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Animal and vegetable fats and oils — Determination of tocopherol and tocotrienol contents by high-performance liquid chromatography

Corps gras d'origines animale et végétale — Détermination des teneurs en tocophérols et en tocotriénols par chromatographie en phase liquide à haute performance

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Foreword

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International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

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

ISO 9936 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 11, *Animal and vegetable fats and oils*.

This second edition cancels and replaces the first edition (ISO 9936:1997), which has been technically revised.

Animal and vegetable fats and oils — Determination of tocopherol and tocotrienol contents by high-performance liquid chromatography

1 Scope

This International Standard specifies a method for the determination of the contents of free α-, β-, γ-, and δ-tocopherols and tocotrienols (referred to jointly as tocols) in animal and vegetable fats and oils (referred to hereinafter as fats) by high-performance liquid chromatography (HPLC).

For products containing tocopherol or tocotrienol esters, it is necessary to carry out a preliminary saponification.

NOTE A suitable method involving a cold saponification procedure is described in Annex B for information only.

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.

ISO 661, *Animal and vegetable fats and oils — Preparation of test sample*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
tocol content
mass fraction of the individual tocols, determined using the method specified in this International Standard

NOTE The content is expressed in milligrams per kilogram as a whole number.

4 Principle

A test portion is dissolved in *n*-heptane and the individual tocols are separated by high-performance liquid chromatography. The content of each tocol is calculated using calibration factors determined from calibration solutions.

5 Reagents

Use only reagents of HPLC grade or equivalent.

5.1 α-, β-, γ- and δ-tocopherol and tocotrienol standards.

If tocopherol standards are not available, a blend of wheat germ and soya bean oil may be used to identify α-, β-, γ- and δ-tocopherols.

If tocotrienol standards are not available, palm oil may be used to identify α- and γ-tocotrienols. The chromatograms obtained can be used to assist peak identification in test sample chromatograms, in which case the calibration factors for the corresponding tocopherols should be used.

NOTE α-, β-, γ- and δ-tocopherol and tocotrienol standards can be obtained from Merck [1)]; α-tocopherol can be obtained from various suppliers. It has been reported that the purity of some commercially available tocopherol standards may vary between 85 % and 100 %. Thus, it is important to determine the concentration of prepared calibration solutions by UV spectrometry (see 9.1.1).

5.2 Tetrahydrofuran, filtered through an HPLC nylon filter (0,45 µm).

5.3 *n*-Heptane, filtered through an HPLC nylon filter (0,45 µm).

5.4 HPLC mobile phase: any suitable mixture of solvents that has been proved to reach a chromatographic resolution of peaks as good as the one presented in Table 2 (relative retention time of tocopherols and tocotrienols) and in Annex A (chromatograms of a mixture of vegetable oils), should be used (see Table C.3).

The preparation of a suitable mobile phase, 3,85 % (volume fraction) tetrahydrofuran solution in *n*-heptane, is as follows. Using a 1 000 ml graduated cylinder (6.5), introduce 1 000 ml of *n*-heptane (5.3) in a 2 litre bottle. Add twice 20 ml of tetrahydrofuran (5.2) using a 20 ml volumetric pipette (6.6). Homogenize the mobile phase by means of an ultrasonic bath (6.8) for 15 min.

5.5 Methanol.

6 Apparatus

Usual laboratory apparatus and, in particular, the following.

6.1 HPLC system, consisting of a high-pressure pump, a sample injection device, column thermostat adjusted to 25 °C (optional), a fluorescence detector with the excitation wavelength set at 295 nm and emission wavelength at 330 nm, and a recording integrator.

An ultraviolet (UV) detector may be used if a fluorescence detector is not available but it is not recommended. However, if a UV detector is used, the wavelength should be set at 292 nm.

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1) Merck Tocopherol set 613424 is available from Calbiochem (www.calbiochem.com). It contains one 50 mg vial each of DL-α-tocopherol, D-β-tocopherol, D-γ-tocopherol, and D-δ-tocopherol with a purity of 95 % by HPLC (for each component). Merck Tocotrienol set 613432 is available from Calbiochem also. It contains one 50 mg vial each of α-tocotrienol, β-tocotrienol, γ-tocotrienol, and δ-tocotrienol with a purity of 95 % by HPLC (75 % for γ-tocotrienol).

This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of these products.

6.2 HPLC analytical column, two types are possible:

— 250 mm × 4 mm, packed with microparticulate **diol** having a mean particle size of about 5 µm, or

— 250 mm × 4,6 mm, packed with microparticulate **silica** having a mean particle size of about 5 µm.

NOTE 1 Suitable diol silica column packing material available commercially is 5 µm LiChrospher 100 Diol; suitable silica column packing materials available commercially are 5 µm LiChrosob SI 60 and Kromasil 100 [2]. When β-tocotrienol is expected in the sample, the diol silica column is preferred as γ-tocopherol and β-tocotrienol are co-eluted when using the silica column.

