

INTERNATIONAL
STANDARD

ISO
23058

IDF
199

First edition
2006-09-15

**Milk and milk products — Ovine and
caprine rennets — Determination of total
milk-clotting activity**

*Lait et produits laitiers — Présures ovines et caprines — Détermination
de l'activité totale de coagulation du lait*

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Reference numbers
ISO 23058:2006(E)
IDF 199:2006(E)

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Published in Switzerland

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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.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

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 23058|IDF 199 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.

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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 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 IDF National Committees casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. IDF shall not be held responsible for identifying any or all such patent rights.

ISO 23058|IDF 199 was prepared by the International Dairy Federation (IDF) and Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*. It is being published jointly by IDF and ISO.

All work was carried out by the Joint ISO-IDF Action Team on *Enzymes in cheesemaking*, of the Standing Committee on *Milk components and characterization of physical properties*, under the aegis of the project leaders Mrs M. Harboe (DK) and Mr C. Repelius (NL).

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Introduction

Animal rennets comprise all milk-coagulating products derived from ruminants, but until now only bovine rennets have been characterized according to the ISO/IDF standards on composition and milk-clotting activity. Ovine and caprine rennets are also available in the market and usually follow the same specifications as for bovine rennets. Each of these enzymes has its own characteristics as far as milk-clotting activity and cheese-making properties are concerned. There are differences in temperature and pH sensitivity and especially in the organoleptical properties of the cheese produced. A limited number of manufacturers produce these specific rennets and in different presentations (liquid, powder or rennet paste). No internationally recognized reference methods specific to the characterization and isolation of samples of these products have been available until now.

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Milk and milk products — Ovine and caprine rennets — Determination of total milk-clotting activity

1 Scope

This International Standard specifies a method for the determination of the total milk-clotting activity of an ovine or caprine rennet, including rennet paste, containing only chymosin and pepsin as the active coagulating enzymes on a standard milk substrate prepared using a calcium chloride solution of 0,5 g per litre (pH \approx 6,5).

The method allows analysis of ovine and caprine rennets in accordance with the relative milk-clotting activity test (REMCAT) for bovine rennets given in ISO 11815|IDF 157.

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 648, *Laboratory glassware — One-mark pipettes*

ISO 1042, *Laboratory glassware — One-mark volumetric flasks*

IDF 110B, *Calf rennet and adult bovine rennet — Determination of chymosin and bovine pepsin contents (chromatographic method)*

3 Terms and definitions

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

3.1

total milk-clotting activity of a standard milk substrate at pH 6,5

amount of activity set relative to the international calf rennet reference standard powder and the adult bovine rennet reference standard powder

NOTE 1 For the first batch of both the calf rennet reference standard powder and the adult bovine rennet reference standard powder (also used for the analysis of bovine and caprine rennets), this was defined as 1 000 International Milk-Clotting Units per gram (IMCU/g). Future preparations of reference standards will be set relative to the previous reference standards.

NOTE 2 The total milk-clotting activity of the microbial coagulant reference standard powder is approximately 1 000 IMCU/g, but the real activity with respect to the international calf rennet control powder is indicated on the glass ampoules.

NOTE 3 The total proteolytic (milk-clotting) activity of the microbial coagulant reference standard powder is checked on a synthetic hexapeptide substrate every second year by NIZO¹⁾.

1) Netherlands Institute for Dairy Research (NIZO), P.O. Box 20, 6710 BA Ede, The Netherlands. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO or IDF of these products.

4 Principle

The time needed for visual flocculation of a standard milk substrate prepared with a calcium chloride solution of 0,5 g per litre (pH \approx 6,5) is determined. The clotting time of the ovine (or caprine) rennet sample is compared to that of a bovine rennet reference standard with a defined enzyme composition of 75:25 and with known milk-clotting activity.

It is measured relative to a reference standard having 75 % calf chymosin and 25 % bovine pepsin because the composition of the test samples is unknown and a reference standard with a ratio 75/25 should cover most samples with enough accuracy. It is not worthwhile to measure the true chymosin/pepsin composition of ovine and caprine rennets as their composition is generally close to the above ratio.

NOTE The composition of ovine and caprine liquid or powder rennets may be determined using IDF 110B, but the accuracy for these rennet types has not been validated.

5 Reagents and materials

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

5.1 Buffer solution, pH 5,5.

Add, using a pipette (6.1), 10,0 ml of 1 mol/l acetic acid (CH_3COOH) to 10,0 g of sodium acetate trihydrate ($\text{CH}_3\text{COONa}\cdot 3\text{H}_2\text{O}$) and mix. Dilute with water to 1 000 ml. Adjust the pH to 5,5 if necessary.

