

Foodstuffs — Determination of vitamin D by high performance liquid chromatography — Measurement of cholecalciferol (D3) or ergocalciferol (D2)

ICS 67.050

National foreword

This British Standard is the UK implementation of EN 12821:2009. It supersedes BS EN 12821:2000 which is withdrawn.

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A list of organizations represented on this committee can be obtained on request to its secretary.

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Produits alimentaires - Dosage de la vitamine D par chromatographie liquide haute performance - Dosage du cholestérol (D3) et de l'ergocalciferol (D2)

Lebensmittel - Bestimmung von Vitamin D mit Hochleistungs-Flüssigchromatographie - Bestimmung von Cholecalciferol (D3) oder Ergocalciferol (D2)

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Foreword

This document (EN 12821:2009) has been prepared by Technical Committee CEN/TC 275 “Food analysis - Horizontal methods”, the secretariat of which is held by DIN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by October 2009, and conflicting national standards shall be withdrawn at the latest by October 2009.

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This document supersedes EN 12821:2000.

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1 Scope

This European Standard specifies a method for the determination of vitamin D₃ (cholecalciferol) or vitamin D₂ (ergocalciferol) in foodstuffs by high performance liquid chromatography (HPLC).

Vitamin D₃ is primary in foodstuffs of animal origin, while vitamin D₂ is primary in wild mushrooms. Both vitamin D₃ and vitamin D₂ can be present in fortified foodstuffs. This European Standard is not applicable for samples with a content of vitamin D₃ and vitamin D₂.

Apart from the vitamin D activity from the parent forms, vitamin D₃ and vitamin D₂, the corresponding metabolites 25-hydroxy vitamin D and 1,25-dihydroxy vitamin D also contribute to the vitamin D activity. This European Standard does only include measurement of vitamin D₃ or vitamin D₂.

This European Standard provides the base for the analytical methods. It is intended to serve as a frame in which the analyst can define his own analytical work in accordance to the standard procedure.

This method has been validated in inter-laboratory tests on fortified and non-fortified samples such as margarine, milk, milk powder, liquid infant formula, infant formula, cooking oil, and fish oil at levels from 0,4 µg/100 g to 14 µg/100 g. Further information on the validation data is given in Annex D.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN ISO 3696, *Water for analytical laboratory use - Specification and test methods (ISO 3696:1987)*.

3 Principle

Vitamin D₃ and vitamin D₂ are saponified in the foodstuffs using alcoholic potassium hydroxide solution and extracted by an appropriate solvent. The determination of vitamin D₃ or vitamin D₂ in an appropriate sample extract solution is carried out by semi-preparative normal phase HPLC followed by reverse-phase analytical HPLC.

If vitamin D₃ is to be determined, then vitamin D₂ is used as an internal standard. If vitamin D₂ is to be determined, then vitamin D₃ is used as an internal standard.

Vitamin D is detected by ultraviolet (UV) spectrometry and peaks are identified on the basis of retention times and additionally by UV spectral profile if diode-array detection is used. The determination is carried out by the internal standard procedure using peak areas or peak heights, see [1] to [8].

4 Reagents

4.1 General

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and water of at least grade 1 according to EN ISO 3696.

4.2 Methanol

4.3 Ethanol, volume fraction $\varphi(\text{C}_2\text{H}_5\text{OH}) = 100 \%$.

4.4 Ethanol, $\varphi(\text{C}_2\text{H}_5\text{OH}) = 96 \%$.

4.5 Sodium sulfate, anhydrous.

4.6 KOH solutions for saponification, in suitable concentrations, e.g. mass concentration $\rho(\text{KOH}) = 50 \text{ g}/100 \text{ ml}$ or $\rho(\text{KOH}) = 60 \text{ g}/100 \text{ ml}$, or alcoholic solutions, e.g. 28 g of KOH in 100 ml of an ethanol and water mixture with a volume fraction of ethanol of 90 %.

4.7 Antioxidants, such as ascorbic acid (AA), sodium ascorbate, pyrogallol, sodium sulfide (Na_2S) or butylated hydroxytoluene (BHT).

4.8 Solvents and extraction solvents, such as diethyl ether (peroxide-free), dichloromethane, light petroleum, *n*-hexane, ethylacetate or appropriate mixtures thereof.

4.9 HPLC Mobile phases

4.9.1 Examples of solvent mixtures for normal phase semi-preparative HPLC

Examples of appropriate solvent mixtures (given as volume fractions) for normal phase semi-preparative HPLC include:

- *n*-hexane and 2-propanol (98 + 2), (99 + 1) or (95 + 5);
- *n*-hexane and isoamyl alcohol (99 + 1);
- *n*-hexane, 2-propanol and tetrahydrofuran (98 + 1 + 1);
- *iso*-octane and iso-butanol (99 + 1);
- *n*-heptane and 2-propanol (97 + 3).

4.9.2 Examples of solvent and solvent mixtures for reverse-phase analytical HPLC

Examples of appropriate solvent and solvent mixtures (given as volume fractions) for reverse-phase analytical HPLC include:

- methanol;
- methanol and water (95 + 5) or (93 + 7);
- acetonitrile and methanol (80 + 20), (90 + 10) or (70 + 30);
- acetonitrile, chloroform and methanol (93 + 4 + 3).

