SLOVENSKI STANDARD
SIST EN 12822:2000

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Živila - Določevanje vitamina E s tekočinsko kromatografijo visoke ločljivosti - Merjenje alfa, beta, gama in delta tokoferolov

Foodstuffs - Determination of vitamin E by high performance liquid chromatography - Measurement of alpha-, beta-, gamma-, and delta-tocopherols

Lebensmittel - Bestimmung von Vitamin E mit Hochleistungs-Fl~ssigchromatographie - Bestimmung von alpha-, beta-, gamma- und delta-Tocopherol

Produits alimentaires - Dosage de la vitamine E par chromatographie liquide haute performance - Dosage des alpha-, béta-, gamma- et delta-tocophérols

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

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Foodstuffs - Determination of vitamin E by high performance liquid chromatography - Measurement of α–, β–, γ– and δ–tocopherols

Produits alimentaires - Dosage de la vitamine E par chromatographie liquide haute performance - Dosage des α–, β–, γ– et δ–tocophérols

Lebensmittel - Bestimmung von Vitamin E mit Hochleistungs-Flüssigchromatographie - Bestimmung von α–, β–, γ— und δ–Tocopherol

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

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of an identical text or by endorsement, at the latest by August 2000, and conflicting national standards shall be withdrawn at the latest by August 2000.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

Introduction

As this European Standard method deals with the measurement of the mass fraction of α-, β-, γ- and δ-tocopherol in foodstuffs, reference is made to the literature for the calculation and expression of the vitamin E content in terms of biological activities [1], [2], [3].

1 Scope

This European Standard specifies a method for the determination of Vitamin E in foodstuffs by high performance liquid chromatography (HPLC). The determination of Vitamin E content is carried out by measurement of α-, β-, γ- and δ-tocopherol.

The vitamin E activity can be calculated from the tocopherol content assuming appropriate factors as given in the introduction.

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 draft 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

Determination of α-, β-, γ- and δ-tocopherol in an appropriate sample solution by high performance liquid chromatographic (HPLC) separation and subsequent photometric (UV-range) or preferably fluorometric detection. In most cases a saponification of the test material followed by an appropriate extraction is necessary. Identification is carried out on the basis of the retention times, and quantitative determination by the external standard method using peak areas or peak heights. Internal standard methods can also be used if the corresponding recovery tests have proven the same behaviour of the internal standard during the analysis as the analyte itself [4] to [13].

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, abs., volume fraction $\varphi(C_2H_5OH) = 100$ %.

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

4.4 Sodium sulfate, anhydrous.

4.5 KOH solutions for saponification, in suitable concentrations, e.g. (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).

4.6 Antioxidants, such as ascorbic acid (AA), sodium ascorbate, pyrogallol, sodium sulfide (Na_2S), hydroquinone 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 phase: Appropriate mixtures expressed as volume fractions of e.g. 3 % *1,4*-dioxane or 0,5 % 2-propanol, 3 % *tert*-butyl methyl ether in *n*-hexane or *n*-heptane for normal phase chromatography (NP) or 1 % to 10 % water in methanol for reversed phase chromatography (RP).

For alternative HPLC-systems see annex C.

4.9 Standard substances

4.9.1 General

β-, γ- and δ-tocopherol can be obtained from Merck[1)]; α-tocopherol can be obtained from various suppliers. The purity of the tocopherol standards can vary between 90 % and 100 %. It is therefore necessary to determine the concentration of the calibration solution by UV-spectrometry (see purity tests [4.10.5]).

4.9.2 α-tocopherol M ($C_{29}H_{50}O_2$) = 430,7 g/mol, with a known mass fraction of at least 95 %. α-tocopheryl acetate, M ($C_{31}H_{52}O_3$) = 472,7 g/mol, may also be used as standard after saponification.

4.9.3 β-tocopherol M ($C_{28}H_{48}O_2$) = 416,7 g/mol, with a known mass fraction of at least 90 %

4.9.4 γ-tocopherol M ($C_{28}H_{48}O_2$) = 416,7 g/mol, with a known mass fraction of at least 90 %

4.9.5 δ-tocopherol M ($C_{27}H_{46}O_2$) = 402,6 g/mol, with a known mass fraction of at least 90 %

4.10 Stock solutions

4.10.1 α-tocopherol stock solution

Dissolve an amount of α-tocopherol standard substance (4.9.2), weighed to the nearest milligram, e.g. approximately 10 mg in a defined volume, e.g. 100 ml of an appropriate solvent, e.g. *n*-hexane for NP-system or methanol for RP-system.

