90 mL of ethanol; to promote dissolution, sonicate if necessary. Mix thoroughly, make up to volume with ethanol. Measure the absorbance of an aliquot at 265 nm. The spectrophotometer should be zeroed against an ethanol blank solution. Calculate and record concentration. Immediately dispense aliquots (~1.3 mL) into cryogenic vials and freeze at < –15 °C for up to 6 months.

(b) Vitamin D_3 Stable Isotope Labelled Stock Standard Solution (~10 µg/mL)—Dispense the contents of a 1 mg vial of d6 vitamin D_3 into a 100 mL volumetric flask. Dissolve

in 90 mL of ethanol; to promote dissolution, sonicate if necessary. Mix thoroughly, make up to volume with ethanol. Measure the absorbance of an aliquot at 265 nm. The spectrophotometer should be zeroed against an ethanol blank solution. Calculate and record concentration. Immediately dispense aliquots (~1.3 mL) into cryogenic vials and freeze at < –15 °C for up to 6 months.

(c) Stable Isotope Labelled Internal Standard Solution (1 µg/mL)—Depending on the number of samples that need to be analyzed in a run, more or less Stable Isotope Labelled Internal Standard Solution needs to be made up. For every 15 samples (or part thereof) in an analytical run, remove 1 vial of Vitamin D_2 Stable Isotope Labelled Stock Standard Solution and/or 1 vial of Vitamin D_3 Stable Isotope Labelled Stock Standard Solution from the freezer and allow to warm to room temperature. Pipette 1.0 mL of Vitamin D_2 Stable Isotope Labelled Stock Standard Solution and/or 1.0 mL of Vitamin D_3 Stable Isotope Labelled Stock Standard into a 10 mL volumetric flask (use a separate 10 mL volumetric flask for each set of 15 samples). Make each 10 mL volumetric flask to volume with acetonitrile, pool together and mix thoroughly. Make fresh daily.

(d) Vitamin D_2 Non-Labelled Stock Standard Solution (~1 mg/mL)—Weigh accurately, approximately 50 mg of vitamin D_2 into a 50 mL volumetric flask. Dissolve in 40 mL of ethanol; to promote dissolution sonicate if necessary. Mix thoroughly, make up to volume with ethanol. Store in freezer at <–15 °C for up to 1 month.

(e) Vitamin D_3 Non-Labelled Stock Standard Solution (~1 mg/mL)—Weigh accurately, approximately 50 mg of vitamin D3 into a 50 mL volumetric flask. Dissolve in 40 mL of ethanol; to promote dissolution sonicate if necessary. Mix thoroughly, make up to volume with ethanol. Store in freezer at <–15 °C for up to 1 month.

(f) Vitamin D_2 Non-Labelled Purity Standard Solution (~10 µg/mL) —Pipette 1.0 mL of Vitamin D_2 Non-Labelled Stock Standard Solution into a 100 ml volumetric flask. Make to volume with ethanol. Measure the absorbance of an aliquot at 265 nm. The spectrophotometer should be zeroed against an ethanol blank solution. Record absorbance and calculate concentration. Make fresh daily.

(g) Vitamin D_3 Non-Labelled Purity Standard Solution (~10 µg/mL)—Pipette 1.0 mL of Vitamin D_3 Non-Labelled Stock Standard Solution into a 100 mL volumetric flask. Make to volume with ethanol. Measure the absorbance of an aliquot at 265 nm.

The spectrophotometer should be zeroed against an ethanol blank solution. Record absorbance and calculate concentration. Make fresh daily.

(h) Non-labelled Working Standard Solution (~1 µg/ml)—Pipette 1.0 mL of Vitamin D_2 non-labelled purity standard solution and/or 1.0 mL of Vitamin D_3 Non-Labelled Purity Standard Solution into a 10 mL volumetric flask. Make to volume with acetonitrile and mix thoroughly. Make fresh daily.

(i) Calibration Standards— See Table 2016.05A for nominal vitamin D concentrations of the Calibration Standard Solutions. Make fresh daily.