NOTE 2 The length and the diameter of the column can be varied according to the HPLC technique used.

6.3 UV spectrometer, capable of absolute measurement of absorbance at precisely defined wavelengths, with a 10-mm path length cell.

6.4 Rotary evaporator.

6.5 Graduated cylinder, of 1 000 ml capacity.

6.6 Volumetric pipettes, of 5 ml, 10 ml and 20 ml capacities.

6.7 Volumetric flasks, 50 ml and 25 ml capacities.

6.8 Ultrasonic bath.

7 Sampling

A representative sample should be sent to the laboratory. It is important that the sample has not been damaged or changed during transport or storage.

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 5555.

8 Preparation of test sample

In the case of liquid laboratory samples, prepare the test sample by homogenization as described in ISO 661, except that filtration should be avoided.

In the case of solid samples, transfer a representative portion (i.e. not less than 10 % by mass of the laboratory sample) to a glass beaker and carefully homogenize by melting, with gentle mixing, in a water bath at a temperature not exceeding 40 °C.

Preparation of the test samples should be carried out, as far as is practicable, in subdued light and in all cases out of direct sunlight.

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2) These types of columns are examples of suitable products which are available commercially.

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9 Procedure

IMPORTANT — In general, the oxidation of tocols during the analysis may lead to low results. The rate of oxidation is increased in the presence of alkalis, or under the influence of heat or light, and measures should be taken to guard against these influences.

9.1 Preparation of calibration solutions

9.1.1 Stock calibration solutions

Prepare a stock solution of each tocol by weighing 10 mg ± 1 mg of the standard (5.1) into a 50 ml volumetric flask and diluting to the mark with *n*-heptane (5.3).

Pipette 5 ml of this solution into an amber glass round-bottomed flask and remove all *n*-heptane on a rotary evaporator (6.4) under vacuum at a temperature not greater than 40 °C. Restore atmospheric pressure with nitrogen and remove the flask from the evaporator as soon as all the solvent has been removed. Pipette into the flask 10 ml of methanol (5.5) and swirl to dissolve the residue. Measure the maximum absorbance of this solution in a wavelength range between 270 nm and 310 nm (see appropriate wavelength in Table 1) using the UV spectrometer (6.3) with a 10-mm path length cell. The measured absorbance should be between 0,2 and 0,8. Calculate the concentration (in micrograms per millilitre) by dividing the absorbance value by the appropriate factor given in Table 1.

Table 1 — Division factors

Wavelength nm	Tocopherol	Division factor
292	α-tocopherol	0,007 6
296	β-tocopherol	0,008 9
298	γ-tocopherol	0,009 1
298	δ-tocopherol	0,008 7
NOTE The factors quoted are derived from the E values (1 %/1 cm) of the tocopherols. For example, the E value (1 %/1 cm) of α-tocopherol is 76 at 292 nm (in methanol); therefore a 1 µg/ml solution of α-tocopherol will have an absorbance of 0,007 6 at 292 nm.		

9.1.2 Standard solution

A suitable standard solution should be prepared, according to the sensitivity of the fluorescence detector used.

The following preparation of working solution is given as an example: mix appropriate volumes, for example 1 ml, of the stock calibration solutions (9.1.1) to obtain a mixed tocol standard solution, and dilute with *n*-heptane to give a solution containing between 1 µg and 5 µg of each standard per millilitre.

The standard solution shall be freshly prepared each working day.

Protect all solutions from light and store them at between 0 °C and 4 °C.

Stock standard solutions can be satisfactorily stored in amber glassware for up to 1 week if refrigerated. Flasks may be wrapped in aluminium foil.

NOTE If a UV detector is used, a more concentrated solution might be needed.

9.2 Optimization of working parameters

9.2.1 If the column (6.2) is new or of unknown history, or if for any other reason it is necessary to condition it, wash and condition it for about 10 min with methanol, then dichloromethane, followed by *n*-heptane at a flow rate of about 1 ml/min.

Pump the HPLC mobile phase (5.4) through the column at a flow rate of 1 ml/min for at least 30 min.

WARNING — Methanol and dichloromethane are hazardous to humans and to the environment. Handle them with care.

9.2.2 Inject 10 µl or 20 µl (according to detector sensitivity) of the standard solution (9.1.2) into the column and, if necessary, adjust the tetrahydrofuran content of the mobile phase and the flow rate to achieve the following conditions:

a) α-tocopherol retention time between 8 min and 12 min;

b) resolution factor RF for the separation of β- and γ-tocopherols of not less than 1,0; i.e. almost baseline separation, where RF is calculated using the following formula:

$$RF = \frac{d_r(\mathrm{I}) - d_r(\mathrm{II})}{0{,}5 \cdot \left[b(\mathrm{I}) + b(\mathrm{II}) \right]}$$

where

d_r(I) is the retention distance of γ-tocopherol;

d_r(II) is the retention distance of β-tocopherol;

b(I) is the width at the base of the γ-tocopherol peak;

b(II) is the width at the base of the β-tocopherol peak.

