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Animal and vegetable fats and oils — Separation of lipid classes by capillary gas chromatography (fingerprint method)

Corps gras d'origines animale et végétale — Séparation des classes lipidiques par chromatographie en phase gazeuse sur colonne capillaire (méthode fingerprint)

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 11, *Animal and vegetable fats and oils,* in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 307, *Oilseeds, vegetable and animal fats and oils and their by-products — Methods of sampling and analysis,* in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Animal and vegetable fats and oils — Separation of lipid classes by capillary gas chromatography (fingerprint method)

1 Scope

This document specifies a method for the semi-quantitative analysis of oils, fats and oil/fat-related samples (deodistillates).

It is applicable to the screening of oils, fats and oil/fat-related samples to obtain main (e.g. triglycerides) and minor (e.g. sterols, sterol esters, tocopherols, wax esters, fatty alcohols, glycerol) component information in one single analysis. For a truly quantitative analysis of pre-identified compound classes, specific methods are more appropriate.

The method can also be used as a useful qualitative screening tool for the relative comparison of sample compositions.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements 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

No terms and definitions are listed in this document.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

4 Principle

The hydroxylated compounds are transformed into silyl derivatives. This operation has no effect on the apolar (non-hydroxylated) compounds also present in the sample. The sample prepared is analysed by gas chromatography (GC) on a high-temperature capillary column with a low film thickness, with an on-column injector and flame-ionization detector.

For quantitative purposes, the compounds are quantified in the presence of an internal standard (1,2,3-tridecanoylglycerol) and the response factors are determined from a reference standard from each class.

5 Reagents

WARNING — Attention is drawn to the regulations which specify the handling of hazardous substances. Technical, organizational and personal safety measures shall be followed.

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and distilled or demineralized water or water of equivalent purity.

5.1 Silylation reagent mixture: Methylimidazole (CAS 616-47-7) and MSHFBA (CAS 53296-64-3).

Prepare a mixture of silylation reagents: 1 ml of MSHFBA + 50 µl of Methylimidazole.

NOTE This mixture cannot be stored more than one week due to moisture absorption. Indeed, silyl derivatives are moisture sensitive.

5.2 Reference substances[1].

5.2.1 Oleic acid (CAS 112-80-1).

5.2.2 1-Monoolein (CAS 111-03-5).

5.2.3 1,3-Diolein (CAS 2465-32-9).

5.2.4 Triolein (CAS 122-32-7).

5.2.5 Tridecanoylglycerol (CAS 621-71-6).

5.2.6 Eicosanol (CAS 629-96-9).

5.2.7 α-Tocopherol (CAS 10191-41-0), **γ tocopherol** (CAS 54-28-4), **δ tocopherol** (CAS 119-13-1).

5.2.8 Cholesterol (CAS 57-88-5).

5.2.9 Cholesterol palmitate (CAS 601-34-3).

5.2.10 Squalene (CAS 111-02-4).

5.2.11 Plant sterol mix[2] (CAS 474-67-9, 474-62-4, 83-48-7, 83-46-5).

5.3 Isooctane, trace organic analysis grade, purity 99 % min.

5.4 Chloroform, chromatographic quality.

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1) Suitable suppliers are Sigma-Aldrich (www.sigmaaldrich.com) or Larodan (www.larodan.com). This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this supplier.

2) A suitable supplier is Larodan (www.larodan.com). This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this supplier.

6 Apparatus

Usual laboratory equipment and, in particular, the following.

6.1 Volumetric flask, 100 ml and 10 ml.

6.2 Oven, at 103 °C ± 1 °C

6.3 Conical glass sample vials, 10 ml capacity.

6.4 Gas chromatograph for capillary columns, equipped with an on-column injector or equivalent device, a temperature-programmable oven and a flame ionization detector (FID).

6.5 Fused silica capillary column, capable of being programmed up to 400 °C ("high temperature" type) for which the following characteristics are advised: 100 % dimethylpolysiloxane or 95 % dimethyl/5 % diphenyl polysiloxane stationary phase, length 30 m, internal diameter 0,32 mm or 0,25 mm, film thickness 0,1 µm. Other columns of similar polarity and selectivity may also be used.