(1) Calibration Standard 1—Pipette 10 µL of Non-Labelled Working Standard Solution and 250 µL of Stable Isotope Labelled Internal Standard Solution into a 25 mL volumetric flask. Add 5 mL of acetonitrile and 75 µL of PTAD Solution, shake to mix and leave in the dark for 5 min. Add 6.25 mL of water then make to volume with acetonitrile, mix, and transfer to HPLC vial ready for analysis.

(2) Calibration Standard 2—Pipette 50 µL of Non-Labelled Working Standard Solution and 250 µL of Stable Isotope Labelled Internal Standard Solution into a 25 mL volumetric flask. Add 5 mL of acetonitrile and 75 µL of PTAD Solution, shake to mix and leave in the dark for 5 min. Add 6.25 mL of water then make to volume with acetonitrile, mix, and transfer to HPLC vial ready for analysis.

(3) Calibration Standard 3—Pipette 250 µL of Non-Labelled Working Standard Solution and 250 µL of Stable Isotope Labelled Internal Standard Solution into a 25 mL volumetric flask. Add 5 mL of acetonitrile and 75 µL of PTAD Solution, shake to mix and leave in the dark for 5 min. Add 6.25 mL of water then make to volume with acetonitrile, mix, and transfer to HPLC vial ready for analysis.

(4) Calibration Standard 4—Pipette 500 µL of Non-Labelled Working Standard Solution and 250 µL of Stable Isotope Labelled Internal Standard Solution into a 25 mL volumetric flask. Add 5 mL of acetonitrile and 75 µL of PTAD Solution, shake to mix and leave in the dark for 5 min. Add 6.25 mL of water then make to volume with acetonitrile, mix, and transfer to HPLC vial ready for analysis.

(5) Calibration Standard 5—Pipette 1250 µL of Non-Labelled Working Standard Solution and 250 µL of Stable Isotope Labelled Internal Standard Solution into a 25 mL volumetric flask. Add 5 mL of acetonitrile and 75 µL of PTAD Solution, shake to mix and leave in the dark for 5 min. Add 6.25 mL of water then make to volume with acetonitrile, mix, and transfer to HPLC vial ready for analysis.

Table 2016.05A. Nominal concentration of Calibration Standards

Calibration Standard	Vitamin D Concentration (ng/mL)	*d6*-Vitamin D Concentration (µg/mL)
1	0.4	10
2	2.0	10
3	10	10
4	20	10
5	50	10

F. Sample Preparation

(a) Powder sample preparation

(1) Accurately weigh 1.8–2.2 g of powder sample into a boiling tube. Record weight.

(b) Powder sample preparation

(1) Accurately weigh 19.0–21.0 g of powder to a disposable slurry container. Record weight.

(2) Accurately weigh ~80 mL water to container. Record weight.

(3) Shake thoroughly until mixed. Place in dark at room temperature for 15 min and shake to mix every 5 min.

(4) Accurately weigh 9.5–10.5 g of slurry or reconstituted powder sample into a boiling tube. Record weight.

(c) Liquid sample preparation

(1) Accurately weigh 10.0 mL of liquid milk into a boiling tube. Record weight.

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G. Extraction and Derivatization

(a) To powder, slurry, or liquid sample in a boiling tube, add 10 mL Ethanolic Pyrogallol Solution, add 0.50 mL of Stable Isotope Labelled Internal Standard Solution, cap and vortex mix.

(b) Add 2 mL of Potassium Hydroxide Solution to boiling tube; cap and vortex mix.

(c) Place boiling tube in water bath at 70°C for 1 h, vortex mix every 15 min.

(d) Place boiling tube in water bath at 70°C until cool.

(e) Add 10 mL isooctane to boiling tube; cap boiling tube tightly and place on horizontal shaker for 10 min.

(f) Add 20 mL of water to boiling tube and invert tube 10 times; place in centrifuge at ≥ 250 × g for 15 min.

