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Milk and milk products — Determination of milk fat purity by gas chromatographic analysis of triglycerides

Lait et produits laitiers — Détermination de la pureté des matières grasses laitières par analyse chromatographique en phase gazeuse des triglycérides

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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.

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).

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. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

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 5, *Milk and milk products*, and the International Dairy Federation (IDF). It is being published jointly by ISO and IDF.

This second edition cancels and replaces the first edition (ISO 17678 | IDF 202:2010), which has been technically revised. The following changes have been made:

— the Scope has been restricted to exclude milk fat obtained from special feeding practices and from whey;

— the Scope has been extended to include milk fat obtained from cheese showing low lipolysis;

— the Normative references have been updated to reflect the modified scope;

— a method has been added for the fat extraction from cheese;

— the Bibliography has been expanded.

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.

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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This document was prepared by the IDF *Standing Committee on Analytical Methods for Composition* and ISO Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products.* It is being published jointly by ISO and IDF.

The work was carried out by the Joint ISO/IDF Action Team C23 of the *Standing Committee on Analytical Methods for Composition* under the aegis of its project leader, Mr J. Molkentin (DE).

Milk and milk products — Determination of milk fat purity by gas chromatographic analysis of triglycerides

1 Scope

This document specifies a reference method for the determination of milk fat purity using gas chromatographic analysis of triglycerides. The method utilizes the differences in triglyceride fingerprint of milk fat from the individual triglyceride fingerprints of other fats and oils to determine samples which are outside the range normally observed for milk fat. This is achieved by using the defined triglyceride formulae based on the normalized weighted sum of individual triglyceride peaks which are sensitive to the integrity of the milk[6][7]. The integrity of the milk fat can be determined by comparing the result of these formulae with those previously observed for a range of pure milk fat samples[12]. Both vegetable fats and animal fats such as beef tallow and lard can be detected.

The method is applicable to bulk milk, or products made thereof, irrespective of the variation in common feeding practices, breed or lactation conditions. In particular, the method is applicable to fat extracted from milk products purporting to contain pure milk fat with unchanged composition, such as butter, cream, milk and milk powder.

Because a false-positive result can occur, the method does not apply to milk fat related to these circumstances:

a) obtained from bovine milk other than cow's milk;

b) obtained from single cows;

c) obtained from cows whose diet contained a particularly high proportion of vegetable oils such as rapeseed, cotton or palm oil, etc.;

d) obtained from cows suffering from serious underfeeding (strong energy deficit);

e) obtained from colostrum;

f) subjected to technological treatment such as removal of cholesterol or fractionation;

g) obtained from skim milk, buttermilk or whey;

h) obtained from cheeses showing increased lipolysis;

i) extracted using the Gerber, Weibull–Berntrop or Schmid–Bondzynski–Ratzlaff methods, or that has been isolated using detergents (e.g. the Bureau of Dairy Industries method).

With the extraction methods specified in i), substantial quantities of partial glycerides or phospholipids can pass into the fat phase.

NOTE 1 In nature, butyric (*n*-butanoic) acid (C4) occurs exclusively in milk fat and enables quantitative estimations of low to moderate amounts of milk fat in vegetable and animal fats to be made. Due to the large variation of C4, for which the approximate content ranges from 3,1 % fat mass fraction to 3,8 % fat mass fraction, it is difficult to provide qualitative and quantitative information for foreign fat to pure milk fat ratios of up to 20 % mass fraction[11].

NOTE 2 In practice, quantitative results cannot be derived from the sterol content of vegetable fats, because they depend on production and processing conditions. Furthermore, the qualitative determination of foreign fat using sterols is ambiguous.

NOTE 3 Due to special feeding practices such as those related to c) and d), false-positive results have sometimes been reported for milk from certain Asian regions[15]. Moreover, grass-only diets such as mountain and, in particular, highland pasture feeding sometimes cause false-positive results, which can be substantiated by a content of conjugated linoleic acid (C18:2 c9t11) of ≥ 1,3 % fatty acid mass fraction[16][17]. Nevertheless, results conforming to the criteria of milk fat purity specified in this document are accepted, even if samples were undoubtedly produced under conditions reported in this note, including those described in h).

NOTE 4 In cases where a positive result is suspected to be caused by circumstances related to c) or d), another analytical method, such as fatty acid or sterol analysis, can be applied to confirm the finding. Due to similar or increased limitations (e.g. as described in NOTE 1 and NOTE 2), a negative result obtained by another method is not appropriate to contrastingly confirm milk fat purity.

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 1211 | IDF 1, *Milk — Determination of fat content — Gravimetric method (Reference method)*

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

ISO 1736 | IDF 9, *Dried milk and dried milk products — Determination of fat content — Gravimetric method (Reference method)*

ISO 2450 | IDF 16, *Cream — Determination of fat content — Gravimetric method (Reference method)*

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

ISO 7328 | IDF 116, *Milk-based edible ices and ice mixes — Determination of fat content — Gravimetric method (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.

