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Heat-treated milk — Determination of lactulose content — Method using high-performance liquid chromatography

Lait traité thermiquement — Détermination de la teneur en lactulose — Méthode par chromatographie liquide à haute performance

Reference number
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Foreword

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International Standard ISO 11868 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Subcommittee SC 5, *Milk and milk products*, in collaboration with the International Dairy Federation (IDF) and AOAC INTERNATIONAL, and will also be published by these organizations.

Annexes A to C of this International Standard are for information only.

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Heat-treated milk — Determination of lactulose content — Method using high-performance liquid chromatography

1 Scope

This International Standard specifies a method for the determination of the lactulose content of heated milk, skimmed, partially skimmed or whole milk, by high-performance liquid chromatography in order to distinguish sterilized milk by Ultra Heat Treatment (UHT) from in-bottle sterilized milk.

The method has been tested over a lactulose content range of 200 mg/l to 1 500 mg/l and is applicable to all heat-treated milks.

The method described in this International Standard is to be used in cases of dispute.

2 Definition

For the purposes of this International Standard the following definition applies.

2.1
lactulose content of skimmed, partially skimmed or whole milk
mass of substances determined by the procedure specified in this International Standard

NOTE — The lactulose content is expressed as milligrams per litre of sample.

3 Principle

Removal of fat and protein, followed by filtration. Determination of lactulose in the filtrate by high-performance liquid chromatography (HPLC). Evaluation of the result obtained for the sample by reference to standard samples consisting of lactulose-free skimmed milk with known amounts of added lactulose.

4 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified, and double-distilled water or water of equivalent purity.

4.1 Lactose monohydrate

4.2 Lactulose, at least 99 % pure.

4.3 Sample pretreatment solution

Dissolve 91,0 g of zinc acetate dihydrate, $Zn(CH_3COO)_2 \cdot 2H_2O$, 54,6 g of phosphotungstic acid tetracosahydrate, $H_3[P(W_3O_{10})_4] \cdot 24H_2O$, and 58,1 ml of glacial acetic acid in water in a 1 000 ml volumetric flask and dilute to the mark with water.

4.4 Eluent

Filter the water, HPLC grade, through a membrane filter with a 0,45 μm pore diameter and, prior to use, boil to remove dissolved air.

NOTE — To remove dissolved air, other methods giving the same results (e.g. helium sparging) may be used instead of boiling water. These alternatives are usually more expensive.

4.5 Standard samples

4.5.1 Lactulose standard solution

Weigh, to the nearest 0,1 mg, about 75 mg of lactulose (4.2) in a 100 ml volumetric flask (5.6). Dissolve in water and make up to 100 ml with water.

4.5.2 Pasteurized skimmed milk, lactulose free, as determined using the method specified below.

Use identical pasteurized skimmed milk samples containing approximately 250 mg, 500 mg, 750 mg and 1 000 mg of lactulose per litre, obtained by the addition of 5 ml, 10 ml, 15 ml and 20 ml respectively of the lactulose standard solution (as described in 8.2) to the pasteurized skimmed milk.

5 Apparatus

Usual laboratory equipment and, in particular, the following.

5.1 Analytical balance, capable of weighing to the nearest 0,1 mg.

5.2 Glass funnels, of diameter about 7 cm.

5.3 Filters

5.3.1 Filter papers, medium grade, of diameter about 12,5 cm.

5.3.2 Cellulose acetate membranes, with 0,45 μm pore diameter.

5.4 Measuring cylinder, of capacity 25 ml.

5.5 Graduated pipette, of capacity 10 ml, graduated in 0,1 ml.

5.6 One-mark volumetric flasks, of capacity 50 ml, 100 ml and 1 000 ml.

5.7 One-mark pipettes, capable of delivering 5 ml, 10 ml, 15 ml and 20 ml.

5.8 Glass filtration equipment, with 0,45 μm pore diameter filter.

5.9 Glass flasks, of capacity 20 ml, with stopcock.

5.10 Ultrasonic water bath

5.11 Water vacuum pump

5.12 HPLC equipment, as follows:

5.12.1 Magnetic stirrer and **heater,** for keeping the eluent at a temperature of 90 °C ± 2 C.

5.12.2 Pump, capable of delivering a volume flow rate of between 0,3 ml/min and 0,6 ml/min with a pulsation of less than 1 % of the pressure drop over the column (1,5 MPa to 4 MPa).

