AOAC Official Method 2011.11
Vitamin D in Infant Formula and Adult/Pediatric Nutritional Formula

Ultra-High-Performance Liquid Chromatography/ Tandem Mass Spectrometry
First Action 2011

(Applicable to the measurement of total vitamin D by UHPLC-MS/MS in infant formula and adult/pediatric nutritional formula.)

Caution: Refer to Material Safety Data Sheets (MSDS) for safety precautions when using chemicals. Use personal protective equipment recommended in MSDS.

A. Principle

Test samples are saponified, extracted, and the solvent evaporated. Vitamin D is determined by UHPLC-MS/MS in reconstituted extracts.

B. Apparatus

(**a**) *MS/MS system.*—MDS SCIEX API, 4000 QTRAP (Applied Biosystems, Concord, Ontario, Canada) or equivalent, atmospheric pressure chemical ionization (APCI) positive ion mode.

(**b**) *UHPLC system.*—Shimadzu LC30AD (Kyoto, Japan) or equivalent.

(**c**) *Hypersil GOLD and GOLD aQ columns.*—100 × 2.1 mm, 1.9 µ particle size (Thermo Scientific, Madison, WI).

C. Chemicals and Reagents

(**a**) *Reagent alcohol.*—Sigma-Aldrich (St. Louis, MO).

(**b**) *Acetonitrile.*—HPLC grade (Burdick & Jackson, Muskegon, MI).

(**c**) *Potassium hydroxide.*—ACS grade (Fisher Scientific, Fairlawn, NJ).

(**d**) *Methanol.*—HPLC grade (Fisher Scientific).

(**e**) *Hexane.*—HPLC grade (Sigma-Aldrich).

(**f**) *Formic acid.*—>95% (Sigma-Aldrich).

(**g**) *Pyrogallic acid.*—ACS grade (J.T. Baker, Phillipsburg, NJ).

(**h**) *Butylated hydroxytoluene (BHT).*—99.8% (ACROS Organics, Morris Plains, NJ).

(**i**) *Water.*—HPLC grade.

D. Reference Standards

(**a**) *Cholecalciferol (vitamin D_3).*—100% (U.S. Pharmacopeial Convention, Rockville, MD).

(**b**) *Ergocalciferol (vitamin D_2).*—100% (U.S. Pharmacopeial Convention).

(**c**) *Isotope vitamin D_2—[$^2H_3$].*—1 mg/mL (IsoScience, King of Prussia, PA).

Table 2011.11B. Parameters for APCI

Nebulizer current	5.0 µA
Temperature	320°C
Ion source gas	50 psi
Collision gas	Medium
Curtain gas	15 psi

(**d**) *Isotope vitamin D_3—[$^2H_3$].*—1 mg/mL (IsoScience).

E. Procedure

(**a**) *Standard solutions preparation.*—(*Notes*: Stock, intermediate, and working standards are stable for 2 months when stored in a freezer set to maintain –20 ± 10°C. Protect standard solutions from actinic light. Calculate standard concentrations in IU/mL.)

(*1*) *Preparation of vitamin D stock solutions.*—(*a*) *Vitamin D_2 stock standard (~12 000 IU/mL).*—Weigh approximately 30 mg vitamin D_2 into a 100 mL volumetric flask. Dilute to volume with hexane.

(*b*) *Vitamin D_3 stock standard (~12 000 IU/mL).*—Weigh approximately 30 mg vitamin D_3 into a 100 mL volumetric flask. Dilute to volume with hexane.

(*c*) *Isotopic vitamin D_3 and/or D_2 stock internal standards (~1600 IU/mL).*—The isotopic vitamin D_3 is supplied in degassed ethanol. Quantitatively transfer the appropriate amount into a 25 mL volumetric flask for a concentration of ~1600 IU/mL.

(*2*) *Intermediate standard solutions.*—(*a*) *Vitamin D_2/ vitamin D_3 intermediate standard (1000 IU/mL).*—Pipet an appropriate amount of vitamin D_2 and/or D_3 stock standard into the same volumetric flask to achieve a final diluted concentration of approximately 1000 IU/mL for each analyte. Evaporate solution under N_2 to near dryness and dilute to volume with ACN.

(*b*) *Vitamin D_2 and vitamin D_3 standard solution (100 IU/mL).*—Pipet an appropriate amount of vitamin D_2/vitamin D_3 intermediate standard into a volumetric flask to achieve a final diluted concentration of approximately 100 IU/mL for each analyte. Dilute to volume with ACN.

(*c*) *Isotope vitamin D_3 internal standard solution (ID_3 and/or ID_2; 100 IU/mL).*—Pipet an appropriate amount of isotopic vitamin D_3 and/or D_2 stock internal standard to achieve a final diluted concentration of approximately 100 IU/mL. The final concentration of internal standard should provide an adequate response of approximately 20 000 peak height units, dependant on instrument.

