
**Milk, milk products, infant
formula and adult nutritionals —
Determination of fatty acids
composition — Capillary gas
chromatographic method**

*Lait, produits laitiers, formules infantiles et produits nutritionnels
pour adultes — Détermination de la composition en acides gras —
Méthode de chromatographie en phase gazeuse sur colonne capillaire*

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Forewords

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 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 on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT) see the following URL: [Foreword - Supplementary information](#)

The committee responsible for this document is ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products* and the International Dairy Federation (IDF), in collaboration with AOAC INTERNATIONAL. It is being published jointly by ISO and IDF and separately by AOAC INTERNATIONAL. The method described in this International Standard is equivalent to the AOAC Official Method 2012.13: *Determination of labeled fatty acids content in milk products and infant formula*.

IDF (the International Dairy Federation) is a non-profit private sector organization representing the interests of various stakeholders in dairying at the global level. IDF members are organized in National Committees, which are national associations composed of representatives of dairy-related national interest groups including dairy farmers, dairy processing industry, dairy suppliers, academics and governments/food control authorities.

ISO and IDF collaborate closely on all matters of standardization relating to methods of analysis and sampling for milk and milk products. Since 2001, ISO and IDF jointly publish their International Standards using the logos and reference numbers of both organizations.

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ISO 16958 | IDF 231 was prepared by the IDF Standing Committee on *Analytical Methods for Composition* and the ISO Technical Committee ISO/TC 34, *Food products*, Subcommittee 5 on *Milk and milk products* (ISO/TC 34/SC 5), in collaboration with AOAC INTERNATIONAL. It is being published jointly by ISO and IDF, and separately by AOAC INTERNATIONAL. The method described in this International Standard is equivalent to the AOAC Official Method 2012.13: *Determination of labeled fatty acids content in milk products and infant formula*

All work was carried out by the ISO-IDF Project Group C11 of the Standing Committee on *Analytical Methods for Composition* under the aegis of its project leader, Mr Pierre-Alain Golay (CH).

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Milk, milk products, infant formula and adult nutritionals — Determination of fatty acids composition — Capillary gas chromatographic method

1 Scope

This International Standard specifies a method for the quantification of individual and/or all fatty acids in the profile of milk, milk products, infant formula and adult nutritional formula, containing milk fat and/or vegetable oils, supplemented or not supplemented with oils rich in long chain polyunsaturated fatty acids (LC-PUFA). This also includes groups of fatty acids often labelled [i.e. *trans* fatty acids (TFA), saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), omega-3, omega-6 and omega-9 fatty acids] and/or individual fatty acids [i.e. linoleic acid (LA), α -linolenic acid (ALA), arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)].

The determination is performed by direct transesterification in food matrices, without prior fat extraction, and consequently it is applicable to liquid samples or reconstituted powder samples with water having total fat $\geq 1,5$ % m/m.

The fat extracted from products containing less than 1,5 % m/m fat can be analysed with the same method after a preliminary fat extraction using methods referenced in [Clause 2](#). Dairy products, like soft or hard cheeses with acidity level ≤ 1 mmol/100 g of fat, can be analysed after a preliminary fat extraction using methods referenced in [Clause 2](#). For products supplemented or enriched with PUFA with fish oil or algae origins, the evaporation of solvents should be performed at the lowest possible temperature (e.g. max. 40 °C) to recover these sensitive fatty acids.

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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.

ISO 1042, *Laboratory glassware — One-mark volumetric flasks*

ISO 1735 | IDF 5, *Cheese and processed cheese products — Determination of fat content — Gravimetric method (Reference method)*

ISO 1740 | IDF 6, *Milk fat products and butter — Determination of fat acidity (Reference method)*

ISO 14156 | IDF 172, *Milk and milk products — Extraction methods for lipids and liposoluble compounds*

3 Terms and definitions

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

3.1

fatty acids content

mass fraction of individual or groups of substances determined by the procedure specified in this International Standard

Note 1 to entry: See [Table A.1](#).

Note 2 to entry: The fatty acid content is expressed as a mass fraction in grams (or in milligrams) of the fatty acids per 100 g of product (see [Table A.1](#)). Fatty acid results can be converted into other results expression formats (see [10.2](#)).