6.6 Microsyringe, 5 µl to 10 µl capacity, suitable for on-column injection in gas chromatography.

6.7 Analytical balance, reading accuracy 0,001 g.

6.8 Ultrasonic bath.

6.9 Nitrogen evaporator.

7 Sample

7.1 Sampling

Sampling is not part of the method specified in this document. A recommended sampling method is given in ISO 5555[1].

It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage.

7.2 Preparation of test sample

Prepare the test sample in accordance with ISO 661.

8 Procedure

8.1 Preparation of the internal standard, tridecanoylglycerol (5.2.5), c = 20 mg/ml

Weigh 200 mg tridecanoylglycerol (5.2.5) into a 10 ml volumetric flask, fill it up with isooctane (5.3).

8.2 Preparation of the individual standard solutions for determination of response factors

A 5 mg/ml standard solution in isooctane (5.3) is prepared for each compound (see Table 1) to determine the response factor for each component group.

Table 1 — List of the standards for the calculation of the response factors

Class determined	Standard
Alcohols	Eicosanol (5.2.6)
Free fatty acids	Oleic acid (5.2.1)
Hydrocarbons	Squalene (5.2.10)
Monoglycerides	Monoolein (5.2.2)
Tocopherols	α-Tocopherol (5.2.7)
Sterols	Cholesterol (5.2.8)
Diglycerides	Diolein (5.2.3)
Sterol esters	Cholesterol palmitate (5.2.9)
Triglycerides	Triolein (5.2.4)

8.3 Silylation of the standards

Introduce 100 µl of the internal standard solution, tridecanoylglycerol (5.2.5), c = 20 mg/ml, and 200 µl of the individual standard solution (each component separately) into a silylation tube (6.3) and evaporate the solvent to dryness under nitrogen (6.9). Introduce 100 µl of the silylation mixture (5.1). Keep at 103 °C for 15 min (6.2), then take up into 5 ml isooctane (5.3).

It is recommended when preparing the following solution to check the method precision (optional).

In a 100 ml volumetric flask (6.1), exactly weigh about 50 mg of each standard (5.2), including 50 mg of the internal standard solution, tridecanoylglycerol (5.2.5), and dilute to 100 ml with chloroform (5.4).

This solution can be stored at ambient temperature for six months.

To keep the solution homogenous over time, the solution has to be placed into an ultrasonic bath for 10 min before use.

Introduce 0,5 ml of the solution into a silylation tube (6.3) and dry under nitrogen (6.9). Introduce 100 µl of the silylation mixture (5.1). Keep at 103 °C for 15 min (6.2), then take up into 10 ml isooctane (5.3).

8.4 Preparation of the sample

If the sample is solid at room temperature, completely melt the sample in a 103 °C oven (6.2) and homogenize it.

8.5 Sample solution

In a 10 ml volumetric flask (6.1), exactly weigh about 100 mg of sample. Introduce exactly 1 ml of internal standard solution (see 8.1) and dilute to 10 ml with isooctane (5.3).

If the sample is not totally soluble in isooctane, chloroform or toluene may be used instead of isooctane.

8.6 Silylation of the sample

Introduce 0,5 ml of the sample solution (see 8.5) into a silylation tube (6.3) and evaporate the solvent to dryness under nitrogen (6.9.). Introduce 100 µl of the silylation mixture (5.1). Keep at 103 °C for 15 min (6.2), then take up into 10 ml isooctane (5.3).

8.7 Gas chromatography

Install the column in the gas chromatograph and check the working conditions by injecting the solvent, isooctane (5.3). The baseline should be straight with a small positive drift. If the drift is high, proceed to condition the column. For a negative drift, check the connections of the column.

If the column is being used for the first time, it is necessary to condition the column by heating it in the oven using a temperature gradient up to 370 °C (depending on the oven temperature chosen for the analysis) in 4 h. Maintain the temperature for 2 h.

A 1 m long, 0,5 mm diameter, empty fused silica capillary column is recommended as a pre-column.

The conditions in Table 2 for the gas chromatograph have been found to give useful chromatograms.

The solvent vent mode is recommended if the inlet in use is capable of using it.

Optimize the temperature programme and the velocity of the carrier gas flow so that chromatograms similar to the ones presented in Figures A.1 to A.10 are obtained.