4.10.2 β-tocopherol stock solution

Dissolve an amount of the β-tocopherol standard substance (4.9.3), weighed to the nearest milligram, e.g. approximately 10 mg in a defined volume, e.g. 100 ml of an appropriate solvent, e.g. *n*-hexane for NP-system or methanol for RP-system.

4.10.3 γ-tocopherol stock solution

Dissolve an amount of the γ-tocopherol standard substance (4.9.4), weighed to the nearest millligram, e.g. approximately 10 mg in a defined volume, e.g. 100 ml of an appropriate solvent, e.g. *n*-hexane for NP-system or methanol for RP-system.

4.10.4 δ-tocopherol stock solution

Dissolve an amount of the δ-tocopherol standard substance (4.9.5), weighed to the nearest milligram, e.g. approximately 10 mg in a defined volume, e.g. 100 ml of an appropriate solvent, e.g. *n*-hexane for NP-system or methanol for RP-system.

4.10.5 Concentration and purity tests

Measure the absorbance of the stock solutions (4.10.1 to 4.10.4) at the appropriate wavelength using an UV-spectrometer (5.1). If the solvent used is *n*-hexane, pipette 10 ml of the stock solution into an amber glass round-bottomed flask and remove the solvent using a rotary evaporator (5.2) under reduced pressure at a temperature not higher than 50 °C. After restoring atmospheric pressure with nitrogen, remove the flask and dissolve the residue in 10 ml of methanol by swirling. Take this solution for the spectrometric measurement.

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1) This information is given for the convenience of users of this standard method and does not constitute an endorsement by CEN ofthe product named.

Calculate the mass concentration of vitamin E, ρ, of the respective α-, β-, γ- or δ-tocopherols, in micrograms per millilitre by using equation (1):

$$\rho = \frac{A \times 10^4}{E^{1\%}_{1cm}} \qquad (1)$$

where:

A is the absorption value of each tocopherol in the respective stock solution;

$E^{1\%}_{1cm}$ is the value for each tocopherol as defined in Table 1.

Table 1 - Examples for $E^{1\%}_{1cm}$ values

Substance	Wavelength (methanol)	$E^{1\%}_{1cm}$	Ref.
α-tocopherol	292 nm	76	[11]
β-tocopherol	296 nm	89	[11]
γ-tocopherol	298 nm	91	[11]
δ-tocopherol	298 nm	87	[11]

In addition to the value for α-tocopherol obtained at a wavelength of 292 nm, the absorbance at 255 nm (minimum) should also be measured. The $E^{1\%}_{1cm}$ value at this wavelength should be in the range of 6 to 8 otherwise the substance has degraded [14], [15].

4.11 Standard solutions

4.11.1 α-tocopherol standard solution

Pipette 10 ml of the α-tocopherol stock solution (4.10.1) into a one-mark 100 ml volumetric flask and dilute to the mark with the appropriate solvent (NP e.g. *n*-hexane, RP e.g. methanol). The standard solution should have a mass concentration of 1 µg/ml to 10 µg/ml of α-tocopherol. If an UV-detector is used to monitor the chromatography, a more concentrated solution has to be used.

The standard solution shall be stored protected from light and at a temperature below 4 °C and should be checked regularly.

4.11.2 Standard solution of a mixture of α-, β-, γ- and δ-tocopherol

Pipette e.g. 10 ml of each of the stock solutions (4.10) into a one-mark 100 ml volumetric flask and dilute to the mark with the appropriate solvent (NP e.g. *n*-hexane, RP e.g. methanol). The standard solution should have a mass concentration of 1 µg/ml to 10 µg/ml of each of the tocopherols.

The standard solution shall be stored protected from light and at a temperature below 4 °C and should be checked before use.

5 Apparatus

Usual laboratory apparatus and, in particular, the following:

5.1 UV Spectrometer, capable of measuring absorbances at defined wavelengths, with appropriate cells, e.g. of 1 cm path length.

5.2 Rotary evaporator, with water bath and vacuum unit.

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

5.3 HPLC-system, consisting of a pump, a sample injecting device, a fluorescence detector with an excitation wavelength set at 295 nm and an emission wavelength set at 330 nm and an evaluation system such as an integrator.

An UV detector may be used. The wavelength shall be set at 292 nm. In this case the standard and the sample solution should be more concentrated. In addition, the possibility of the detection of interfering compounds is increased.

