



BSI Standards Publication

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

National foreword

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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*



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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).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

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]].

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**, $\geq 99\%$ pure.
- 5.1.2 **Vitamin D₂**, ergocalciferol.
- 5.1.3 **Vitamin D₃**, cholecalciferol.
- 5.1.4 ***d*6-Vitamin D₂**, 26,26,26,27,27,27-*d*6 ergocalciferol.
- 5.1.5 ***d*6-Vitamin D₃**, 26,26,26,27,27,27-*d*6 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**, $c(4\text{-phenyl-1,2,4-triazoline-3,5-dione}) = 10 \text{ mg/ml}$. Dissolve 50 mg PTAD ([5.1.6](#)) in 5,0 ml acetone ([5.1.13](#)).
- 5.2.2 **Potassium hydroxide solution**, $c(\text{KOH}) = 8,9 \text{ mol/l}$. Dissolve 100 g potassium hydroxide ([5.1.8](#)) in 200 ml water.
- 5.2.3 **Ethanolic pyrogallol solution**, $c(\text{C}_6\text{H}_3(\text{OH})_3) = 0,079 \text{ mol/l}$. Dissolve 5 g pyrogallol ([5.1.9](#)) in 500 ml ethanol ([5.1.10](#)).
- 5.2.4 **Mobile phase A**, $c(\text{HCO}_2\text{H}) = 0,0265 \text{ mol/l}$. 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 ($\sim 1,3 \text{ ml}$) into cryogenic vials and freeze at $< -15^\circ\text{C}$ 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 ($\sim 1,3 \text{ ml}$) into cryogenic vials and freeze at $< -15^\circ\text{C}$ 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 $< -15^\circ\text{C}$ 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 $< -15^\circ\text{C}$ 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