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Infant formula and adult nutritionals — Determination of vitamin D by liquid chromatography- mass spectrometry

*Formules infantiles et produits nutritionnels pour adultes —
Détermination de la teneur en vitamine D par chromatographie
liquide couplée à la spectrométrie de masse*



Reference number
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ISO 20636:2018(E)**Foreword**

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 34, *Food products*, in collaboration with AOAC INTERNATIONAL. It is being published by ISO and separately by AOAC INTERNATIONAL. The method described in this document is equivalent to the AOAC Official Method 2016.05, *Analysis of Vitamin D₂ and Vitamin D₃ in Fortified Milk Powders, Infant Formulas, and Adult/Pediatric Nutritional Formulas*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Infant formula and adult nutritionals — Determination of vitamin D by liquid chromatography-mass spectrometry

WARNING — The use of this method can involve hazardous materials, operations and equipment. This method does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this method to establish appropriate safety and health practices.

1 Scope

This document specifies a method for the quantitative determination of vitamin D₂ and/or vitamin D₃ in infant formula, and adult nutritionals in solid (i.e. powders) or liquid (i.e. ready-to-feed liquids and liquid concentrates) forms using liquid chromatography-mass spectrometry. The application range runs from 0,15 µg/100 g (limit of quantification) to 59 µg/100 g for vitamin D₂ and from 0,25 µg/100 g to 65 µg/100 g for vitamin D₃.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <http://www.electropedia.org/>

3.1

adult nutritional

nutritionally complete, specially formulated food, consumed in liquid form, which may constitute the sole source of nourishment, made from any combination of milk, soy, rice, whey, hydrolysed protein, starch and amino acids, with and without intact protein

3.2

infant formula

breast-milk substitute specially manufactured to satisfy, by itself, the nutritional requirements of infants during the first months of life up to the introduction of appropriate complementary feeding

[SOURCE: CODEX STAN 72-1981]

4 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 (PTAD) is added to derivatise 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 reversed-phase liquid chromatography. Detection is by mass spectrometry using multiple reaction monitoring (MRM). Stable isotope labelled *d*6-vitamin D₂ and *d*6-vitamin D₃ internal standards are used for quantitation to correct for losses in extraction and any variation in derivatisation and ionisation efficiencies^[2].

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5 Reagents and materials

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and distilled or demineralized water or water of equivalent purity.

5.1 General

- 5.1.1 **Standards**, ≥ 99 % pure.
- 5.1.2 **Vitamin D₂**, ergocalciferol.
- 5.1.3 **Vitamin D₃**, cholecalciferol.
- 5.1.4 **d6-Vitamin D₂**, 26,26,26,27,27,27-d6 ergocalciferol.
- 5.1.5 **d6-Vitamin D₃**, 26,26,26,27,27,27-d6 cholecalciferol.
- 5.1.6 **PTAD** (4-phenyl-1,2,4-triazoline-3,5-dione).
- 5.1.7 **Formic acid** (HCO₂H), LC-MS grade.
- 5.1.8 **Potassium hydroxide** (KOH).
- 5.1.9 **Pyrogallol** (C₆H₃(OH)₃).
- 5.1.10 **Ethanol** (C₂H₅OH).
- 5.1.11 **Methanol** (CH₃OH), LC-MS grade.
- 5.1.12 **Isooctane** ((CH₃)₃CCH₂CH(CH₃)₂).
- 5.1.13 **Acetone** (CH₃COCH₃).
- 5.1.14 **Acetonitrile** (CH₃CN), LC-MS grade.

5.2 Reagent preparation

- 5.2.1 **PTAD solution**, $c(4\text{-phenyl-1,2,4-triazoline-3,5-dione}) = 10$ mg/ml. Dissolve 50 mg PTAD (5.1.6) in 5,0 ml acetone (5.1.13).
- 5.2.2 **Potassium hydroxide solution**, $c(\text{KOH}) = 8,9$ mol/l. Dissolve 100 g potassium hydroxide (5.1.8) in 200 ml water.
- 5.2.3 **Ethanolic pyrogallol solution**, $c(\text{C}_6\text{H}_3(\text{OH})_3) = 0,079$ mol/l. Dissolve 5 g pyrogallol (5.1.9) in 500 ml ethanol (5.1.10).
- 5.2.4 **Mobile phase A**, $c(\text{HCO}_2\text{H}) = 0,0265$ mol/l. To 500 ml of water, add 0,5 ml formic acid (5.1.7).
- 5.2.5 **Mobile phase B**, methanol, 500 ml (5.1.11).

