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Liquid milk — Determination of acid-soluble β-lactoglobulin content — Reverse-phase HPLC method

Lait liquide — Détermination de la teneur en β-lactoglobuline soluble dans l'acide — Méthode par chromatographie liquide haute performance en phase inverse

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ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
Web www.iso.org

International Dairy Federation
Diamant Building • Boulevard Auguste Reyers 80 • B-1030 Brussels
Tel. + 32 2 733 98 88
Fax + 32 2 733 04 13
E-mail info@fil-idf.org
Web www.fil-idf.org

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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 13875 | IDF 178 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 the National Committees casting a vote.

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All work was carried out by the Joint ISO/IDF/AOAC Action Team, *Characterization of heat treatment*, of the Standing Committee on *Minor components and characterization of physical properties*, under the aegis of its project leader, Prof. L. Pellegrino (IT).

Liquid milk — Determination of acid-soluble β-lactoglobulin content — Reverse-phase HPLC method

1 Scope

This International Standard specifies a method for the quantitative determination of the β-lactoglobulin content, soluble at pH 4,6, in liquid milk. The method has been tested over a range between 0 mg and 3 500 mg of β-lactoglobulin per litre of milk. It is suitable for distinguishing different categories of heat-treated liquid milk.

2 Normative references

The following referenced documents are indispensable for the application 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 8968-1 | IDF 20-1, *Milk — Determination of nitrogen content — Part 1: Kjeldahl method*

ISO 8968-2 | IDF 20-2, *Milk — Determination of nitrogen content — Part 2: Block-digestion method (Macro method)*

3 Terms and definitions

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

3.1
β-lactoglobulin content
β-LG content
mass fraction of substance determined by the procedure specified in this International Standard

NOTE It is expressed in milligrams per litre of test sample.

4 Principle

Casein and denatured whey protein are precipitated isoelectrically from milk at pH 4,6. The acid whey is separated by centrifuging and filtering. The acid-soluble β-LG content in the acid whey is determined by reverse-phase HPLC. The soluble β-LG content in the test sample is quantified by single-point or a multi-point calibration using a reference sample.

5 Reagents

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

5.1 Standard sample, pure β-lactoglobulin (A+B genetic variants).

Test the chromatographic purity of the *β*-LG standard sample by the HPLC procedure described in Clause 10. Determine its precise concentration as described in 10.2.

5.2 Reference sample

The reference sample is reconstituted from a freeze-dried raw bulk milk sample which was originally prepared from a skimmed raw bulk milk sample. It contains a known amount of soluble *β*-LG (A+B), which is determined by the HPLC procedure described in Clause 10.

The freeze-dried reference sample may be stored at 4 °C for 6 months, preventing hydration.

5.3 Reagents for sample preparation

5.3.1 Hydrochloric acid, dilute, c(HCl) = 2 mol/l.

5.3.2 Phosphate buffer solution, of pH 6,7 (final concentration 0,1 mol/l).

Add 57 ml of 0,2 mol/l sodium dihydrogen orthophosphate (NaH_2PO_4) solution to a 200 ml volumetric flask (6.10). Add 43 ml of 0,2 mol/l disodium hydrogen orthophosphate (Na_2HPO_4) solution and mix the phosphate solutions. Dilute to the mark with water and mix again.

5.4 HPLC elution solvents

Use elution solvents prepared from reagents of recognized HPLC-grade.

SAFETY PRECAUTIONS — Take appropriate safety precautions when handling the elution solvents as the chemicals may be carcinogenic.

5.4.1 Water, of HPLC-grade.

Laboratory-prepared water may be not sufficiently pure. Impure water produces column contamination and loss of resolution. If impure or improperly stored trifluoracetic acid is used, peaks can be unresolved or absent from the chromatogram.

5.4.2 Acetonitrile (CH_3CN).

5.4.3 Trifluoracetic acid (CF_3COOH), of the highest purity.

6 Apparatus

Usual laboratory equipment and, in particular, the following.

