SLOVENSKI STANDARD
SIST EN 12821:2000

01-november-2000

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

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

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

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EUROPEAN STANDARD

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Foodstuffs - Determination of vitamin D by high performance liquid chromatography - Measurement of cholecalciferol (D_3) and ergocalciferol (D_2)

Produits alimentaires - Dosage de la vitamine D par chromatographie liquide haute performance - Dosage du cholécalciférol (D_3) et de l'ergocalciférol (D_2)

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

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Contents

	Page
Foreword	2
1 Scope	3
2 Normative references	3
3 Principle	3
4 Reagents	3
5 Apparatus	6
6 Sampling	6
7 Procedure	6
8 Calculation	9
9 Precision	9
10 Test Report	9
Annex A (informative) Examples of suitable HPLC-systems	10
Annex B (informative) Examples of suitable extraction and saponification conditions	12
Annex C (informative) Examples of suitable semi-preparative and analytical HPLC chromatograms	13
Annex D (informative) Precision data	14
Bibliography	15

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SIST EN 12821:2000
https://standards.iteh.ai/catalog/standards/sist/bbacf698-d806-4722-83a9-029da3d0127f/sist-en-12821-2000

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of an identical text or by endorsement, at the latest by August 2000, and conflicting national standards shall be withdrawn at the latest by August 2000.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

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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 D_3, and this is the form of the vitamin determined. Vitamin D_2, ergocalciferol, is sometimes present in fortified foodstuffs and can also be determined using this European Standard. Some foods will contain both vitamin D_3 and D_2. 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 D_3 and D_2 are saponified in the foodstuffs using alcoholic potassium hydroxide solution and extracted by an appropriate solvent. The determination of vitamin D_3 or D_2 in an appropriate sample extract solution is carried out by semi-preparative normal phase HPLC followed by reversed-phase analytical HPLC.

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If vitamin D_3 is to be determined, then vitamin D_2 is used as an internal standard. If vitamin D_2 is to be determined, then vitamin D_3 is used as an internal standard.

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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 (C_2H_5OH) = 100 %.

4.3 Ethanol, φ (C_2H_5OH) = 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 (Na_2S) 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.

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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 D_2), M ($C_{28}H_{44}O$) = 396,7 g/mol

Vitamin D_2 (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 D_3), M ($C_{27}H_{44}O$) = 384,6 g/mol

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Vitamin D_3 (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).

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4.10 Stock solutions

4.10.1 Vitamin D_2 stock solution

Weigh about 100 mg of vitamin D_2 (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 D_2. Store below 4 °C and protect from light.

Calculate the mass concentration of the stock solution and the mass fraction of the vitamin D_2 standard by the procedure described in 4.11.1.

4.10.2 Vitamin D_3 stock solution

Weigh about 100 mg of vitamin D_3 (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 D_3. Store below 4 °C and protect from light.

Calculate the mass concentration of the stock solution and the mass fraction of the vitamin D_3 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 D_2 standard solution

Pipette 1 ml of the vitamin D_2 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 D_2. Prepare this solution on the day of use.

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Measure the absorption of the vitamin D_2 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 D_2, ρ_{D_2}, of the standard solution using equation (1):

$$\rho_{D_2} = \frac{A_{265} \times 10^4}{475} \qquad (1)$$

where:

A_{265} is the absorption value of the vitamin D_2 standard solution at 265 nm;

475 is the $E^{1\%}_{1cm}$ value, see [10].

4.11.2 Vitamin D_3 standard solution

Pipette 1 ml of the vitamin D_3 stock solution (4.10.2) 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 D_3. Prepare this solution on the day of use.

Measure the absorption of the vitamin D_3 standard solution in a 1 cm quartz cell at a wavelength of 265 nm using ethanol (4.3) in the reference path. Calculate the mass concentration of vitamin D_3, ρ_{D_3}, of the standard solution using equation (2):

$$\rho_{D_3} = \frac{A_{265} \times 10^4}{480} \qquad (2)$$

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where:

A_{265} is the absorption value of the vitamin D_3 standard solution at 265 nm;

480 is the $E^{1\%}_{1cm}$ value, see [10].