4 Principle

Addition of the internal standard solution to the sample, preparation of fatty acid methyl esters (FAMES) by direct transesterification with methanolic sodium methoxide for liquid samples; dissolution (i.e. reconstitution) in water for powder sample and direct transesterification with methanolic sodium methoxide. The same transesterification procedure is applied to fat extracted from various foods (e.g. low fat products, cheeses).

Separation of FAMES using capillary gas-liquid chromatography. Identification of FAMES by comparison with the retention time of pure standards and quantification as fatty acids by reference to an internal standard (C11:0 FAME) and instrument response factors. Verification of the transesterification performance using a second internal standard (C13:0 TAG).

5 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified.

5.1 *n*-Hexane, $[\text{CH}_3(\text{CH}_2)_4\text{CH}_3]$, chromatography grade.

5.2 Methanol, $[\text{CH}_3\text{OH}]$, chromatography grade.

5.3 Water, HPLC grade or equivalent purity quality.

5.4 Sodium methoxide solution, $[\text{CH}_3\text{ONa}]$, dissolved in methanol 30 % m/v, or 25 % m/v, depending on local availability.

5.5 Transesterification solution, (sodium methoxide solution 5 % m/v in methanol).

Into a 300 ml volumetric flask, pipette 50 ml (or 60 ml) of sodium methoxide solution 30 % m/v (or 25 % m/v) and mix gently with 250 ml of methanol using a magnetic stirrer. Remove the magnetic stirrer, then cool to room temperature and make up to the mark with methanol.

Stored in the dark at 4 °C, this solution is stable for one week. Allow the solution to come to room temperature before use. This solution volume is sufficient to analyse approximately 40 samples. In case of a smaller number of analyses, the reagent volume can be adapted accordingly.

Perform the transesterification reaction at ambient temperature (between 20 °C and 25 °C).

NOTE Value indicated in brackets () corresponds to sodium methoxide solution with 25 % m/v concentration

5.6 di-Sodium hydrogen citrate sesquihydrate, $[\text{HOC}(\text{COOH})(\text{CH}_2\text{COONa})_2 \cdot 1,5 \text{H}_2\text{O}]$.

5.7 Sodium chloride, $[\text{NaCl}]$.

5.8 Neutralization solution, (di-sodium hydrogen citrate sesquihydrate 10 % m/v, sodium chloride 15 % m/v in water).

Weigh 50,0 g of di-sodium hydrogen citrate sesquihydrate and 75,0 g of sodium chloride in a 500 ml volumetric flask. Dissolve in 450 ml of water using a magnetic stirrer. Remove the magnetic stirrer, then make up to the mark with water.

Stored in the dark at 4 °C, this solution is stable for one month. Presence of salt crystals may appear in the solution during storage, but disappear after shaking.

Allow the solution to come to room temperature before use. This solution volume is sufficient to analyse approximately 40 samples or more. In case of a smaller number of analyses (or single analysis), the mass and volume of solution can be adapted accordingly.

5.9 Tert-butyl methyl ether (MTBE), chromatography grade.

5.10 Methyl undecanoate (C11:0 FAME), of purity ≥ 99 % mass fraction.

5.11 Tritridecanoin (C13:0 TAG), of purity ≥ 99 % mass fraction.

5.12 C11:0 FAME/C13:0 TAG standard solution.

Into a 250 ml volumetric flask, weigh to the nearest 0,1 mg about 500 mg of tritridecanoin and 500 mg of methyl undecanoate. Dissolve and make up to the mark with MTBE.

Stored in the dark at 4 °C, this solution is stable for one week. Allow the solution to come to room temperature before use.

This solution volume is sufficient to analyse approximately 40 samples or more. In case of a smaller number of analyses, standard mass and volume of solvent can be adapted accordingly.

5.13 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 methylene chloride.

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

5.14 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 methylene chloride.