6.1 pH-meter, calibrated over the pH range 4,0 to 7,0, and accurate to 0,1 pH units.

6.2 Centrifuge, capable of operating at 2 000 g.

6.3 Centrifuge glass tubes, of capacity about 30 ml.

6.4 Glass vials, of capacity about 5 ml.

6.5 Glass funnels, of diameter about 7 cm.

6.6 Filter paper, fast grade, of diameter about 11 cm.

6.7 Glass test tubes, of capacity about 30 ml.

6.8 One-mark pipettes, capable of delivering 1 ml, 2 ml and 5 ml.

6.9 Beakers, of capacities 50 ml and 100 ml.

6.10 One-mark volumetric flasks, of capacities 10 ml, 20 ml, 25 ml, 50 ml and 200 ml.

6.11 Microfiltration tools.

6.11.1 Glass syringe, of capacity 5 ml.

6.11.2 Disposable syringe filter units, of pore size 0,22 µm, used with aqueous solutions.

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

6.13 Magnetic stirrer.

6.14 HPLC equipment.

6.14.1 Elution gradient pumping system, capable of operating at 1,0 ml/min at 200 bar.

6.14.2 Manual or **automatic injector**, capable of injecting 20 µl.

6.14.3 Column heater, capable of maintaining the column at 40 °C ± 1°C.

6.14.4 UV detector, capable of operating at 205 nm or at 280 nm wavelength and 0,1 AUFS.

6.14.5 Integrator or **data-reprocessing software**, capable of measuring peak areas.

6.14.6 PLRP-S column[1)], of length 150 mm and internal diameter 4,6 mm, of particle size 5 µm or 8 µm, and pore size 30 nm; or an equivalent column packed with underivatized polystyrene divinyl benzene, giving an equivalent chromatographic pattern.

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 International Standard. A recommended sampling method is given in ISO 707.

8 Procedure

8.1 Preparation of test portion

8.1.1 Check that the reported expiry date of the test sample has not been passed. Bring the closed package of the test sample to 20 °C ± 2 °C. Just before opening, shake the package and its contents carefully by inversion.

Open the package and transfer about 50 ml of test sample to a 100 ml beaker (6.9).

The test sample package should not be opened until just before starting the preparation.

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1) PLRP-S column is the trade name of a product supplied by Polymer Laboratories Ltd, Church Stretton, United Kingdom.

This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO or IDF of this product. Equivalent products may be used if they can be shown to lead to the same results.

8.1.2 Adjust the pH of the test portion to 4,6 by dropwise addition of the dilute hydrochloric acid (5.3.1) while stirring continuously. Allow the test portion to stand for 20 min at room temperature.

Transfer the prepared test portion to a centrifuge tube (6.3) and centrifuge at 2 000 *g* for 20 min. Filter the supernatant through filter paper (6.6), collecting the casein-free acid whey in a test tube (6.7).

The undiluted acid whey test portion may be stored for 24 h at 4 °C or for 2 weeks at −18 °C. Once defrosted, the acid whey test portion shall not be refrozen.

8.2 Preparation of test solution

After defrosting, carefully mix the acid whey test portion. Using a pipette (6.8), transfer suitable amounts of the acid whey test portion, depending on the type of test sample, to a 10 ml volumetric flask (6.10):

a) 1 ml, if from a test sample of raw or pasteurized or high-temperature pasteurized milk (final dilution 1:10);

b) 2 ml, if prepared from a test sample of UHT milk (final dilution 1:5);

c) 5 ml, if prepared from a test sample of bottle-sterilized milk (final dilution 1:2).

Dilute the test portion to the mark with the phosphate buffer solution (5.3.2). Mix carefully by inversion and allow to stand for 1 h. Mix again and filter using the microfiltration tools (6.11). Discard the first few millilitres of filtrate. Collect the rest of the filtrate in a glass vial (6.4). The diluted acid whey solution may be stored at 4 °C but shall be analysed within 24 h.