5.2 Calcium chloride stock solution, $c(\text{CaCl}_2) = 500 \text{ g/l}$.

Calcium chloride solutions with the required accurate concentration of 500 g/l of calcium chloride and the actual density stated are commercially available²⁾. Store the solution as described by the manufacturer.

Prior to use, bring the calcium chloride stock solution to room temperature (18 °C to 22 °C). Check the concentration of the solution by titration with EDTA (ethylenediaminetetraacetic acid) every year.

5.3 Calcium chloride working solution, $c(\text{CaCl}_2) = 0,5 \text{ g/l}$.

Use the density of the calcium chloride stock solution (5.2) to calculate the mass of calcium chloride needed to obtain a final amount of 0,5 g/l of calcium chloride in the calcium chloride working solution.

The mass of the solution should be equivalent to the addition of 2,00 ml of the stock solution with the exact concentration required, $c(\text{CaCl}_2) = 500 \text{ g/l}$; in that case, the solution mass is \approx 2,70 g.

Weighing the calcium chloride stock solution (5.2) is recommended in order to be able to prepare the calcium chloride working solution, as the viscous solution is difficult to pipette.

Weigh, to the nearest 0,01 g, about 2,70 g of the calcium chloride stock solution (5.2) of exactly known concentration at room temperature (18 °C to 22 °C) in a 2 000 ml one-mark volumetric flask. Dilute to the mark with water and mix. The calcium chloride solution shall be freshly prepared on the day of use.

Alternatively, an intermediate calcium chloride solution of 50 g/l may be prepared and further diluted before use.

2) Ordering address: Chr. Hansen A/S, 1-27 Jernholmen, 2650 Hvidovre, Denmark (Fax: +45 36 86 77 76). This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO or IDF of these products.

5.4 Low-heat, low-fat, spray-dried milk powders, of good renneting and bacteriological quality.

NOTE Low-heat, low-fat, spray-dried milk powders meeting the requirements are commercially available²⁾, ³⁾.

5.5 Calf rennet reference standard powder⁴⁾, in pouches containing 2,7 g of powder, containing > 98 % chymosin and < 2 % bovine pepsin in terms of enzyme activity, as determined according to IDF 110B.

The exact total milk-clotting activity is indicated on the certificate of analysis and should be about 1 000 IMCU/g.

The calf rennet reference standard powder is a primary reference standard; a secondary liquid standard may be made and used if it has been ensured that the same result is obtained.

Store the calf rennet reference standard powder in the dark at –18 °C, protected against moisture. For short periods, for example during transport, it may be kept at ambient temperatures.

5.6 Adult bovine rennet reference standard powder⁴⁾, in pouches containing 2,7 g of powder, containing < 2 % chymosin and > 98 % bovine pepsin in terms of enzyme activity, as determined according to IDF 110B.

The exact total milk-clotting activity is indicated on the certificate of analysis and should be about 1 000 IMCU/g.

The adult bovine rennet reference standard powder is a primary reference standard; a secondary liquid standard may be made and used if it has been ensured that the same result is obtained.

Store the adult bovine reference standard powder in the dark at –18 °C, protected against moisture. For short periods, for example during transport, it may be kept at ambient temperatures.

6 Apparatus

Usual laboratory equipment and, in particular, the following.

6.1 Micropipette, or any other pipette capable of delivering 0,5 ml in less than 1 s with a repeatability of 0,2 % or better.

6.2 One-mark pipettes, in accordance with ISO 648, to deliver appropriate amounts.

Alternatively, a dilutor (e.g. a Hamilton diluter) with the same high precision may be used for diluting the coagulants. For measuring the substrate, a syringe or a dispenser delivering the appropriate amount with a repeatability of 0,4 % may also be used.

6.3 One-mark volumetric flasks, in accordance with ISO 1042, of the required capacities.

6.4 Thermometer, calibrated, graduated between 20 °C and 45 °C, with a precision of ± 0,1 °C.

6.5 pH-meter, capable of measuring the pH in 0,01 units.

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

3) Institut national de la Recherche agronomique, France, which is dependent on the Station expérimentale laitière, P.P. No. 94, 39800 Poligny, France. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO or IDF of these products.

4) AMAFE. Ordering address: Chr. Hansen A/S, 1-27 Jernholmen, 2650 Hvidovre, Denmark (Fax: +45 36 86 77 76). This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO or IDF of these products.

6.7 Stopwatch, capable of reading in seconds.

6.8 Flasks or test tubes, for milk-clotting testing, with a suitable capacity (see 6.9.1 and 9.5.1).

6.9 Water bath, capable of maintaining a temperature of $32\text{ °C} \pm 1\text{ °C}$ but also capable of keeping the maintained temperature constant within limits of $\pm 0,2\text{ °C}$ throughout the bath (see also 9.5.1), with the following attachments.

