DRAFT INTERNATIONAL STANDARD ISO/DIS 15304

ISO/TC **34**/SC **11** Secretariat: **BSI**

Voting begins on: **2007-10-26**

Voting terminates on: **2008-03-26**

INTERNATIONAL ORGANIZATION FOR STANDARDIZATION • МЕЖДУНАРОДНАЯ ОРГАНИЗАЦИЯ ПО СТАНДАРТИЗАЦИИ • ORGANISATION INTERNATIONALE DE NORMALISATION

Animal and vegetable fats and oils — Determination of cis-, trans-, saturated, mono- and polyunsaturated fatty acids in vegetable or non-ruminant oils and fats — Capillary gas chromatographic method

Corps gras d'origines animale et végétale — Détermination des acides gras saturés, mono- et poly-insaturés, cis ou trans, dans les corps gras d'origines végétale ou animale (non ruminant) — Méthode par chromatographie en phase gazeuse sur colonne capillaire

[Revision of first edition (ISO 15304:2002 and of ISO 15304:2002/Cor.1:2003)]

ICS 67.200.10

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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 15304 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 11, Animal and vegetable fats and oils.

This second/third/... edition cancels and replaces the first/second/... edition (ISO 15304:2002), [clause(s) / subclause(s) / table(s) / figure(s) / annex(es)] of which [has / have] been technically revised.

Animal and vegetable fats and oils — Determination of cis-, trans-, saturated, mono- and polyunsaturated fatty acids in vegetable or non-ruminant oils and fats — Capillary gas chromatographic method

1 Scope

This international standard determines, by a single capillary GLC procedure, the levels of trans isomers, saturated fatty acid (SAFA), cis- and trans-monounsaturated fatty acid (MUFA), and cis- and trans-polyunsaturated fatty acid (PUFA) levels in a sample of fat or oil. For nutritional labelling purposes the total fat, saturated, cis-monounsaturated, cis-polyunsaturated and trans fatty acid contents may be determined.

The method is applicable to crude, refined, partially hydrogenated or fully hydrogenated oils and fats derived from vegetable or non-ruminant animal sources. This method is not suitable for the analysis of dairy, ruminant, marine, long chain polyunsaturated (PUFA) fats and oils, or products supplemented with conjugated linoleic acid (CLA). There is minor co-elution of *cis*- and *trans*- fatty acid isomers, particularly in the C18:1 (oleic acid) region, using this technique.

This international standard is based upon *AOCS Official Method Ce 1h-05* [1].

NOTE During (high temperature) refining (deacidification and deodorization), only geometrical isomers are formed of the mono- and poly-unsaturated fatty acids: i.e. the double bond(s) remain(s) at the same natural position. During hydrogenation, both positional and geometrical isomers are formed.

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*

ISO 3696, *Water for Analytical Laboratory Use —Specifications and Test Methods*

ISO 5509, *Animal and vegetable fats and oils – Preparation of methyl esters of fatty acids*

ISO 6353, *Reagents for chemical analysis – Part 2: Specifications*

ISO 6353, *Reagents for chemical analysis – Part 3: Specifications*

3 Terms and definitions

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

3.1
total fat content
is the sum of all fatty acids, expressed as triacylglycerol.

3.2
saturated fat
text of the definition is the sum of all saturated fatty acids.

3.3
***trans* fat**
is the sum of all *trans* fatty acids excluding *trans* isomers with a conjugated double bond.

3.4
***cis* monounsaturated fat**
is the sum of all fatty acids containing one double bond in the cis configuration.

3.5
***cis* polyunsaturated fat**
is the sum of all fatty acids containing two or more double bonds in the *cis* configuration.

3.6
n-6 polyunsaturated fat
is the sum of all fatty acids containing cis n-6 polyunsaturated fatty acids.

3.7
n-3 polyunsaturated fat
is the sum of all fatty acids containing cis n-3 polyunsaturated fatty acids.

4 Principle

Methylated fatty acids of the sample are separated on a capillary gas chromatograph with a highly polar stationary phase, with respect to their chain length, degree of (un)saturation and geometry and position of the double bonds.

5 Reagents and materials

Unless otherwise stated, use only reagents as specified in ISO 6353 parts 2 and 3 if listed there; if not, then use reagents of recognized analytical grade and water of at least grade 3 as defined in ISO 3696.

WARNING — attention is drawn to the regulations which specify the handling of dangerous matter. Technical, organizational and personal safety measures shall be followed.

5.1 Reference fatty acid methyl esters (FAME)

5.1.1 Reference materials and chromatograms[1] with peaks identified can be used for the identification of fatty acids analyzed under the test conditions of this method.