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

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

- *cis*-9,*cis*-12,*cis*-15-octadecatrienoic acid methyl ester (approximately 3 % m/m),
- *cis*-9,*cis*-12,*trans*-15-octadecatrienoic acid methyl ester (approximately 7 % m/m),
- *cis*-9,*trans*-12,*cis*-15-octadecatrienoic acid methyl ester (approximately 7 % m/m),
- *cis*-9,*trans*-12,*trans*-15-octadecatrienoic acid methyl ester (approximately 15 % m/m),
- *trans*-9,*cis*-12,*cis*-15-octadecatrienoic acid methyl ester (approximately 7 % m/m),
- *trans*-9,*cis*-12,*trans*-15-octadecatrienoic acid methyl ester (approximately 15 % m/m),
- *trans*-9,*trans*-12,*cis*-15-octadecatrienoic acid methyl ester (approximately 15 % m/m), and
- *trans*-9,*trans*-12,*trans*-15-octadecatrienoic acid methyl ester (approximately 30 % m/m).

Concentration 10 mg/ml in methylene chloride.

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.

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

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.

NOTE This standard is commercially available from Supelco Inc, brand of Sigma Aldrich (Cat. 05507)¹⁾. This standard contains the two principal CLA isomers, but isomer ratio may vary from lot to lot.

5.17 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 5.13 to 5.16. All standards that are commercially available could be used. Into a 50 ml volumetric flask, add each standard isomer solution in equal proportion. Dissolve and make up to the mark with hexane. Dilute in accordance with the type of injector used.

5.18 FAME standards calibration solution

5.18.1 Preparation with individual FAME standards

5.18.1.1 Individual FAME standards

Purchase individual FAME standards as follows (purity $\geq 99\%$):

Butyric acid methyl ester (C4:0), caproic acid methyl ester (C6:0), caprylic acid methyl ester (C8:0), capric acid methyl ester (C10:0), undecanoic acid methyl ester (C11:0), lauric acid methyl ester (C12:0), tridecanoic acid methyl ester (C13:0), myristic acid methyl ester (C14:0), myristoleic acid methyl ester (C14:1 *cis*-9 or n-5), pentadecanoic acid methyl ester (C15:0), *cis*-10-pentadecenoic acid methyl ester (C15:1 *cis*-10 n-5), palmitic acid methyl ester (C16:0), palmitoleic acid methyl ester (C16:1 *cis*-9 or n-7), heptadecanoic acid methyl ester (C17:0), *cis*-10-heptadecenoic acid methyl ester (C17:1 *cis*-10 or n-7), stearic acid methyl ester (C18:0), elaidic acid methyl ester (C18:1 *trans*-9 or n-9), oleic acid methyl ester (C18:1 *cis*-9 or n-9), linolelaidic acid methyl ester (C18:2 all *trans*-9,12 or n-6), linoleic acid methyl ester (C18:2 all *cis*-9,12 or n-6), arachidic acid methyl ester (C20:0), gamma-linoleic acid methyl ester (C18:3 all *cis*-6,9,12 or n-6), *cis*-11-eicosenoic acid methyl ester (C20:1 *cis*-11 or n-9), linolenic acid methyl ester (C18:3 all *cis*-9,12,15 or n-3), heneicosanoic acid methyl ester (C21:0), *cis*-11,14-eicosadienoic acid methyl ester (C20:2 all *cis*-11,14 or n-6), behenic acid methyl ester (C22:0), *cis*-8,11,14-eicosatrienoic acid methyl ester (C20:3 all *cis*-8,11,14 or n-6 *cis*), erucic acid methyl ester (C22:1 *cis*-13 or n-9), *cis*-11,14,17-eicosatrienoic acid methyl ester (C20:3 all *cis*-11,14,17 or n-3), arachidonic acid methyl ester (C20:4 all *cis*-5,8,11,14 or n-6), *cis*-13,16-docosadienoic acid methyl ester (C22:2 all *cis*-13,16 or n-6), lignoceric acid methyl ester (C24:0), *cis*-5,8,11,14,17-eicosapentaenoic acid methyl ester (C20:5 all *cis*-5,8,11,14,17 or n-3), nervonic acid methyl ester (C24:1 *cis*-15 or n-9), *cis*-4,7,10,13,16,19-docosahexaenoic acid methyl ester (C22:6 all *cis*-4,7,10,13,16,19 or n-3).

