International Standard

ISO 12966-4

Second edition
2026-03

Animal and vegetable fats and oils — Gas chromatography of fatty acid methyl esters —

Part 4: Determination by capillary gas chromatography

Corps gras d'origines animale et végétale — Chromatographie en phase gazeuse des esters méthyliques d'acides gras —

Partie 4: Détermination par chromatographie capillaire en phase gazeuse

Reference number
ISO 12966-4:2026(en)

ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Email: copyright@iso.org
Website: www.iso.org

Published in Switzerland

Contents

Page

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.

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 document 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).

ISO draws attention to the possibility that the implementation of this document may involve the use of (a) patent(s). ISO takes no position concerning the evidence, validity or applicability of any claimed patent rights in respect thereof. As of the date of publication of this document, ISO had not received notice of (a) patent(s) which may be required to implement this document. However, implementers are cautioned that this may not represent the latest information, which may be obtained from the patent database available at www.iso.org/patents. ISO shall not be held responsible for identifying any or all such patent rights.

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

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).

This second edition cancels and replaces the first edition (ISO 12966-4:2015), which has been technically revised.

The main changes are as follows:

— the Scope has been extended to the separation of fatty acid methyl esters from C4 to C24;

— ruminant fat has been added to the Scope,

— quantification by area (%) or by mass (g/100 g) using internal standards and corrections factors calculated with a quantitative fatty acid methyl esters standard mixture containing *cis* and *trans* fatty acid methyl esters from C4:0 to C22:6; has been added

— quantification of total *trans* fatty acid methyl esters by mass (g/100 g) has been added;

— the use of 100 m, 0,25 mm ID, 0,20 µm film thickness columns are now required to separate most C18:1 *trans*- and *cis*-isomers;

— a method has been added for determination of the composition of fatty acid methyl esters expressed by area % in liquid vegetable oils.

A list of all parts in the ISO 12966 series can be found on the ISO website.

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.

Introduction

This document is one part of a series of four International Standards for the preparation and determination of fatty acid methyl esters (FAMEs) by gas chromatography in animal and vegetable fats and oils. The ISO 12966 series is applicable to crude, refined, partially hydrogenated, or fully hydrogenated fats, oils, and fatty acids derived from animal and vegetable sources, and fats extracted from foodstuff.

The ISO 12966 series is not suitable for milk and milk products (or fat coming from milk and milk products), or products supplemented with conjugated linoleic acid (CLA). Furthermore, it is not intended to be applied to polymerized and oxidized fats and oils.

This document gives the conditions for the analysis of FAMEs by capillary gas chromatography, while ISO 12966-2 and ISO 12966-3 cover the preparation of FAMEs by different methods. ISO 12966-1 is a guideline to the modern gas chromatography of FAMEs.

Animal and vegetable fats and oils — Gas chromatography of fatty acid methyl esters —

Part 4: Determination by capillary gas chromatography

1 Scope

This document specifies a method for the determination of fatty acid methyl esters (FAMEs) derived by transesterification or esterification from fats, oils, and fatty acids by capillary gas chromatography (GLC). FAMEs from C4 to C24 can be separated using this document including saturated FAMEs, *cis*- and *trans*-monounsaturated FAMEs, and *cis*- and *trans*-polyunsaturated FAMEs.

This document is applicable to crude, refined, partially hydrogenated or fully hydrogenated fats, oils and fatty acids derived from animal and vegetable sources, and fats extracted from foodstuff.

This document does not apply to milk and milk products (or fat coming from milk and milk products) or products supplemented with conjugated linoleic acid (CLA).

This document does not apply to di-, tri-, polymerized, hydroxylated and oxidized fatty acids, and fats and oils.

A method for the determination of the composition of FAMEs expressed by area % in liquid vegetable oils is proposed in Annex E.