5.12.3 HPX-87 P column (Bio-Rad, 30 cm x 0,78 cm)[1], or an equivalent column packed with sulfonic ion exchanger in the lead form, based on a polystyrene divinylbenzene 8 % crosslinked polymer. The pre-column consists of the Bio-Rad de-ashing system[1)] (a cartridge, 3 cm x 0,46 cm, packed with a cation-exchange resin in the hydrogen form and a cartridge, 3 cm x 0,46 cm, packed with an anion-exchange resin in the carbonate form) or a system of equivalent effectiveness.

NOTE — The pre-columns extend both the life and the length of the analytical column, minimizing separation problems and substantially reducing quantization errors. When the HPLC system begins to lose resolution, replace the spent pre-column before contamination extends to the main column.

5.12.4 Thermostatic column oven, capable of being maintained at a temperature of 75 °C ± 1 °C.

NOTE — The pre-columns should be placed outside the oven. The inlet tubing to the main column should have a length of 10 cm to 15 cm in the oven to equilibrate the eluent temperature to 75 °C, otherwise peak distortion may occur.

5.12.5 Refractive index detector, highly sensitive, with a noise level less than 5×10^{-9} Refractive Index Units (RIU), measured in water. The internal thermostat should be set at a temperature above room temperature, sufficient to obtain a stable baseline. A temperature of 35 °C to 40 °C is advisable in most cases.

NOTE — Highly sensitive monitoring of the refractive index is hampered by baseline drift due to thermal changes. To minimize the baseline drift, it is advisable to locate the HPLC equipment in a conditioned room to avoid temperature changes.

5.12.6 Integrator, capable of peak height measurements.

The integration control parameters should be carefully chosen (e.g. peak width, slope drift, peak threshold, etc.). The integrator should be forced to drop a perpendicular between the lactose and the lactulose peaks. (Skimming leads to inaccuracy due to the presence of varying amounts of glucose in the milk.) The integrator shall be inhibited against finding the baseline between the lactose and the lactulose peaks, unless the valley reaches the baseline at all lactulose concentrations.

Many integrators automatically vary peak integration parameters during the run. If possible, this feature should be disconnected in order to obtain more repeatable results.

6 Sampling

6.1 Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 707 [1].

It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage.

6.2 Store the sample in such a way that deterioration and change in composition are prevented.

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1) The HPX-87P column (Bio-Rad, 30 cm x 0,78 cm) and Bio-Rad de-ashing system are examples of suitable products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of these products.

7 Preparation of test sample

Bring the sample to 25 °C ± 5 °C and mix carefully. If the fat is not evenly dispersed, heat the sample slowly to 40°C, mix gently by inversion only and cool quickly to 20 °C ± 2 °C.

8 Procedure

8.1 Test portion

8.1.1 Pipette 15 ml of the test sample (clause 7) into a 50 ml volumetric flask (5.6). Add 20 ml of water using a graduated cylinder (5.4) and swirl. Add 5,5 ml of the sample pretreatment solution (4.3) with a graduated pipette (5.5) and swirl. Dilute to the mark with water and mix.

8.1.2 After leaving to stand for 1 h at 25 °C ± 5 °C, filter (5.3) using a glass funnel (5.2). Discard the first 5 ml of filtrate. Collect the rest of the filtrate in the glass funnel.

8.2 Preparation of calibration samples

8.2.1 Pipette 15 ml of lactulose-free skimmed milk (4.5.2) into each of four 50 ml volumetric flasks.

8.2.2 Standard A

8.2.2.1 Pipette 5 ml of the standard lactulose solution (4.5.1) into the first 50 ml volumetric flask containing the lactulose-free skimmed milk (8.2.1) and swirl.