4.10 Standard substances

4.10.1 Ergocalciferol standard substance (vitamin D₂), $M(C_{28}H_{44}O) = 396,7$ g/mol

Vitamin D₂ standard substance shall be of the highest purity obtainable (having a mass fraction of greater than 98 %) and shall be stored according to the supplier's instructions (in the absence of light, typically less than 4 °C).

4.10.2 Cholecalciferol standard substance (vitamin D₃), $M(C_{27}H_{44}O) = 384,6$ g/mol

Vitamin D₃ standard substance shall be of the highest purity obtainable (having a mass fraction of greater than 98 %) and shall be stored according to the supplier's instructions (in the absence of light, typically less than 4 °C).

4.11 Stock solutions

4.11.1 Vitamin D₂ stock solution

Weigh about 100 mg of vitamin D₂ (4.10.1) to the nearest milligram into a one mark 100 ml volumetric flask, dissolve in ethanol (4.4) and dilute to the mark with ethanol. This solution contains approximately 1 mg/ml of vitamin D₂. Store below 4 °C and protect from light.

Calculate the mass concentration of the stock solution and the mass fraction of the vitamin D₂ standard by the procedure described in 4.12.1.

This solution is stable for 6 months at - 18 °C.

4.11.2 Vitamin D₃ stock solution

Weigh about 100 mg of vitamin D₃ (4.10.2) to the nearest milligram into a one mark 100 ml volumetric flask, dissolve in ethanol (4.4) and dilute to the mark with ethanol. This solution contains approximately 1 mg/ml of vitamin D₃. Store below 4 °C and protect from light.

Calculate the mass concentration of the stock solution and the mass fraction of the vitamin D₃ standard by the procedure described in 4.12.2.

This solution is stable for 6 months at - 18 °C.

4.12 Standard solutions

4.12.1 Vitamin D₂ standard solution

Pipette 1 ml of the vitamin D₂ stock solution (4.11.1) into a one mark 100 ml volumetric flask and dilute to the mark with ethanol (4.4). This solution contains approximately 10 µg/ml of vitamin D₂. Prepare this solution on the day of use.

NOTE The mass concentration of the standard solution can be adjusted if necessary to suit the analytical requirements.

Measure the absorption of the vitamin D₂ standard solution in a 1 cm quartz cell at a wavelength of 265 nm using ethanol in the reference path. Calculate the mass concentration of vitamin D₂, ρ_{D2} , in microgram per millilitre of the standard solution using Equation (1):

$$\rho_{D2} = \frac{A_{265} \times M_{D2} \times 1000}{\epsilon \times b} \quad (1)$$

where:

- A_{265} is the absorption of the vitamin D₂ standard solution at 265 nm;
- M_{D_2} is the molar mass of vitamin D₂ ($M_{D_2} = 396,7$ g/mol);
- ϵ is the molar absorption coefficient of vitamin D₂ (here: $\epsilon = 18\,843$ m²/mol, calculated from the $E_{1\text{cm}}^{1\%}$ value, see [9]);
- b is the optical path length of the quartz cell in centimetres.

4.12.2 Vitamin D₃ standard solution

Pipette 1 ml of the vitamin D₃ stock solution (4.11.2) into a one mark 100 ml volumetric flask and dilute to the mark with ethanol (4.4). This solution contains approximately 10 µg/ml of vitamin D₃. Prepare this solution on the day of use.

NOTE The mass concentration of the standard solution can be adjusted if necessary to suit the analytical requirements.

Measure the absorption of the vitamin D₃ standard solution in a 1 cm quartz cell at a wavelength of 265 nm using ethanol (4.4) in the reference path. Calculate the mass concentration of vitamin D₃, ρ_{D_3} , in microgram per millilitre of the standard solution using Equation (2):

$$\rho_{D_3} = \frac{A_{265} \times M_{D_3} \times 1000}{\epsilon \times b} \quad (2)$$

where:

- A_{265} is the absorption of the vitamin D₃ standard solution at 265 nm;
- M_{D_3} is the molar mass of vitamin D₃ ($M_{D_3} = 384,6$ g/mol);
- ϵ is the molar absorption coefficient of vitamin D₃ (here: $\epsilon = 18\,461$ m²/mol, calculated from the $E_{1\text{cm}}^{1\%}$ value, see [9]);
- b is the optical path length of the quartz cell in centimetres.

4.13 Internal standard solutions

4.13.1 Vitamin D₂ internal standard solution

Pipette 10 ml of the vitamin D₂ standard solution (4.12.1) into a one mark 100 ml volumetric flask and dilute to the mark with ethanol (4.4). Prepare this solution on the day of use.

4.13.2 Vitamin D₃ internal standard solution

Pipette 10 ml of the vitamin D₃ standard solution (4.12.2) into a one mark 100 ml volumetric flask and dilute to the mark with ethanol (4.4). Prepare this solution on the day of use.

NOTE If vitamin D₃ is to be determined, then vitamin D₂ is used as an internal standard. If vitamin D₂ is to be determined, then vitamin D₃ is used as an internal standard.