BS EN 12822:2014

BSI Standards Publication

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

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National foreword

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The UK participation in its preparation was entrusted to Technical Committee AW/275, Food analysis - Horizontal methods.

A list of organizations represented on this committee can be obtained on request to its secretary.

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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

This European Standard was approved by CEN on 17 April 2014.

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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of an identical text or by endorsement, at the latest by December 2014 and conflicting national standards shall be withdrawn at the latest by December 2014.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 $E_{1cm}^{1\%}$ values and calculated ε

Substance	Wavelength (in methanol)	$E_{1cn}^{1\%}$	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

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 potassium hydroxide solution (4.5) shall be added and saponification time shall be extended.

6.2.3.2 Extraction

In order to avoid emulsions, add an amount of water 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, add a certain amount of a more polar solvent 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 (for more information see [16] and [17]).

Repeat the extraction procedure three to four 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).

The extraction may also be performed by solid supported liquid/liquid technique (e.g. EXtrelut®[3)] when the content of vitamin E is not too low (for more information see [18], [22]).

6.2.3.3 Evaporation

Evaporate the extract using a rotary evaporator (5.2). Remove traces of water by drying with sodium sulfate (4.4) or by azeotropic distillation with ethanol (4.2) or toluene. Other equivalent techniques such as phase separation filter paper to eliminate traces of water may be used provided they have been proven not to affect the result.

6.2.3.4 Dilution

Redissolve the residue using the mobile phase (4.8) or another HPLC compatible solvent to a final concentration of 1 µg/ml to 10 µg/ml for each of the tocopherols.

6.3 Identification

Identify the tocopherols by comparison of the retention times of the individual peaks in the chromatograms obtained with the sample test solution and with the standard solution. Peak identification can also be performed by adding small amounts of the appropriate standard solutions to the sample test solution.

NOTE The separation and the quantification have proven to be satisfactory if the following experimental conditions are followed (see also Figure A.1 and Figure A.2). For alternative HPLC systems, see Table C.1.

Stationary phase:	Lichrosorb® Si 60, 5 µm;
Column dimension:	125 mm x 4 mm;
Mobile phase:	a volume fraction of 3 % 1,4-dioxane in *n*-hexane;
Flow rate:	1,0 ml/min;
Injection volume:	10 µl to 100 µl;
Detection:	fluorometric, excitation: 295 nm, emission: 330 nm.

3) EXtrelut® is an example of a suitable product available commercially. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN.

6.4 Determination

Inject appropriate volumes (up to 100 µl) of the standard solution as well as the sample test solution into the HPLC system. To carry out a quantitative determination by the external standard method, integrate the peak areas or determine the peak heights and compare the results with the corresponding values for the standard substance.

Inject equal volumes of the sample and of the standard solutions or compensate with a corresponding factor in the calculation of the results (see Clause 7). Check the linearity of the calibration function.

7 Calculation

Base the calculation on a calibration curve or use the corresponding programmes of the integrator or use the following simplified procedure. Calculate the mass fraction, *w*, of α-, β- γ- and δ-tocopherol in mg/100 g of the sample using Formula (2):

$$w = \frac{A_S \cdot \rho \cdot V \cdot V_{ST}}{A_{ST} \cdot m \cdot V_S \cdot 1000} \cdot 100 \qquad (2)$$

where

A_S is the peak area or peak height for α-, β- γ- or δ-tocopherol obtained with the sample test solution;

A_{ST} is the peak area or peak height for α-, β- γ- or δ-tocopherol obtained with the standard solution;

V is the total volume of sample test solution (see 6.2.2 and 6.2.3), in millilitre;

ρ is the concentration of α-, β- γ- or δ-tocopherol in the standard solution (see 4.11.1 and 4.11.2), corrected for purity (see 4.10.5), in micrograms per millilitre;

m is the sample mass, in gram;

V_{ST} is the injection volume of the standard solution, in microlitres;

V_S is the injection volume of the sample test solution, in microlitres;

1 000 is the conversion factor for microgram to milligram;

100 is the conversion factor for the mass fraction per 100 g.

Report the result for α-, β- γ- or δ-tocopherol in mg/100 g. For vitamin E activity, see the Introduction and [1], [2] and [3].