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*

ISO 3696, *Water for analytical laboratory use — Specification and test methods*

ISO 6353-2, *Reagents for chemical analysis — Part 2: Specifications — First series*

ISO 6353-3, *Reagents for chemical analysis — Part 3: Specifications — Second series*

ISO 12966-2:2017, *Animal and vegetable fats and oils — Gas chromatography of fatty acid methyl esters — Part 2: Preparation of methyl esters of fatty acids*

ISO 12966-3, *Animal and vegetable fats and oils — Gas chromatography of fatty acid methyl esters — Part 3: Preparation of methyl esters using trimethylsulfonium hydroxide (TMSH)*

3 Terms and definitions

No terms and definitions are listed in this document.

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

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

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

4 Principle

Using capillary gas chromatography, FAMEs are separated on a highly polar stationary phase with respect to their chain length, degree of (un)saturation, and geometry and position of the double bonds. Peaks are identified by comparison with the retention time of pure standards and quantified as fatty acids methyl esters by reference to internal standards and instrument correction factors.

5 Reagents and materials

Unless otherwise stated, use only reagents as specified in ISO 6353-2 and ISO 6353-3 (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 standards.

5.1.1 Reference FAMEs.

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* methyl ester isomers are available on the market. The following are examples of suitable products available commercially.

5.1.1.1 Octadecenoic acid methyl esters,*cis* and *trans* isomers mixture of C18:1 with *trans*-4 to *trans*-16 octadecenoic (all isomers) and principal *cis* isomers. Concentration 2,5 mg/ml in iso-octane (5.3) or MTBE (5.4) or *n*-heptane (5.6) or *n*-hexane (5.5) or dichloromethane (5.8).

NOTE This standard is commercially available from Supelco Inc, brand of Sigma-Aldrich (Cat. 40495-U)[1)].

5.1.1.2 Linoleic acid methyl esters,*cis* and *trans* isomers mixture of C18:2 with *trans*-9, *trans*-12-octadecadienoic acid (approximately 50 %), *cis*-9,*trans*-12-octadecadienoic acid (approximately 20 %), *trans*-9,*cis*-12-octadecadienoic acid (approximately 20 %) and *cis*-9,*cis*-12-octadecadienoic acid (approximately 10 %). Concentration 10 mg/ml in iso-octane (5.3) or MTBE (5.4) or *n*-heptane (5.6) or *n*-hexane (5.5) or dichloromethane (5.8).

NOTE This standard is commercially available from Supelco Inc, brand of Sigma-Aldrich (Cat. 47791)[1)].

5.1.1.3 Linolenic acid methyl esters, *cis* and *trans* isomers mixture of C18:3 with:

— *cis*-9,*cis*-12,*cis*-15-octadecatrienoic acid methyl ester (approximately a mass fraction of 3 %);

— *cis*-9,*cis*-12,*trans*-15-octadecatrienoic acid methyl ester (approximately a mass fraction of 7 %);

— *cis*-9,*trans*-12,*cis*-15-octadecatrienoic acid methyl ester (approximately a mass fraction of 7 %);

— *cis*-9,*trans*-12,*trans*-15-octadecatrienoic acid methyl ester (approximately a mass fraction of 15 %);

— *trans*-9,*cis*-12,*cis*-15-octadecatrienoic acid methyl ester (approximately a mass fraction of 7 %);

— *trans*-9,*cis*-12,*trans*-15-octadecatrienoic acid methyl ester (approximately a mass fraction of 15 %);

— *trans*-9,*trans*-12,*cis*-15-octadecatrienoic acid methyl ester (approximately a mass fraction of 15 %);

1) Supelco Inc., brand of Sigma Aldrich, is an example of suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by either ISO or IDF of the product named. Equivalent products may be used if they can be shown to lead to the same results.

— *trans*-9,*trans*-12,*trans*-15-octadecatrienoic acid methyl ester (approximately a mass fraction of 30 %).