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

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

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

3.1
milk fat purity
absence of vegetable and animal fats determined by the procedure specified in this document

Note 1 to entry: The purity is determined using *S*-values, which are calculated from the content of triglycerides. Triglyceride mass fractions are expressed as percentages.

4 Principle

Fat extracted from milk or milk products is analysed by gas chromatography (GC) using a packed or a short capillary column to determine triglycerides (TGs), separated by total carbon numbers. By inserting the mass fraction, expressed as a percentage, of fat molecules of different sizes (C24 to C54, using even C numbers only) into suitable TG formulae, *S*-values are calculated. If the *S*-values exceed the limits established with pure milk fat, the presence of foreign fat is detected.

NOTE 1 The suitability and equivalence of both packed and capillary columns have been demonstrated previously[8][9][10].

NOTE 2 An *S*-value is the sum of weighted TG mass fractions.

5 Reagents

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade.

5.1 Water, conforming to the requirements of ISO 3696, grade 2.

5.2 Carrier gas, nitrogen or, alternatively, helium or hydrogen, all with a purity of at least 99,995 % volume fraction.

5.3 Fat standards, as described in 5.3.1 and 5.3.2.

5.3.1 Triglyceride standards, saturated, with a purity of at least 99 % mass fraction, for standardizing the milk fat standard described in 8.3.3 (suitable products are available commercially).

5.3.2 Cholesterol standard, with a purity of at least 99 % mass fraction, for standardizing the milk fat standard described in 8.3.3.

5.4 Methanol, with a water content of not more than 0,05 % mass fraction.

5.5 *n*-Hexane.

5.6 *n*-Heptane.

5.7 Other gases, hydrogen, purity at least 99,995 % volume fraction, free from organic impurities (C_nH_m < 1 µl/l); synthetic air, free from organic impurities (C_nH_m < 1 µl/l).

5.8 Anhydrous sodium sulfate.

6 Apparatus

Usual laboratory equipment and, in particular, the following.

6.1 High-temperature gas chromatograph, suitable for use at temperatures of at least 400 °C and equipped with a flame ionization detector (FID). For capillary GC, an on-column or a programmed temperature vaporization injector is indispensable while a split injector is unsuitable.

Septa used in the injector shall withstand high temperatures and exhibit a very low degree of "bleeding". Always use graphite seals to connect the column as well as injector and/or detector inserts (where applicable).

6.2 Packed chromatography column, glass, of internal diameter 2 mm and length 500 mm, packed with a stationary phase of 3 % OV-1 on 125 µm to 150 µm (100 to 120 mesh) Gas ChromQ[1)].

The preparation, silanization, packing and conditioning of the packed column are described in Annex A.

Alternatively, a capillary column (6.3) may be used.

1) Example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or by IDF of this product.

6.3 Capillary chromatography column, short, e.g. of length 5 m, with a non-polar stationary phase that can withstand temperatures up to 400 °C or more[2)].

Condition the column by performing 20 analyses of a milk fat solution (see 8.2) within no more than two days using the settings given in 8.3.4.3. After that, ensure that the response factors (see 8.3.3) are close to 1 and not higher than 1,250 0.

Because of the variable overlap between C24 and cholesterol, a higher response factor may be accepted for C24.

Columns with different dimensions and a different non-polar, highly temperature-resistant phase may be used as long as their performance is consistent with this document. However, the column length is restricted by the indispensable limitation in resolution as shown in Figure 1. See also 8.3.4.3.

6.4 Extrelut column[1)], capacity 1 ml to 3 ml, filled with silica gel, for the extraction of milk fat in accordance with 8.1.4 only.

6.5 Graphite seals, capable of withstanding temperatures of at least 400 °C; for the connection of the GC column as well as for the injector and/or detector inserts.

6.6 Water bath, capable of being maintained at 50 °C ± 2 °C.

6.7 Oven, capable of operating at 50 °C ± 2 °C and 100 °C ± 2 °C.

6.8 Micropipette.

6.9 Graduated pipette, capacity 5 ml, in accordance with ISO 835[2], class A.

6.10 Round-bottomed flask, capacity 50 ml.

6.11 Erlenmeyer flask, nominal capacity 250 ml.

6.12 Funnel.

6.13 Fine-pored filter paper.

6.14 Rotary evaporator.

6.15 Ampoules, nominal capacity 1 ml, fitted with a polytetrafluoroethylene-lined aluminium crimp cap or screw cap.

6.16 Injection syringe, with syringe plunger not reaching into the tip of the needle (packed column GC).

NOTE With these syringes, better repeatability of the results is obtained.

6.17 Analytical balance, capable of weighing to the nearest 1 mg, with a readability of 0,1 mg.

7 Sampling

Sampling is not part of the method specified in this document. A recommended sampling method is given in ISO 707 | IDF 50[1].

2) CP-Ultimetal SimDist (5 m, 0,53 mm, 0,17 µm) is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or by IDF of this product.

