SLOVENSKI STANDARD

SIST EN 12821:2009

01-julij-2009

Nadomeġļ a:

SIST EN 12821:2000

Ģivila - Doloļ evanje vitamina D s tekoļ insko kromatografijo visoke loļ ljivosti - Merjenje holekalciferola (D3) in ergokalciferola (D2)

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

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

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

Ta slovenski standard je istoveten z: **EN 12821:2009**

ICS:

67.050	Splošne preskusne in analizne metode za živilske proizvode	General methods of tests and analysis for food products

SIST EN 12821:2009 **en,fr,de**

EUROPEAN STANDARD

NORME EUROPÉENNE

EUROPÄISCHE NORM

EN 12821

April 2009

ICS 67.050

Supersedes EN 12821:2000

English Version

Foodstuffs - Determination of vitamin D by high performance liquid chromatography - Measurement of cholecalciferol (D3) or 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) oder Ergocalciferol (D2)

This European Standard was approved by CEN on 21 February 2009.

CEN members are bound to comply with the CEN/CENELEC Internal Regulations which stipulate the conditions for giving this European Standard the status of a national standard without any alteration. Up-to-date lists and bibliographical references concerning such national standards may be obtained on application to the CEN Management Centre or to any CEN member.

This European Standard exists in three official versions (English, French, German). A version in any other language made by translation under the responsibility of a CEN member into its own language and notified to the CEN Management Centre has the same status as the official versions.

CEN members are the national standards bodies of Austria, Belgium, Bulgaria, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland and United Kingdom.

EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

Management Centre: Avenue Marnix 17, B-1000 Brussels

Contents

Page

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.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. CEN [and/or CENELEC] shall not be held responsible for identifying any or all such patent rights.

This document supersedes EN 12821:2000.

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

1 Scope

This European Standard specifies a method for the determination of vitamin D_3 (cholecalciferol) or vitamin D_2 (ergocalciferol) in foodstuffs by high performance liquid chromatography (HPLC).

Vitamin D_3 is primary in foodstuffs of animal origin, while vitamin D_2 is primary in wild mushrooms. Both vitamin D_3 and vitamin D_2 can be present in fortified foodstuffs. This European Standard is not applicable for samples with a content of vitamin D_3 and vitamin D_2.

Apart from the vitamin D activity from the parent forms, vitamin D_3 and vitamin D_2, 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 D_3 or vitamin D_2.

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 D_3 and vitamin 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 vitamin D_2 in an appropriate sample extract solution is carried out by semi-preparative normal phase HPLC followed by reverse-phase analytical HPLC.

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.

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 $\varphi(C_2H_5OH)$ = 100 %.

4.4 Ethanol, $\varphi(C_2H_5OH)$ = 96 %.

4.5 Sodium sulfate, anhydrous.

4.6 KOH solutions for saponification, in suitable concentrations, e.g. mass concentration ρ(KOH) = 50 g/100 ml or ρ(KOH) = 60 g/100 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 D_2), $M(C_{28}H_{44}O)$ = 396,7 g/mol

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

Vitamin D_3 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 D_2 stock solution

Weigh about 100 mg of vitamin D_2 (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 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 4.12.1.

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

4.11.2 Vitamin D_3 stock solution

Weigh about 100 mg of vitamin D_3 (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 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 4.12.2.

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

4.12 Standard solutions

4.12.1 Vitamin D_2 standard solution

Pipette 1 ml of the vitamin D_2 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 D_2. 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 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_{D2}, in microgram per millilitre of the standard solution using Equation (1):

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

where:

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

M_{D2} is the molar mass of vitamin D_2 (M_{D2} = 396,7 g/mol);

ε is the molar absorption coefficient of vitamin D_2 (here: ε = 18 843 m^2/mol, calculated from the $E_{1cm}^{1\%}$ value, see [9]);

b is the optical path length of the quartz cell in centimetres.

4.12.2 Vitamin D_3 standard solution

Pipette 1 ml of the vitamin D_3 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 D_3. 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 D_3 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 D_3, ρ_{D3}, in microgram per millilitre of the standard solution using Equation (2):

$$\rho_{D3} = \frac{A_{265} \times M_{D3} \times 1000}{\varepsilon \times b} \qquad (2)$$

where:

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

M_{D3} is the molar mass of vitamin D_3 (M_{D3} = 384,6 g/mol);

ε is the molar absorption coefficient of vitamin D_3 (here: ε = 18 461 m^2/mol, calculated from the $E_{1cm}^{1\%}$ value, see [9]);

b is the optical path length of the quartz cell in centimetres.

4.13 Internal standard solutions

4.13.1 Vitamin D_2 internal standard solution

Pipette 10 ml of the vitamin D_2 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 D_3 internal standard solution

Pipette 10 ml of the vitamin D_3 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 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.

4.14 Vitamin D_2 and vitamin D_3 semi-preparative standard solution

Pipette 5 ml of the vitamin D_2 standard solution (4.12.1) and 5 ml of the vitamin D_3 standard solution (4.12.2) into a rotary evaporator flask and carefully remove the solvent (at not more than 40 °C). Re-dissolve the residue in 100 ml of the semi-preparative HPLC mobile phase (4.9.1).

The concentration of the semi-preparative standard may be adjusted if necessary to suit the HPLC system in use (5.4 or 5.5).

4.15 Vitamin D_2 and vitamin D_3 analytical standard solution

Pipette 5 ml of the vitamin D_2 standard solution (4.12.1) and 5 ml of the vitamin D_3 standard solution (4.12.2) into a rotary evaporator flask and carefully remove the solvent (at not more than 40 °C). Re-dissolve the residue in 50 ml of the analytical HPLC mobile phase (4.9.2).

5 Apparatus

5.1 General

Usual laboratory apparatus and, in particular, the following.

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

5.3 Rotary evaporator, with water bath and vacuum unit

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

5.4 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.

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

5.6 HPLC columns

5.6.1 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.

5.6.2 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.

5.6.3 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.

5.7 Filter device

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.

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 Procedure

6.1 General

Vitamin D_2 and vitamin D_3 are 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 ºC.

6.2 Preparation of the test sample

Homogenize the test sample. Comminute coarse material thoroughly and homogenize in a food blender or liquidiser. 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.

6.3 Preparation of the sample test solution

6.3.1 Saponification

Saponify 10 g to 30 g of the test sample by refluxing, preferably under nitrogen, using suitable amounts of ethanol (4.4), water, an antioxidant (4.7) such as ascorbic acid, sodium ascorbate or pyrogallol and one of the potassium hydroxide solutions (4.6). Add the antioxidants to the sample prior to the addition of potassium hydroxide. Sodium sulfide (4.7) may also be added to obviate the oxidative catalytic effects of traces of metals.

If vitamin D_3 is to be determined, pipette an appropriate amount of vitamin D_2 internal standard solution (4.13.1) 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 (4.13.2) shall be added as the internal standard.

A sample that does not contain the internal standard should be taken through the analytical procedure to ensure that there is no sample matrix interference at the internal standard retention time.

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

Table 1 — Examples of suitable ratios of reagents

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.