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**Milk and milk products — Microbial
coagulants — Determination of total
milk-clotting activity**

*Lait et produits laitiers — Coagulants microbiens — Détermination de
l'activité totale de coagulation du lait*

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

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

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 3.

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 International Standard may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 15174|IDF 176 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.

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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 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 National Committees casting a vote.

ISO 15174|IDF 176 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.

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

This second edition cancels and replaces the first edition of IDF 176.

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Introduction

Microbial coagulants are derived from various microbial sources, the most common sources being *Rhizomucor miehei* (EC 3.4.23.23), *Rhizomucor pusillus* (EC 3.4.23.23) and *Cryphonectria parasitica* (EC 3.4.23.22, formerly named *Endothia parasitica*). Each of these enzymes has its own characteristics as far as milk-clotting activity and cheese-making properties are concerned. These are differences in temperature sensitivity, pH sensitivity, sensitivity to calcium ions, and the effect on the rheology of the milk-gel formed. The microbial coagulants are produced by a limited number of manufacturers, each having their own reference standard for measuring the milk-clotting activity of their product. No internationally recognized reference standard to enable the characterization of samples of these microbial products relative to a standard with known milk-clotting activity has been available up to now. For economic reasons a method for the determination of the total milk-clotting activity of microbial coagulants with respect to an internationally recognized reference standard is therefore highly desirable. For practical reasons it was decided to use the *Rhizomucor miehei* enzyme as a microbial coagulant reference standard for all types of microbial coagulants.

The method is in accordance with the relative milk-clotting activity test for bovine rennets as described in IDF 157.

A qualitative determination of the microbial coagulants in a sample is given in IDF 110B:1997, appendix A. In the case of mixtures of different clotting enzymes, no correct determination of the total milk-clotting activity for the sample can be obtained.

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Milk and milk products — Microbial coagulants — Determination of total milk-clotting activity

1 Scope

This International Standard describes a method to compare the total milk-clotting activity of a microbial coagulant sample with the milk-clotting activity of an international microbial coagulant reference standard on a standard milk substrate prepared with a calcium chloride solution containing 0,5 g/l of calcium chloride (pH \approx 6,5).

2 Normative references

The following normative documents contain provisions which, through reference in this text, constitute provisions of this International Standard. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

ISO 648, *Laboratory glassware — One-mark pipettes*

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

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3 Term and definition

For the purposes of this International Standard, the following term and definition applies.

3.1

total milk-clotting activity of the international microbial coagulant reference standard powder (*Rhizomucor miehei*)

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

NOTE 1 For the first batch, this was defined as 1 000 International Milk-Clotting Units per gram (IMCU/g) relative to a standard milk substrate at pH 6,5 (see IDF 157). 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 labelled 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), PO Box 20, 6710 BA Ede, The Netherlands.

4 Principle

The time needed for visual flocculation of renneted milk is determined. The total milk-clotting activity of a microbial coagulant sample is compared to the microbial coagulant reference standard powder on a standard milk substrate prepared with a calcium chloride solution containing 0,5 g/l of calcium chloride (pH \approx 6,5).

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

Using a pipette (6.1), add 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 with 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 calcium chloride 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 exact concentration of $c(\text{CaCl}_2) = 500 \text{ g/l}$; in that case, the solution mass is \approx 2,70 g.)

Weighing of the calcium chloride stock solution (5.2) is recommended in order 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 exact known concentration at room temperature (18 °C to 2 °C) in a 2 000 ml one-mark volumetric flask. Dilute with water to the mark and mix. The calcium chloride solution shall be freshly prepared on the day of its use.

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

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 available^{1), 2), 3)}.

5.5 Microbial coagulant reference standard powder (*Rhizomucor miehei*), in glass ampoules containing 2,7 g.

The exact total milk-clotting activity is labelled on the ampoules (\approx 1 000 IMCU/g).

Store the microbial coagulant reference standard powder in the dark at $-18 \text{ }^\circ\text{C}$, protected against moisture. For short periods, for example during transport, the powder may be kept at ambient temperatures.

2) Chr. Hansen's A/S, 1-27 Jernholmen, 2650 Hvidovre, Denmark.

3) DSM Food Specialities, Dairy Ingredients Group, P.O. Box 1, 2600 MA Delft, 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.

The total proteolytic activity of the microbial coagulant reference standard powder is checked on a synthetic hexapeptide every second year by the NIZO¹⁾.

The microbial coagulant reference standard powder is a primary reference standard; a secondary liquid standard may be made and used if it is assured that the same result is obtained.

The international microbial coagulant reference standard powder is commercially available from DSM Food Specialities³⁾.

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, conforming to ISO 648, to deliver appropriate amounts.

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

6.3 One-mark volumetric flasks, conforming to ISO 1042, of required capacities.

6.4 Thermometer, calibrated, graduated between 20 °C and 45 °C, with a precision $\pm 0,1$ °C.

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

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

6.7 Stopwatch, capable of reading in seconds.

6.8 Flasks (conforming to ISO 1042) or **test tubes**, for milk-clotting testing, with suitable capacity (see 6.9.1 and 9.4).

6.9 Water bath, capable of maintaining a temperature of $32\text{ °C} \pm 0,2\text{ °C}$ throughout, 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 (2 to 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 observation of the milk-clotting in the flask or test tube.

7 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.