8 Precision

8.1 General

The precision data of different HPLC methods for the determination of α-tocopherol were established in 1994 by an international comparison study, see [18], organized on behalf of the European Commission's Standards, Measurement and Testing Programme on a sample of margarine (Certified Reference Material (CRM) 122 and milk powder CRM 421 and provided the statistical information shown in Annex B. The data derived from these comparison studies may not be applicable to analyte concentration ranges and sample matrices other than those given in Annex B.

The precision data for milk powder and oat powder have been established in an interlaboratory study according to ISO 5725:1986, see [19], carried out by the Max von Pettenkofer-Institute of the Federal Health Office (today: Federal Institute for Health Protection of Consumers and Veterinary Medicine), Food Chemistry

Departement, Berlin, Germany, see [20]. The data derived from this interlaboratory study may not be applicable to analyte concentration ranges and sample matrices other than those given in Annex B.

8.2 Repeatability

The absolute difference between two single test results found on identical test material by one operator using the same apparatus within the shortest feasible time interval will exceed the repeatability limit *r* in not more than 5 % of the cases. The repeatability is dependent on the concentration level of the analyte in the sample.

The values are:

Margarine	α-tocopherol	$\bar{x}$ = 24,09 mg/100 g	*r* = 2,765 mg/100 g
Milk powder	α-tocopherol	$\bar{x}$ = 9,89 mg/100 g	*r* = 1,130 mg/100 g
Milk powder	α-tocopherol	$\bar{x}$ = 10,2 mg/100 g	*r* = 0,853 mg/100 g
	β-tocopherol	$\bar{x}$ = 0,081 mg/100 g	*r* = 0,025 mg/100 g
	γ-tocopherol	$\bar{x}$ = 1,989 mg/100 g	*r* = 0,311 mg/100 g
	δ-tocopherol	$\bar{x}$ = 0,280 mg/100 g	*r* = 0,082 mg/100 g
Oat powder	α-tocopherol	$\bar{x}$ = 0,279 mg/100 g	*r* = 0,071 mg/100 g
	β-tocopherol	$\bar{x}$ = 0,057 mg/100 g	*r* = 0,017 mg/100 g

8.3 Reproducibility

The absolute difference between two single test results obtained on identical material reported by two laboratories will exceed the reproducibility limit *R* in not more than 5 % of the cases.

The values are:

Margarine	α-tocopherol	$\bar{x}$ = 24,09 mg/100 g	*R* = 4,18 mg/100 g
Milk powder	α-tocopherol	$\bar{x}$ = 9,89 mg/100 g	*R* = 1,96 mg/100 g
Milk powder	α-tocopherol	$\bar{x}$ = 10,2 mg/100 g	*R* = 3,705 mg/100 g
	β-tocopherol	$\bar{x}$ = 0,081 mg/100 g	*R* = 0,046 mg/100 g
	γ-tocopherol	$\bar{x}$ = 1,989 mg/100 g	*R* = 0,978 mg/100 g
	δ-tocopherol	$\bar{x}$ = 0,280 mg/100 g	*R* = 0,134 mg/100 g
Oat powder	α-tocopherol	$\bar{x}$ = 0,279 mg/100 g	*R* = 0,133 mg/100 g
	β-tocopherol	$\bar{x}$ = 0,057 mg/100 g	*R* = 0,030 mg/100 g

9 Test report

The test report should comply with EN ISO/IEC 17025 [23] and shall contain at least the following data:

a) all information necessary for the identification of the sample;

b) a reference to this European Standard or to the method used;

c) the date and time of sampling procedure (if known);

d) the date of receipt;

e) the date of test;