Concentration 10 mg/ml in iso-octane (5.3) or MTBE (5.4) or *n*-heptane (5.6) or *n*-hexane (5.5) or dichloromethane (5.8).

NOTE This standard is commercially available from Supelco Inc, brand of Sigma Aldrich (Cat. 47792)[1)]. This standard contains all *trans* isomers of C18:3 (eight in total) but their abundance and ratio are different to those observed in refined/deodorized oils and fats.

5.1.1.4 Methyl octadecadienoate conjugated acids (CLA), mixture of C18:2 *cis*-9,*trans*-11 and *cis*-10,*trans*-12-octadecadienoate conjugated acids, of purity ≥ 99 % mass fraction.

NOTE This standard is commercially available from Supelco Inc, brand of Sigma Aldrich (Cat. O5507)[1)]. This standard contains the two principal CLA isomers, but isomer ratio can vary from lot to lot.

5.1.2 Fats and oils with certified fatty acid composition.

Fats and oils with certified fatty acid composition (e.g. external reference materials or certified reference material). This type of sample is accompanied with a certificate that provides mean values and its associated uncertainty for each fatty acid. These products are often, for example, commercially available from proficiency testing programmes (BIPEA, AOCS, FAPAS, JRC, etc).

5.1.3 Quantitative FAME standard mixture containing *cis* and *trans* FAMEs from C4:0 to C22:6.

This type of FAME mixture is commercially available[2)]. It is also possible to prepare the FAME standard mixture from individual and pure FAME standards, but the purchasing of individual FAME standards is more expensive and the preparation is time consuming and requires high precision.

The amount of each FAME standard present in the mixture is necessary for determining the correction factor (area/amount) for each FAME (see Figure B.1).

5.1.4 Calibration FAME standard solution at 2 mg/ml for the calculation of the correction factors.

Allow quantitative FAME standard mixture (5.1.3) to come to room temperature in the dark without heating. Using a Pasteur pipet, rapidly transfer the content of the vial into a 50 ml volumetric flask, and dilute to the mark with iso-octane (5.3), or MTBE (5.4), or *n*-heptane (5.6), or *n*-hexane (5.5). Dilute accordingly to the type of injector used.

5.2 Internal standards.

For the quantification of the fatty acids, in grams per 100 g, the use of a FAME as an internal standard (IS) is mandatory.

If it is necessary to check the recovery and the effectiveness of the derivatization method, then either or both a triacylglycerol (TAG) and a FAME internal standard should be used. While the TAG-IS is added to the sample prior to the FAME preparation, the FAME-IS is added before or after the FAME preparation. The FAME-IS is used to calculate the recovery of the FAME from the TAG-IS and therefore, the efficiency of the derivatisation procedure. In this case, a different chain length of the standards is required.

For the quantification of all fatty acids in vegetable oils, animal fats or extracted fats, C21:0 FAME or C19:0-FAME are the recommended internal standards, depending on the risk of coelutions such as C21:0/C18:2 conjugated.

For the quantification of all fatty acids in fish oil, C23:0 FAME is the recommended internal standard.

For the quantification of butyric acid (C4:0) and caproic acid (C6:0) only, in fat containing short chain fatty acids, C5:0 FAME is the recommended internal standard.

2) Examples of suitable products available commercially: Nu-Check-Prep, Cat. No. GLC 36 or GLC 37 (including C23:0 FAME). This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products. Equivalent products may be used if they can be shown to lead to the same results.

For the quantification of *trans* fatty acids only, in vegetable oils, animal fats or extracted fats, C19:0-FAME is the recommended internal standard. The concentration of the internal standard should be adapted to the expected content of *trans* fatty acids.

Depending on the capillary column and carrier gas used, the coelutions can be different. It is recommended to carry out further analysis of the sample without the addition of the internal standard to check the natural content of the fatty acid, which is used as the internal standard (C19:0 or C21:0 or C23:0). The content shall be considered in the calculation.