NOTE Purchasing of individual FAME standards is more expensive than a single FAME standard mixture. In addition, weighing each FAME standard individually could give imprecision and requires high precision of weighing.

5.18.1.2 Stock solution 1 – Saturated

Into a 100 ml volumetric flask, accurately weigh to the nearest 0,1 mg about 25 mg of Lignoceric acid methyl ester (C24:0), 25 mg of behenic acid methyl ester (C22:0), 25 mg of heneicosanoic acid methyl ester (C21:0), 25 mg of arachidic acid methyl ester (C20:0), 25 mg of stearic acid methyl ester (C18:0), 25 mg of heptadecanoic acid methyl ester (C17:0), 50 mg of palmitic acid methyl ester (C16:0), 25 mg of pentadecanoic acid methyl ester (C15:0), 25 mg of myristic acid methyl ester (C14:0), 25 mg of tridecanoic acid methyl ester (C13:0), 25 mg of lauric acid methyl ester (C12:0), 25 mg of undecanoic acid methyl ester (C11:0), 25 mg of capric acid methyl ester (C10:0), 25 mg of caprylic acid methyl ester (C8:0), 25 mg of caproic acid methyl ester (C6:0) and 25 mg butyric acid methyl ester (C4:0). Make up to the mark with *n*-hexane.

Palmitic acid is weighed in double amount. Short chain fatty acid methyl esters (i.e. C4:0, C6:0 and C8:0) are volatile and shall be weighed at the end of the procedure.

5.18.1.3 Stock solution 2 – Monounsaturated

Into a 100 ml volumetric flask, accurately weigh to the nearest 0,1 mg about 25 mg of nervonic acid methyl ester (C24:1 *cis*-15 or *n*-9), 25 mg of erucic acid methyl ester (C22:1 *cis*-13 or *n*-9), 25 mg of *cis*-11-eicosenoic acid methyl ester (C20:1 *cis*-11 or *n*-9), 25 mg of oleic acid methyl ester (C18:1 *cis*-9 or *n*-9), 25 mg of elaidic acid methyl ester (C18:1 *trans*-9 or *n*-9 *trans*), 25 mg of *cis*-10-heptadecenoic acid methyl ester (C17:1 *cis*-10 or *n*-7), 25 mg of palmitoleic acid methyl ester (C16:1 *cis*-9 or *n*-7), 25 mg of *cis*-10-pentadecenoic acid methyl ester (C15:1 *cis*-10 or *n*-5) and 25 mg of myristoleic acid methyl ester (C14:1 *cis*-9 or *n*-5). Make up to the mark with *n*-hexane.

5.18.1.4 Stock solution 3 – Polyunsaturated

Into a 100 ml volumetric flask, accurately weigh to the nearest 0,1 mg about 25 mg of Linolelaidic acid methyl ester (C18:2 all *trans*-9,12 or *n*-6 *trans*), 25 mg of linoleic acid methyl ester (C18:2 all *cis*-9,12 or *n*-6), 25 mg of gamma-linoleic acid methyl ester (C18:3 all *cis*-9,12 or *n*-6), 25 mg of linolenic acid methyl ester (C18:3 all *cis*-12,15 or *n*-3), 25 mg of *cis*-11,14-eicosadienoic acid methyl ester (C20:2 all *cis*-11,14 or *n*-6), 25 mg of *cis*-8,11,14-eicosatrienoic acid methyl ester (C20:3 all *cis*-8,11,14 or *n*-6), 25 mg of *cis*-11,14,17-eicosatrienoic acid methyl ester (C20:3 all *cis*-11,14,17 or *n*-3), 25 mg of arachidonic acid methyl ester (C20:4 all *cis*-5,8,11,14 or *n*-6), 25 mg of *cis*-13,16-docosadienoic acid methyl ester (C22:2 *cis*-13,16 or *n*-6), 25 mg of *cis*-5,8,11,14,17-eicosapentaenoic acid methyl ester (C20:5 all *cis*-5,8,11,14,17 or *n*-3) and 25 mg of *cis*-4,7,10,13,16,19-docosahexaenoic acid methyl ester (C22:6 *cis*-4,7,10,13,16,19 or *n*-3). Make up to the mark with *n*-hexane.