9.2.3 Select the optimum settings for the detection and integration system. Inject 10 µl or 20 µl of the standard solution (9.1.2). Repeat the injection and check that reproducible chromatograms are obtained.

9.3 Preparation of test solution

Depending on the tocol concentration (9.1.2), weigh, to the nearest 1 mg, 0,25 g ± 0,1 g of the test sample (Clause 8) into a 25 ml one-mark volumetric flask. Add a quantity of *n*-heptane (5.3), swirling to dissolve the test portion, and dilute to the mark with the same solvent. Filter the solution with an HPLC nylon filter 0,45 µm if not clear.

It is important that the test solutions be protected from light prior to analysis, and analysed on the day of preparation.

NOTE It may be necessary to prepare a more concentrated solution or to dilute the solution further prior to chromatography.

9.4 Determination

9.4.1 Inject 10 µl or 20 µl (according to detector sensitivity) of the standard solution (9.1.2) into the column and record the areas of the peaks.

9.4.2 Inject 10 µl or 20 µl (according to detector sensitivity) of the test solution (9.3) into the column and identify the tocols present by reference to the calibration chromatograms. Record the areas of the peaks.

Repeat the injection of the test solution and the measurement. Use the mean values of the two measurements as the result of one determination.

Inject a further 10 µl or 20 µl (according to detector sensitivity) of the standard solution (9.1.2) and record the areas of the peaks.

The relative retention times shown in Table 2 have been found to be typical.

Table 2 — Example of relative retention time of tocopherols and tocotrienols

Silica column (α-tocopherol as reference substance)		Diol column (α-tocopherol as reference substance)	
α-tocopherol = 1,00	α-tocotrienol = 1,19	α-tocopherol = 1,00	α-tocotrienol = 1,24
β-tocopherol = 1,34	β-tocotrienol = 1,63	β-tocopherol = 1,59	β-tocotrienol = 2,03
γ-tocopherol = 1,63	γ-tocotrienol = 2,00	γ-tocopherol = 1,74	γ-tocotrienol = 2,22
δ-tocopherol = 2,24	δ-tocotrienol = 2,79	δ-tocopherol = 2,46	δ-tocotrienol = 3,19

10 Expression of results

The α-tocopherol content, w, of the sample, expressed in milligrams per kilogram (mg/kg), is given by the formula:

$$w = \frac{\rho \times \bar{A}_t \times V}{\bar{A}_s \times m}$$

where

ρ is the concentration, in micrograms per millilitre, of α-tocopherol in the standard solution (9.1.2);

$\bar{A}_s$ is the mean of the peak areas obtained for the α-tocopherol standard;

$\bar{A}_t$ is the mean of the peak areas obtained for the α-tocopherol in the test sample;

m is the mass, in grams, of the test sample (9.3);

V is the volume of test solution prepared (= 25 ml).

Calculate the remaining tocol contents of the test sample in the same way using the data obtained from the corresponding standard.

If the only standard available is α-tocopherol, relate all tocopherols to this standard, but make this clear when reporting the results. If UV detection is used, again relate all tocopherols to the α-tocopherol standard, but normalize the peak areas to α-tocopherol using the division factors given in 9.1.1.

NOTE The fluorescence intensity of tocotrienols is the same as of the corresponding tocopherols, and the UV absorbencies are similar.

The content is expressed in milligrams per kilogram as a whole number.

11 Precision

11.1 Interlaboratory test

Details of an interlaboratory test on the precision of the method are summarized in Annex C. The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given.

11.2 Repeatability

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5 % of cases be greater than the value of r given in Table 3.

11.3 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will in not more than 5 % of cases be greater than the value of R given in Table 3.

Table 3 — Repeatability limit (r) and reproducibility limit (R)

Tocol content mg/kg	**Range of concentration** mg/kg	r mg/kg	R mg/kg
T_1 = mean value of individual **tocopherol** content	0 to 2 220	0,082 5 T_1	0,209 4 T_1
T_2 = mean value of individual **tocotrienol** content	10 to 210	0,090 0 T_2	0,255 2 T_2
T_3 = mean value of **total content** (tocopherols + tocotrienols)	200 to 3 250	0,071 8 T_3	0,255 7 T_3

12 Test report

The test report shall specify:

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

b) the sampling method used, if known;

c) the test method used, together with mention of this International Standard;

d) all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the test result(s);

e) the test result(s) obtained;

f) if the repeatability has been checked, the final quoted result obtained.