The internal standard solutions are stable if precautions are taken to eliminate the loss of solvent and therefore, a change in the concentration of the IS. For example, store the solution in a refrigerator in a well-sealed amber bottle when not in use. Pure standards are available on the market. Purity of the IS shall be confirmed by thin-layer chromatography, high-performance liquid chromatography, gas chromatography analysis or by any other appropriate technique.

The following are examples of suitable standard solutions:

— Internal standard solution of C5:0 FAME: valeric acid methyl ester (purity ≥ 99 % mass fraction), mass concentration 0,5 mg/ml in iso-octane (5.3) or MTBE (5.4) or *n*-heptane (5.6) or *n*-hexane (5.5) can be used as the internal standard.

— Internal standard solution of C19:0 FAME: nonadecanoic acid methyl ester (purity ≥ 99 % mass fraction), mass concentration 5 mg/ml in iso-octane (5.3) or MTBE (5.4) or *n*-heptane (5.6) or *n*-hexane (5.5) can be used as the internal standard.

— Internal standard solution of C21:0 FAME: heneicosanoic acid methyl ester (purity ≥ 99 % mass fraction), mass concentration 5 mg/ml in iso-octane (5.3) or MTBE (5.4) or *n*-heptane (5.6) or *n*-hexane (5.5) can be used as the internal standard.

— Internal standard solution of C23:0 FAME: tricosanoic acid methyl ester (purity ≥ 99 % mass fraction), mass concentration 5 mg/ml in iso-octane (5.3) or MTBE (5.4) or *n*-heptane (5.6) or *n*-hexane (5.5) can be used as the internal standard.

In order to check the recovery and the effectiveness of the derivatization method, the suitable standard solution is the following:

— Internal standard solution of C13:0 TAG: tritridecanoin (purity > 99 % mass fraction), mass concentration 5 mg/ml in iso-octane (5.3) or MTBE (5.4) or *n*-heptane (5.6) or *n*-hexane (5.5) can be used as the internal standard.

If the TAG-IS is hard to dissolve in the cold, a hot methylation procedure, as specified in ISO 12966-2:2017, 4.3, 4.4, and 4.5, shall be used.

5.3 Iso-octane (2,2,4-trimethyl pentane).

5.4 Methyl tert-Butyl ether (MTBE) (2-Methoxy-2-methylpropane).

5.5 *n*-Hexane.

5.6 *n*-Heptane.

5.7 Qualitative *cis* and *trans* isomers standard mixture solution.

For the retention time (RT) identification of *cis* and *trans* isomers (i.e. C18:1, C18:2, C18:3 and CLA), prepare a qualitative standard solution with the standards listed in 5.1.1.1 to 5.1.1.4. All standards that are commercially available can be used. Into a 50 ml volumetric flask, add each standard isomer solution in equal proportion. Dissolve and make up to the mark with iso-octane (5.3) or MTBE (5.4) or *n*-heptane (5.6) or *n*-hexane (5.5). Dilute in accordance with the type of injector used.

It is also possible to identify the retention times of *cis* and *trans* isomers using a fat or an oil with certified fatty acid composition (5.1.2), for example, reference hydrogenated oil samples.

5.8 Dichloromethane (methylene chloride).

6 Apparatus

The usual laboratory equipment and, in particular, the following shall be used.

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

NOTE The use of on-column and programmable temperature vaporizer (PTV) injectors are also possible.

6.2 Capillary column, fused silica capillary 100 m and 0,25 mm i.d. coated with cyanopropyl-polysiloxane stationary phase, such as SP-2560 or CP-Sil 88[3)] to a thickness of 0,20 µm. Commercially prepared columns are available from different suppliers.

