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NORME POUR LE LAIT ET PRODUITS LAITIERS — DETERMINATION DE LA TENEUR EN LACTOSE PAR CHROMATOGRAPHIE LIQUIDE HAUTE PERFORMANCE (METHODE DE REFERENCE)

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Milk and milk products — Determination of lactose content by high-performance liquid chromatography (reference method)

*Lait et produits laitiers — Détermination de la teneur en lactose
par chromatographie liquide haute performance (méthode de
référence)*

**Second edition
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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 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).

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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 22662 | IDF 198:2007), which has been technically revised.

The main changes are as follows:

- the Scope has been extended to dairy permeate powders and dairy permeates, including validation data;
- test portions have been adapted accordingly.

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.

ISO 22662:2024(en)
IDF 198:2024(en)

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 IDF/ISO Action Team C52 of the *Standing Committee on Analytical Methods for Composition* under the aegis of its project leader P. Trossat (FR).

Milk and milk products — Determination of lactose content by high-performance liquid chromatography (reference method)

1 Scope

This document specifies a reference method for the determination of lactose content of raw milk, heat-treated milks, dried milk, dairy permeate, dairy permeate powder, and raw and pasteurized cream.

The method does not apply to fermented milks and milks to which oligosaccharides have been added, nor to low-lactose or lactose-free milks.

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 648, *Laboratory glassware — Single-volume pipettes*

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

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

lactose content

mass fraction of substances

Note 1 to entry: The lactose content is determined according to the procedure specified in this document.

Note 2 to entry: The mass fraction can be expressed as a percentage.

4 Principle

An internal standard [D(+)-melezitose] is added to a weighed volume of test sample and to lactose standards. A chemical reagent (Biggs-Szijarto solution) is added to precipitate out the fat and the protein component fractions of milk. The filtrate is filtered twice prior to injection, first through paper filter and then through a 0,45 µm nylon filter. The lactose and the internal standard are separated by a cation exchange column in the lead form and detected by a differential refractometer detector or other suitable detector. As mobile phase, high-performance liquid chromatography (HPLC) grade water is used.

5 Reagents

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

5.1 Degassed HPLC grade water.

Filter the water, conforming to the requirements of ISO 3696, grade 1, obtained from the water purification unit (6.9) using the solvent filtration unit (6.10). To improve the pump performance and to obtain a stable baseline, degas the mobile phase daily by selecting one of the available techniques such as sparging with helium, sonication, vacuum or in-line degassing system.

5.2 D(+)-Melezitose hydrate solution, purity $\geq 99\%$, $c(\text{C}_{18}\text{H}_{32}\text{O}_{16} \cdot \text{H}_2\text{O}) = 50 \text{ mg/ml}$.

Dissolve an amount of D(+)-melezitose hydrate in water (5.1) to give a final concentration equivalent to 50 mg/ml of the anhydrous form. The D(+)-melezitose solution can be stored at 4 °C for no longer than one week.

5.3 α -Lactose monohydrate, $\text{C}_{12}\text{H}_{22}\text{O}_{11} \cdot \text{H}_2\text{O}$, purity $\geq 99\%$.

Before use, dry the α -lactose monohydrate at 70 °C for 4 h. Cool it to room temperature in a desiccator.

NOTE After drying, the lactose remains in the monohydrate form.

5.4 Biggs-Szijarto solution.

Dissolve 25 g of zinc acetate dihydrate, $\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$ and 12,5 g of phosphotungstic acid monohydrate ($\text{W}_{12}\text{O}_{36} \cdot \text{H}_3\text{PO}_4 \cdot \text{H}_2\text{O}$) in about 100 ml of HPLC grade water (5.1) in a 200 ml one-mark volumetric flask.

Add 20 ml of glacial acetic acid (CH_3COOH). Dilute to the 200 ml mark with HPLC grade water (5.1) and mix. After use, the solution may be stored at 4 °C for no longer than one week.

SAFETY PRECAUTIONS — Follow prevailing health and safety guidelines for the storage and handling of these chemicals.

6 Apparatus

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

6.1 HPLC ion exchange resin column, of length 300 mm, of internal diameter 7,8 mm, with 8 % cross-linked copolymer, based on polystyrene-divinylbenzene cation exchange resin, packed in the lead form allowing the required separation.