(g) 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 (discard boiling tube with lower layer).

(h) Add 5 mL of water to centrifuge tube; cap and vortex mix; place in centrifuge at 2000 × g for 5 min.

(i) Transfer 4–5 ml of 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 (discard centrifuge tube with lower layer).

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

(k) Allow to stand in dark for 5 min to allow for derivatization reaction to complete.

(l) Add 1 mL acetonitrile to centrifuge tube, cap and vortex mix; place in centrifuge at 2000 × g for 5 min.

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

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

(o) Using a syringe filter, transfer an aliquot from Eppendorf vial to an amber HPLC vial; cap ready for analysis.

H. Chromatography

(a) Set-up the UHPLC system with the following configuration shown in Table 2016.05B.

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Table 2016.05B. Chromatographic instrument settings

Instrument Parameter	Value
mobile phase	A = formic acid, 0.1% B = methanol, 100%
column	Kinetex C_{18} core-shell, 2.6 µm, 2.1 × 50 mm
oven temperature	40 °C
chiller temperature	15 °C
injection volume:	3 µL
initial flow rate:	0.6 mL/min (see gradient details below)

(b) Form high pressure gradients by mixing of the two mobile phases, A and B, using the procedure given in Table 2016.05C.

Table 2016.05C. Gradient procedure for chromatographic separation

Time (min)	Flow rate (mL/min)	Mobile Phase A (%)	Mobile Phase 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

(a) Set-up the mass spectrometer with the instrument setting shown in Table 2016.05D (note settings may be different instruments other than the one described in this method).

Table 2016.05D. Mass spectrometer settings

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

(b) Compound specific parameters to be used are shown in Table 2016.05E and Table 2016.05F.

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Table 2016.05E. Compound parameters (vitamin D_2 instrument method only)

Vitamin D2 ion[a]	Precursor Ion	Product Ion	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 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 D_3 instrument method only)

Vitamin D3 ion[a]	Precursor Ion	Product Ion	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 D3-PTAD adduct
[b] DP = declustering potential
[c] EP = entrance potential
[d] CE = collision energy
[e] CXP = collision cell exit potential

J. Calculations

(a) Concentration of d6-vitamin D2 in Vitamin D2 Stable Isotope Labelled Stock Standard Solution:

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

where: $SILD_2SS_{D2conc}$ = conc. of SIL *d6*-vitamin D2 in stock standard (units: µg/mL)

$SILD_2SS_{abs(\lambda max)}$ = UV absorbance of stock standard at 265 nm (units: none)

$E^{1\%}_{1cm}$ = extinction coefficient for vitamin D_2 in ethanol [461] (units: dL/g.cm)

10000 = concentration conversion factor (units: g/dL to µg/mL)

(b) Concentration of *d6*-vitamin D*3* in Vitamin D3 Stable Isotope Labelled Stock Standard Solution:

$$SILD_3SS_{D2conc} = \frac{SILD_3SS_{abs(\lambda max)}}{E^{1\%}_{1cm}} \times 10000$$

where: $SILD_3SS_{D3conc}$ = conc. of SIL *d6*-vitamin D_3 in stock standard (units: µg/mL)

$SILD_3SS_{abs(\lambda\ max)}$ = UV absorbance of stock standard at 265 nm (units: none)

$E^{1\%}_{1cm}$ = extinction coefficient for vitamin D_3 in ethanol [485] (units: dL/g.cm)

10000 = concentration conversion factor (units: g/dL to µg/mL

(c) Concentration of *d6*-vitamin D_2 in Internal Standard:

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

where: $SILIS_{D2conc}$ = conc. of SIL *d6*-vitamin D_2 in internal standard (units: ng/mL)

$SILD_2SS_{D3conc}$ = conc.of SIL *d6*-vitamin D_2 in stock standard (units: µg/mL)

1000 = concentration conversion factor (units: µg/mL to ng/mL)

(d) Concentration of *d6*-vitamin D_3 in Internal Standard:

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

where: $SILIS_{D3conc}$ = conc. of SIL *d6*-vitamin D_3 in internal standard (units: ng/mL)

$SILD_3SS_{D3conc}$ = conc. of SIL *d6*-vitamin D_3 in stock standard (units: µg/mL)

1000 = concentration conversion factor (units: µg/mL to ng/mL)

(e) Concentration of non-labelled vitamin D_2 in Vitamin D_2 Non-Labelled Purity Standard Solution:

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

where: NLD_2PS_{D2conc} = conc. of vitamin D_2 in purity standard (units: µg/mL)

$NLD_2PS_{abs(\lambda\ max)}$ = UV absorbance of purity standard at 265 nm (units: none)

$E^{1\%}_{1cm}$ = extinction coefficient for vitamin D2 in ethanol [461] (units: dL/g.cm)

10000 = concentration conversion factor (units: g/dL to µg/mL)

(f) Concentration of non-labelled vitamin D_3 in Vitamin D_3 Non-Labelled Purity Standard Solution:

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

where: NLD_3PS_{D3conc} = conc. of vitamin D3 in purity standard (units: µg/mL)

NLD_3PS= UV absorbance of purity standard at 265 nm (units: none)

$E^{1\%}_{1cm}$ = extinction coefficient for vitamin D3 in ethanol [485] (units: dL/g.cm)

10000 = conc. conversion factor (units: g/dL to µg/mL)

(g) Concentration of non-labelled vitamin D2 in Non-Labelled Working Standard Solution:

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

where: $NLWS_{D2conc}$ = conc. of vitamin D_2 in working standard (units: ng/mL)

NLD_2PS_{D2conc} = conc. of vitamin D_2 in purity standard (units: µg/mL)

1000 = concentration conversion factor (units: µg/mL to ng/mL)

(h) Concentration of non-labelled vitamin D3 in Non-Labelled Working Standard Solution:

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

where: $NLWS_{D3conc}$ = conc. of vitamin D_3 in working standard (units: ng/mL)

NLD_3PS_{D3conc} = conc. of vitamin D_3 in purity standard (units: µg/mL)

1000 = concentration conversion factor (units: µg/mL to ng/mL)

(i) Concentration of vitamin D2 and vitamin D3 in Calibration Standards:

$$CS1_{D2conc} = NLWS_{D2conc} \times \frac{0.01}{25} \qquad CS1_{D3conc} = NLWS_{D3conc} \times \frac{0.01}{25}$$

$$CS2_{D2conc} = NLWS_{D2conc} \times \frac{0.05}{25} \qquad CS2_{D3conc} = NLWS_{D3conc} \times \frac{0.05}{25}$$

$$CS3_{D2conc} = NLWS_{D2conc} \times \frac{0.25}{25} \qquad CS3_{D3conc} = NLWS_{D3conc} \times \frac{0.25}{25}$$

$$CS4_{D2conc} = NLWS_{D2conc} \times \frac{0.5}{25} \qquad CS4_{D3conc} = NLWS_{D3conc} \times \frac{0.5}{25}$$

$$CS5_{D2conc} = NLWS_{D2conc} \times \frac{1.25}{25} \qquad CS5_{D3conc} = NLWS_{D3conc} \times \frac{1.25}{25}$$

where: $CS1\text{-}5_{D2conc}$ = conc. of vitamin D_2 in Calibration Standards (units: ng/mL)

$CS1\text{-}5_{D3conc}$ = conc. of vitamin D_3 in Calibration Standards (units: ng/mL)

$NLWS_{D2conc}$ = conc. of vitamin D_2 in Working Standard (units: ng/mL)

$NLWS_{D3conc}$ = conc. of vitamin D_3 in Working Standard (units: ng/mL)

