SLOVENSKI STANDARD
SIST EN 12822:2014

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Nadomešča:
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Živila - Določevanje vitamina E s tekočinsko kromatografijo visoke ločljivosti - Merjenje α-, β-, γ- in δ-tokoferolov

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

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

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

Produits alimentaires - Détermination de la teneur en vitamine E par chromatographie liquide haute performance - Dosage des α-, ß-, γ- et δ-tocophérols

Lebensmittel - Bestimmung von Vitamin E mit Hochleistungs-Flüssigchromatographie - Bestimmung von α-, ß-, γ- und δ-Tocopherol

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
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Foreword

This document (EN 12822:2014) 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of an identical text or by endorsement, at the latest by December 2014 and conflicting national standards shall be withdrawn at the latest by December 2014.

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This document supersedes EN 12822:2000.

Annexes A, B and C are informative.

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WARNING — The use of this standard can involve hazardous materials, operations and equipment. This standard does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.

Introduction

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.

As the method in this European Standard deals with the measurement of the mass fraction of α-, β-, γ- and δ-tocopherol in food, reference is made to the literature for the calculation and expression of the vitamin E content in terms of biological activities. For further information see [1], [2], [3] and [4]. The differentiation of *RRR*-tocopherol and all racemic tocopherols is not possible with this method.

1 Scope

This European Standard specifies a method for the determination of vitamin E in foods by high performance liquid chromatography (HPLC). The determination of vitamin E content is carried out by measurement of α-, β-, γ- and δ-tocopherol. This method has been validated in two interlaboratory studies. The first study was for the analysis of α-tocopherol in margarine and milk powder ranging from 9,89 mg/100 g to 24,09 mg/100 g. The second study was for the analysis of α-, β-, γ- and δ-tocopherol in milk powder and of α-, and β-tocopherol in oat powder ranging from 0,057 mg/100 g (β-tocopherol) to 10,2 mg/100 g (α-tocopherol).

NOTE The vitamin E activity can be calculated from the tocopherol content assuming appropriate factors as given in [1], [2], [3] and [4].

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. 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)*

3 Principle

α-, β-, γ- and δ-tocopherol are determined in a sample solution by HPLC separation and subsequent photometric (UV-range) or preferably fluorometric detection. In most cases a saponification of the test material followed by an extraction is necessary. Identification is carried out on the basis of retention times and quantitative determination by the external standard method using peak areas of 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, for more information see [4] to [14].

NOTE Using normal phase columns, the separation of tocopherols and tocotrienols is also feasible.

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

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

4.4 Sodium sulfate, anhydrous.

4.5 KOH solution, for saponification, in suitable mass concentrations, for example ρ(KOH) = 50 g/100 ml or ρ(KOH) = 60 g/100 ml or alcoholic solutions, for example 28 g of KOH in 100 ml of a mixture of 9 parts per volume of ethanol and 1 part per volume of water.

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), dicholormethane, 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 for example 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 Calbiochem [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 (for purity tests, see 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-tocopherol 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

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

4.10.2 β-tocopherol stock solution

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

4.10.3 γ-tocopherol stock solution

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

4.10.4 δ-tocopherol stock solution

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

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1) This information is given for convenience of users of this European Standard and does not and does not constitute and endorsement by CEN. Equivalent products may be used if they can be shown to lead to the same results.

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.

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

$$\rho = \frac{A \cdot M \cdot 1000}{\varepsilon} \quad (1)$$

where

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

ε is the molar absorption coefficient in methanol in l x mol^{-1} x cm^{-1} at the specific wavelength as given in Table 1;

M is the molar mass, in grams per mol, of each tocopherol as given in Table 1.

Table 1 — Examples for $E^{1\%}_{1cm}$ values and calculated ε

Substance	Wavelength (in methanol)	$E^{1\%}_{1cm}$	Molar mass (in g · mol^{-1})	ε (in l · mol^{-1} · cm^{-1})	Reference
α-tocopherol	292 nm	76	430,7	3 273,3	[12], [13], [15]
β-tocopherol	296 nm	89	416,7	3 708,6	[12], [13], [15]
γ-tocopherol	298 nm	91	416,7	3 782	[12], [13], [15]
δ-tocopherol	298 nm	87	402,6	3 502,6	[12], [13], [15]

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 ratio at this wavelength should not exceed E_{255}/E_{292} = 0,18. Otherwise the substance has degraded (for more information see [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 (for NP e.g. *n*-hexane, for 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 shall be used.

The standard solution shall be stored protected from light and at a temperature below 4 °C and should be checked as described in 4.10.5.

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 (for NP e.g. *n*-hexane, for 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 as described in 4.10.5.

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.

The use of nitrogen is recommended for releasing the vacuum.

5.3 HPLC system

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. diameter of 4,0 mm to 4,6 mm, length of 100 mm to 250 mm, filled with silica, particle size 5 µm. Other particle sizes or column dimensions that those specified in this European Standard may be used. Separation parameters shall 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 packaging materials are Lichrosorb® Si 60 [2)], Spherisorb® Si[2)], Hypersil® Si[2)] and Lichrospher® 100 DIOL[2)].

Analytical reversed phase columns, e.g. C18, particle size of 5 µm, diameter of 4,0 mm to 4,6 mm, length of 100 mm to 250 mm may also be used. Suitable RP column packaging 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 and may provide values for the sum of β- + γ-tocopherol.

5.5 Filter device

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

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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 European Standard and does not constitute an endorsement by CEN.

6 Procedure

6.1 Preparation of the test sample

Homogenize the test sample. Grind coarse material with an appropriate mill and mix again. Measures shall be taken to avoid exposing the sample to high temperatures for longer periods of time.

6.2 Preparation of the sample test solution

6.2.1 Precautions

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

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

6.2.2.1 Oil and fat with low water content

This procedure is applicable only to samples containing unesterified tocopherols. If this is not the case, proceed according to 6.2.3.

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 can 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.

6.2.2.2 Margarine and butter

The isolation of 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.

6.2.3 Other samples

6.2.3.1 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 potassium hydroxide solution (4.5). 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 — Suitable ratios of reagents

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 to 20 g	150 ml ethanol	1,0 g AA	50 ml of a 60 g/100 ml solution