



Analysis of Vitamin D₂ and Vitamin D₃ by LC-MS/MS in Milk Powders, Infant Formulas, and Adult Nutritionals

Brendon D. Gill^{1*}, Grant A. Abernethy², Rebecca J. Green¹,
and Harvey E. Indyk¹

¹ Fonterra Co-operative Group Ltd, P.O. Box 7, Waitoa, New Zealand

² Fonterra Research and Development Centre, Dairy Farm Rd, Palmerston North 4442, New Zealand

* Corresponding author

Stakeholder Panel on Dietary Supplements

Expert Review Panel for Nutrient Methods

Darryl Sullivan, Chair, Covance Labs

John Austad, Covance Labs

Sneh Bhandari, Silliker Labs

Esther Campos-Gimenez, Nestlé

Scott Christiansen Perrigo

Hans Crujisen, FrieslandCampina

Jon DeVries, General Mills/Medallion Labs

Brendon Gill, Fonterra

Don Gilliland, Abbott Nutrition

Min Huang, Frontage Labs

Estela Kneeteman, INTI

Adrienne McMahon, Nestlé

Bill Mindak, FDA

Maria Ofitserova, Pickering Lab

Melissa Phillips, NIST

Shay Phillips, Mead Johnson

Karen Schimpf, Abbott Nutrition

Guenther Raffler, CLF-Eurofins

Kate Rimmer, NIST

Wil van Loon, FrieslandCampina

Jinchuan Yang, Waters Corp.

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 D₂ and vitamin D₃ 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.

B. 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 C₁₈ core-shell, 2.6 µm, 2.1 × 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 \times 47 mm, nylon.
- (o) Cryogenic vials, 2 mL.
- (p) Schott bottles, 1 L, 100 mL.
- (q) HPLC vials, septa, and caps.

C. Reagents

- (a) Vitamin D₂ (ergocalciferol).—CAS No. 50-14-6, purity: $\geq 99\%$.
- (b) Vitamin D₃ (cholecalciferol).—CAS No. 67-97-0, purity: $\geq 99\%$.
- (c) *d6*-Vitamin D₂.—(26,26,26,27,27,27-*d6* ergocalciferol), CAS No. 1311259-89-8, enrichment: $\geq 99\%$, purity: $\geq 99\%$.
- (d) *d6*-Vitamin D₃.—(26,26,26,27,27,27-*d6* cholecalciferol), CAS No. 118584-54-6, enrichment: $\geq 99\%$, purity: $\geq 99\%$.
- (e) PTAD.—Reagent grade (store in desiccator at 2–8 °C).
- (f) Formic acid.—LC–MS grade.
- (g) Potassium hydroxide.—Reagent grade.
- (h) Magnesium chloride anhydrous.—Reagent grade.
- (i) Pyrogallol.—Reagent grade.
- (j) Ethanol.—LC grade.
- (k) Methanol.—LC–MS grade.
- (l) Isooctane (2,2,4-trimethylpentane).—LC grade.
- (m) Acetone.—LC grade.
- (n) Acetonitrile.—LC–MS grade.
- (o) Water.—Reagent grade ($\geq 18 \text{ M}\Omega$).

D. Reagent Preparation

- (a) Acetone (dry).—To a 100 mL Schott bottle, add 50 mL acetone, then add ~10 g magnesium chloride to remove traces of moisture. Cap the bottle and seal with parafilm and wait for the magnesium chloride to settle before use (~24 h). Expiry: 1 month.
- (b) PTAD solution (10 mg/mL).—To a 5 mL volumetric flask, add 50 mg PTAD, then add 4 mL dry acetone, and dissolve; dilute to volume with acetone. Expiry: 1 day.
- (c) Potassium hydroxide solution (50% w/v).—Dissolve 100 g potassium hydroxide in 200 mL water. Expiry: 1 month.

- (d) Ethanolic pyrogallol solution (1% w/v).—Dissolve 5 g pyrogallol in 500 mL ethanol. Expiry: 1 day.
- (e) Mobile phase A (formic acid; 0.1% v/v).—To 500 mL water, add 0.5 mL formic acid. Expiry: 1 week.
- (f) 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 D₃ is exclusively required for analysis, then standards pertaining to vitamin D₂ need not be used and vice versa.

