International Standard

ISO 16756

IDF 259

First edition
2024-09

Milk and milk products — Guidance for the application of Carr-Purcell-Meiboom-Gill (CPMG) pulsed time-domain nuclear magnetic resonance (TD-NMR) spectroscopy for fat determination

Laits et produits laitiers — Lignes directrices pour l'application de la spectroscopie par résonance magnétique nucléaire dans le domaine temporel (TD-RMN) à impulsions Carr-Purcell-Meiboom-Gill (CPMG) pour le dosage de la matière grasse

Reference numbers
ISO 16756:2024(en)
IDF 259:2024(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

International Dairy Federation
Silver Building • Bd Auguste Reyers 70/B
B-1030 Brussels
Phone: +32 2 325 67 40
Fax: +32 2 325 67 41
Email: info@fil-idf.org
Website: www.fil-idf.org

Published in Switzerland

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Forewords

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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.

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.

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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 IDF/ISO Action Team C56 of the *Standing Committee on Analytical Methods for Composition* under the aegis of its project leaders Mr P.A. Golay (CH) and Mr D. Darrell (US).

Milk and milk products — Guidance for the application of Carr-Purcell-Meiboom-Gill (CPMG) pulsed time-domain nuclear magnetic resonance (TD-NMR) spectroscopy for fat determination

1 Scope

This document gives guidance on the determination of total fat content in milk and milk-based products, such as milk, cream, yogurt, ice cream, processed dairy, cheese and dairy powders by low-resolution nuclear magnetic resonance (NMR) using Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence to optimize the specific response of fat molecules.

This document is applicable to the analysis of any milk and milk-based products, regardless of source (species or region). It is applicable to dry samples (i.e. moisture content ≤ 10 %) and liquid or wet samples which have been pre-dried such that all appreciable water has been removed. The NMR with CPMG pulse sequence analyses glycerolipids, which produces fat results comparable to the total fat result of standard fat extraction techniques, without the need for matrix specific calibrations while meeting the precision criteria listed in Clause 12.

The application is not limited by sample viscosity, colour or particle size.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

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

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/

3.1
time-domain nuclear magnetic resonance spectroscopy
TD-NMR spectroscopy
technique where the intensity and speed of magnetic resonance signal decay of atomic nuclei are used to determine physical and chemical properties of atoms and molecules

Note 1 to entry: Intensity of magnetic resonance should correlate to quantity of the atomic nuclei present in the sample, whereas speed of magnetic resonance signal decay inversely correlates to the movement of the molecule where the signal originated, which can be aligned with the type of molecule.

Note 2 to entry: When used under the conditions defined in this document, NMR spectroscopy is able to provide the *total fat* (3.2) value.

3.2
total fat
class of compounds that are generally soluble in organic solvents and largely insoluble in water

Note 1 to entry: Fats are principally composed of triesters of glycerol and fatty acids (i.e. triacylglycerols), but contain also minor lipids (i.e. diacylglycerols, monoacylglycerols, phospholipids, sterols). Fats can be either solid or liquid, though commonly the term fat is used to refer to the solid form and oil is used for the liquid form at room temperature.

Note 2 to entry: Glycerolipids is the term used to describe triesters of glycerol and fatty acids (triacylglycerols), diesters of glycerol and fatty acids (diacylglycerols) and monoesters of glycerol and fatty acids (monoacylglycerols). Non-glycerolipids refer to all other lipids present in the samples (i.e. phospholipids, cholesterol, sterols, free fatty acids, etc.).