8.2.2.2 Add 15 ml of water using a measuring cylinder (5.4) and swirl.

8.2.2.3 Add 5,5 ml of the sample pretreatment solution (4.3) with a graduated pipette (5.5) and swirl. Dilute to the mark with water and mix. After allowing the solution to stand for 1 h at 25 °C ± 5 °C, filter in a glass funnel (5.2) through a filter (5.3). Discard the first 5 ml of filtrate.
Collect the rest of the filtrate in the glass funnel.

8.2.3 Standard B

8.2.3.1 Pipette 10 ml of the standard lactulose solution (4.5.1) into the second 50 ml volumetric flask containing the lactulose-free skimmed milk (8.2.1) and swirl.

8.2.3.2 Add 10 ml of water using a measuring cylinder (5.4) and swirl.

8.2.3.3 Proceed as in 8.2.2.3.

8.2.4 Standard C

8.2.4.1 Pipette 15 ml of the standard lactulose solution (4.5.1) into the third 50 ml volumetric flask containing the lactulose-free skimmed milk (8.2.1) and swirl.

8.2.4.2 Add 5 ml of water using a measuring cylinder (5.4) and swirl.

8.2.4.3 Proceed as in 8.2.2.3.

8.2.5 Standard D

8.2.5.1 Pipette 20 ml of the standard lactulose solution (4.5.1) into the fourth 50 ml volumetric flask containing the lactulose-free skimmed milk (8.2.1) and swirl.

8.2.5.2 Proceed as in 8.2.2.3.

8.3 Chromatographic determination

8.3.1 Sample degassing

Pipette about 3 ml of filtrate from the test portion (8.1.2) and from the calibration samples (8.2.2.3) into separated glass flasks (5.9). Remove dissolved air from the filtrate by attaching the flask stopcock to the water vacuum pump (5.11), with ultrasonic bath treatment (5.10) for about 30 s at room temperature. If possible, avoid foaming.

NOTE — Inclusion of air in the sample may cause the appearance of a negative peak after the lactulose retention time.

8.3.2 Inject 10 µl to 30 µl (accurately measured) of filtrate into the HPLC apparatus (5.12) operating at a volume flow rate of 0,3 ml/min.

When the lactulose content is less than 200 mg/kg of milk, the analysis should be performed using two columns in series, increasing the flow rate to 0,6 ml/min.

NOTE — The chromatogram (annex A) shows a large off-scale peak of lactose with a retention time of about 19 min and, in the case of standard samples (8.2.2, 8.2.3, 8.2.4 and 8.2.5) and with heated milk, a relatively small peak of lactulose with a retention time of about 24 min.

Choose a plotter setting which provides a minimum height for the lactulose peak of 5 mm for the standard sample A (8.2.2). Depending on the quality of the column and pre-column used (5.12.3), a well-separated or less well-separated lactulose peak is obtained (5.12.6). In order to determine the minimum resolution required between lactose and lactulose, a standard solution containing 0,69 g lactose (4.1) and 3,75 mg lactulose (4.2) per 50 ml is prepared.

The following separation parameter, R_S, shall be not less than 5:

$$R_S = \frac{h_2}{h_v}$$

where

h_2 is the height of the lactulose peak;
h_v is the height of the valley between the lactose and lactulose peaks.

A period of 60 min between injection of consecutive samples is recommended.

8.3.3 The integrator reports the height of each peak h_1 and h_2, where h_1 is the height of the lactose peak and h_2 is the height of the lactulose peak.

The sample should be re-injected if the baseline drift exceeds 10 % of the full scale.

It is essential to examine the appearance of the chromatogram prior to quantification, in order to detect any abnormalities due either to malfunctioning of the apparatus or to the origin and nature of the sample analysed.

If in doubt, repeat the analysis. The lactose peak height in a test sample should not deviate by more than 10 % from that of the standards. If this occurs, other standard samples should be prepared.

Always include calibration samples with every series of samples. Recalibrate every 10 to 15 samples.

8.4 Recovery

If required, test the recovery by a standard addition procedure. If the recovery is less than 99 % in samples with a lactulose content equal to or exceeding 200 mg/l, the analysis should be repeated.