5.1.2 Reference fatty acid methyl esters (FAME) - Methyl esters of pure fatty acids, in particular *cis*- and *trans* isomers of octadecenoic (oleic), *trans* isomers of octadecadienoic (linoleic) and octadecatrienoic (α-linolenic) acids. Wide ranges of *cis*- and *trans* octadecenoic methyl ester isomers are available on the

[1] Reference standards are available form AOCS, 2211 W. Bradley Ave., Champaign, IL USA 61821 (www.aocs.org/tech/samples.asp).

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market[2]. *Trans* geometrical isomers of linoleic and α-linolenic acids can be prepared in the laboratory with the aid of p-toluenesulfonic acid [2].

5.2 Triacylglycerol (TAG) internal standard (IS) solution for calculating fatty acid data as milligrams per 100 g oil or 100 g of food sample

C21:0 TAG - triheneicosanoin (purity > 99 %), mass concentration 5,0 mg/mL in chloroform. The TAG internal standard solution is stable indefinitely if precautions are taken to eliminate the loss of chloroform and therefore a change in the concentration of the IS. For example, store the solution in a refrigerator in well-sealed amber bottle when not in use. Pure triheneicosanoin is available on the market (e.g., from Nu-Chek-Prep). Purity of the IS shall be confirmed by thin-layer chromatography, high-performance liquid chromatography, and gas chromatography analysis or by any other appropriate technique.

NOTE Toluene can be used in place of chloroform with the following considerations. Triheneicosanoin is not as soluble in toluene as it is in chloroform. A solution with a mass concentration of 2 mg/ml can be prepared in toluene. It is necessary to warm the solution slightly to get it to dissolve, but once in solution it will stay dissolved if kept at room temperature. If the solution is stored is a refrigerator, it will crystallize out, but can be dissolved again by slight warming of the solution. Care must be taken so none of the toluene is evaporated during this warming procedure. Care must also be taken to prevent the loss of toluene during storage.

5.3 Micro syringe, for gas chromatography, 10 μL delivery, with a hardened needle.

5.4 Carrier gas, Hydrogen or helium, 99,9995 % pure or better, gas chromatography quality, dried, and oxygen removed by suitable filters.

NOTE Nitrogen gas is not acceptable as a carrier gas for this method

WARNING — Hydrogen, which is used with capillary columns, can double the speed of the analysis (in comparison with helium) but is hazardous. Safety devices are available and it is essential that a suitable device be incorporated in the apparatus.

5.5 Flame gases, hydrogen and air, gas chromatography quality.

5.6 Make-up gas, nitrogen or helium, gas chromatography quality.

6 Apparatus

Usual laboratory equipment and, in particular, the following.

6.1 Gas chromatograph, equipped with flame ionization detector, split injector and data acquisition system.

6.2 Injection port split liner, i. d. 4 mm, o. d. 6,3 mm, length 78,5 mm split liner with glass wool[3]

NOTE No other liner types (e.g., straight through, mixing, etc.) shall be used with this method.

6.3 Capillary Column, Fused silica capillary 100 m, and 0,25 mm i. d., coated with SP-2560 or CP-Sil 88, 100 % cyanopropylsilicone stationary phase, to a thickness of 0,20 μm. Commercially prepared columns are available[4] from different suppliers.

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[2] Suppliers, e.g.: Alltech Associates, Inc., Deerfield, IL, Nu-Chek-Prep, Elysian, MN, Supelco Inc., Bellefonte, PA, and Sigma Chemical Co., St. Louis, MO.

[3] Agilent part No 5183-4647 is the trade name of a product supplied.

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NOTE Shorter columns (60 m) give poorer resolution but may be appropriate for faster routine analyses.

7 Sampling

A representative sample should have been sent to the laboratory. It should not have 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

Prepare the test sample in accordance with ISO 661.

9 Preparation of methyl esters from oils and fats

The fatty acid methyl esters are prepared according to ISO 5509.

Prior to methylation, add enough internal standard solution to the reaction flask, so that after the oil or fat is added, the mass fraction is between 0,05 and 0,10 mg IS/mg oil or fat. Since chloroform is used in the IS solution, it shall be evaporated from the flask prior to the methylation procedure.

Dissolve the prepared FAME in *n*-hexane or *n*-heptane, the mass concentration should be approximately (15 to 20) mg/mL in *n*-hexane or heptane.

10 Procedure

10.1 General

The first sample in an analysis batch shall always be a blank (n-heptane or n-hexane). No peaks shall be detected in this blank run. Repeat this test after every ten samples.

10.2 GC Conditions

The following conditions have been proved to give the chromatograms shown in Annex B:

Injector temperature	250 °C
Detector temperature:	250°C
Oven temperature:	180°C
Carrier gas hydrogen:	column head pressure, 170 kPa (25 psi) flow rate, 1,0 mL/min; linear velocity; 26 cm/s split ratio, 100:1.
Carrier gas helium:	column head pressure, 286 kPa (41 psi)

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[4] Possible products and suppliers are: SP-2560 (part # 24056, Supelco) and CP-Sil 88 (part # CP7489, Varian); Chrompack.

