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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 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 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 D_2 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 C_{18} 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 × 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 D_2 (ergocalciferol).—CAS No. 50-14-6, purity: ≥ 99%.

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

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

(d) *d6*-Vitamin D3.—(26,26,26,27,27,27-*d6* cholecalciferol), CAS No. 118584-54-6, enrichment: ≥ 99%, purity: ≥ 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 (≥ 18 MΩ).

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 D_3 is exclusively required for analysis, then standards pertaining to vitamin D_2 need not be used and vice versa.

(a) Stable isotope-labelled vitamin D_2 or vitamin D_3 stock standard ($SILD_2SS$ or $SILD_3SS$; ~10 µg/mL).—

(1) 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.

(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 $SILD_2SS$ or $SILD_3SS$ at 265 nm. The spectrophotometer should be zeroed against an ethanol blank solution. Calculate and record the concentration.

(4) Immediately dispense aliquots of $SILD_2SS$ or $SILD_3SS$ (~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 $SILD_2SS$ and one vial of $SILD_3SS$ from the freezer and allow to warm to room temperature.

(2) Pipet 1.0 mL each of $SILD_2SS$ and $SILD_3SS$ 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 D_2 or vitamin D_3 stock standard (NLD_2SS or NLD_3SS; ~1 mg/mL).—

(1) Accurately weigh approximately 50 mg vitamin D_2 or vitamin D_3 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 D_2 or vitamin D_3 purity standard (NLD_2PS or NLD_3PS; ~10 µg/mL).—Make fresh daily.—

(1) Pipet 1.0 mL NLD_2SS or NLD_3SS 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 NLD_2PS and 1.0 mL NLD_3PS 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 *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.

(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 weigh19.0–21.0 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 × 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	N2
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 2016.05F Compound parameters (vitamin D_3 instrument method only)

Vitamin D_3 ion[a]	Precursor ion, m/z	Product ion, m/z	DPb, V	EPc, V	CEd, V	CXPe, 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_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

J. Calculations

(a) Concentration of stable isotope-labelled vitamin D_2 in the stock standard, $SILD_2SS$.—

$$SILD_2SS_{D2concn} = \frac{SILD_2SS_{abs(\lambda\ max)}}{E^{1\%}_{1\ cm}} \times 10000$$

where

$SILD_2SS_{D2concn}$ is the concentration of *d6*-vitamin D_2 in the stock standard (µg/mL),

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

E1% is the extinction coefficient for vitamin D_2 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 D_3 in the stock standard, $SILD_3SS$.—

$$SILD_3SS_{D3concn} = \frac{SILD_3SS_{abs(\lambda\ max)}}{E_{1\ cm}^{1\%}} \times 10000$$

where

$SILD_3SS_{D3concn}$ is the concentration of *d6*-vitamin D_3 in the stock standard (µg/mL),

$SILD_3SS_{abs(\lambda\ max)}$ is the UV absorbance of the stock standard at 265 nm (cm^{-1}),

$E^{1\%}$ is the extinction coefficient for vitamin D_3 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 D_2 in the internal standard, SILIS.—

$$SILIS_{D2concn} = SILD_2SS_{D2concn} \times \frac{1.0}{10} \times 1000$$

where

$SILIS_{D2concn}$ is the concentration of *d6*-vitamin D_2 in the internal standard (ng/mL),

$SILD_2SS_{D2concn}$ is the concentration of *d6*-vitamin D_2 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 D_3 in the internal standard, SILIS.—

$$SILIS_{D3concn} = SILD_3SS_{D3concn} \times \frac{1.0}{10} \times 1000$$

where

$SILIS_{D3concn}$ is the concentration of *d6*-vitamin D_3 in the internal standard (ng/mL),

$SILD_3SS_{D3concn}$ is the concentration of *d6*-vitamin D_3 in the stock standard (µg/mL),