The use of 100 m, 0,25 mm ID, 0,20 µm film thickness columns with SP-2560 or CP-Sil 88 as the stationary phase are required as the separation capacity of these columns is sufficient to separate most C18:1 *trans*- and *cis*-isomers and is in accordance with resolution specification indicated in 10.3. If this separation is not required, a 50 m or 60 m column can also be used. However, it is possible that some 50 m or 60 m long columns can also achieve this separation, mostly for vegetable oils. Other types of columns (BPX70, DB-23, HP-23, Rtx-2330, SP-2330, SP-2380, etc.) are also possible, but a shift in the elution order is possible. For fast GC analysis, short columns are also possible (10 m to 15 m), but with limited information which in certain cases, will not be a problem.

6.3 Micro syringe, for gas chromatography, 10 µl delivery with a hardened needle.

6.4 Carrier gas, hydrogen (recommended), nitrogen or helium, 99,999 5 % pure or better, gas chromatography quality, dried, oxygen removed by suitable filters (<0,1 mg/kg), free from organic impurities.

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

6.5 Flame gases, hydrogen and air, gas chromatography quality, free from organic impurities.

6.6 Make-up gas, nitrogen or helium, gas chromatography quality, free from organic impurities.

7 Sampling

A representative sample should be sent to the laboratory. It should not be damaged or changed during transport or storage.

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

8 Preparation of test sample

Prepare the test sample in accordance with ISO 661.

3) Examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products. Equivalent products may be used if they can be shown to lead to the same results.

9 Preparation of methyl esters from fats, oils, and fatty acids

The FAMEs shall be prepared in accordance with ISO 12966-2 or ISO 12966-3. The use of ISO 12966-3 is not recommended for FAMEs quantification by mass (g/100 g) because only 70 % to 80 % of free fatty acids are esterified and this preparation can lead to an isomerization of unsaturated fatty acids such as methyl octadecadienoate conjugated acids (CLA).

Prior to methylation, the internal standard solution, is added to the reaction flask so that after the oil or fat is added, the mass fraction is between 0,01 and 0,20 mg IS/mg oil or fat.

For example, 2 ml of internal standard solution (5.2) can be added to 100 mg test portion of fat prior to methylation.

Dissolve the prepared FAMEs in iso-octane (5.3) or MTBE (5.4) or *n*-heptane (5.6) or *n*-hexane (5.5). The mass concentration should be approximately 15 mg/ml to 20 mg/ml for split injection. For on-column injection, the mass concentration should be adapted.

It is recommended to carry out further analysis of the sample without the addition of the internal standard to check the natural content of the fatty acid which is used as the internal standard. This natural content shall be considered in the calculation.

10 Procedure

WARNING — Due to the toxic character of some solvents, a ventilated hood shall be used.

10.1 General

The first sample in an analysis batch shall always be a blank FAME dissolution solvent. No peaks shall be detected in this blank run.

10.2 GC conditions

Adapt the temperatures and GC conditions considering the type of fat, oil, or fatty acid analysed and the apparatus used. The following conditions have been proven to be suitable for the separation of FAMEs (C4 to C24) on 100 m columns. However, other conditions are also possible and can be used.

Injector temperature:	250 °C
Detector temperature:	250 °C
Oven temperature:	60 °C (2 min) to 172 °C with 30 °C/min, hold 5 min at 172 °C, 172 °C to 210 °C with 1 °C/min, hold 22 min at 210 °C
Carrier gas hydrogen:	column head pressure, 150 kPa (constant pressure)
	linear velocity; (30 to 40) cm/s, flow rate approximately 1,0 ml/min
	split ratio, 1:25 or 1:100 depending on the dilution
Injection volume:	1 µl (equivalent to 15 µg to 20 µg FAME)

Examples of chromatograms are shown in Annex B.

NOTE For the analysis of animal fats, the complete elution of all FAMEs can be checked with certified reference standards.

10.3 Performance check

Column performance is checked using a suitable mixture of FAMEs covering the range of fatty acids under investigation. Since commercial GC designs are different and the separation obtained is not identical to the