4 Principle

A test sample is dried (i.e. moisture content ≥ 10 %), and then equilibrated to a defined temperature prior to being inserted into the magnetic field of a low-resolution, pulsed NMR instrument. A sequence of radiofrequency pulses is then applied, which enables the observation of signal related to the quantity of hydrogen nuclei present in the sample. The CPMG pulse sequence is optimized such that the resulting signal enables measurement of the spin-spin relaxation time (T_2) of hydrogen nuclei only associated with liquid glycerolipid fat. Signal data are then isolated using the principles of TD-NMR, identifying the signal attributable to glycerolipid fats within the sample while ignoring interferences occurring from the signal of other constituents such as carbohydrates, proteins and bound water. The signal data is then processed by a multi-exponential fitting such that the resulting data produces a single linear calibration line when compared to data from reference methods. This universal calibration incorporates a statistically significant number of data points, a broad range of sample types or matrices, and a full range of fat content, while meeting the terms of repeatability and reproducibility given in this document.

5 Principal characteristics of NMR instruments

A TD-NMR instrument is an apparatus which, when used under the conditions defined in this document, enables fat determination in a range of milk and milk-based products without the requirement for multiple calibrations according to matrix type or fat composition.

The chosen data processing methods may vary but should enable fat determinations with the accuracy and precision demonstrated in Clause 12.

6 Apparatus

The usual laboratory apparatus and, in particular, the following should be used.

6.1 TD-NMR instrument, pulsed low-resolution TD-NMR spectrometer (benchtop) utilizing a CPMG radio frequency pulse sequence which, when used under the conditions defined in this document, provides quantitative results of fat content comparable to reference standards (i.e. chemical methods).

6.2 Sample tubes and consumables, proton-free material, capable of fitting the test sample in the probe and suitable for use with the NMR spectrometer as described in Clause 10.

6.3 Analytical balance, electronic, capable of weighing to four decimal places.

6.4 Temperature conditioning block (heating block), programmable to two decimal places and accurate to ±1 °C.

6.5 Drying system. Traditional oven drying system or comparable equipment which follows the procedures of an validated loss-on-drying method.

NOTE The drying method (traditional or rapid) has been studied and found to have no effect on the results of the NMR fat analysis, so long as drying performance is in accordance with results obtained with the adapted reference method.

7 Factors affecting the measurements

7.1 Instrument factors

7.1.1 Stability

The reliability of fat determinations is critically dependent on the overall stability of the system, which can be broadly assessed through routine analysis of standards as well as through repeat stability testing over the period of hours to days. Recommended stability assessment protocols and specifications can vary with instrument manufacturer. A general recommendation is that signal for a standard sample of oil should not vary by more than approximately 0,05 % to 0,075 % over a period of 12 h.

A variety of environmental factors can affect stability, including air drafts (due to proximity to doors/windows and air conditioning vents), ambient temperature fluctuations, electromagnetic interference and physical shocks/movement. Installation site preparation requirements and recommendations for minimizing the effect of environmental factors can vary depending on the instrument manufacturer. Instrument stability issues, despite controlling for environmental factors, should be addressed according to the manufacturer's recommendations.

7.1.2 Homogeneity of the magnetic field

While NMR provides a bulk measurement, considering the sample in its entirety, reliable results are dependent on a homogeneous magnetic field within the sampling region of the instrument. An insufficiently homogeneous magnetic field will result in degraded measurement sensitivity and precision. Recommended homogeneity specifications can vary depending on instrument manufacturer, but a general recommendation is < 10 µg/g (< 10 ppm[1)]). If homogeneity sufficiently exceeds the specified limits, the instrument should be re-shimmed according to the manufacturer's recommendations.

Metallic objects, and particular sources of electromagnetic radiation in proximity to the magnet, can adversely affect homogeneity. The instrument manufacturer should be consulted regarding specific requirements in terms of proper isolation.

7.1.3 Magnet temperature

The chosen equilibration temperature of the magnet (and sampling region of sample probe) and its associated stability over time can directly affect fat determinations. The magnet equilibration temperature should be chosen by the NMR supplier such that it sufficiently exceeds the ambient temperature. Ideally, the magnet temperature should also exceed the melting point of the fat contained within each sample.