- (a) Stable isotope-labelled vitamin D₂ or vitamin D₃ stock standard (SILD₂SS or SILD₃SS; ~10 µg/mL).—
 - (1) 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.
 - (2) Dissolve in ~90 mL ethanol. To promote dissolution, sonicate if necessary. Mix thoroughly; dilute to volume with ethanol.
 - (3) 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.
 - (4) Immediately dispense aliquots of SILD₂SS or SILD₃SS (~1.3 mL) into cryogenic vials and freeze at ≤ 15 °C.
- (b) Stable isotope-labelled internal standard (SILIS; ~1 µg/mL).—Make fresh daily.—
 - (1) 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.
 - (2) Pipet 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.
 - (3) Pool all 10 mL volumetric flasks together and mix thoroughly.
- (c) Non-labelled vitamin D₂ or vitamin D₃ stock standard (NLD₂SS or NLD₃SS; ~1 mg/mL).—
 - (1) Accurately weigh approximately 50 mg vitamin D₂ or vitamin D₃ into separate 50 mL volumetric flasks.
 - (2) 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.
- (d) Non-labelled vitamin D₂ or vitamin D₃ purity standard (NLD₂PS or NLD₃PS; ~10 µg/mL).—Make fresh daily.—

- (1) Pipet 1.0 mL NLD₂SS or NLD₃SS into separate 100 mL volumetric flasks. Dilute to volume with ethanol.
 - (2) 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.
- (e) Non-labelled working standard (NLWS; ~1 µg/mL).—Make fresh daily.—
- (1) Pipet 1.0 mL NLD₂PS and 1.0 mL NLD₃PS into a single 10 mL volumetric flask. Dilute to volume with acetonitrile.
- (f) Calibration standards (CS).—Make fresh daily. See Table 2016.05A for concentrations of the calibration standard solutions.—
- (1) Calibration standard 1 (CS1).—Pipet 10 µL NLWS and 250 µL SILIS into a 25 mL volumetric flask.
 - (2) Calibration standard 2 (CS2).—Pipet 50 µL NLWS and 250 µL SILIS into a 25 mL volumetric flask.
 - (3) Calibration standard 3 (CS3).—Pipet 250 µL NLWS and 250 µL SILIS into a 25 mL volumetric flask.
 - (4) Calibration standard 4 (CS4).—Pipet 500 µL NLWS and 250 µL SILIS into a 25 mL volumetric flask.
 - (5) Calibration standard 5 (CS5).—Pipet 1250 µL NLWS and 250 µL SILIS into a 25 mL volumetric flask.
 - (6) To each calibration standard, add 5 mL acetonitrile and 75 µL PTAD solution; shake to mix.
 - (7) Leave the calibration standards in the dark for 5 min.
 - (8) Add 6.25 mL water to each calibration standard and then dilute to volume with acetonitrile; shake to mix.
 - (9) Transfer ~1 mL of each calibration standard to an HPLC vial ready for analysis.

Table 2016.05A. Nominal concentrations of calibration standards

Calibration standard	Concentration, ng mL ⁻¹	
	Vitamin D	SIL <i>d6</i> -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.

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

G. Extraction and Derivatization

- (a) 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.
- (b) Add 2 mL potassium hydroxide solution to the boiling tube; cap and vortex mix.
- (c) Place the boiling tube in a water bath at 70 °C for 1 h; vortex mix every 15 min.
- (d) Place the boiling tube in a water bath at room temperature until cool.
- (e) Add 10 mL isooctane to the boiling tube; cap the boiling tube tightly and place on a horizontal shaker for 10 min.
- (f) Add 20 mL water to the boiling tube and invert the tube 10 times; place in a 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 pipet, taking care not to transfer any of the lower layer.
- (h) Add 5 mL water to the centrifuge tube; cap and vortex mix; then place in a centrifuge at 2000 × g for 5 min.
- (i) 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.

- (j) Add 75 μL PTAD solution to the centrifuge tube; cap and immediately vortex mix.
- (k) Allow to stand in the dark for 5 min to allow the derivatization reaction to complete.
- (l) Add 1 mL acetonitrile to the centrifuge tube; cap and vortex mix; then place in a centrifuge at $2000 \times g$ for 5 min.
- (m) Using a variable volume pipet, transfer 500 μL lower layer into an Eppendorf vial, taking care not to transfer any of the upper layer.
- (n) Add 167 μL water to the Eppendorf vial; cap and vortex mix.
- (o) Using a syringe filter, transfer an aliquot from the Eppendorf vial to an amber HPLC vial; then cap.

H. Chromatography

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

I. Mass Spectrometry

- (a) Set up the mass spectrometer with the instrument settings in Table 2016.05D.
- (b) The specific compound parameters to be used are listed in Tables 2016.05E and 2016.05F

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	Mobile phase composition	
		% 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

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.