(j) Concentration of stable isotope labelled vitamin D_3 in Calibration Standards:

$$CS1\text{-}5_{D2conc} = SILIS_{D2conc} \times \frac{0.25}{25} \qquad CS1\text{-}5_{D3conc} = SILIS_{D3conc} \times \frac{0.25}{25}$$

where: $CS1\text{-}5_{D2conc}$ = conc. of SIL *d6*-vitamin D_2 in Calibration Stds (units: ng/mL)

$CS1\text{-}5_{D3conc}$ = conc. of SIL d6-vitamin D_3 in Calibration Stds (units: ng/mL)

$SILIS_{D2conc}$ = conc. of SIL *d6*-vitamin D_2 in Internal Standard (units: ng/mL)

$SILIS_{D3conc}$ = conc. of SIL *d6*-vitamin D*3* in Internal Standard (units: ng/mL)

(k) Determine the linear regression curve y = mx + c (using the "least squares" method) for the ratio of peaks areas (non-labelled vitamin D/stable isotope labelled vitamin D) vs. the ratio of concentrations (non-labelled vitamin D/stable isotope labelled vitamin D) for five calibration standards with the y-intercept forced through zero.

(l) The concentration of vitamin D_2 in the sample is calculated as:

$$\text{Result } D_2 = \frac{PA_{NLD2}}{PA_{SILD2}} \times \frac{SILIS_{D2conc}}{L} \times \frac{SILIS_{vol}}{S_{mass}} \times \frac{100}{1000}$$

where: Result D_2 = vitamin D_2 concentration in sample (units: µg/hg)

PA_{NLD2} = peak area of vitamin D_2 in sample (units: none)

PA_{SILD2} = peak area of SIL *d6*-vitamin D_2 in sample (units: none)

$SILIS_{D2conc}$ = conc. of *d6*-vitamin D_2 in Internal Standard (units: ng/mL)

L = slope of calibration curve (units: none)

$SILIS_{vol}$ = volume of Internal Standard spiked to sample [0.5] (units: mL)

1000 = mass conversion factor (units: µg/g to mg/g)

100 = mass conversion factor (units: mg/g to mg/hg)

S_{mass} = mass of sample (units: g)

(m) The concentration of vitamin D_3 in the sample is calculated as:

$$\text{Result } D_3 = \frac{PA_{NLD3}}{PA_{SILD2}} \times \frac{SILIS_{D3conc}}{L} \times \frac{SILIS_{vol}}{S_{mass}} \times \frac{100}{1000}$$

where: Result D_3 = vitamin D_3 concentration in sample (units: µg/hg)

$PANL_{D3}$ = peak area of vitamin D_3 in sample (units: none)

$PASIL_{D3}$ = peak area of SIL *d6*-vitamin D_3 in sample (units: none)

$SILIS_{D3conc}$ = conc.of *d6*-vitamin D_3 in Internal Standard (units: ng/mL)

L = slope of calibration curve (units: none)

$SILIS_{vol}$ = volume of Internal Standard spiked to sample [0.5] (units: mL)

1000 = mass conversion factor (units: µg/g to mg/g)

100 = mass conversion factor (units: mg/g to mg/hg)

S_{mass} = mass of sample (units: g)

(n) The concentration of vitamin D3 in the sample is calculated as:

$$\text{Result (IU/hg)} = \text{Result (}\mu\text{g/hg)} \times 40$$

where: 40 = dietary conversion factor (units: µg/hg to IU/hg)

K. Data Handling

Report result as µg/hg to 1 decimal place or IU/hg to 0 decimal places.

L. References

(1) Gill, B.D.; Zhu, X.; Indyk, H.E. (2015) A rapid method for the determination of vitamin D3 in milk and infant formula by liquid chromatography/tandem mass spectrometry. *Journal of AOAC International* 98, 431–435.

(2) Gill, B.D.; Abernethy, G.A.; Green, R.J.; Indyk, H.E. (2016) Analysis of vitamin D2 and vitamin D3 in fortified milk powders and infant and nutritional formulas by liquid chromatography-tandem mass spectrometry: single laboratory validation, First Action 2016.05. *Journal of AOAC International* 99, 1321–1330.