5.3 Standard preparation

5.3.1 Vitamin D is sensitive to light. Perform all steps under low-level incandescent lighting. If exclusively vitamin D₃ is required for analysis, then standards pertaining to vitamin D₂ need not be used and vice versa. Calibration standards should be bracketed at the beginning and at the end of an analytical run.

5.3.2 Vitamin D₂ stable isotope labelled stock standard solution, $\rho \approx 10 \mu\text{g/ml}$. Dispense the contents of a 1 mg vial of *d6*-vitamin D₂ (5.1.4) into a 100 ml volumetric flask. Dissolve in 90 ml of ethanol (5.1.10). To promote dissolution, sonicate if necessary. Mix thoroughly, make up to volume with ethanol (5.1.10). Measure the absorbance of an aliquot at 265 nm. The spectrophotometer should be zeroed against an ethanol (5.1.10) blank solution. Calculate and record concentration. Immediately dispense aliquots (~1,3 ml) into cryogenic vials and freeze at $< -15 \text{ }^\circ\text{C}$ for up to 6 months.

5.3.3 Vitamin D₃ stable isotope labelled stock standard solution, $\rho \approx 10 \mu\text{g/ml}$. Dispense the contents of a 1 mg vial of *d6*-vitamin D₃ (5.1.5) into a 100 ml volumetric flask. Dissolve in 90 ml of ethanol (5.1.10). To promote dissolution, sonicate if necessary. Mix thoroughly, make up to volume with ethanol (5.1.10). Measure the absorbance of an aliquot at 265 nm. The spectrophotometer should be zeroed against an ethanol (5.1.10) blank solution. Calculate and record concentration. Immediately dispense aliquots (~1,3 ml) into cryogenic vials and freeze at $< -15 \text{ }^\circ\text{C}$ for up to 6 months.

5.3.4 Stable isotope labelled internal standard solution, $\rho \approx 1 \mu\text{g/ml}$. Depending on the number of samples that need to be analysed 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 D₂ stable isotope labelled stock standard solution (5.3.2) and/or 1 vial of Vitamin D₃ stable isotope labelled stock standard solution (5.3.3) from the freezer and allow to warm to room temperature. Pipette 1,0 ml of vitamin D₂ stable isotope labelled stock standard solution (5.3.2) and/or 1,0 ml of vitamin D₃ stable isotope labelled stock standard solution (5.3.3) 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.

5.3.5 Vitamin D₂ non-labelled stock standard solution, $\rho \approx 1 \text{ mg/ml}$. Weigh accurately, approximately 50 mg of vitamin D₂ (5.1.2) into a 50 ml volumetric flask. Dissolve in 40 ml of ethanol (5.1.10). To promote dissolution, sonicate if necessary. Mix thoroughly, make up to volume with ethanol (5.1.10). Store in freezer at $< -15 \text{ }^\circ\text{C}$ for up to 1 month.

5.3.6 Vitamin D₃ non-labelled stock standard solution, $\rho \approx 1 \text{ mg/ml}$. Weigh accurately, approximately 50 mg of vitamin D₃ (5.1.3) into a 50 ml volumetric flask. Dissolve in 40 ml of ethanol (5.1.10). To promote dissolution, sonicate if necessary. Mix thoroughly, make up to volume with ethanol (5.1.10). Store in freezer at $< -15 \text{ }^\circ\text{C}$ for up to 1 month.

5.3.7 Vitamin D₂ non-labelled purity standard solution, $\rho \approx 10 \mu\text{g/ml}$. Pipette 1,0 ml of vitamin D₂ non-labelled stock standard solution (5.3.5) into a 100 ml volumetric flask. Make to volume with ethanol (5.1.10). Measure the absorbance of an aliquot at 265 nm. The spectrophotometer should be zeroed against an ethanol (5.1.10) blank solution. Record absorbance and calculate concentration. Make fresh daily.

5.3.8 Vitamin D₃ non-labelled purity standard solution, $\rho \approx 10 \mu\text{g/ml}$. Pipette 1,0 ml of vitamin D₃ non-labelled stock standard solution (5.3.6) into a 100 ml volumetric flask. Make to volume with ethanol (5.1.10). Measure the absorbance of an aliquot at 265 nm. The spectrophotometer should be zeroed against an ethanol (5.1.10) blank solution. Record absorbance and calculate concentration. Make fresh daily.