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flow rate, 1,0 mL/min; linear velocity; 19 cm/s
split ratio, 100:1.

10.3 Injection volume/amount

Inject 1 μl, which is equivalent to 15 to 20 μg of fatty acids.

10.4 Performance check

Column performance is checked using a suitable mixture of fatty acid methyl esters covering the range of fatty acids under investigation. Figure B.1 shows the separation of a mixture containing C12:0; 9*c*-18:1; 11*c*-18:1, 9*c*,12*c*,15*c*-18:3; 11*c*-20:1; and the Internal Standard C21:0. Since commercial GC designs are different, and the separation obtained is not identical to the example chromatograms, small changes in the sample size, sample concentration or oven temperature may be required. If so, adjust the sample size, sample concentration or oven temperature until the best separation results are obtained. If column oven temperature needs to be adjusted, it should be adjusted with small increments, preferably by steps of 1°C. Note: on SP-2560, CP-Sil 88 or any other cyanopropylsilicone capillary columns, the column temperature has profound effect on the elution pattern of 13*t* and 14*t*-C18:1, 16*t*-C18:1, 14*c*-C18:1, 9*c*,12*c*,15*t*-C18:3, 11*c*-C20:1 and 9*c*,12*c*,15*c*-C18:3 [3]. The best isothermal resolution of these and other fatty acids is obtained when the column is operated at 180ºC.

10.5 Peak identification

The individual FAMEs are identified by their retention times, and by comparison with the FAME reference standards and the reference hydrogenated oil samples. See Figures B.1 - B.5 for examples of chromatograms of the reference samples. When unknown peaks are observed, attempt to identify such peaks using appropriate procedures such as GC-MS, FTIR, silver-ion chromatography and classical chemical methods [4]. Peaks of unknown identity shall not be included in the summation of peak areas when quantifying the concentrations of total fat, saturated, *cis*-monounsaturated, *cis*-polyunsaturated and *trans* fatty acids unless they have been confirmed to be fatty acids.

NOTE External reference – the performance of the method and peak identification may be checked by participation in a suitable Proficiency Testing Scheme[5], and reference materials, with chromatograms, are usually available from the same sources.

11 Quality Assurance and Control

11.1 Blank sample

The first sample in an analysis batch is always a blank (*n*-heptanes or *n*-hexane). No peaks shall be detected in the blank run. Repeat this test after every ten samples.

11.2 Proficiency test

For external reference, the method is tested each year by participation in a Laboratory Proficiency Program e.g. AOCS, or any other suitable ring test.

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[5] see www.aocs.org/tech/lpp.asp or www.dgfett.de/lvu/index.htm or www.fapas.com

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11.3 Reference materials

Reference materials with chromatograms, and samples from the collaborative study are available from sources mentioned on page 6 of this international standard.

12 Calculations

12.1 Calculation of individual fatty acids and total fat per 100 g of test sample.

12.1.1 Calculation of the amount (in g) of individual fatty acids, expressed as FAMEs (W_{FAMEx}) or as TAG (W_{TAGx}) in test sample.

$$W_{FAMEx} = \frac{A_x \times W_{is} \times 1{,}0040 \times R_x}{A_{is}}$$

$$W_{TAGx} = W_{FAMEx} \cdot F_{TAGx}$$

Where:

W_{FAMEx} is the amount of individual fatty acids in the test sample, expressed as fatty acid methyl esters in grams;

W_{TAGx} is the amount of individual fatty acids in the test sample, expressed as triacylglycerols in grams;

A_x is the area counts for fatty acid x;

W_{is} = is the weight of C21:0 internal standard, added to the test portion, in grams;

A_{is} is the peak area counts of the IS;

1,0040 is conversion of IS in test.

R_x is the theoretical flame ionization detector correction factor (TCF) for FAMEs relative to C21:0 TAG IS.

NOTE TCF shall be applied to the analytical data for optimum accuracy [8, 9] and to minimize variation between laboratories because of differences in calculating response factors. The TCF of a number of FAME that are commonly encountered in dietary fats are listed in Table A.2. They were calculated using the following formula:

$$TCF_x = \frac{MW_x}{(N_x - 1) \cdot AW_C \cdot 1{,}3503}$$

Where

TCF_x is the theoretical flame ionization detector response factor for fatty acid x (as methyl ester) with respect to C21:0 FAME (internal standard);

MW_x is the molecular weight of component x;

N_x *ist the* number of carbon atoms in the FAME of component x;

AW_C is the atomic weight of carbon (12,011);

1,3503 is the TCF for C21:0 FAME.