6.2 Guard column

In order to prolong ion exchange resin column life, replace the guard column after about 200 injections.

6.3 Micro-guard holder.

6.4 Column heater, capable of maintaining a constant temperature of $85\text{ °C} \pm 1\text{ °C}$.

6.5 HPLC pump, capable of maintaining a flow rate of between 0 ml/min and 10 ml/min.

6.6 HPLC autosampler.

NOTE Manual injection can also be used.

6.7 Differential refractometer detector, highly sensitive.

NOTE Other detectors (e.g. an evaporative light scattering detector) can also be used.

6.8 Software, capable of automating injections, performing data acquisition, processing and managing chromatographic information.

6.9 Water purification unit, capable of providing water conforming to the grade 1 requirements of ISO 3696, with a resistivity of between 10 M Ω ·cm and 18 M Ω ·cm.

6.10 Solvent filtration unit, including a vacuum source, with a membrane filter of 0,45 μ m pore size and of diameter 47 mm.

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

6.12 Water bath, capable of maintaining a temperature of between 38 °C and 40 °C.

6.13 Accurate dispenser, accurate automatic pipette or one-mark pipettes conforming to the requirements of ISO 648, Class A, of capacity 2 ml.

6.14 Filter funnel, of diameter 75 mm.

6.15 Filter paper, of diameter 110 mm, Whatman¹⁾ No 1 or equivalent.

6.16 Nylon syringe filter, of porosity 0,45 μ m.

NOTE An in-line filter of the same porosity can also be used.

6.17 Syringe, with Luer-lock, of capacity 5 ml.

6.18 HPLC vials, with caps.

6.19 One-mark volumetric flasks, of capacity 10 ml $\pm$ 0,02 ml.

NOTE Flasks with a capacity of more than 10 ml can also be used by taking into account the concentration factor.

7 Sampling

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

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

8 Preparation of test sample

For fluid milk and cream, warm the test sample in the water bath (6.12) to between 38 °C and 40 °C. Gently mix the test sample by repeatedly inverting the bottle. Cool the sample quickly to 20 °C $\pm$ 1 °C while gently mixing the sample immediately prior to weighing the test portion (see 9.2).

1) Whatman 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 IDF of this product

9 Procedure

9.1 Preparation of the standard solution

9.1.1 In a 10 ml one-mark volumetric flask ([6.19](#)), weigh, to the nearest 1 mg, the appropriate amount of α -lactose monohydrate ([5.3](#)) to give the equivalent of a 20 mg/ml anhydrous α -lactose solution.

9.1.2 Dissolve the α -lactose monohydrate (see [9.1.1](#)) in about 5 ml of HPLC grade water ([5.1](#)). Add 2 ml of D(+)-melezitose solution ([5.2](#)), used as internal standard, to the flask. Make up to the mark with HPLC grade water ([5.1](#)) and mix by inverting the flask. Express the final α -lactose concentration in milligrams of the anhydrous form per millilitre.

9.1.3 Filter the standard solution through a filter paper ([6.15](#)) using a filter funnel ([6.14](#)). Aspirate the filtrate into a syringe ([6.17](#)). Screw the nylon syringe filter ([6.16](#)) to the syringe and then transfer each filtrate into an HPLC vial ([6.18](#)). Inject each standard solution twice in accordance with the requirements of [9.4.2](#).

The standard solution thus prepared can provide three sets of calibration solutions. Use each set once only to calibrate the HPLC column. Store non-used sets of lactose standard solution at 4 °C for no longer than one week. Before use, bring all refrigerated standard solutions to approximately 20 °C.

In order to monitor the calibration, inject the standard solution as unknown sample at the beginning and at the end of the set of the test portions.

NOTE The filtration step through the filter paper can be replaced by centrifugation of the test sample.

9.2 Preparation of test portion

9.2.1 Fluid milk or dairy permeate test sample

Weigh, to the nearest 1 mg, about 3 ml of prepared test sample fluid milk (see [Clause 8](#)) or about 2 ml of dairy permeate into a 10 ml one-mark volumetric flask ([6.19](#)). Proceed as in [9.3](#).