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			21	18	120
Analyte qualifier	560.2	280.0	151	10	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

(a) Concentration of stable isotope-labelled vitamin D₂ in the stock standard, SILD₂SS.—

$$\text{SILD}_2\text{SS}_{\text{D2concn}} = \frac{\text{SILD}_2\text{SS}_{\text{abs}(\lambda, \text{max})}}{E_{1\text{cm}}^{1\%}} \times 10000$$

where

SILD₂SS_{D2concn} is the concentration of *d6*-vitamin D₂ in the stock standard (µg/mL),

SILD₂SS_{abs(λ max)} is the UV absorbance of the stock standard at 265 nm (cm⁻¹),

E₁^{1%} is the extinction coefficient for vitamin D₂ in ethanol (461 dL/g.cm),

and 10 000 is the concentration conversion factor (g/dL to µg/mL).

- (b) Concentration of stable isotope-labelled vitamin D₃ in the stock standard, SILD₃SS.—

$$\text{SILD}_3\text{SS}_{\text{D3concn}} = \frac{\text{SILD}_3\text{SS}_{\text{abs}(\lambda \text{ max})}}{E_1^{1\%}} \times 10000$$

where

SILD₃SS_{D3concn} is the concentration of *d6*-vitamin D₃ in the stock standard (µg/mL),

SILD₃SS_{abs(λ max)} is the UV absorbance of the stock standard at 265 nm (cm⁻¹),

E₁^{1%} is the extinction coefficient for vitamin D₃ in ethanol (485 dL/g.cm),

and 10 000 is the concentration conversion factor (g/dL to µg/mL).

- (c) Concentration of stable isotope-labelled vitamin D₂ in the internal standard, SILIS.—

$$\text{SILIS}_{\text{D2concn}} = \text{SILD}_2\text{SS}_{\text{D2concn}} \times \frac{1.0}{10} \times 1000$$

where

SILIS_{D2concn} is the concentration of *d6*-vitamin D₂ in the internal standard (ng/mL),

SILD₂SS_{D2concn} is the concentration of *d6*-vitamin D₂ in the stock standard (µg/mL),

and 1000 is the concentration conversion factor (µg/mL to ng/mL).

- (d) Concentration of stable isotope-labelled vitamin D₃ in the internal standard, SILIS.—

$$\text{SILIS}_{\text{D3concn}} = \text{SILD}_3\text{SS}_{\text{D3concn}} \times \frac{1.0}{10} \times 1000$$

where

SILIS_{D3concn} is the concentration of *d6*-vitamin D₃ in the internal standard (ng/mL),

SILD₃SS_{D3concn} is the concentration of *d6*-vitamin D₃ in the stock standard (µg/mL),

and 1000 is the concentration conversion factor (µg/mL to ng/mL).

- (e) Concentration of non-labelled vitamin D₂ in purity standard NLD2PS.—

$$\text{NLD}_2\text{PS}_{\text{D2concn}} = \frac{\text{NLD}_2\text{PS}_{\text{abs}(\lambda \text{ max})}}{E_1^{1\%}} \times 10000$$

where

NLD₂PS_{D2concn} is the concentration of vitamin D₂ in the purity standard (µg/mL),

NLD₂PS_{abs(λ max)} is the UV absorbance of the purity standard at 265 nm (cm⁻¹),

E₁^{1%} is the extinction coefficient for vitamin D₂ in ethanol (461 dL/g.cm),

and 10 000 is the concentration conversion factor (g/dL to µg/mL).

- (f) Concentration of non-labelled vitamin D₃ in the purity standard, NLD₃PS.—

$$\text{NLD}_3\text{PS}_{\text{D3concn}} = \frac{\text{NLD}_3\text{PS}_{\text{abs}(\lambda \text{ max})}}{E_{1\text{ cm}}^{1\%}} \times 10000$$

where

NLD₃PS_{D3concn} is the concentration of vitamin D₃ in the purity standard (µg/mL),

NLD₃PS_{abs(λ max)} is the UV absorbance of the purity standard at 265 nm (cm⁻¹),

E^{1%} is the extinction coefficient for vitamin D₃ in ethanol (485 dL/g.cm),

and 10 000 is the concentration conversion factor (g/dL to µg/mL).

- (g) Concentration of non-labelled vitamin D₂ in the working standard, NLWS.—

$$\text{NLWS}_{\text{D2concn}} = \text{NLD}_2\text{PS}_{\text{D2concn}} \times \frac{1.0}{10} \times 1000$$

where

NLWS_{D2concn} is the concentration of vitamin D₂ in the working standard (ng/mL),

NLD₂PS_{D2concn} is the concentration of vitamin D₂ in the purity standard (µg/mL),

and 1000 is the concentration conversion factor (µg/mL to ng/mL).

- (h) Concentration of non-labelled vitamin D₃ in the working standard NLWS.—

$$\text{NLWS}_{\text{D3concn}} = \text{NLD}_3\text{PS}_{\text{D3concn}} \times \frac{1.0}{10} \times 1000$$

where

NLWS_{D3concn} is the concentration of vitamin D₃ in working standard (ng/mL),

NLD₃PS_{D3concn} is the concentration of vitamin D₃ in purity standard (µg/mL),

and 1000 is the concentration conversion factor (µg/mL to ng/mL).