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4.12 Internal standard solutions

NOTE: If vitamin D_3 is to be determined then vitamin D_2 is used as an internal standard. If vitamin D_2 is to be determined then vitamin D_3 is used as an internal standard.

4.12.1 Vitamin D_2 internal standard solution

Pipette 10 ml of the vitamin D_2 standard solution (4.11.1) into a one-mark 100 ml volumetric flask and dilute to the mark with ethanol (4.3). Prepare this solution on the day of use.

4.12.2 Vitamin D_3 internal standard solution

Pipette 10 ml of the vitamin D_3 standard solution (4.11.2) into a one-mark 100 ml volumetric flask and dilute to the mark with ethanol (4.3). Prepare this solution on the day of use.

4.13 Vitamin D_2 and vitamin D_3 semi-preparative standard solution

Pipette 5 ml of the vitamin D_2 standard solution (4.11.1) and 5 ml of the vitamin D_3 standard solution (4.11.2) into a rotary evaporator flask and carefully remove the solvent (at not more than 40 °C). Re-dissolve the residues in 50 ml of the semi-preparative HPLC mobile phase (4.8.1).

The concentration of the semi-preparative standard may be adjusted if necessary to suit the HPLC-system in use (5.3 or 5.4).

4.14 Vitamin D_2 and vitamin D_3 analytical standard solution

Pipette 5 ml of the vitamin D_2 standard solution (4.11.1) and 5 ml of the vitamin D_3 standard solution (4.11.2) into a rotary evaporator flask and carefully remove the solvent (at not more than 40 °C). Re-dissolve the residues in 10 ml of the analytical HPLC mobile phase (4.8.2).

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5 Apparatus

Usual laboratory apparatus and, in particular, the following

5.1 UV spectrometer, capable of measuring at a wavelength of 265 nm.

5.2 Rotary evaporator, with water bath and vacuum unit

NOTE: The use of nitrogen is recommended for releasing the vacuum.

5.3 Semi-preparative HPLC-system consisting of a pump, sample injection device, UV detector, a means of collecting a defined aliquot portion of column eluent, and a recorder or integrator.

5.4 Analytical HPLC-system consisting of a pump, sample injection device, UV detector, recorder/integrator or similar data capture device.

5.5 HPLC columns

5.5.1 Semi-preparative normal phase column, e.g. silica or bonded cyano-amino, particle size 5 µm, diameter 4,0 mm to 8,0 mm, length 250 mm to 300 mm. See annex A for more information.

5.5.2 Analytical reverse phase column, e.g. C_{18} reverse phase, particle size 5 µm, diameter 4,0 mm to 4,6 mm, length 250 mm. See annex A for more information.

5.5.3 Packing materials, particle sizes and column dimensions other than those specified in this European Standard may be used, but the analyst has to ensure that they provide adequate separation of the vitamins D from matrix interferences if equivalent results are to be obtained.

5.6 Filter device

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Large and small scale filter devices to filter HPLC mobile phases and sample solutions respectively, e.g. of 0,45 µm pore size or similar is appropriate.

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NOTE: Filtering of the mobile phase as well as of the sample test solution through a membrane filter prior to use or injection usually increases longevity of the columns.

6 Sampling

Sampling shall be in accordance with EN ISO 5555, if appropriate.

7 Procedure

7.1 General

Vitamin D is sensitive to UV radiation and to oxidizing agents (e.g. atmospheric oxygen). It is therefore necessary to exclude UV light by using amber glassware, aluminium foil or UV absorbing materials. Antioxidants need to be added to solutions containing extracted vitamin, and nitrogen flushing should be used. The solvents shall be evaporated under reduced pressure using a rotary evaporator at not more than 40 °C.