9.2.2 Dairy powder test sample

Weigh, to the nearest 1 mg, about 0,300 g of dried milk or 0,200 g of dairy permeate powder into a 10 ml one-mark volumetric flask ([6.19](#)). Add about 5 ml of HPLC grade water ([5.1](#)) pre-warmed to between 50 °C and 60 °C. Mix thoroughly until the solution becomes homogenous. Allow the test solution thus obtained to cool to 20 °C $\pm$ 1 °C. Proceed as in [9.3](#).

9.2.3 Cream test sample

Weigh, to the nearest 1 mg, about 1 g of prepared test sample (see [Clause 8](#)) into a 10 ml one-mark volumetric flask ([6.19](#)). Proceed as in [9.3](#).

9.3 Preparation of filtrate

Add 2 ml of D(+)-melezitose internal standard solution ([5.2](#)) and 1,2 ml of Biggs-Szijarto solution ([5.4](#)) to the content of the flask obtained in accordance with the procedures of [9.2.1](#), [9.2.2](#) or [9.2.3](#), as appropriate. Dilute to the mark with HPLC grade water ([5.1](#)).

Gently mix the contents by inverting the flask five times. Allow to stand at room temperature for 10 min. Repeat the mixing and standing process two more times.

Filter the contents of the flask through a filter paper (6.15) using a filter funnel (6.14). Collect the filtrate with a syringe (6.17). Screw the nylon syringe filter (6.16) to the syringe and then transfer the filtrate into a HPLC vial (6.18). Inject the test solution in accordance with the requirements of 9.4.2.

NOTE The filtration step through the filter paper can be replaced by centrifugation of the test sample.

9.4 HPLC determination

9.4.1 Preliminary preparation of HPLC

In order to get a stable baseline, turn on the detector (6.7) at least 24 h before starting the analysis. Set the internal temperature at 35 °C. Set the HPLC pump (6.5) to deliver a flow rate of 0,2 ml/min for at least 20 min while the column heater (6.4) is set to room temperature.

Increase the column heater temperature to 85 °C. When that temperature is reached, gradually increase the flow rate from 0,2 ml/min to 0,6 ml/min. Allow the system to equilibrate at a flow rate of 0,6 ml/min and at 85 °C for 2 h or until a stable baseline is obtained.

NOTE Checking and recording the pressure of the system from day to day can help to detect whether abnormal pressure changes occur.

9.4.2 Chromatographic conditions

The chromatographic conditions are given in Table 1.

Table 1 — Chromatographic conditions

Condition	Details
Mobile phase	degassed HPLC grade water
Internal detector temperature	35 °C
Guard column temperature	ambient temperature
Column temperature	85 °C
Flow rate	0,6 ml/min
Volume to be injected	20 µl
Run time	15 min
Retention time of D(+)-melezitose	9 min ± 1 min
Retention time of lactose	11 min ± 1 min

Carefully choose the acquisition and integration parameters such as sensitivity, scale factor, time constant, peak width and threshold. See Figure 1 for an example of a chromatogram.

Measure the column efficiency, also called theoretical plate count, N , at least once per week. A decrease in N is related to the band spreading of the peak which is often due to a loss in column performance. Calculate N by using Formula (1):