- (i) Concentrations of vitamin D₂ and vitamin D₃ in calibration standards, CS1–CS5.—

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

$$\text{CS2}_{\text{Deconcn}} = \text{NLWS}_{\text{Deconcn}} \times \frac{0.05}{25}$$

$$\text{CS3}_{\text{Deconcn}} = \text{NLWS}_{\text{Deconcn}} \times \frac{0.25}{25}$$

$$\text{CS4}_{\text{Deconcn}} = \text{NLWS}_{\text{Deconcn}} \times \frac{0.5}{25}$$

$$\text{CS5}_{\text{Deconcn}} = \text{NLWS}_{\text{Deconcn}} \times \frac{1.25}{25}$$

where

CS1 through CS5_{Dconcn} are the concentrations of vitamin D₂ or vitamin D₃ in the calibration standards (ng/mL),

and NLWS_{Dconcn} is the concentration of vitamin D₂ or vitamin D₃ in the working standard (ng/mL).

- (j) Concentrations of stable isotope-labelled *d6*-vitamin D₂ and *d6*-vitamin D₃ in the calibration standards, CS1–CS5.—

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

where

CS1 through CS5_{Dconcn} are the concentrations of *d6*-vitamin D₂ or *d6*-vitamin D₃ in calibration standards (ng/mL),

and SILIS_{Dconcn} is the concentration of *d6*-vitamin D₂ or *d6*-vitamin D₃ in internal standard (ng/mL).

- (k) Mass of powder in slurried sample.—

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

where

S_{mass} is the mass of the sample (g),

D_{mass} is the mass of the dry powder used to make the slurry (g),

W_{mass} is the mass of the water used to make the slurry (g),

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

- (l) Determine the linear regression curves (vitamin D₂ and vitamin D₃) $y = mx + c$ (using the least-squares method) for the ratio of peak areas (non-labelled vitamin D/stable isotope-labelled *d6*-vitamin D) versus the ratio of concentrations (non-labelled vitamin D/stable isotope-labelled *d6*-vitamin D) for the five calibration standards, with the y-intercept forced through zero.
- (m) The concentration (w/w) of vitamin D₂ or vitamin D₃ in the dry powders is calculated as

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

where

Result D is the vitamin D₂ or vitamin D₃ concentration in the sample (µg/hg),

PA_{NLD} is the peak area of vitamin D₂ or vitamin D₃ in the sample,

PA_{SILD} is the peak area of *d6*-vitamin D₂ or *d6*-vitamin D₃ in the sample,

SILIS_{Dconcn} is the concentration of *d6*-vitamin D₂ or *d6*-vitamin D₃ in the SILIS (ng/mL),

L is the slope of the calibration curve,

SILIS_{alqt} is the volume of the SILIS aliquot spiked into the sample (0.5 mL),

S_{mass} is the mass of the sample (g),

1000 is the mass conversion factor (ng/g to µg/g),

and 100 is the mass conversion factor ($\mu\text{g/g}$ to $\mu\text{g/hg}$).

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

$$\text{Result D} = \frac{\text{PA}_{\text{NLD}}}{\text{PA}_{\text{SILD}}} \times \frac{\text{SILIS}_{\text{Dconc}}}{L} \times \frac{\text{SILIS}_{\text{alqt}}}{S_{\text{vol}}} \times \frac{100}{1000}$$

where

Result D is the vitamin D₂ or vitamin D₃ concentration in the sample ($\mu\text{g/dL}$),

PA_{NLD} is the peak area of vitamin D₂ or vitamin D₃ in the sample,

PA_{SILD} is the peak area of *d6*-vitamin D₂ or *d6*-vitamin D₃ in the sample,

SILIS_{Dconc} is the concentration of *d6*-vitamin D₂ or *d6*-vitamin D₃ in the SILIS (ng/mL),

L is the slope of the calibration curve,

SILIS_{alqt} is the volume of the SILIS aliquot spiked into the sample (0.5 mL),

S_{vol} is the volume of the sample (g),

1000 is the mass conversion factor (ng/g to $\mu\text{g/g}$),

and 100 is the mass conversion factor ($\mu\text{g/g}$ to $\mu\text{g/hg}$).

- (o) The concentration of vitamin D₂ or vitamin D₃ as IU/hg in the sample is calculated as

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

where

40 is the dietary conversion factor ($\mu\text{g/hg}$ to IU/hg).

K. Data Handling

Report results as $\mu\text{g/hg}$ to one decimal place or as IU/hg to zero decimal places.