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Milk — Determination of lactulose content — Enzymatic method

*Lait — Détermination de la teneur en lactulose — Méthode
enzymatique*



Reference numbers
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IDF 175:2004(E)

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Foreword

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The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

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.

ISO 11285|IDF 175 was prepared by Technical Committee 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.

Foreword

IDF (the International Dairy Federation) is a worldwide federation of the dairy sector with a National Committee in every member country. Every National Committee has the right to be represented on the IDF Standing Committees carrying out the technical work. IDF collaborates with ISO and AOAC International in the development of standard methods of analysis and sampling for milk and milk products.

Draft International Standards adopted by the Action Teams and Standing Committees are circulated to the National Committees for voting. Publication as an International Standard requires approval by at least 50 % of IDF National Committees casting a vote.

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All work was carried out by the Joint ISO/IDF/AOAC Action Team, *Characterization of milk and milk products according to heat treatment*, of the Standing Committee on *Minor components and characterization of physical properties*, under the aegis of its project leader, Mrs E. Lechner (DE).

This edition of ISO 11285|IDF 175 cancels and replaces IDF 175:1995, which has been technically revised.

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Milk — Determination of lactulose content — Enzymatic method

1 Scope

This International Standard specifies an enzymatic method for the determination of the lactulose content of milk.

2 Terms and definitions

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

2.1

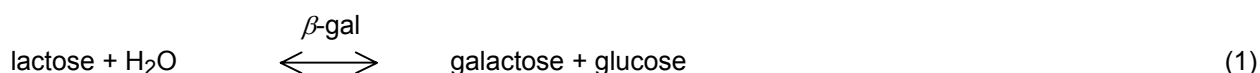
lactulose content

mass of substances determined by the procedure specified in this International Standard

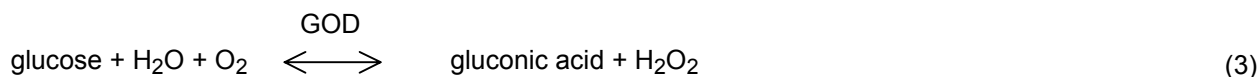
NOTE The lactulose content is expressed as milligrams per kilogram.

3 Principle

Fat and protein are precipitated by the addition of zinc sulfate and potassium hexacyanoferrate(II) solution and are then removed by filtration. Lactose and lactulose are hydrolysed to galactose and glucose, or galactose and fructose, respectively, in the presence of the enzyme β -D-galactosidase (β -gal). The amount of liberated fructose is stoichiometric with the amount of lactulose:



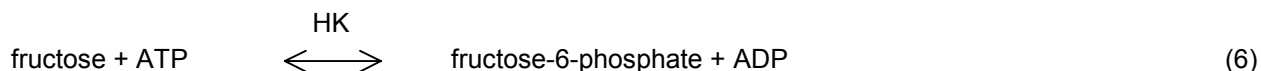
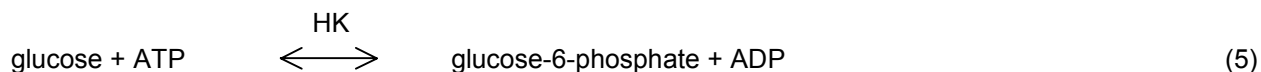
Lactose is present in milk in significantly higher amounts than lactulose. Thus, the glucose present after hydrolysis would interfere with the determination of fructose. Therefore, the glucose is mostly oxidized by glucose oxidase (GOD) to gluconic acid in the presence of oxygen:



By this means it is possible to determine small amounts of lactulose in the presence of excess lactose. The hydrogen peroxide formed in reaction (3) is destroyed by catalase. This reaction is used to provide the oxygen which is required for the oxidation of the glucose:



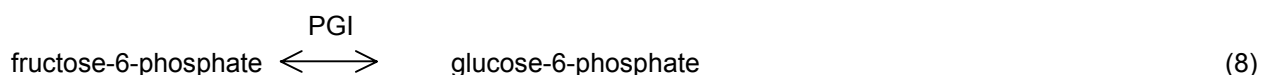
The remaining unoxidized glucose and the fructose produced by the hydrolysis of the lactulose are phosphorylated by means of adenosine triphosphate (ATP) in the presence of hexokinase (HK) to yield glucose-6-phosphate and fructose-6-phosphate, respectively.