6.9.1 Electric motor, provided with a rotating spindle to which the flask or test tube (6.8) can be attached, capable of rotating at a suitable angle of about 30° with the water surface of the water bath.

NOTE The rotation speed is not very important for this method; a speed of between 2 r/min and 4 r/min is suitable.

6.9.2 Electric lamp, placed in such a position as to illuminate the flask or test tube (6.8) effectively.

A screen with a dark background, placed in the water bath, may be used to improve the determination of the flocculation point in the flask or test tube.

6.10 Stomacher, for dissolving rennet paste, e.g. from Seward⁵⁾.

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. Sampling of liquid rennet (8.1) and powdered rennet (8.3) should be carried out following the instructions given in ISO 707|IDF 50 for "Milk and liquid milk products" and for "Dried milk and dried milk products", respectively.

Store the test samples in the dark at a temperature of between 0 °C and 5 °C .

8 Preparation of test sample

8.1 Liquid ovine (or caprine) rennet

Mix the test sample by swirling while avoiding the formation of foam. Bring the sample to room temperature (18 °C to 22 °C) prior to starting preparation of the coagulant test solution (9.4).

8.2 Ovine (or caprine) rennet paste

Mix the rennet paste test sample to obtain a homogeneous paste. Dissolve a sample of known mass of $8\text{ g} \pm 2\text{ g}$ in a plastic bag with 200 ml of buffer solution (5.1) by using a stomacher (6.10) set at a recommended speed of 230 r/min for 60 s.

Alternatively, the paste dissolution in the plastic bag may be done manually in the stomacher bag for 60 s.

Record the exact amount of sample taken, in grams, to three significant digits. Filter the extraction solution through gauze or a Buchner filter.

5) Seward Ltd., London, United Kingdom. (www.seward.co.uk). 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.

Set the dilution factor of the paste to the liquid extract at (200 g + mass of the paste) divided by the mass of paste to be used in the calculation of the final dilution factor (see 9.4).

NOTE Alternatively, a Bagfilter^{®6)} can be used to allow extraction and filtration in one operation.

8.3 Powdered ovine (or caprine) rennet

Mix the test sample thoroughly to obtain a homogeneous powder. Bring the sample to room temperature (18 °C to 22 °C) prior to starting the preparation of the rennet test solution (9.4).

NOTE 1 Note that powdered products can separate rapidly.

NOTE 2 Consider the amount of test sample to be taken out. Often sample amounts of 3 g to 5 g are sufficient, but when testing inhomogeneous samples or when very accurate results are desired, then larger sample sizes such as 10 g are needed for analysis.

9 Procedure

9.1 Preparation of substrate

Measure 1 000 ml of calcium chloride working solution (5.3) into a 1 000 ml volumetric flask (6.3). Weigh, to the nearest 0,1 g, 110 g of low-heat, low-fat, spray-dried milk powder (5.4) into a 2 000 ml beaker. Add about 100 ml of calcium chloride working solution (5.3) to the powder in the beaker. Stir manually to obtain a homogeneous mixture. Add the remaining 900 ml of calcium chloride working solution to the contents of the beaker, allowing the 1 000 ml volumetric flask to drain. Stir the substrate thus obtained with a magnetic stirrer for 30 min, taking care to avoid the formation of foam.

Leave the substrate at room temperature for 30 min. The substrate may be kept at room temperature for no longer than 4 h, but it may be kept refrigerated during the day of preparation.

The pH of the prepared substrate will be approximately 6,50 and should not be adjusted.

9.2 Preparation of the calf and adult bovine rennet reference solutions

9.2.1 Preparation of the calf rennet reference standard solution

Bring the pouch with the calf rennet reference standard powder (5.5) to room temperature (18 °C to 22 °C) before opening it, to avoid moisture getting into the powder.

Open the pouch and weigh, to the nearest 1 mg, 2,500 g of calf rennet reference standard powder (5.5) into a 50 ml volumetric flask (6.3). Add 15 ml to 20 ml of buffer solution (5.1) and mix by swirling, while avoiding foam formation, to dissolve the powder. Dilute to the 50 ml mark with buffer solution (5.1) and mix well again.

9.2.2 Preparation of the adult bovine rennet reference standard solution

Repeat the preparation in 9.2.1 by replacing the calf rennet reference powder (5.5) with the adult bovine rennet reference standard powder (5.6).

6) Bagfilter[®] is available from Interscience, 30, chemin du Bois-des-Arpents, 78860 St-Nom-la-Bretèche, France (www.interscience.fr). 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.