7.2 Preparation of the test sample

Homogenize the test sample. Comminute coarse material thoroughly and homogenize in a food blender or liquidizer. Precautions such as pre-cooling the sample shall be taken to avoid exposure to high temperatures. After this preparation the test sample shall be analysed without delay. Protect samples from light.

7.3 Preparation of the sample test solution

7.3.1 Saponification

Saponify 10 g to 30 g of the test sample by refluxing, preferably under nitogen, using suitable amounts of ethanol (4.3), water, an antioxidant (4.6) such as ascorbic acid, sodium ascorbate or pyrogallol and one of the potassium hydroxide solutions (4.5.1). Add the antioxidants to the sample prior to the addition of potassium hydroxide. Sodium sulfide (4.6) may also be added to obviate the oxidative catalytic effects of traces of metals.

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If vitamin D_3 is to be determined, pipette an appropriate amount of vitamin D_2 internal standard solution (4.12.1) into the saponification flask. The amount of vitamin D_2 internal standard solution added shall be similar to the amount of vitamin D_3 expected in the sample. If vitamin D_2 is to be determined then vitamin D_3 standard solution (4.12.2) shall be added as the internal standard. A sample that does not contain the internal standard shall be taken through the analytical procedure to ensure that there is no sample matrix interference in the chromatographs at the internal standard retention time or that both vitamins D_3 and D_2 are present in the sample.

Examples of suitable ratios of reagents are given in Table 1:

Table 1

Sample	Ethanol	Pyrogallol	Ascorbic Acid / Na Ascorbate	Potassium Hydroxide
10 g to 30 g	100 ml	0,5 g to 1 g	1,0 g to 2,5 g	50 ml of a 50 g/100 ml solution

The usual time of saponification ranges from 20 min to 45 min with temperatures of 70 °C to 100 °C. Saponification may also be carried out at room temperature overnight (approximately 16 h) under otherwise same conditions.

If after saponification and cooling, fat or oil is present on the surface of the saponification mixture, additional ethanolic potassium hydroxide has to be added and saponification time extended.

NOTE: Conditions found suitable for saponification of a margarine and a milk powder are shown in annex B.

7.3.2 Extraction

In order to avoid emulsions, an amount of water has to be be added to the saponified sample solution so that the ratio of alcohol to water in the resulting solution is 1:1.

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Extract the vitamins D_2 and D_3 from the cooled saponification mixture using a suitable solvent, or mixture of solvents (4.7), and repeat the procedure two to four times with volumes ranging from 100 ml to 200 ml. Wash the combined solvent extracts to neutral pH with water (typically 5 times with 50 ml to 100 ml).

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NOTE: Some methods prescribe washing to neutrality with 3 % or 5 % potassium hydroxide in 0,9 % sodium chloride solution buffered in 2,6 mol/l sodium acetate (pH 7), or similar mixtures. Annex B shows extraction conditions found suitable for a margarine and a milk powder.

7.3.3 Evaporation

Evaporate sample extracts using a rotary evaporator (5.2) under reduced pressure, and at a temperature not exceeding 40 °C. Prior to evaporation it is good practice to add an antioxidant (e.g. 2 ml of 1 mg/ml BHT in *n*-hexane) to the sample extract.

Absolute ethanol (4.2) or anhydrous sodium sulfate (4.4) should be added to the concentrated sample extract to assist in the removal of traces of water (azeotropic distillation).

At this stage in the analytical procedure solid-phase clean-up of the sample extract may be employed to remove potential interferences. Commercially available silica, alumina and C_{18} cartridges can be used singly or in series. If this type of additional clean-up is employed, the procedure has to be fully validated before use.

7.3.4 Dilution

Re-dissolve the residue in a small, known volume of solvent which is compatible with the semi-preparative HPLC-system. Addition of a small amount of anhydrous sodium sulfate will remove residual traces of water.

7.4 Calibration

Use standard solutions of vitamins D_2 (4.11.1) and D_3 (4.11.2) to calibrate the semi-preparative (5.5.1) and analytical HPLC (5.5.2) systems and assess system suitability.

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