Glucose-6-phosphate is oxidized by means of NADP^+ in the presence of glucose-6-phosphate dehydrogenase with consequent production of 6-phosphogluconate and NADPH. After completion of the reaction, the NADPH formed is measured by means of its absorbance at 340 nm.



Fructose-6-phosphate is isomerized to glucose-6-phosphate in the presence of the enzyme phosphoglucose-isomerase (PGI). The glucose-6-phosphate is oxidized as given in reaction (7). The increase in NADPH is measured by means of its absorbance at 340 nm and is proportional to the fructose and lactulose content.



In order to compensate for any free fructose which may originally be present, a blank determination is performed with the addition of β -galactosidase omitted. This blank value is taken into account in the final calculation of the lactulose content of the sample.

4 Reagents

Use only reagents of recognized analytical grade.

The reagents for the fructose determination (4.15 to 4.18) are commercially available as test combinations. Take due account of manufacturer's instructions.

4.1 Water used in the preparation of the enzyme solutions and buffer solutions shall be fresh and of at least double glass-distilled purity. Water used for other purposes shall be glass-distilled or of at least equivalent purity.

4.2 Zinc sulfate solution, $\rho(\text{ZnSO}_4) = 300 \text{ g/l}$.

Dissolve 30,0 g of zinc sulfate heptahydrate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) in water in a 100 ml one-mark volumetric flask (5.2). Dilute to the mark with water and mix.

4.3 Potassium hexacyanoferrate(II) solution, $\rho(\text{K}_4[\text{Fe}(\text{CN})_6]) = 150 \text{ g/l}$.

Dissolve 15,0 g of potassium hexacyanoferrate(II) trihydrate ($\text{K}_4[\text{Fe}(\text{CN})_6] \cdot 3\text{H}_2\text{O}$) in water in a 100 ml one-mark volumetric flask (5.2). Dilute to the mark with water and mix.

4.4 Buffer solution A, pH 7,5.

Dissolve 4,80 g of disodium hydrogenphosphate (Na_2HPO_4), 0,86 g of sodium dihydrogenphosphate monohydrate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) and 0,10 g of magnesium sulfate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) in 80 ml of water (4.1). Adjust the pH to $7,5 \pm 0,1$ at 20°C , if necessary, with 1 mol/l sodium hydroxide solution (4.10). Dilute with water to 100 ml and mix (sufficient for approximately 15 analyses).

4.5 Buffer solution B, pH 7,6.

Dissolve 14,00 g of triethanolamine hydrochloride [$\text{N}(\text{CH}_2\text{CH}_2\text{OH})_3 \cdot \text{HCl}$] and 0,25 g of magnesium sulfate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) in 80 ml of water (4.1).

Adjust the pH to $7,6 \pm 0,1$ at 20 °C, if necessary, with 1 mol/l sodium hydroxide solution (4.10). Dilute with water to 100 ml and mix (sufficient for approximately 60 analyses).

4.6 Buffer solution C.

Dilute 40,0 ml of buffer solution B (4.5) to 100 ml with water and mix (sufficient for approximately 50 analyses).

4.7 Sodium hydrogen carbonate (NaHCO₃).

4.8 Hydrogen peroxide (H₂O₂), 30 % (mass fraction).

4.9 Octan-1-ol (C₈H₁₈O).

4.10 Sodium hydroxide solutions, $c(\text{NaOH}) = 0,33 \text{ mol/l}$ and 1 mol/l respectively.

4.11 β -D-Galactosidase (β -gal), from *E. coli* (β -gal EC 3.2.1.23), 5 mg/ml suspension in 3,2 mol/l ammonium sulfate [(NH₄)₂SO₄] solution, 30 units/mg (at 25 °C, with lactose as substrate); 300 units/mg (at 37 °C, with 2-nitrophenyl- β -D-galactoside (*o*-nitrophenyl- β -D-galactopyranoside) as substrate), respectively.