5.4 HPLC column

Analytical normal phase column, e.g. of diameter 4,0 mm to 4,6 mm, length 100 mm to 250 mm, filled with silica, particle size 5 µm.

Particle sizes and column dimensions other than those specified in this European Standard may be used. Separation parameters have to be adapted to such materials to guarantee equivalent results.

The performance criterion for suitable analytical columns is the baseline resolution of the analytes concerned.

Suitable silica column packing materials available commercially are Lichrosorb® Si 60[2)], Spherisorb® Si[2)], Hypersil® Si[2)] and Lichrospher® 100 DIOL[2)].

Analytical reversed phase columns, e.g. C_{18}, particle size 5 m, diameter 4,0 mm to 4,6 mm, length 100 mm to 250 mm may also be used. Suitable RP column packing materials are Spherisorb® ODS[2)] and Hypersil® ODS[2)]. Most RP-columns do not separate β-tocopherol and γ-tocopherol. However, these columns may be used for the quantification of α- and δ-tocopherol.

5.5 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 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.

5.6 Phase separation filter (optional).

6 Sampling

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

7 Procedure

7.1 Preparation of the test sample

Homogenize the test sample. Grind coarse material with an appropriate mill and mix again. Measures such as pre-cooling have to be taken to avoid exposing the sample to high temperatures for long periods of time.

7.2 Preparation of sample test solution

NOTE: It is important that the sample test solutions are protected from light prior to analysis.

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2) Lichrosorb® Si 60, Spherisorb® Si, Hypersil® Si, Lichrospher® 100 DIOL, Spherisorb® ODS and Hypersil® ODS are examples of suitable products available commercially. This information is given for the convenience of users of this standard and does not constitute an endorsement by CEN.

7.2.1 Oil and fat samples with low water content containing unesterified tocopherols

7.2.1.1 **Oil and fat** (low water content)

This procedure is applicable only to samples containing unesterified tocopherols. If this is not the case, proceed according to 7.2.2.

Weigh 2 g of the test sample to the nearest 1 mg into a one-mark 25 ml volumetric flask. Add *n*-hexane or another appropriate solvent (4.7) and dissolve the test portion by swirling. Sonication of the solution may support the dissolution process. Dilute to the mark with the same solvent. This sample test solution shall be used only on NP-systems.

It may be necessary to dilute this solution further prior to chromatography or to use a smaller sample mass.

7.2.1.2 Margarine, butter

The isolation of the fat is necessary for margarine and butter prior to the dilution step. It can be performed e.g. by mixing the sample with anhydrous sodium sulfate (4.4), adding *n*-hexane (4.7) and treating the mixture in an ultrasonic bath. Filter off the solids and wash at least two times with *n*-hexane. Remove the solvent using a rotary evaporator (5.2) and reduced pressure, dissolve the residue in a defined volume of *n*-hexane and quantify by NP-HPLC.

7.2.2 Other samples

7.2.2.1 Saponification

Saponify 2 g to 10 g of the test sample by refluxing preferably under nitrogen using suitable amounts of ethanol (4.3) or methanol (4.1), water, an antioxidant such as ascorbic acid, hydroquinone, pyrogallol or BHT (4.6) and aqueous potassium hydroxide (4.5.1). Add alcohol and antioxidants to the sample prior to the addition of the potassium hydroxide.

Examples of suitable ratios of reagents are given in Table 2:

Table 2

Sample mass	Alcohol	Antioxidant	Potassium hydroxide
2 g to 5 g	50 ml methanol	0,25 g AA	5 ml of a 50 g/100 ml solution
5 g to 10 g	100 ml ethanol	1,0 g AA + 0,04 g Na_2S	20 ml of a 60 g/100 ml solution
10 g	150 ml ethanol	1,0 g AA	50 ml of a 60 g/100 ml solution

Usual times of saponification range from 15 min to 40 min at temperatures of 80 °C to 100 °C.

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.

7.2.2.2 Extraction

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

Extract the tocopherols by means of a suitable solvent (4.7). If *n*-hexane is used as solvent for the extraction of γ-tocopherol and δ-tocopherol, a certain amount of a more polar solvent has to be added to avoid the unsatisfactory recovery which has been reported in this case. Use a mixture e.g. of light petroleum and 20 % diethyl ether to achieve a quantitative extraction of these compounds. Check the recovery in order to identify possible losses [16], [17].

Repeat the extraction procedure 3 to 4 times with volumes ranging from 50 ml to 150 ml. Wash the combined extracts to neutral with water (2 to 4 times 50 ml to 150 ml).