9 Calculation and expression of results

9.1 Calibration

After injection of each of the test standards and separation by the HPLC apparatus, the integrator has recorded the height of the following peaks:

h_{2a}, the numerical value of the height of the lactulose peak of standard A;
h_{2b}, the numerical value of the height of the lactulose peak of standard B;
h_{2c}, the numerical value of the height of the lactulose peak of standard C;
h_{2d}, the numerical value of the height of the lactulose peak of standard D.

Calculate the concentration, c_{a-d}, of the lactulose in the standards A, B, C and D (8.2) in milligrams per 50 ml as follows:

$$c_a = m_1 \times 5/100$$
$$c_b = m_1 \times 10/100$$
$$c_c = m_1 \times 15/100$$
$$c_d = m_1 \times 20/100$$

where m_1 is the numerical value of the mass of lactulose of the standard solution (4.5.1).

Least-squares linear regression analysis for the pairs $h_{2a}-c_a$, $h_{2b}-c_b$, $h_{2c}-c_c$ and $h_{2d}-c_d$, with c_a, c_b, c_c and c_d as the independent variables, gives the coefficients of regression, a and b, of the following equation:

$$h_2 = a + b \times c_L$$

where

h_2 is the numerical value of the height of the lactulose peak assigned to the dependent variable in the regression;

c_L is the numerical value of the concentration of lactulose, in milligrams per 50 ml, assigned to the independent variable in the regression.

9.2 Calculation of lactulose content

Calculate the lactulose content, w_l , expressed in milligrams per litre, of the test sample, using the following equation:

$$w_l = \frac{(h_{2s} - a)}{b.V_s}.d$$

where

h_{2s} is the numerical value of the height of lactulose peak of the sample;
V_s is the numerical value of the volume of the test sample, in millilitres (8.1.1);
d is the numerical value of the dilution factor to obtain an expression in milligrams per litre ($d = 10^3$).

10 Precision

10.1 Interlaboratory test

The values given for repeatability and reproducibility were derived from the results of interlaboratory tests. Details of these tests on the precision of the method are summarized in annex B and reported in reference [4].

10.2 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 6,0 % (relative) of the arithmetic mean of the two results.

The coefficient of variability of the repeatability, which expresses the variability of independent analytical results obtained under repeatability conditions, should not be greater than 2 %.

10.3 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 20 % (relative) of the arithmetic mean of the two results.

The coefficient of variability of the reproducibility which expresses the variability of independent analytical results obtained under reproducibility conditions, should not be greater than 7 %.

11 Test report

The test report shall specify:

— all information required for the complete identification of the sample;

— the sampling method used, if known;

— the test method used, with reference to this International Standard;