4.12 Glucose oxidase (GOD), from *Aspergillus niger* (EC 1.1.3.4), degree of purity II, lyophilizate, 200 units/mg (at 25 °C) or 230 units/mg (at 37 °C, both with glucose as substrate), respectively.

4.13 Oxidation solution.

Dissolve 20 mg of glucose oxidase (4.12) in 1 ml of water. Prepare this solution freshly just before use.

4.14 Catalase, from beef liver (EC 1.11.1.6), suspension of 20 mg/ml in water (stabilized), 65 000 units/mg (at 25 °C; with H₂O₂ as substrate).

4.15 Hexokinase/glucose-6-phosphate-dehydrogenase (HK/G6P-DH), mixture of enzymes from yeast (EC 2.7.1.1 and EC 1.1.1.49), suspension in 3,2 mol/l ammonium sulfate [(NH₄)₂SO₄] solution, HK and G6P-DH:

— HK: 2 mg/ml; 140 units/mg (at 25 °C; with glucose and ATP as substrate);

— G6P-DH: 1 mg/ml, 140 units/mg (at 25 °C; with glucose-6-phosphate as substrate).

4.16 Phosphoglucose isomerase (PGI), from yeast (EC 5.3.1.9).

Suspension in 3,2 mol/l ammonium sulfate [(NH₄)₂SO₄] solution:

— PGI: 2 mg/ml 350 units/mg (at 25 °C; with fructose-6-phosphate as substrate).

NOTE 1 The EC numbers in 4.11, 4.12, 4.14 to 4.16 refer to the Enzymatic Classification number given by the Nomenclature Committee of the International Union of Biochemistry (see [4]).

NOTE 2 The unit in mentioned 4.11, 4.12, 4.14 to 4.16 (often called the International Unit or Standard Unit) is defined as the amount of enzyme which will catalyse the transformation of 1 μmol of substrate per minute under standard conditions.

4.17 ATP solution

Dissolve 50 mg of adenosine-5'-triphosphate disodium salt (5'-ATP-Na₂) and 50 mg of sodium hydrogen carbonate (NaHCO₃) in 1 ml of water.

4.18 NADP solution

Dissolve 10 mg of β -nicotinamide adenine dinucleotide phosphate disodium salt (β -NADP-Na₂) in 1 ml of water.

5 Apparatus

Usual laboratory equipment and, in particular, the following.

- 5.1 **Analytical balance**, capable of weighing to the nearest 0,001 g.
- 5.2 **One-mark volumetric flasks**, of capacity 10 ml, 20 ml and 100 ml.
- 5.3 **Conical flask**, with ground glass stopper, of capacity 50 ml.
- 5.4 **Funnel**, of diameter 50 mm.
- 5.5 **Fluted filter**, medium flow rate, of diameter 125 mm and 70 mm.
- 5.6 **Water bath or drying oven**, capable of maintaining a temperature of $40\text{ °C} \pm 2\text{ °C}$.
- 5.7 **Spectrometer**, suitable for making measurements at 340 nm.

Spectral line filter photometers suitable for making measurements at 365 nm and 334 nm (mercury lamps) may also be used. The molar absorption coefficients of NADPH (see 9.1) are then 3,4 (365 nm) or 6,18 (334 nm) ($\text{l}\cdot\text{mmol}^{-1}\cdot\text{cm}^{-1}$).

- 5.8 **Cuvettes**, made of glass or plastic, with optical path length 10,0 mm.

In the case of using plastic cuvettes, check the path length.

- 5.9 **Pipettes**, of capacity 20 μl , 50 μl , 100 μl , 500 μl , 1 ml, 2 ml, 5 ml and 10 ml, suitable for enzymatic analysis.
- 5.10 **Graduated pipettes**, of capacity 2 ml and 10 ml.
- 5.11 **Stirrer rod**, for blending the cuvette solutions.

6 Sampling

It is important that the laboratory receive a sample which is truly representative and has not 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.