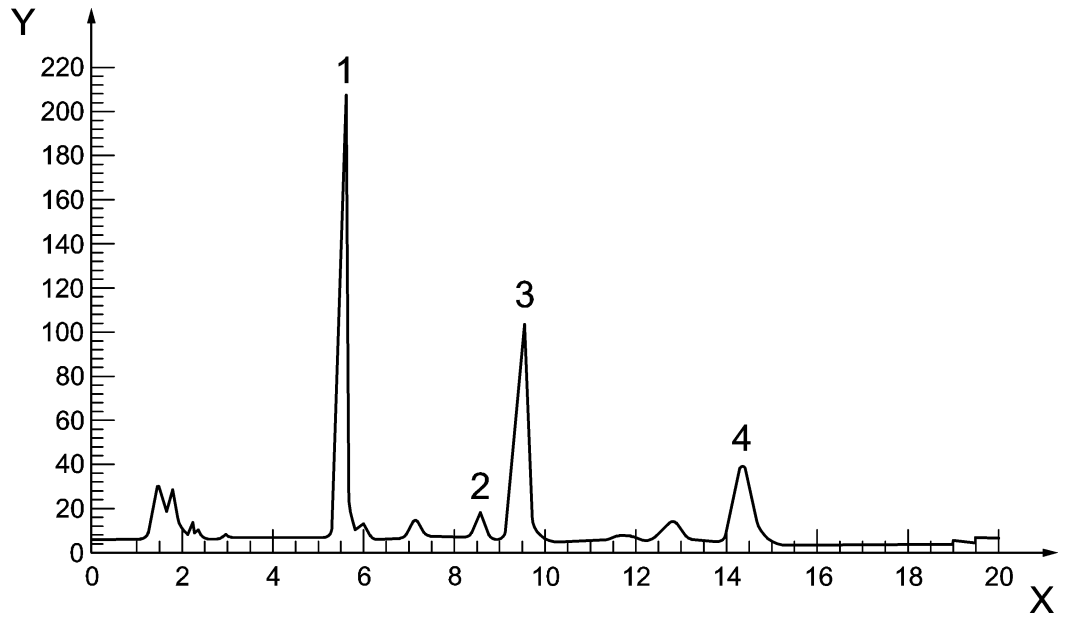
f) the results and the units in which the results have been expressed;

g) any particular points observed in the course of the test;

h) any operations not specified in the method or regarded as optional which might have affected the results.

Annex A
(informative)

Examples of HPLC chromatograms

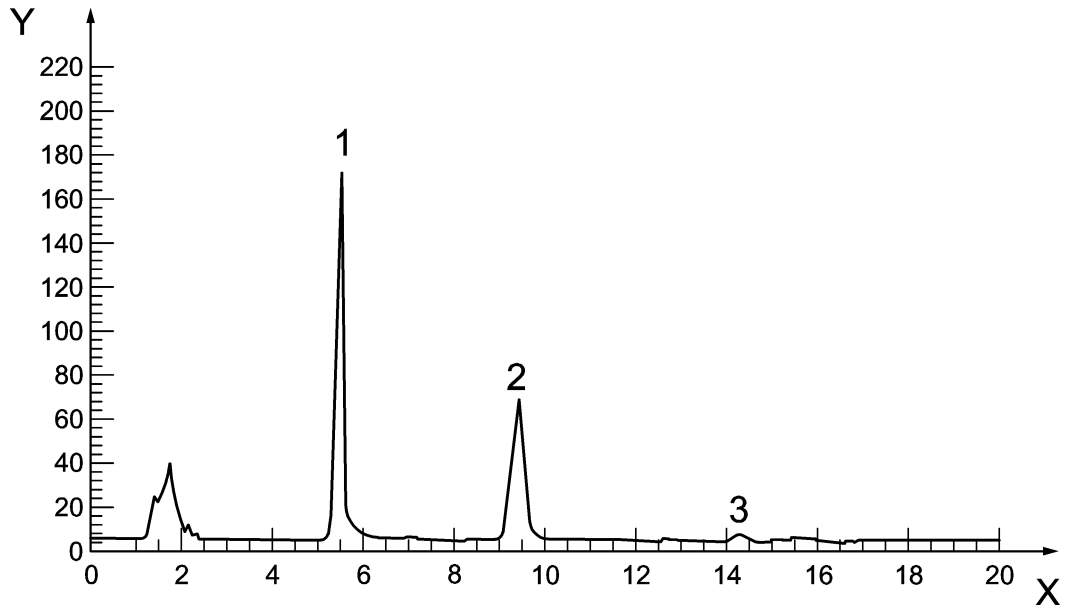


Key

Y	fluorescence
X	time (min)
1	α-tocopherol
2	β-tocopherol
3	γ-tocopherol
4	δ-tocopherol

Stationary phase:	Lichrosorb® Si 60, 5 µm
Column dimension:	125 mm x 4 mm
Mobile phase:	a volume fraction of 3 % 1,4-dioxane in *n*-hexane
Flow rate:	1,0 ml/min
Injection volume:	20 µl
Detection:	fluorometric, excitation of 295 nm, emission of 330 nm

Figure A.1 — Example of a HPLC separation of α-, β-, γ- and δ-tocopherol from a margarine sample (CRM 122)