5.18.1.5 Preparation of FAME standards calibration solution

Into a 100 ml volumetric flask, pipette 25,0 ml of the calibration standard stock solution 1 (5.18.1.2), 25,0 ml of the calibration standard stock solution 2 (5.18.1.3) and 25,0 ml of the calibration standard stock solution 3 (5.18.1.4). Then make up to the mark with *n*-hexane. Dilute in accordance with the type of injector used.

Stored in the dark at -20 °C, this solution is stable for about six months. To prevent contamination of the standard solution, distribute the solution into different vials (ready to inject) and store them at -20 °C before use. Use each vial once, then discard it.

5.18.2 Preparation from a quantitative FAME standard mixture

5.18.2.1 Quantitative FAME standard mixture

Purchase a quantitative FAME standard mixture: Nu-Check-Prep, Cat. Number GLC- Nestle-36²⁾.

The FAME calibration standard mixture is carefully prepared by mass by the supplier. The mass percentage of each component is indicated in the accompanying certificate. Each ampoule contains approximately 100 mg of the FAME calibration standard mix. All individual FAME standards are distributed in equal proportions in the standard mixture, except for palmitic acid methyl ester (C16:0) which is added in double amount.

5.18.2.2 Preparation of FAME standards calibration mixture

Before use, allow the ampoule to come to room temperature (maximum 25 °C) in the dark without heating. Cut the ampoule with a glass knife, using a Pasteur pipette, rapidly transfer the content of the ampoule into a 50 ml pre-tarred volumetric flask, weigh and make up to the mark with *n*-hexane. Dilute in accordance with the type of injector used.

2) Nu-Check-Prep GLC-Nestle36 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.

Stored in the dark at $-20\text{ }^{\circ}\text{C}$, this solution is stable for about six months. To prevent contamination of the standard solution, distribute the solution into different vials (ready to inject) and store them at $-20\text{ }^{\circ}\text{C}$ before use. Use each vial once, then discard it.

6 Apparatus

WARNING — Since the determination involves the use of volatile flammable solvents, all electrical apparatus employed shall comply with legislation relating to the hazards in using such solvents.

Usual laboratory equipment and, in particular, the following.

- 6.1 **Analytical balance**, capable of weighing to the nearest 1 mg, with a readability of 0,1 mg.
- 6.2 **Volumetric flasks**, of capacities 50 ml, 100 ml, 250 ml, 300 ml and 500 ml.
- 6.3 **Volumetric pipettes, with one mark**, of capacities 2 ml, 5 ml, 10 ml, 25 ml and 50 ml, class AS (ISO 1042).
- 6.4 **Volumetric pipettes, with two marks**, of capacities 2 ml and 5 ml, class AS (ISO 1042).
- 6.5 **Micropipette**, of capacity 200 μl .
- 6.6 **Dispensers**, of capacities 2 ml, 5 ml and 10 ml.
- 6.7 **Test tube, of diameter 26 mm, of length 100 mm**, fitted with PTFE-lined screw cap.
- 6.8 **Test tube mixer vortex-genie**, or equivalent.
- 6.9 **Laboratory centrifuge**, equipped with adapters for test tubes with external diameter of 26 mm.
- 6.10 **Gas-liquid chromatograph**, equipped with flame ionization detector and split/splitless or on-column injector. Auto-sampler and integration system preferably computerized.

Use of the cleanest possible glassware and caps is required to avoid impurities in the FAME chromatogram.

- 6.10.1 **Carrier gas**, hydrogen or helium, purity $\geq 99,9997\text{ } \%$.

NOTE The use of hydrogen or helium as carrier gas affects principally the chromatography duration (i.e. increase of time between 10 min to 15 min with helium) but does not have significant impact on the chromatographic resolution with optimized conditions.

The other gases necessary for the detector (FID) should be free from organic impurities (i.e. C_nH_m of below 1 ppm) and have purity at least $\geq 99,995\text{ } \%$. Synthetic air or compressed air can be used. The use of gas generator is also possible.