7 Preparation of test sample

Thoroughly mix the test sample to obtain a representative sample.

See 8.6 for the preparation of test samples for the blank test.

8 Procedure

8.1 Preparation of test solution

Weigh, to the nearest 0,001 g, about 50 g of prepared test sample (Clause 7) into a 100 ml one-mark volumetric flask (5.2). Dilute to the mark with water and mix.

8.2 Clarification

Add, using a pipette (5.10), 10,0 ml of the test solution (8.1) into a 50 ml conical flask (5.3). Using each time another pipette, add the following in the given order: 1,75 ml of potassium hexacyanoferrate(II) solution (4.3), 1,75 ml of zinc sulfate solution (4.2) and 6,5 ml of buffer solution A (4.4), swirling thoroughly after each addition. Filter the obtained solution through a fluted filter (5.5) of diameter 125 mm. Discard the first few millilitres of filtrate.

8.3 Hydrolysis of lactose and lactulose

Pipette 5,0 ml of the obtained filtrate (8.2) into a 10 ml one-mark volumetric flask (5.2). Using a pipette (5.9), add 50 μ l of β -D-galactosidase (4.11). Mix and stopper the flask. Incubate the contents of the flask in the water bath or drying oven (5.6) set at 40 °C for at least 10 h (overnight).

8.4 Oxidation of glucose

Using the required pipette (5.9), add the following in the given order: 2,0 ml of buffer solution C (4.6), 100 μ l of oxidation solution (4.13), 1 drop of octan-1-ol (4.9) as antifoaming agent, 0,5 ml of 0,33 mol/l sodium hydroxide solution (4.10) to neutralize the nascent gluconic acid, 50 μ l of hydrogen peroxide (4.8) and 0,1 ml of catalase (4.14) to the hydrolysed solution (8.3), mixing after each addition. Incubate the thus-obtained mixture in the water bath or drying oven (5.6) set at 40 °C for a further 3 h.

8.5 Filtration

After incubation (8.4), dilute with water to the 10 ml mark and mix. Filter through a fluted filter of diameter 70 mm (5.5). Discard the first few millilitres of filtrate.

8.6 Blank test

Carry out a blank test. Proceed as specified in 8.2 to 8.5 for the test sample but without the addition of β -D-galactosidase.

8.7 Determination of glucose and fructose

Proceed as specified in Table 1.

Table 1 — Determination of glucose and fructose

Procedure	Blank	Sample
Pipette into cuvettes:		
buffer solution B (4.5)	1,00 ml	1,00 ml
ATP solution (4.17)	0,100 ml	0,100 ml
NADP solution (4.18)	0,100 ml	0,100 ml
filtrate (8.5)	1,00 ml	1,00 ml
water, double distilled (4.1)	1,00 ml	1,00 ml
Mix. ^a After 3 min read the absorbance (A_1) of the solutions.		
Start reaction by addition of hexokinase/glucose-6-phosphatase-dehydrogenase (4.15)	20 μ l	20 μ l
Mix ^a , wait until reaction has stopped (approx. 10 min). Read the absorbance (A_2) of the solutions		
Start reaction by addition of phosphoglucose isomerase (4.16)	20 μ l	20 μ l
Mix ^a , wait until reaction has stopped (approx. 10 min to 15 min). Read the absorbance (A_3) of the solutions.		
^a Mix using the same stirrer rod (5.11) for one cuvette.		

If the increase in absorbance with 1 ml of the filtrate exceeds 1,3, reduce the volume of the sample filtrate (8.5) and increase the addition of the water so that the whole volume of filtrate and water is again 2,00 ml. Change the volume for the blank filtrate (8.6) the same way as that of the sample filtrate.

Preferably, the absorbance range should be between 0,1 to 1,0 units, as 0,05 is the minimum acceptable unit.