5.3.9 Non-labelled working standard solution, $\rho \approx 1 \mu\text{g/ml}$. Pipette 1,0 ml of vitamin D₂ non-labelled purity standard solution (5.3.7) and/or 1,0 ml of vitamin D₃ non-labelled purity standard solution (5.3.8) into a 10 ml volumetric flask. Make to volume with acetonitrile (5.1.14) and mix thoroughly. Make fresh daily.

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5.4 Calibration standard solutions

5.4.1 See [Table 1](#) for nominal vitamin D concentrations of the calibration standard solutions. Make fresh daily.

5.4.2 **Calibration standard 1.** Pipette 10 µl non-labelled working standard solution ([5.3.9](#)) and 250 µl stable isotope labelled internal standard solution ([5.3.4](#)) into a 25 ml volumetric flask. Add 5 ml of acetonitrile ([5.1.14](#)) and 75 µl of PTAD solution ([5.2.1](#)), shake to mix and leave in the dark for 5 min. Add 6,25 ml of water then make to volume with acetonitrile ([5.1.14](#)), mix, and transfer to HPLC vial ready for analysis.

5.4.3 **Calibration standard 2.** Pipette 50 µl non-labelled working standard solution ([5.3.9](#)) and 250 µl stable isotope labelled internal standard solution ([5.3.4](#)) into a 25 ml volumetric flask. Add 5 ml of acetonitrile ([5.1.14](#)) and 75 µl of PTAD solution ([5.2.1](#)), shake to mix and leave in the dark for 5 min. Add 6,25 ml of water then make to volume with acetonitrile ([5.1.14](#)), mix, and transfer to HPLC vial ready for analysis.

5.4.4 **Calibration standard 3.** Pipette 250 µl non-labelled working standard solution ([5.3.9](#)) and 250 µl stable isotope labelled internal standard solution ([5.3.4](#)) into a 25 ml volumetric flask. Add 5 ml of acetonitrile ([5.1.14](#)) and 75 µl of PTAD solution ([5.2.1](#)), shake to mix and leave in the dark for 5 min. Add 6,25 ml of water then make to volume with acetonitrile ([5.1.14](#)), mix, and transfer to HPLC vial ready for analysis.

5.4.5 **Calibration standard 4.** Pipette 500 µl non-labelled working standard solution ([5.3.9](#)) and 250 µl stable isotope labelled internal standard solution ([5.3.4](#)) into a 25 ml volumetric flask. Add 5 ml of acetonitrile ([5.1.14](#)) and 75 µl of PTAD solution ([5.2.1](#)), shake to mix and leave in the dark for 5 min. Add 6,25 ml of water then make to volume with acetonitrile ([5.1.14](#)), mix, and transfer to HPLC vial ready for analysis.

5.4.6 **Calibration standard 5.** Pipette 1 250 µl non-labelled working standard solution ([5.3.9](#)) and 250 µl stable isotope labelled internal standard solution ([5.3.4](#)) into a 25 ml volumetric flask. Add 5 ml of acetonitrile ([5.1.14](#)) and 75 µl of PTAD solution ([5.2.1](#)), shake to mix and leave in the dark for 5 min. Add 6,25 ml of water then make to volume with acetonitrile ([5.1.14](#)), mix, and transfer to HPLC vial ready for analysis.

Table 1 — Nominal concentration of calibration standards

Calibration solution	Concentration of vitamin D ng/ml	Concentration of <i>d6</i> -vitamin D ng/ml
1	0,4	10
2	2,0	10
3	10	10
4	20	10
5	50	10

6 Apparatus

Usual laboratory glassware and equipment and, in particular, the following.

6.1 **Ultra high performance liquid chromatography (UHPLC) system**, consisting of dual pump system, a sample injector unit, a degasser unit, and a column oven.

6.2 **Triple quadrupole mass spectrometer**, with sufficient sensitivity to detect and quantify vitamin D in PTAD adduct at 0,4 ng/ml.