(*d*) *Dilution solvent.*—Pipet 2.0 mL of the 100 IU/mL ID_3 solution into a glass vial and dilute with 18 mL ACN. Cap and mix

Table 2011.11A. Preparation of working standard solutions

Standard solution	Concentration, IU/mL	ID_3 (100 IU/mL), mL	D_2/D_3 (100 IU/mL), mL	ACN	STD3, mL	STD4, mL	Dilution solvent, mL
STD1	200	1.00	2.0[a]	7.00	—	—	—
STD2	50	1.00	5.0	4.00	—	—	—
STD3	10	1.00	1.0	8.00	—	—	—
STD4	1	—	—	—	1.00	—	9.00
STD5	0.2	—	—	—	—	2.00	8.00

[a] Use vitamin D_2 and D_3 intermediate stock solutions (1000 IU/mL).

Table 2011.11C. UHPLC gradient elution program

Time, min	Flow rate, mL/min	Phase B, %
0	0.25	60
0.4	0.25	90
0.7	0.25	100
5.55	0.25	100
5.56	0.5	100
8.50	0.5	100
8.51	0.5	60
9.3	0.5	60
9.31	0.25	60
10.0	0.25	60

well. Prepare fresh before use.

(*3*) *Working standard solutions*.—Prepare the working standard solutions according to Table **2011.11A**. *See* Table **2011.11B** for APCI parameters and Table **2011.11C** for UHPLC gradient elution program.

(**b**) *Sample preparation.*—(*1*) *Saponification*.—(*a*) Weigh 1 to 10 g or 30 g of a reconstitution or ready-to-feed in an Erlenmeyer flask, depending on the vitamin D concentration in the samples.

(*b*) Add 40 mL reagent alcohol with 2% pyrogallic acid, 0.6 mL 100 IU/mL isotope D_3 internal standard, and 20 mL KOH (50%).

(*c*) Set for overnight saponification at 25°C with magnetic stirring after removing air with nitrogen flow.

(*d*) Transfer to a separatory funnel and extract with 30 mL hexane containing 12.5 mg/L BHT. Shake for approximately 1 min. Allow phase separation of two layers to occur and drain off lower layer. Add approximately 20 mL washing solvent (85% water/15% ROH) to the separatory funnel and shake for approximately 5 s. Allow phase separation of two layers to occur and drain off lower layer. Dry down approximately 10 mL extract for reconstitution in 1 mL acetonitrile–water (70 + 30, v/v) with 5 min sonication.

(*e*) Filter sample solution through a 0.45 µm PTFE membrane before injection.

(**c**) *Instrument parameters (see Table* ***2011.11D****)*.—Mobile phases A and B were methanol–water (20 + 80, v/v/v) and methanol (100%), respectively, with both mobile phases including 0.1% formic acid. Other parameters include mobile phase flow rate, 0.25 to 0.50 mL/min; column temperature, 29°C; sample injection volume, 5 µL; collision energy, 21 V; ion gas pressure, 50 psi; ionization temperature, 320°C.

F. Calculations

The vitamin D_3 standard linear curve with a 1/x weighting is calculated using the total area of the vitamin D_3 and the previtamin D_3 (if present) with the total area of the isotope vitamin D_3 and the isotope previtamin D_3 as the internal standard.

The sample vitamin D_3 concentration is calculated from the above curve using the sample total area of the isotope vitamin D_3 and the isotope previtamin D_3 as the sample internal standard. The sample previtamin D_3 concentration is calculated from the same curve and the same total area of the isotope vitamin D_3 and the isotope previtamin D_3 as the sample internal standard.

$$\frac{[SCVitD3(IU/mL)+SCPreD3(IU/mL)]\times ISS(mL)\times CISS(IU/mL)}{weight(g)\times FCIS(IU/mL)}\times 100$$

$$= IU/100g$$

where SCVitD3 = sample concentration vitamin D_3, IU/mL, from linear curve; SCPreD3 = sample concentration previtamin D_3, IU/mL, from linear curve; ISS = amount of internal standard spiked in sample, mL; CISS = concentration of internal standard spiked in sample, IU/mL; weight = sample size taken, g; and FCIS = final concentration of internal standard in working standards, IU/mL.

Vitamin D_2 is calculated in the same way.

Reference: *J. AOAC Int*. **95**, 319(2012); AOAC SMPR 2011.004, *J. AOAC Int*. **95**, 292(2012)

Table 2011.11D. Parameters for MS/MS measurement

Analyte	Q1[a], amu	Q3[b], amu	DP[c]	EP[d]	CE[e]	CXP[f]
D_3	385.4	259.1	55.0	14.5	21.0	17.3
D_3	385.4	107.1	55.0	14.5	31.9	5.20
D_3	385.4	159.1	55.0	14.5	32.7	9.10
D_2	397.4	125.1	43.0	9.5	19.1	6.9
D_2	397.4	107.0	43.0	4.5	36.0	19.7
D_2	397.4	271.1	43.0	9.0	17.3	18.6
Isotope D_3	388.4	259.1	55.0	14.5	21.0	19.1

[a] Q1 = Quadrupole mass filter 1.

[b] Q3 = Quadrupole mass filter 3.

[c] DP = Declustering potential.

[d] EP = Entrance potential.

[e] CE = Collision energy.

[f] CXP = Collision cell exit potential.