9 Calculation and expression of results

9.1 Calculation

9.1.1 Difference in absorbance

9.1.1.1 Test sample

Calculate the difference in absorbance for the test sample, ΔA_S , by using the formula

$$\Delta A_S = (A_3 - A_2)$$

where

A_2 is the numerical value of the obtained absorbance after the addition of the hexokinase/glucose-6-phosphate-dehydrogenase (see 8.7);

A_3 is the numerical value of the obtained absorbance after the isomerization of fructose to glucose (see 8.7).

9.1.1.2 Blank test

Calculate the difference in absorbance for the blank sample, ΔA_B , using the formula

$$\Delta A_B = (A_3 - A_2)$$

9.1.1.3 Absorbance

Calculate the net absorbance difference related to the lactulose content, ΔA_L , using the formula

$$\Delta A_L = \Delta A_S - \Delta A_B$$

9.1.2 Lactulose content

Calculate the lactulose content, w , expressed as milligrams of lactulose per kilogram of sample, by using the following equation

$$w = \frac{M_L \times V_1 \times 4 \times 10\,000}{\varepsilon \times d \times V_2 \times 100 \times m} \times \Delta A_L$$

where

ΔA_L is the numerical value of the increase of absorbance by the fructose formed out of lactulose (9.1.1.3);

M_L is the numerical value of the relative molecular mass of lactulose ($M_L = 342,3 \text{ g}\cdot\text{mol}^{-1}$);

- ε is the numerical value of the molar absorption of NADPH at 340 nm, $\varepsilon = 6,3$ (l·mmol⁻¹·cm⁻¹); see 5.7 for the molar absorption at 334 nm or 365 nm;
- V_1 is the numerical value of the total volume of liquid in the cuvette (i.e. $V_1 = 3,240$ ml) (8.7), in millilitres;
- V_2 is the numerical value of the volume of the filtrate in the cuvette (i.e. $V_2 = 1,00$ ml) (8.7), in millilitres;
- d is the numerical value of the optical path length of the cuvette (i.e. $d = 1,000$ cm) (5.8), in centimetres;
- m is the numerical value of the mass of the test portion (8.1), in grams.

9.2 Expression of results

Express the results to one decimal place.

10 Precision

10.1 Interlaboratory test

The values for the repeatability and reproducibility were derived from the result of an interlaboratory test carried out in accordance with ISO 5725:1986¹⁾ and have been published (see [5] and [6]). The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given.

10.2 Repeatability

The absolute difference between two individual single test results, obtained with 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:

- for UHT milk with a mean lactulose content of 278 mg/kg: $r = 10,6$ mg/kg; $s_r = 3,8$ mg/kg;
- for sterilized milk with a mean lactulose content of 1 044 mg/kg: $r = 24,0$ mg/kg; $s_r = 8,5$ mg/kg.

10.3 Reproducibility

The absolute difference between two single test results, obtained with 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:

- for UHT milk with a mean lactulose content of 278 mg/kg: $R = 32,3$ mg/kg; $s_R = 11,4$ mg/kg;
- for sterilized milk with a mean lactulose content of 1044 mg/kg: $R = 77,2$ mg/kg; $s_R = 27,3$ mg/kg.

¹⁾ ISO 5725:1986, *Precision of test methods — Determination of repeatability and reproducibility for a standard test method by interlaboratory test* (now withdrawn), was used to obtain the precision data.

11 Test report

The test report shall specify:

- a) all information necessary for the complete identification of the sample;
- b) the sampling method used, if known;
- c) the test method used, with reference to this International Standard;
- d) all operational details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the test result(s).
- e) the test result(s) obtained and, if the repeatability has been checked, the final quoted result obtained.

Bibliography

- [1] ISO 707²⁾, *Milk and milk products — Guidance on sampling*
- [2] ISO 5725-1, *Accuracy (trueness and precision) of measurement methods and results — Part 1: General principles and definitions*
- [3] ISO 5725-2, *Accuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method*
- [4] *Enzyme Nomenclature Recommendations*, Academic Press, New York, 1984
- [5] FEIER and GOETSCH. *Milchwissenschaft*, **45**(7), 1990, pp. 440-441
- [6] *Bundesgesundheitsblatt (BGB)*, April 1990, Nr. 4, p. 176

2) Equivalent to IDF 50.

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