- 6.10.2 **Capillary column**, bonded with cyanopropyl-polysiloxane phase or equivalent (100 m length, 0,25 mm internal diameter, 0,2 micron film thickness), that elutes the FAMEs primarily by carbon chain length and secondarily by the number of double bonds.

Traces of oxygen and humidity will damage the polar phase of the column. If pure gas is not available, use a gas purifying filter device.

6.10.3 Flame ionization detector, capable of being heated to a temperature 50 °C above the final temperature of the column oven.

6.10.4 Split/splitless injector, capable of being heated to a temperature 30 °C above the final temperature of the column oven.

6.10.5 On-column injector, capable of being not heated (cold), or being heated to a temperature 30 °C above the final temperature of the column oven.

NOTE The installation of one single injector (i.e. split/splitless or on-column) on the GC instrument is sufficient.

6.10.6 Injection syringe, capacity 10 µl.

6.10.7 Integration system.

6.11 Gas chromatographic conditions

The oven temperature and the carrier gas flow depend on the column selected, and on the carrier gas used (i.e. hydrogen or helium). In any case, the selected conditions shall produce the separation between *cis* and *trans* zone for C18:1, C18:2, C18:3 and conjugated linoleic acids (CLA), as shown in [Annex B, Figures B.1, B.2 and B.3](#).

The examples in [6.11.1](#) and [6.11.2](#) list applicable conditions for a correct separation/identification of *cis* and *trans*.

6.11.1 Example 1 – Split injection mode

- Column: 100 m length, 0,25 mm internal diameter, 0,2 µm film thickness, fused silica capillary column.
- Stationary phase: cyanopropyl-polysiloxane.
- Carrier gas type: helium.
- Column head carrier gas pressure: 225 kPa (175 kPa – 225 kPa).
- Split flow: 25,5 ml/min.
- Split ratio: 10:1.
- Injector temperature: 250 °C.
- Detector temperature: 275 °C.
- Oven temperature programme: initial temperature of 60 °C, maintained for 5 min, raised at a rate of 15 °C min⁻¹ up to 165 °C, maintained at this temperature for 1 min and then raised at a rate of 2 °C min⁻¹ up to 225 °C for 20 min.
- Amount of sample injected: 1,0 µl.

An example of the full GC profile obtained with these conditions is shown in [Annex B, Figure B.4](#).

6.11.2 Example 2 – On-column injection mode

- Column: 100 m length, 0,25 mm internal diameter, 0,2 µm film thickness, fused silica capillary column.
- Stationary phase: cyanopropyl-polysiloxane.
- Carrier gas type: hydrogen.
- Column head carrier gas pressure: 210 kPa (175 kPa – 225 kPa).

- Injector temperature: cold.
- Detector temperature: 275 °C.
- Oven temperature programme: initial temperature of 60 °C, maintained for 5 min, raised at a rate of 15 °C min⁻¹ up to 165 °C, maintained at this temperature for 1 min and then raised at a rate of 2 °C min⁻¹ up to 225 °C for 17 min.
- Amount of sample injected: 1,0 µl.

An example of the full GC profile obtained with these conditions is shown in [Annex B, Figure B.5](#).

6.12 Resolution between C18:1 *cis* and *trans*

For the accurate quantification of C18:1 TFA (level ≥ 0,5 g/100 g fat), a sufficient resolution between C18:1 *trans*-13/14 and C18:1 *cis*-9 (oleic acid) is required. The resolution is determined with the injection of the qualitative *cis* and *trans* C18:1 FAME isomers standard mixture solution ([5.17](#)).

Inject into the gas chromatograph 1,0 µl of the calibrating solution ([5.13](#)). Determine peak width at half height and distance between the left of the chromatogram and the top of peak for C18:1 *trans*-13/14 and C18:1 *cis*-9 (oleic acid methyl ester). The resolution criteria *R* is calculated by using Formula (1):

$$R = 1,18 \times (t_{R2} - t_{R1}) / (W_{\left(\frac{1}{2}\right)_1} + W_{\left(\frac{1}{2}\right)_2}) \quad (1)$$

where

t_{R1} is the distance, in centimetres, between the left of the chromatogram and the top of peak 1 (C18:1 *trans*-13/14);

t_{R2} is the distance, in centimetres, between the left of the chromatogram and the top of peak 2 (C18:1 *cis*-9);

$W_{\left(\frac{1}{2}\right)_1}$ is the peak width, in centimetres, at half height of peak 1 (C18:1 *trans*-13/14);

$W_{\left(\frac{1}{2}\right)_2}$ is the peak width, in centimetres, at half height of peak 2 (C18:1 *cis*-9).