Key

Y	fluorescence
X	time (min)
1	α-tocopherol
2	γ-tocopherol
3	δ-tocopherol

Stationary phase:	Lichrosorb® Si 60, 5 µm
Column dimension:	125 mm x 4 mm
Mobile phase:	a volume fraction of 3 % 1,4-dioxane in *n*-hexane
Flow rate:	1,0 ml/min
Injection volume:	20 µl
Detection:	fluorometric, excitation of 295 nm, emission of 330 nm

Figure A.2 — Example of a HPLC separation of α-, β-, γ- and δ-tocopherol from a milk powder sample (CRM 421)

Annex B
(informative)

Precision data

The data in Table B.1 of different methods for the determination of vitamin E (*α*-tocopherol) have been defined in an international comparison study organized by the Standard, Measurement and Testing Programme of the European Commission, see [18]. The data in Table B.2 and Table B.3 have been defined in an interlaboratory study in accordance with ISO 5725:1986 [19], conducted by the Max von Pettenkofer-Institute of the Federal Health Office, Food Chemistry Department, Berlin, Germany [20].

Table B.1 — Precision data for margarine and milk powder

Sample	CRM 122 Margarine	CRM 421 Milk Powder
Analyte	α-tocopherol	α-tocopherol
Year of interlaboratory study	1994	1994
Number of laboratories	9	10
Number of samples	1	1
Number of laboratories retained after elimination of outliers	9	10
Number of outliers	0	0
Number of data sets	9	10
Number of replicate measurement	45	50
Mean value, $\bar{x}$, mg/100 g	24,09	9,89
Repeatability standard deviation s_r, mg/100 g	0,977	0,399
Repeatability relative standard deviation RSD_r, %	4,1	4,0
Repeatability value, r [r = 2,83 x s_r], mg/100 g	2,765	1,130
Reproducibility standard deviation, s_R, mg/100 g	1,477	0,693
Reproducibility relative standard deviation RSD_R, %	6,1	7,0
Reproducibility value R [R = 2,83 x s_R], mg/100 g	4,180	1,960
HorRat value, according to [21]	0,87	0,87

NOTE The data obtained in this international comparison study have been produced using established methods being identical with in-house routine assay procedures of the participant laboratories with the HPLC systems described in Annex C.

Table B.2 — Precision data for milk powder

Analyte	**α-tocopherol**	**β-tocopherol**	**γ-tocopherol**	**δ-tocopherol**
Year of interlaboratory study	1993	1993	1993	1993
Number of laboratories	13	12	13	10
Number of samples	5	5	5	5
Number of laboratories retained after eliminating outliers	12	9	11	8
Number of outliers	1	3	2	2
Number of accepted results	66	51	65	40
Mean value, $\bar{x}$, mg/100 g	10,2	0,081	1,989	0,280
Repeatability standard deviation, s_r, mg/100 g	0,301	0,009	0,110	0,029
Repeatability relative standard deviation RSD_r, %	3,0	11,1	5,5	10,4
Repeatability value, r [$r = 2{,}83 \times s_r$], mg/100 g	0,853	0,025	0,311	0,082
Reproducibility standard deviation, s_R, mg/100 g	1,31	0,016	0,346	0,047
Reproducibility relative standard deviation RSD_R, %	12,8	19,8	17,4	16,8
Reproducibility value R [$R = 2{,}83 \times s_R$], mg/100 g	3,705	0,046	0,978	0,134
HorRat value, according to [21]	1,1	1,2	1,7	1,2

Table B.3 — Precision data for oat powder

Analyte	**α-tocopherol**	**β-tocopherol**
Year of interlaboratory study	1993	1993
Number of laboratories	13	13
Number of samples	5	5
Number of laboratories retained after eliminating outliers	12	11
Number of outliers	1	2
Number of accepted results	70	64
Mean value, $\bar{x}$, mg/100 g	0,279	0,057
Repeatability standard deviation, s_r,, mg/100 g	0,025	0,006
Repeatability relative standard deviation RSD_r, %	9,0	10,5
Repeatability value, r [$r = 2{,}83 \times s_r$], mg/100 g	0,071	0,016
Reproducibility standard deviation, s_R, mg/100 g	0,047	0,011
Reproducibility relative standard deviation RSD_R, %	16,8	19,3
Reproducibility value R [$R = 2{,}83 \times s_R$], mg/100 g	0,133	0,030
HorRat value, according to [21]	1,2	1,1

Annex C
(informative)

Alternative HPLC systems

The separation and quantification has been proven to be satisfactory in the following chromatographic conditions are being applied [18].