Magnet temperature fluctuations during routine analysis should be monitored such that fat determinations are only permitted if the magnet temperature is within a particular range. As the difference between magnet temperature during the initial linear calibration and the temperature during the routine analysis increases, the precision of the calibration will deteriorate. The exact level of acceptable temperature fluctuation should be determined by the NMR supplier during initial development.

7.1.4 Carryover

Carryover can be considered as the presence of residual matter from previous samples in the sampling region of the NMR probe. The potential presence of carryover should be routinely assessed though analyses of blank samples. The instrument manufacturer should be consulted for best practices in cleaning and/or removing the NMR probe.

1) ppm = parts per million.

Carryover risk can be largely mitigated by carefully placing samples entirely within a container that is discarded after use.

7.1.5 Non-glycerolipids

This document outlines the NMR technique for fat determination in milk and milk-based products, defining fat as glycerolipids, which make up a large portion of total lipids in milk and milk-based products (i.e. ≥ 98 %). If a significant amount (> 10 mg/g of total sample mass) of non-glycerolipids are present, then the mass fraction of fat, in g/100 g, determined with NMR should be validated against a known sample with similar concentration of non-glycerolipids, or an established method for determination of the mass fraction of fat. Any discrepancies should be coordinated with the supplier of the NMR instrument for options.

7.2 Physicochemical factors

7.2.1 Sample temperature

For the NMR instrument to produce an accurate result, the sample temperature should adhere to the following:

a) It should exceed the melting point range of the fat within the sample.

b) It should be equilibrated to a defined temperature prior to analysis. This can be accomplished by either (or both) the use of a precision block heater (uniformity and stability of approximately ±0,1 °C) or by an extended residence time in the magnet (if the magnet temperature is equal to the desired sample temperature).

7.2.2 Sample pre-drying

While various pulse sequences (and pulsed-field gradients) can allow for the suppression of water signal, samples should be carefully pre-dried (using validated methodology) for optimal fat determination accuracy and reliability. Determined moisture content should be monitored for accuracy and repeatability, and as an indicator of representative sampling.

8 Validation and routine stability of the instrument

8.1 Standard reference material comparison

It is recommended to confirm NMR instrument performance by analysing a set of standard reference materials (SRMs) purchased from a reliable and accredited source. The exact details of the SRMs to be tested (product type, fat level, etc.) should be dependent on the primary products the NMR instrument will be used to analyse.

Some NMR manufacturers can have additional validation tools or processes that can be implemented to confirm initial setup or continued NMR instrument performance.

NOTE In-house reference material obtained with suitable/adapted chemical reference standard for fat extraction can be used in absence of SRMs.

8.2 System settings

Internal settings and checks can vary depending on the instrument manufacturer, including:

a) instrument stability;

b) magnet homogeneity;

c) pulse width;

d) voltages and internal temperatures of control modules;

e) signal/noise ratio.

9 Sampling

Sampling is not part of the method specified in this document. A recommended sampling method is given in ISO 707 | IDF 50. A general recommendation is that it is important that a representative sample should be obtained for analysis. For liquid milk and milk-based products, it is commonly recommended that samples should be tempered at approximately 38 °C ± 2 °C to aid in the even distribution of fat globules prior to sampling.

Care should be exercised that sample bottles are leak proof and that a proper empty volume is left. A too-large empty volume can facilitate churning. A too-small empty volume can cause problems with mixing.

10 Procedure

10.1 Preparation of test samples

10.1.1 General aspects

Sample preparation should be adapted according to product type (i.e. solid, powder, wet, liquid), fat content and instructions given with the NMR instrument used for the determination. Subclauses 10.1.2 and 10.1.3 are aspects to consider regarding sample preparation.

10.1.2 Pre-drying of the sample

Pre-drying samples containing moisture content higher than approximately 10 % (can be adapted according to matrix) is mandatory because a higher moisture content in the sample can significantly influence the result (i.e. overestimation of the fat content). Products containing moisture content equal to approximately 10 % and lower can be analysed directly without a pre-drying step in most cases.

