



National Standards Authority of Ireland

IRISH STANDARD

I.S. EN 12821:2000

ICS 67.040

**FOODSTUFFS - DETERMINATION OF VITAMIN
D BY HIGH PERFORMANCE LIQUID
CHROMATOGRAPHY - MEASUREMENT OF
CHOLECALCIFEROL (D₃) AND
ERGOCALCIFEROL (D₂)**

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ICS 67.040

English version

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

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

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

This European Standard was approved by CEN on 2 January 2000.

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Foreword

This European Standard 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of an identical text or by endorsement, at the latest by August 2000, and conflicting national standards shall be withdrawn at the latest by August 2000.

According to the CEN/CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard: Austria, Belgium, Czech Republic, Denmark, Finland, France, Germany, Greece, Iceland, Ireland, Italy, Luxembourg, Netherlands, Norway, Portugal, Spain, Sweden, Switzerland and the United Kingdom.

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.

1 Scope

This European Standard specifies a method for the determination of vitamin D in foodstuffs by high performance liquid chromatography (HPLC).

In the majority of foodstuffs vitamin D is naturally present as cholecalciferol, vitamin D₃, and this is the form of the vitamin determined. Vitamin D₂, ergocalciferol, is sometimes present in fortified foodstuffs and can also be determined using this European Standard. Some foods will contain both vitamin D₃ and D₂. This method is not applicable to these samples.

2 Normative references

This European Standard incorporates by dated or undated reference, provisions from other publications. These normative references are cited at the appropriate places in the text, and the publications are listed hereafter. For dated references, subsequent amendments to, or revisions of any of these publications apply to this European Standard only when incorporated in it by amendment or revision. For undated references, the latest edition of the publication referred to applies.

EN ISO 3696 Water for analytical laboratory use - Specification and test methods (ISO 3696:1987).
EN ISO 5555 Animal and vegetable fats and oils - Sampling (ISO 5555:1991)

3 Principle

Vitamin D₃ and D₂ are saponified in the foodstuffs using alcoholic potassium hydroxide solution and extracted by an appropriate solvent. The determination of vitamin D₃ or D₂ in an appropriate sample extract solution is carried out by semi-preparative normal phase HPLC followed by reversed-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 [9].

4 Reagents

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.

4.1 Methanol

4.2 Ethanol, volume fraction φ (C₂H₅OH) = 100%

4.3 Ethanol, φ (C₂H₅OH) = 96%

4.4 Sodium sulfate, anhydrous

4.5 Potassium hydroxide (KOH) solutions,

4.5.1 KOH solutions for saponification, in suitable concentrations, e.g. ρ (KOH) = 50 g/100 ml or 60 g/100 ml, or alcoholic solutions, e.g. 28 g KOH in 100 ml of an ethanol/water mixture (9+1)(V+V).

4.5.2 KOH solutions for extraction, in suitable concentrations, e.g. 5 g/100 ml.

4.6 Antioxidants, such as ascorbic acid (AA), sodium ascorbate, pyrogallol, sodium sulfide (Na₂S) or butylated hydroxytoluene (BHT).

4.7 Solvents and extraction solvents such as diethyl ether (peroxide-free), dichloromethane, light petroleum (boiling range of 40 °C to 60 °C), *n*-hexane, ethylacetate or appropriate mixtures thereof.

4.8 HPLC Mobile phases

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

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

4.8.2 Examples of appropriate solvent and solvent mixtures (given as volume parts) for reversed-phase analytical HPLC include:

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

4.9 Standard substances

4.9.1 Ergocalciferol standard substance (Vitamin D₂), M (C₂₈H₄₄O) = 396,7 g/mol

Vitamin D₂ (ergocalciferol) 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).

4.9.2 Cholecalciferol standard substance (Vitamin D₃), M (C₂₇H₄₄O) = 384,6 g/mol

Vitamin D₃ (cholecalciferol) 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 Stock solutions

4.10.1 Vitamin D₂ stock solution

Weigh about 100 mg of vitamin D₂ (4.9.1) to the nearest milligram into a one mark 100 ml volumetric flask, dissolve in ethanol (4.3) 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 4.11.1.

4.10.2 Vitamin D₃ stock solution

Weigh about 100 mg of vitamin D₃ (4.9.2) to the nearest milligram into a one mark 100 ml volumetric flask, dissolve in ethanol (4.3) 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 4.11.2.

4.11 Standard solutions

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

4.11.1 Vitamin D₂ standard solution

Pipette 1 ml of the vitamin D₂ stock solution (4.10.1) into a one mark 100 ml volumetric flask and dilute to the mark with ethanol (4.3). This solution contains approximately 10 µg/ml of vitamin D₂. Prepare this solution on the day of use.

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