Table C.1 — Alternative HPLC conditions

Stationary Phase[a]	**Column Dimension** (mm x mm)	**Mobile Phase** (volume parts)	**Flow** ml/min	**Detection** (nm)
Knauer polygosil® 60–5	250 × 4,6	*n*-hexane + di-isopropylether (80 + 20)	1,5	F: Ex: 296 Em: 320
Si 60	250 × 4,6	*n*-hexane + 2-propanol (98 + 2)	1,5	F: Ex: 284 Em: 330
Silica, 5 µm	100 × 8	iso-octane + di-isopropylether (with 0,15 % propanol) (97,5 + 2,5)	2,0	F: Ex: 295 Em: 330
Lichrospher®Si 100, 5 µm	250 × 4	*n*-hexane + 2-propanol (99,85 + 0,15)	2,5	F: Ex: 290 Em: 330
Lichrospher®Si 60, 5 µm	250 × 4,6	*n*-hexane + 2-propanol (99,3 + 0,7)	1,2	F: Ex: 290 Em: 330
Lichrospher®Si 60, 5 µm	250 × 4	*n*-hexane + dioxane (97 + 3)	1,0	F: Ex: 293 Em: 326
Lichrospher®Si 60, 5 µm	250 × 4	Gradient of 1 % 2-propanol in *n*-heptane in 7 min to 1,5 % 2-propanol in *n*-heptane	1,0	F: Ex: 290 Em: 327
Amino, 3 µm	100 × 4,6	iso-octane + iso-butanol (98 + 2)	1,5	F: Ex: 290 Em: 330
Nucleosil® C18, 5 µm	250 × 4	methanol + water (97 + 3)	2,0	UV: 292
RP-8, 10 µm	250 × 4,6	acetonitrile + methanol + water (50 + 45 + 5)	2,0	F: Ex: 293 Em: 326 UV: 290 nm

[a] Trademarks of the listed products are examples of suitable products available commercially. This information is given for the convenience of the users of this European Standard and does not constitute an endorsement by CEN.

F: Fluorometric

UV: Ultraviolet

Ex: Excitation wavelength

Em: Emission wavelength

NOTE The reversed phase columns Nucleosil C18 and RP-8 have been used in the interlaboratory study of *α*-tocopherol of margarine and milk powder (see Table B.1).

Bibliography

[1] *Dietary Reference Intakes for vitamin C, vitamin E, selenium and carotenoids*. Institute of Medicine. Nat. Acad. Press, Washington, 2000

[2] Deutsche gesellschaft für Ernährung (DGE): Referenzwerte für Nährstoffzufuhr; 5. Überarbeitung 1991

[3] Brubacher, g. and Wiss, O. (1972), Vitamin E active compounds, synergists and antagonists in: Sebrell, W.H. Jr. and Harris eds The Vitamins, Chemistry, Physiology, Pathology, Methods 2nd edn Vol 5, Academic Press, New York, 255 – 258

[4] DAB 10 (1991), Deutsches Arzneibuch 10. Ausgabe 1991, Stand 1997; T 5.6/0692 int-rac-*α*-Tocopherolum Comment to Ph. Eur. 5.6. Deutscher Apotheker Verlag Stuttgart

[5] Brubacher, g. et al., (1985). Methods for the Determination of Vitamins in Food, Elsevier App. Science Publishers, London, 97 – 106

[6] GERTZ C., KERRMAN K. Zur Analytik der Tocopherole und Tocotrienole in Lebensmitteln. *Z. Lebensmittelunters. Forsch.* 1982, **174** pp. 390–394

[7] NOBILE S., MOOR H. Analysenmethode zur Bestimmung von Vitamin E in Lebensmitteln und Futtermitteln. *Mitt. Lebensmittel Unters. Hyg.* 1953, **44** p. 396

[8] Determination of Tocopherols in fats and oils. L-13.03/04. September 1987 in: Amtliche Sammlung von Untersuchungsverfahren nach § 64 LFGB: Verfahren zur Probenahme und Untersuchung von Lebensmitteln, Tabakerzeugnissen, kosmetischen Mitteln und Bedarfsgegenständen/Bundesgesundheitsamt (In: Collection of official methods under article 64 of the german Federal Foods and Feeds Act, Methods of sampling and analysis of foods, tobacco products, cosmetics and commodity goods/Federal Health Office), Loseblattausgabe September 1998, Bd. 1 (Loose leaf edition as of 1998-09, Vol. 1) Berlin, Köln: Beuth Verlag gmbH