- 6.3 Solid core silica column**, e.g. Phenomenex Kinetex¹⁾ C₁₈ 2,6 µm, 2,1 mm × 50 mm, or equivalent.
- 6.4 Spectrophotometer**, capable of digital readout to three decimal places.
- 6.5 Centrifuge tubes**, polypropylene, 15 ml.
- 6.6 Boiling tubes**, glass, 60 ml.
- 6.7 Water bath**, 20 °C to 70 °C.
- 6.8 Disposable syringes**, capacity 1 ml.
- 6.9 Syringe filters**, PTFE, 0,2 µm, 13 mm.
- 6.10 Centrifuges**, suitable for 60 ml boiling tubes, and 15 ml centrifuge tubes.
- 6.11 Pasteur pipettes**, glass, ~140 mm.
- 6.12 Horizontal shaker**.
- 6.13 Micro centrifuge tubes**, 2 ml.
- 6.14 Filter membranes**, 0,45 µm polyamide.
- 6.15 Cryogenic vials**, 2 ml.
- 6.16 High performance liquid chromatography (HPLC) vials, septa, and caps**.

7 Sample preparation

7.1 Powder sample preparation

Accurately weigh 1,8 g to 2,2 g of powder sample into a boiling tube. Record mass.

7.2 Slurry sample preparation

Accurately weigh 19,0 g to 21,0 g of powder to a disposable slurry container. Record mass.

Accurately weigh ~80 ml water to container. Record mass.

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

Accurately weigh 9,5 g to 10,5 g of slurry or reconstituted powder sample into a boiling tube. Record mass.

7.3 Liquid sample preparation

Accurately weigh 10,0 ml of liquid milk into a boiling tube. Record mass.

1) This is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

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8 Procedure

8.1 Extraction and derivatisation

To powder, slurry, or liquid sample in a boiling tube, add 10 ml of ethanolic pyrogallol solution (5.2.3), and 0,5 ml of stable isotope labelled internal standard solution (5.3.4), cap and vortex mix.

Add 2 ml of potassium hydroxide solution (5.2.2) to boiling tube, cap and vortex mix.

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

Place boiling tube in water bath at room temperature until cool.

Add 10 ml of isooctane (5.1.12) to the boiling tube; cap boiling tube tightly and place on horizontal shaker for 10 min.

Add 20 ml of water to boiling tube and invert tube 10 times; place in centrifuge at $\geq 250g$ for 15 min.

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).

Add 5 ml of water to centrifuge tube, cap and vortex mix and place in centrifuge at 2 000g for 5 min.

Transfer 4 ml to 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).

Add 75 μ l of PTAD solution (5.2.1) to centrifuge tube, cap and immediately vortex mix.

Allow to stand in the dark for 5 min to allow for derivatization reaction to complete.

Add 1 ml acetonitrile to centrifuge tube, cap and vortex mix, place in centrifuge at 2 000g for 5 min.

Using a variable volume pipette, transfer 500 μ l of the lower layer into a micro centrifuge tube (6.13) taking care not to transfer any of the upper layer.

Add 167 μ l of water to the micro centrifuge tube (6.13), cap and vortex mix.

Using a syringe filter, transfer an aliquot from the micro centrifuge tube (6.13) to an amber HPLC vial, cap ready for analysis.

8.2 Chromatography

Form high pressure gradients by mixing the two mobile phases, A and B, using the procedure given in Table 2. Information on expected retention times and product ion spectra are given in Annex A.

Table 2 — Gradient procedure for chromatographic separation

Time min	Flow rate ml/min	Mobile phase A %	Mobile phase 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

8.3 Mass spectrometry

Set up the mass spectrometer with the instrument setting shown in [Table 3](#). These values are indicative and need to be optimized for each instrument used. Examples of alternative instrument settings are given in [Annex C](#).

Table 3 — Mass spectrometer instrument settings

Instrument parameter	Value
ionization mode	ESI ⁺
curtain gas	207 kPa (30 psi)
nebulizer gas	277 kPa (40 psi)
heater gas	277 kPa (40 psi)
collision gas	N ₂
source temperature	300 °C
ion spray voltage	5 500 V

Settings are applicable to Sciex 6500 mass spectrometer. Sciex 6500 mass spectrometer is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

Compound specific parameters to be used are shown in [Table 4](#) and [Table 5](#).

Table 4 — Compound parameters (vitamin D₂ instrument method only)

Vitamin D ₂ ion ^a	Precursor ion <i>m/z</i>	Product ion <i>m/z</i>	DP V	EP V	CE V	CXP 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

Key

DP: declustering potential

EP: entrance potential

CE: collision energy

CXP: collision cell exit potential

^a Analyte = vitamin D₂-PTAD adduct, Internal standard ion = *d*6-vitamin D₂-PTAD adduct.