8.3 Preparation of reference portion

Weigh, to the nearest 0,01 g, 2,50 g of reference sample (5.2) into a 30 ml beaker (6.9). Add 10 ml of distilled water at 40 °C. Stir using a stirring rod (6.13) in order to dissolve any lumps. Quantitatively transfer the reconstituted reference sample to a 25 ml volumetric flask (6.10). Dilute to the mark with distilled water and mix thoroughly.

Quantitatively transfer the 25 ml of reference solution to a 50 ml beaker (6.9). Prepare the acid whey reference portion as described in 8.1.2 for the test portion.

Standardize the reference sample periodically as described in Clause 10.

8.4 Preparation of reference solutions for the multi-point calibration

After defrosting, carefully mix the acid whey reference portion. Pipette 2 ml of the reference portion into a 20 ml volumetric flask (6.10) (marked D). Dilute to the mark with phosphate buffer solution (5.3.2) and mix.

Immediately pipette 1 ml, 2 ml and 5 ml of this solution into three different 10 ml volumetric flasks (6.10) (marked A, B, C). Dilute to the mark with the phosphate buffer solution (5.3.2). Stopper the flasks and mix carefully.

Filter each reference solution (A, B, C and D) using the microfiltration tools (6.11). Discard the first few millilitres of each filtrate and collect the rest.

Using this procedure, obtain the following four diluted reference solutions:

— reference solution A with dilution ratio 1:100;

— reference solution B with dilution ratio 1:50;

— reference solution C with dilution ratio 1:20;

— reference solution D with dilution ratio 1:10.

The diluted reference solutions may be stored at 4 °C but shall be analysed within 24 h.

8.5 Preparation of the reference solution for the "single-point" calibration procedure

Prepare a reference solution which produces a β-LG peak area close (i.e. ± 20 %) to that of the test solution. Do not use reference solutions containing a β-LG content lower than 50 mg/l.

Usually reference solution B (8.4) is used for the quantification of β-LG in bottle-sterilized milk and in UHT milk. Use reference sample solution D (8.4) for the quantification of raw milk, pasteurized milk and high-temperature pasteurized milk.

8.6 HPLC determination

8.6.1 Elution solvents

Use the following elution solvents:

a) elution solvent X1: a volume fraction of 0,1 % trifluoracetic acid (5.4.3) in water (5.4.1);

b) elution solvent Y: a volume fraction of 0,1 % trifluoracetic acid (5.4.3) in acetonitrile (5.4.2);

c) elution solvent X2: mix volumes of water (5.4.1), acetonitrile (5.4.2) and trifluoracetic acid (5.4.3) in the following ratio: 65:35:0,1.

A low-pressure gradient system may produce a baseline noise due to poor mixing of the elution solvents X1 and Y. In that case, change the elution solvent X1 to the solution X2. Elution solvent Y may remain the same. Calculate the new elution gradient on the basis of that reported in Table 1, taking into account that solvent X2 already contains 35 % acetonitrile.

8.6.2 Elution gradient

Table 1 — Suggested elution gradient

Time min	Elution solvent X1 %	Elution solvent Y %
Initial	65	35
1,0	65	35
8,0	62	38
16,0	58	42
22,0	54	46
22,5	0	100
23,0	0	100
23,5	65	35
NOTE The elution gradient might require slight modification in order to achieve the resolution shown in Figure 1.		

Set the flowrate of the elution gradient pumping system (6.14.1) of the HPLC equipment (6.14) at 1,00 ml/min. Set the temperature of the column heater (6.14.3) at 40 °C.

Determine the equilibration time by monitoring the column elution. The detector response at the end of the run (baseline) should be equal to its initial value. An isocratic flushing of 15 min is usually sufficient.