$$N = 5,54 \times \left(\frac{t_R}{w} \right)^2 \quad (1)$$

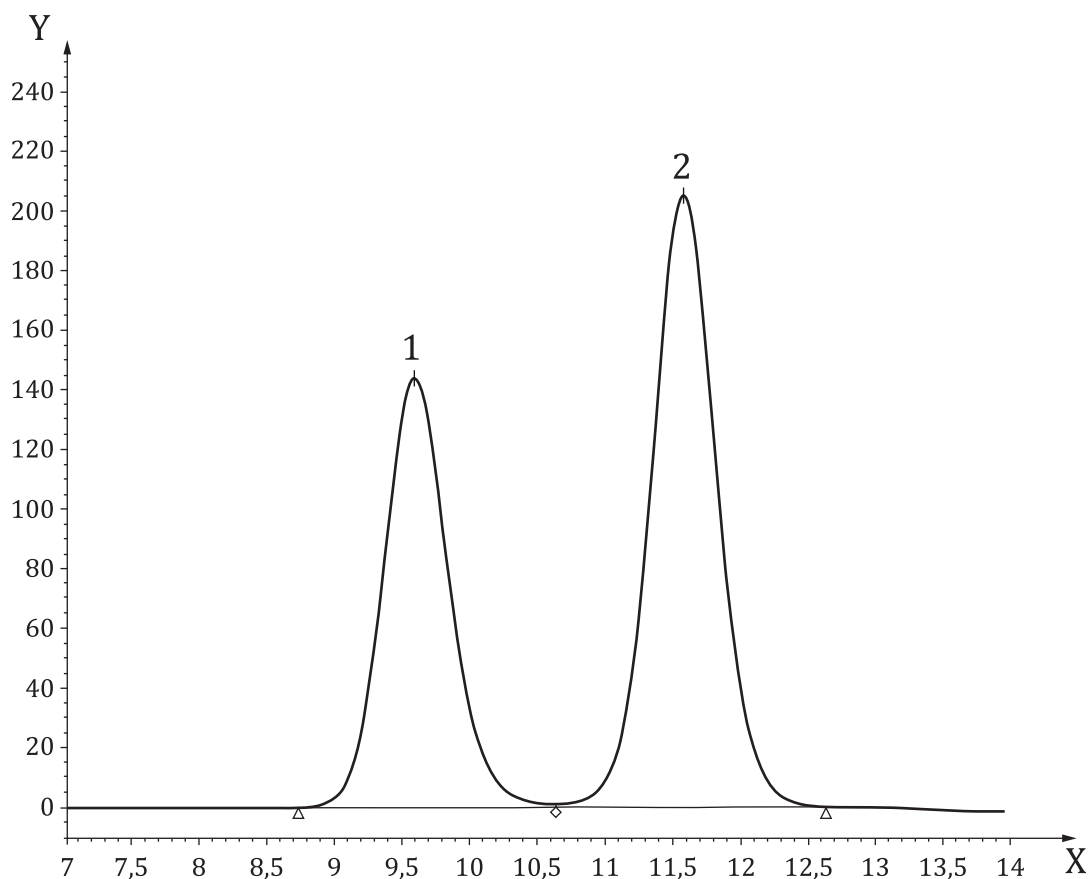
where

t_R is the retention time, in minutes, of the lactose peak;

w is the width of the lactose peak, equivalent to time difference in minutes, at 50 % of its height.

When the theoretical plate count decreases by more than 25 % compared to the original measurement, a replacement of the column is recommended.

NOTE In most cases, a used column performing with low efficiency can be restored to its original form by back washing with an appropriate regenerating solvent described in the manufacturer's documentation.



Key

- X retention time, in min
- Y potential difference, in mV
- 1 D(+)-melezitose
- 2 α -lactose

Figure 1 — Example of a chromatogram from a raw milk sample containing the internal standard

10 Calculation and expression of results

10.1 Calculation

A computer performs the calculations as follows:

- First, the software (6.8) generates a curve by plotting the response ratio of the lactose standard peak area, A_s , to that of the internal standard, A_{is} , multiplied by the internal standard concentration, c_{is} , i.e. $(A_s/A_{is}) \times c_{is}$, against lactose concentration, c_l . The curve fit is linear through the origin.
- To quantify an unknown test sample, the software divides the concentration derived from the calibration curve by the mass of the test sample to calculate the anhydrous lactose concentration expressed in g/100 g.

10.2 Expression of results

Express the test results to three decimal places.

11 Precision

11.1 Interlaboratory test

Details of the interlaboratory test on the precision of the method are summarized in [Annex A](#).

The values derived from this interlaboratory test are not necessarily applicable to concentration ranges and matrices other than those given.

11.2 Repeatability

The absolute difference between two independent single test results, 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, will in not more than 5 % of cases be greater than:

- a) for fluid milk and dairy permeate: 0,06 g/100 g;
- b) for cream: 0,06 g/100 g;
- c) for dried milk and dairy permeate powder: 0,37 g/100 g.

11.3 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will in not more than 5 % of cases be greater than:

- a) for fluid milk and dairy permeate: 0,13 g/100 g;
- b) for cream: 0,38 g/100 g;
- c) for dried milk and dairy permeate powder: 2,94 g/100 g.