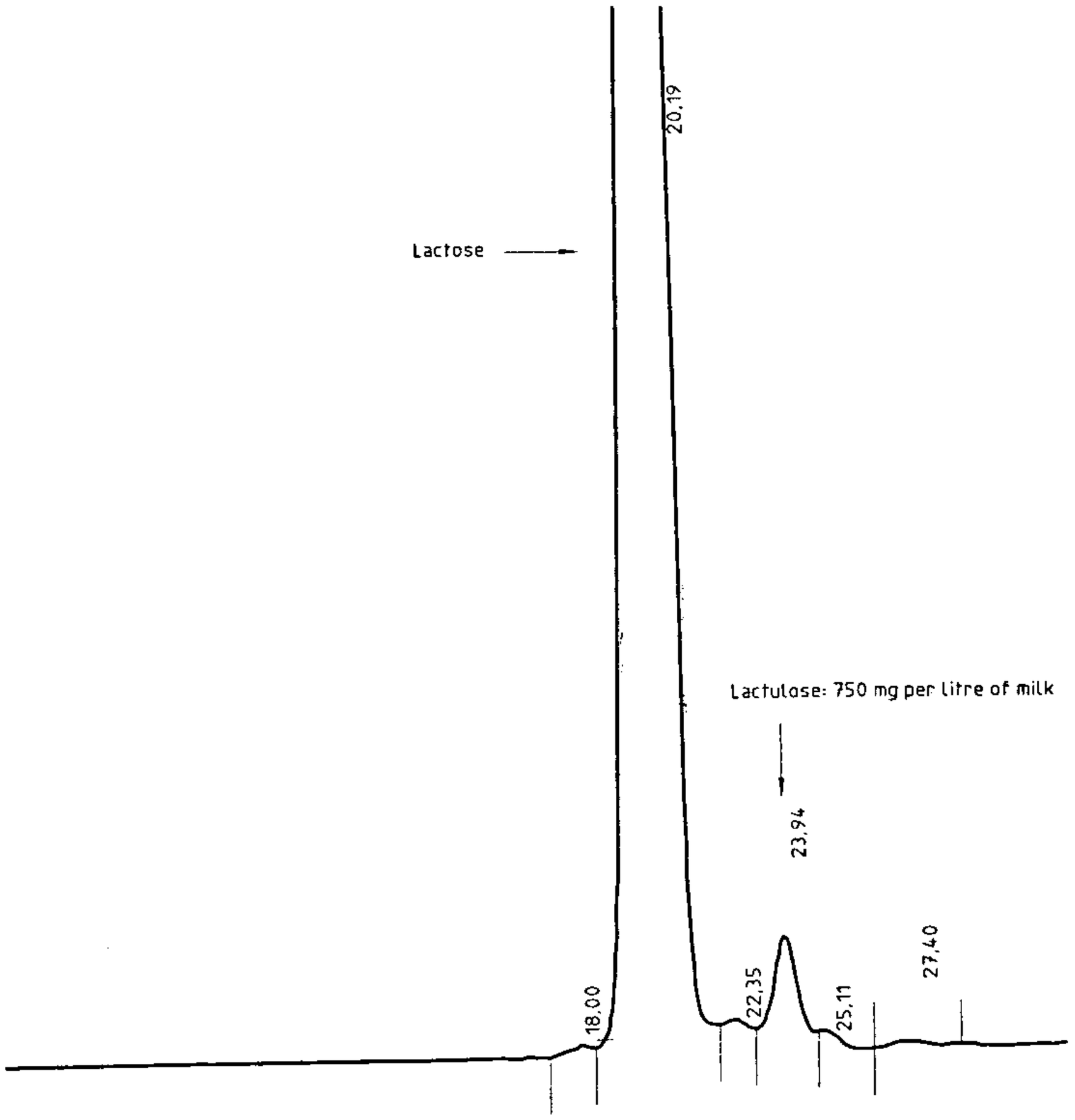
— all operating details not specified in this International Standard, or regarded as optional, together with details of any incident that may have influenced the test result(s);

— the test result(s) obtained;

— if the repeatability has been checked, the final quoted result obtained.

Annex A
(informative)

Example of a chromatogram



Annex B
(informative)

Results of interlaboratory tests

Sample level	Number of participants	Mean mg/100 ml	r	s_r	CV_r	R	s_R	CV_R	Remarks
	8	012,41	1,32	0,47	3,80	07,06	2,52	10,31	
Low	7	014,89	0,69	0,24	1,65	04,20	1,50	10,07	Lab 2[1)]
	7	014,67	5,48	1,96	3,34	07,06	2,52	07,19	Lab 2[1)]
	8	049,44	4,17	1,48	3,01	12,14	4,34	08,77	
Medium	8	094,08	1,62	0,58	0,62	08,36	2,99	03,17	
	7	099,35	6,04	2,16	2,17	08,28	1,09	07,16	Lab 2[1)]
High	7	131,36	2,34	0,84	0,64	05,50	9,82	05,09	Lab 2[1)]
	8	134,72	3,29	1,17	0,87	06,27	5,81	04,31	
Means			3,12	1,11	2,01	07,61	3,82	07,00	

1) Laboratory is deleted (Cochran outlier test).

Annex C
(informative)

Bibliography

[1] ISO 707:1997, *Milk and milk products — Guidance on sampling.*

[2] ISO 5725-1:1994, *Accuracy (trueness and precision) of measurement methods and results — Part 1: General principles and definitions.*

[3] ISO 5725-2:1994, *Accuracy (trueness and precision) of measurement methods and results — Part 2: A basic method for the determination of repeatability and reproducibility of a standard measurement method.*

[4] Mottar, J. Milk. *Bulletin of the International Dairy Federation*, No. 285 (1993), pp.86-97.

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