Table 2 — Gas chromatographic conditions

Function	Conditions
Column	HP1 (30 m long, 0,25 mm i.d., 0,10 µm film thickness)
Oven temperature	Initial temperature 60 °C, programmed at 20 °C/min to 170 °C, 10 °C/min to 360 °C (hold 10 min)
Carrier gas	Linear velocity 87,7 cm/sec (carrier gas used H_2)
Detector temperature	370 °C
Injector	Cold on-column
Injection volume	1 µl of the solution prepared in 8.6

8.8 Peak identification and integration

Identify the peaks with the standard solution prepared in 8.2. Typical chromatograms are presented in Annex A. The relative retention times of identified compounds or families of compounds are listed in Table 3.

When the compound of interest is present in the form of a single peak, integration according to the conventional criteria is used. By family, the resolution level is not always obtained as there can be some minor compounds. It is then recommended to integrate the set of peaks together or to combine them in a sum.

Table 3 — Indicative relative retention times of identified compounds or families of compounds

Relative retention time (RRT) to tridecanoylglycerol	Determination or class of compounds
< 0,53	Alcohols *(silyl derivative)*
0,57 to 0,63	Free fatty acids *(silyl derivative)*
0,87	Squalene
0,82 to 0,86	Monoglycerides *(silyl derivative)*
0,90	Delta-tocopherol *(silyl derivative)*
0,94	Gamma-tocopherol *(silyl derivative)*
0,99	α-Tocopherol *(silyl derivative)*
0,98 to 1,05	Sterols *(silyl derivative)*
1,00	Tridecanoylglycerol *(internal standard)*
1,30 to 1,36	Diglycerides *(silyl derivative)*
1,52 to 1,57	Sterol esters
> 1,72	Triglycerides

9 Result of the determination

9.1 Calculation of the response factor

Calculate the response factor, *F*, for each family representative compound *x* as given in Formula (1):

$$F_x = \frac{A_{IS} \cdot m_x}{m_{IS} \cdot A_x} \quad (1)$$

where

F_x is the response factor of compound *x* associated with the family X;

m_{IS} is the mass of tridecanoylglycerol (internal standard), in mg;

m_x is the mass of reference compound *x*, in mg;

A_{IS} is the area of tridecanoylglycerol (internal standard);

A_x is the area of reference compound *x*.

Examples of indicative response factors are listed in Table 4.

If very different response factors are observed from those given in Table 4, for example, this can occur in the case of squalene, it is recommended that an alternative internal standard molecule is used that is chemically similar to the compound in question.

Table 4 — Indicative response factors of standards relative to tridecanoylglycerol (internal standard)

Class determined	Standard	Response factor
Alcohols	Eicosanol	0,70
Free fatty acids	Oleic acid	1,04
Hydrocarbons	Squalene	0,89
Monoglycerides	Monoolein	1,19
Tocopherols	α-Tocopherol	0,90
Sterols	Cholesterol	0,75
Internal standard	Tridecanoylglycerol	1,00
Diglycerides	Diolein	1,03
Sterol esters	Cholesterol palmitate	1,05
Triglycerides	Triolein	1,14

9.2 Quantitative determination

The mass fraction of the compounds from the family X is calculated as given in Formula (2):

$$w_x = \frac{F_x \cdot m_{IS} \cdot \sum A_x \cdot 100}{A_{IS} \cdot m} \quad (2)$$

where

w_x is the mass fraction of the compounds of the family X (in mg/100 mg);

F_x is the response factor of compound x associated with the family;

m_{IS} is the mass of tridecanoylglycerol (internal standard), in mg;

A_{IS} is the area of tridecanoylglycerol (internal standard);

$\sum A_x$ is the sum of the areas of the compounds of the family X.

m is the sample mass, in mg.

10 Precision of the method

10.1 Interlaboratory test

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

10.2 Repeatability

The absolute difference between two independent single test results, obtained with 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, shall in not more than 5 % of cases exceed the value of *r* given in Table B.1.

10.3 Reproducibility

The absolute difference between two single test results, obtained with the same method on identical test material in different laboratories by different operators using different equipment, shall in not more than 5 % of cases exceed the value of *R* given in Table B.1.

11 Test report

The test report shall contain at least the following information:

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

b) the sampling method used, if known;

c) the test method used, with reference to this document, i.e. ISO/TS 22115:2021;

d) all operating details not specified in this document, or regarded as optional, together with details of any incidents occurred when performing the method, which can have influenced the test result(s);

e) the test result obtained;

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