12 Test report

The test report shall specify:

- a) all the information required for the complete identification of the sample;
- b) the sample method used, if known;
- c) the method used, with reference to this document, i.e. ISO 22662 | IDF 198;
- d) all operating details not specified in this document, or regarded as optional, together with details of any incident which can have influenced the results;
- e) the test result(s) obtained and, if the repeatability has been checked, the final quoted result obtained;
- f) the date of the test.

Annex A (informative)

Results of interlaboratory test

An international collaborative test involving eight laboratories from five countries was carried out on 18 samples (six of fluid milk, six of cream and six of milk powder), all obtained from three dairy plants in Québec, Canada. The test was organized by the Programme des Analyses des Troupeaux Laitiers du Québec.

The results obtained were subjected to statistical analysis in accordance with ISO 5725-1 and ISO 5725-2 to give the precision data shown in [Tables A.1](#) to [A.3](#). All values expressed as percentages, except coefficients of variation, are mass fractions.

Table A.1 — Fluid milk results

Parameter	Test sample						Mean
	1	2	3	4	5	6	
No. of participating laboratories after eliminating outliers	8	8	7	8	7	8	
Mean lactose mass fraction, g/100 g	4,530	4,466	4,718	4,644	4,364	4,551	
Repeatability standard deviation, s_r , g/100 g	0,025	0,023	0,017	0,035	0,011	0,018	0,022
Repeatability limit, $r = 2,8 \times s_r$, g/100 g	0,071	0,065	0,047	0,098	0,031	0,051	0,061
Coefficient of variation of repeatability, $C_{V,r}$, g/100 g	0,559	0,523	0,360	0,751	0,258	0,397	0,474
Reproducibility standard deviation, s_R , g/100 g	0,042	0,056	0,044	0,046	0,030	0,058	0,046
Reproducibility limit, $R = 2,8 \times s_R$, g/100 g	0,118	0,156	0,123	0,128	0,085	0,162	0,129
Coefficient of variation of reproducibility, $C_{V,R}$, g/100 g	0,931	1,249	0,929	0,986	0,692	1,273	1,010

Table A.2 — Cream results

Parameter	Test sample						Mean
	1	2	3	4	5	6	
No. of participating laboratories after eliminating outliers	8	8	7	8	6	6	
Mean lactose mass fraction, g/100 g	1,461	3,822	3,686	2,886	3,256	3,103	
Repeatability standard deviation, s_r , g/100 g	0,030	0,015	0,025	0,032	0,009	0,007	0,020
Repeatability limit, $r = 2,8 \times s_r$, g/100 g	0,084	0,041	0,069	0,090	0,026	0,020	0,055
Coefficient of variation of repeatability, $C_{V,r}$, g/100 g	2,066	0,381	0,672	1,117	0,283	0,224	0,790
Reproducibility standard deviation, s_R , g/100 g	0,171	0,108	0,136	0,118	0,134	0,136	0,134
Reproducibility limit, $R = 2,8 \times s_R$, g/100 g	0,479	0,302	0,380	0,331	0,375	0,382	0,375
Coefficient of variation of reproducibility, $C_{V,R}$, g/100 g	11,721	2,824	3,686	4,090	4,113	4,397	5,139

Table A.3 — Milk powder results

Parameter	Test sample						Mean
	1	2	3	4	5	6	
No. of participating laboratories after eliminating outliers ^a	6	7	7	6	6	7	
Mean lactose mass fraction, g/100 g	48,721	36,348	35,087	52,306	46,460	48,171	
Repeatability standard deviation, s_r , g/100 g	0,110	0,040	0,111	0,260	0,157	0,118	0,133
Repeatability limit, $r = 2,8 \times s_r$, g/100 g	0,308	0,111	0,312	0,729	0,440	0,331	0,372
Coefficient of variation of repeatability, $C_{V,r}$, g/100 g	0,226	0,109	0,318	0,497	0,339	0,245	0,289
Reproducibility standard deviation, s_R , g/100 g	1,130	0,801	0,773	0,709	1,461	1,419	1,049
Reproducibility limit, $R = 2,8 \times s_R$, g/100 g	3,165	2,241	2,166 3	1,986	4,091	3,974	2,937
Coefficient of variation of reproducibility, $C_{V,R}$, g/100 g	2,322	2,202	2,202	1,356	3,145	2,946	2,362
^a Only seven laboratories provided results for the tests on milk powder.							

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