The sample should be contained within a consumable vessel according to the NMR instrument manufacturer's recommendations. The sample mass should be recorded to four decimal places. Drying should be performed using a drying system as described in 6.5. While drying efficiency does not need to be in full conformity with food reference standards used for moisture determination, the drying method should remove the moisture to a constant or stable end mass. In the event of batch drying multiple samples of varying moisture content, a suitable analysis time should be used to ensure any remaining moisture content is well below 10 % of initial sample mass (i.e. 4 h at 102 °C, or 87 °C for some matrices with high lactose content).

In the instance that complete drying cannot be guaranteed or is possibly not necessary if the moisture content is near the 10 % limit but still within specification, it is recommended to confirm NMR fat results for the product against a similar reference or standard material to confirm the efficacy of the drying procedure.

10.1.3 Preparation of the sample for NMR analysis

10.1.3.1 Dried liquid sample

After drying, the sample mass should be recorded to four decimal places, then the dried sample should be carefully introduced into the NMR tube (using a dedicated tool to avoid contamination according to the manufacturer's recommendations). The sample should be contained within the NMR tube such that it is fully within the NMR measurement region when placed into the instrument. It is recommended to input the pre-dried initial mass for total fat content, or the final dry mass for fat by dry mass value.

10.1.3.2 Solid or sample in powder format (< 10 % moisture content)

The particle size should be reduced as needed, then the sample should be homogenized and prepared as directed. The sample should be contained within a consumable vessel according to the NMR instrument manufacturer's recommendations, and sample mass should be recorded to four decimal places. Then, the

sample should be carefully introduced into the NMR tube (using a dedicated tool if necessary, and according to the manufacturer's recommendations). The sample should be contained within the NMR tube such that it is fully within the NMR measurement region when placed into the instrument.

10.2 Measurement

The sample on the NMR instrument should be analysed immediately after the sample has reached the target temperature (in accordance with 7.2.1). The target sample temperature may be reached by incubating the sample for at least 45 min in a block heater (e.g. 45 °C).

The total fat content is automatically calculated by the NMR using the sample mass prior to drying (if required) and reported as the mass fraction of fat, in g/100 g, of the analysed sample.

For any sample being pre-dried, the NMR result may be expressed as the mass fraction of fat, in g/100 g, by dried mass by inputting the mass of the sample after the drying method has been completed.

11 Checking instrument stability

11.1 Control sample

The precision and trueness of measurements obtained by NMR can be tested with suitable quality control (QC) samples (i.e. certified reference material, proficiency test, in-house control samples) having a similar composition to the analysed samples. Sample preparation for NMR analysis should be the same as for the test sample.

11.2 Instrument diagnostics

Benchtop NMR instruments are typically robust, but it is highly recommended to check the instrument performance (according to the manufacturer's guidelines) after electric shutdown or when the NMR system is moving to a new location.

12 Precision and accuracy

12.1 General

The indicative target values for precision and accuracy given in this document were extracted from internal data made available and from available results of proficiency studies[2)]. Within the two reports, the total range was defined as 0,09 g/100 g to 77,81 g/100 g fat content (though other independent studies have confirmed applicability outside this range from 0,05 g/100 g to 100,00 g/100 g fat), with a linearity for both sample sets defined as R^2 = 1,000. These reports serve as a guidance for the expectations on various samples types that can be validated against the traditional, existing reference methods described in the second column.

12.2 Limit of detection (LoD) and limit of quantification (LoQ)

The LoD and LoQ can vary by matrix but are generally close or equivalent to 0,05 g/100 g and 0,10 g/100 g, respectively.

12.3 Repeatability

The absolute difference between two independent single test results (r), obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, should in no more than 5 % of cases be greater than the values listed in Table 1.

2) Performance data were obtained on an Oracle™ NMR instrument (CEM), courtesy of Fonterra and Actalia-Cecalait. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of the product named. Equivalent products may be used if they can be shown to lead to the same results.