The resolution is sufficient when *R* criterion ≥ 1,00 ± 5 % (see [Annex B, Figure B.3](#)).

NOTE In case of insufficient resolution, but with *R* close to the target value, the fine tuning of chromatography conditions (i.e. slight modification of carrier gas pressure/flow, or oven temperature programme) can give an acceptable *R* value.

7 Sampling

It is important that the laboratory receives a sample that is representative and 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 707 | IDF 50.

8 Preparation of test sample

8.1 Liquid and powder milk and infant formula with a fat content $\geq 1,5$ % m/m

Bring the sample to room temperature and shake vigorously before use. Ensure that the sample is homogeneous (i.e. mix well).

8.2 Liquid and powder milk and infant formula with a fat content $< 1,5$ % m/m

Bring the sample to room temperature and shake vigorously before use. Ensure that the sample is homogeneous (i.e. mix well).

Extract the fat in accordance with ISO 14156 | IDF 172 taking care to evaporate completely the extraction solvent(s) by heating to a temperature not higher than 40 °C to avoid the degradation of long chain polyunsaturated fatty acids (LC-PUFA).

NOTE See also ISO 1211 | IDF 1, ISO 1737 | IDF 13, ISO 8381 | IDF 123 and ISO 8262-1 | IDF 124-1 for useful guidance on fat extraction methods.

8.3 Cheese

Bring the sample to room temperature. Ensure that the sample is homogeneous (i.e. mix well).

Extract the fat in accordance with ISO 1735 | IDF 5 taking care to remove completely the extraction solvent by heating the fat to a temperature not higher than 60 °C.

Verify the acidity of the fat in accordance with ISO 1740 | IDF 6 (acceptance criteria ≤ 1 mmol/100 g of fat).

NOTE In the presence of methanolic sodium methoxide, free fatty acids are not converted into methyl esters (FAME). In case of higher acidity (i.e. free fatty acids), these fatty acids are not quantified with the others.

9 Procedure

9.1 Test portion

Into a 25 ml centrifuge tube with a screw cap, weigh to the nearest 0,1 mg an equivalent quantity of sample (8.1) in order to have approximately 50 mg of fat in the tube. (For example, for a sample containing 26 g fat per 100 g from a product, the corresponding sample mass is approximately 190 mg.)

NOTE 1 For fatty acid analysis on fat extracted from foods, the same amount of fat sample is required (i.e. approximately 50 mg).

For a powder sample, add 2,0 ml of water using a micropipette. For a liquid sample, water addition is not required. Close the tube, then dissolve gently using a vortex mixer. Wait for 15 min at room temperature.

For the fat extracted from product (8.2 and 8.3), weigh to the nearest 0,1 mg 50 mg of melted fat into a 25 ml centrifuge tube. Water addition is not required for fatty acid analysis of fat sample.

Pipette 5 ml of internal standard solution (5.12). Add with a pipette 5 ml of 5 % (m/v) methanolic sodium methoxide solution (5.5). The transesterification time starts with the addition of the first drop of reagent. Close the tube hermetically and shake well for 10 s using a vortex mixer.

180 s after the start time, open the tube and add 2 ml of hexane. 210 s after the start time add 10 ml of disodium hydrogen citrate and sodium chloride aqueous solution (5.8). The transesterification time stops after the addition of the last drop of neutralization solution. Shake gently using a vortex mixer for 30 s. The transesterification time should not exceed 240 s after the start time.

NOTE 2 It is important to respect the transesterification time (240 s). The number of tubes cannot exceed six tubes at the same time under these conditions. Rapid delivery system (dispenser) can be used to add reagents, but not for the addition of internal standard solution which requires high precision.