[9] BALZ M., SCHULTE E., THIER H.P. Trennung von Tocopherolen und Tocotrienolen durch HPLC. *Fat. Sci. Technol.* 1992, **6** pp. 209–213

[10] SPEEK A.J., SCHRIJVER J., SCHREURS W.H.P. Vitamin E composition of some seed oils as determined by high-performance liquid chromatography with fluorimetric detection. *J. Food Sci.* 1985, **50** pp. 121–124

[11] MANZ U., PHILLIP K. A method for the routine determination of tocopherols in animal feed and human foodstuffs. *Int. J. Vitam. Nutr. Res.* 1981, **51** pp. 342–348

[12] Pocklington, W.D. and Diefenbacher, A. (1988). Determination of tocopherols and tocotrienols in vegetable oils and fats by high performance liquid chromatography: results of a collaborative study and the standardised method

[13] BOURGEOIS C. *Determination of Vitamin E: Tocopherols and Tocotrienols, Elsevier App*. Science Publishers, London, 1992

[14] LUMLEY I.D. (1993), Vitamin analysis in food in: The Technology of Vitamins in Food, ed. by P.B. Ottaway, Blacie Academic & Professional, glasgow, 186-190

[15] BALZ M., SCHULTE E., THIER H.P. A new parameter for checking the suitability of tocopherol standards. *Z. Lebensm. Unters. Forsch.* 1996, **202** pp. 80–81

[16] VDLUFA-Methodenbuch: Die chemische Untersuchung von Futtermitteln (The chemical analysis of animal feeding stuffs), Band III (Vol iii), Bestimmung der Tocopherole, HPLC-Verfahren (Determination of Tocopherols HPLC-method) 4. Erg (4th Add), Kapitel 13.5.5 (Chapter 13.5.5), VDLUFA-Verlag, Darmstadt

[17] KONINGS E.J.M., ROOMANS H.H.S., BELJAARS P.R. Liquid Chromatographic Determination of Tocopherols and Tocotrienols in Margarine. *Infant Foods and Vegetables, JAOAC*. 1996, **79** (4) pp. 902–906

[18] FINGLAS P.M., VAN DEN BERG H., DE FROIDMONT-GORTZ I. *The certification of the mass fractions of vitamins in three reference materials: margarine (CRM 122), milk powder (CRM 421), and lyophilized Brussels sprouts (CRM 431). EUR-Report 18039*. Commission of the European Union, Luxembourg, 1997

[19] ISO 5725:1986, *Precision of test methods — Determination of repeatability and reproducibility for a standard test method by inter-laboratory tests*

[20] Untersuchung von Lebensmitteln – Bestimmung von Tocopherolen und Tocotrienolen in dietätischen Lebensmitteln L 49.00-5 September 1998 (Food analysis – Determination of tocopherols and tocotrienols in dietetic foodstuffs L 49.00-5 September 1998) in: Amtliche Sammlung von Untersuchungsverfahren nach § 64 LFGB: Verfahren zur Probenahme und Untersuchung von Lebensmitteln, Tabakerzeugnissen, kosmetischen Mitteln und Bedarfsgegenständen/Bundesgesundheitsamt (In: Collection of official methods under article 64 of the german Federal Foods and Feed Act, Methods of sampling and analysis of foods, tobacco products, cosmetics and commodity goods/Federal Health Office), Loseblattausgabe September 1998, Bd. 1 (Loose leaf edition as of 1998-09, Vol. 1) Berlin, Köln: Beuth Verlag gmbH

[21] HORWITZ W., ALBERT R., The Horwitz Ration. (HorRat): A useful Index of Method Performance with Respect to Precision. *J. AOAC Int.* 2006, **89** pp. 1095–1109

[22] Bourgeois, C.F. and Ciba, N., 1988. Disposal cartridge extraction of retinol and alpha-tocopherol from fatty samples. J.A.O.A.C. 71 (1), 12-15

[23] EN ISO/IEC 17025:2005, *General requirements for the competence of testing and calibration laboratories (ISO/IEC 17025:2005)*

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