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

(e) Concentration of non-labelled vitamin D_2 in purity standard NLD2PS.—

$$NLD_2PS_{D2concn} = \frac{NLD_2PS_{abs(\lambda\ max)}}{E_{1\ cm}^{1\%}} \times 10000$$

where

$NLD_2PS_{D2concn}$ is the concentration of vitamin D2 in the purity standard (µg/mL),

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

$E^{1\%}$ is the extinction coefficient for vitamin D_2 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 D_3 in the purity standard, NLD_3PS.—

$$NLD_3PS_{D3concn} = \frac{NLD_3PS_{abs(\lambda\ max)}}{E^{1\%}_{1\ cm}} \times 10000$$

where

$NLD_3PS_{D3concn}$ is the concentration of vitamin D_3 in the purity standard (µg/mL),

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

$E^{1\%}$ is the extinction coefficient for vitamin D_3 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 D_2 in the working standard, NLWS.—

$$NLWS_{D2concn} = NLD_2PS_{D2concn} \times \frac{1.0}{10} \times 1000$$

where

$NLWS_{D2concn}$ is the concentration of vitamin D_2 in the working standard (ng/mL),

$NLD_2PS_{D2concn}$ is the concentration of vitamin D_2 in the purity standard (µg/mL),

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

(h) Concentration of non-labelled vitamin D_3 in the working standard NLWS.—

$$NLWS_{D3concn} = NLD_3PS_{D3concn} \times \frac{1.0}{10} \times 1000$$

where

$NLWS_{D3concn}$ is the concentration of vitamin D_3 in working standard (ng/mL),

$NLD_3PS_{D3concn}$ is the concentration of vitamin D_3 in purity standard (µg/mL),

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

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

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

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

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

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

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

where

$CS1$ through $CS5_{Dconcn}$ are the concentrations of vitamin D_2 or vitamin D_3 in the calibration standards (ng/mL),

and $NLWS_{Dconcn}$ is the concentration of vitamin D_2 or vitamin D_3 in the working standard (ng/mL).

(j) Concentrations of stable isotope-labelled *d6*-vitamin D_2 and *d6*-vitamin D_3 in the calibration standards, CS1–CS5.—

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

where

$CS1$ through $CS5_{Dconcn}$ are the concentrations of *d6*-vitamin D_2 or *d6*-vitamin D_3 in calibration standards (ng/mL),

and $SILIS_{Dconcn}$ is the concentration of *d6*-vitamin D_2 or *d6*-vitamin D_3 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),

Dmass 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 D_2 and vitamin D_3) $y = mx + c$ (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 D_2 or vitamin D_3 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 D_2 or vitamin D_3 concentration in the sample (µg/hg),

PA_{NLD} is the peak area of vitamin D_2 or vitamin D_3 in the sample,

PA_{SILD} is the peak area of *d6*-vitamin D_2 or *d6*-vitamin D_3 in the sample,

$SILIS_{Dconcn}$ is the concentration of *d6*-vitamin D_2 or *d6*-vitamin D_2 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 (µg/g to µg/hg).

(n) The concentration (w/v) of vitamin D_2 or vitamin D_3 in ready-to-feed (RTF) liquids is calculated as

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

where

Result D is the vitamin D_2 or vitamin D_3 concentration in the sample (µg/dL),

PA_{NLD} is the peak area of vitamin D_2 or vitamin D_3 in the sample,

PA_{SILD} is the peak area of *d6*-vitamin D_2 or *d6*-vitamin D_3 in the sample,

$SILIS_{Dconcn}$ is the concentration of *d6*-vitamin D_2 or *d6*-vitamin D_2 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 µg/g),

and 100 is the mass conversion factor (µg/g to µg/hg).

(o) The concentration of vitamin D_2 or vitamin D_3 as IU/hg in the sample is calculated as

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

where

40 is the dietary conversion factor (µg/hg to IU/hg).

K. Data Handling

Report results as µg/hg to one decimal place or as IU/hg to zero decimal places.