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

8 Procedure

8.1 Preparation of test samples

8.1.1 General

For the preparation of test samples, use one of the milk fat isolation or extraction methods specified in 8.1.2 to 8.1.5.

8.1.2 Isolation from butter or butteroil

Melt 50 g to 100 g of test sample in the water bath (6.6) or the oven (6.7) at 50 °C.

Add 0,5 to 1,0 g of sodium sulfate (5.8) to a folded filter paper (6.13). Preheat a 250 ml Erlenmeyer flask (6.11) and a funnel (6.12) with the filter paper inserted, containing the sodium sulfate, in the oven (6.7) at 50 °C.

When a limited amount of test sample is available, use a smaller test sample and adapt the procedure accordingly.

However, note that the handling of a smaller test portion involves a higher risk of obtaining a non-representative sample.

While keeping the preheated flask, funnel and inserted filter device in the oven, filter the fat layer of the molten sample without transferring any serum.

NOTE 1 Butter can be obtained from cream by churning and thorough washing of the resulting butter grains.

NOTE 2 The milk fat obtained using the procedure in this subclause is almost free of phospholipids.

8.1.3 Extraction according to the Röse–Gottlieb gravimetric method

Extract the fat fraction from the test sample using the gravimetric method specified in one of ISO 1211 | IDF 1, ISO 1736 | IDF 9, ISO 2450 | IDF 16 or ISO 7328 | IDF 116.

8.1.4 Extraction from milk using silica gel columns

Temper the milk to 20 °C. Using a micropipette (6.8), add 0,7 ml of the sample thus prepared into a 1 ml to 3 ml Extrelut column (6.4). Allow the sample to distribute uniformly on the silica gel for approximately 5 min.

To denature the protein–lipid complexes, using the graduated pipette (6.9), add 1,5 ml of methanol (5.4) into the Extrelut column. Subsequently, extract the fat fraction from the test sample with 20 ml of *n*-hexane (5.5). Add the *n*-hexane slowly in small amounts. Collect the solvent draining off in a 50 ml round-bottomed flask (6.10) previously dried to a constant, known mass weighed to the nearest 1 mg and record the mass to 0,1 mg.

Allow the column to drain until empty after the extraction. Distil off the solvents from the eluate on a rotary evaporator (6.14) with its water bath maintained at between 40 °C and 50 °C.

After distilling off the solvents, dry and subsequently weigh the round-bottomed flask and its contents to the nearest 1 mg, recording the mass to 0,1 mg. Determine the fat mass yield by subtracting the mass of the dried empty round-bottomed flask from the mass obtained.

Depending on the fat content of the milk and the required concentration of the sample solution, decide whether it is necessary to combine the yield of two or more extractions to obtain an adequate amount of fat.

8.1.5 Extraction from cheese

Extract the cheese fat fraction from the test sample using the method specified in ISO 14156 | IDF 172. With cheeses typically showing increased lipolysis, which often occurs with mould-ripened and long-ripened cheeses, determine the fat acidity using the method specified in ISO 1740 | IDF 6. If the acidity is higher than 8 mmol/100 g of fat, the standard is not applicable[18][19].

8.2 Preparation of fat sample solution

For gas chromatography with a packed column, prepare a 5 % volume fraction solution of the melted fat obtained in 8.1.2, 8.1.3, 8.1.4 or 8.1.5 in *n*-hexane (5.5) or *n*-heptane (5.6). Depending on the column dimensions, use a concentration of 1 % [0,53 mm internal diameter (ID), wide-bore] or lower for on-column injection with a capillary column.

When using the fat sample prepared in 8.1.4, calculate the amount of solvent (5.5 or 5.6) to be added to the test sample in the flask by equating the mass of fat obtained with its volume.

Completely dissolve the fat in the solvent used. Transfer approximately 0,5 ml to 1 ml of the obtained fat sample solution into an ampoule (6.15).

8.3 Chromatographic triglyceride determination

8.3.1 Baseline drift

To minimize baseline rising, condition the column as specified in 6.3 (capillary column) or in A.5 (packed column).

NOTE Because of the high column temperature, the analysis of TGs is particularly susceptible to a rise of the baseline in the high carbon-number range.

8.3.2 Injection technique

8.3.2.1 Packed column

To avoid discrimination effects and to improve the quantification of the high-boiling TG components, apply the hot-needle technique.

Fill the needle with air by drawing up the fat solution into the body of the syringe. Insert the needle into the injector. Heat the needle prior to injection for about 3 s. Then, rapidly inject the syringe contents.

8.3.2.2 Capillary column

When using cool on-column injection (see 8.3.4.3), insert the needle of the syringe and inject immediately. Choose a suitable subsequent dwell time of the needle in the injector so as to avoid broad tailing of the solvent peak.

NOTE The optimum dwell time is typically about 3 s.

8.3.3 Calibration

8.3.3.1 General

For the calibration of test samples, perform two to three analyses of standardized milk fat at the beginning of each working day. Use the last analysis of the standardized milk fat to determine the

