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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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Foreword

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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 ≈ 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*

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 dilutor) 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 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.

Sampling of liquid microbial coagulant (8.1) and powdered microbial coagulant (8.2) should be carried out following the instructions given in ISO 707 for milk and liquid milk products, and for dried 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 microbial coagulant

Mix the test sample by swirling while avoiding foam formation. Bring the sample to room temperature (18 °C to 22 °C) prior to starting the preparation of the coagulant test solution (9.3).

8.2 Powdered microbial coagulant

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 coagulant test solution (9.3).

NOTE 1 Note that powdered products can rapidly separate.

NOTE 2 Consider the amount of test sample to be taken out. Often sample amounts of (3 to 5) g are sufficient. However, when testing inhomogeneous test samples and accurate test results are desired, larger sample sizes are necessary.

9 Procedure

9.1 Preparation of substrate

Add 1 000 ml of the calcium chloride working solution (5.3) to the mark of a 1 000 ml one-mark volumetric flask (6.3). Weigh, to the nearest 0,1 g, 110 g of the low-heat, low-fat, spray-dried milk powder (5.5) into a 2 000 ml beaker. Add about 100 ml of the 1 000 ml calcium chloride working solution to the powder in the beaker. Stir manually to obtain a homogeneous mixture. Add the remaining 900 ml of the calcium chloride working solution to the contents of the beaker allowing the volumetric flask to drain. Stir the thus-obtained substrate with a magnetic stirrer for 30 min while taking care to avoid the formation of foam.

Leave the obtained substrate in the dark at room temperature for 30 min. If necessary, the substrate may be kept in the dark at room temperature for no longer than 4 h or may be refrigerated during the day of preparation.

NOTE The pH of the prepared substrate will be approximately 6,50. The pH is not critical and need not be adjusted.

9.2 Preparation of microbial coagulant reference solution

9.2.1 Microbial coagulant reference standard solution

Dissolve the microbial coagulant reference standard powder (5.5) according to the following instructions.

Allow the glass ampoule with the microbial coagulant reference standard powder to reach room temperature (18 °C to 22 °C) before opening it to avoid moisture getting into the powder.

Weigh from the appropriate ampoule, to the nearest 1 mg, 2,500 g of microbial coagulant reference standard powder into a 50 ml one-mark volumetric flask (6.3). Add 15 ml to 20 ml of the buffer solution (5.1) and mix by swirling to dissolve the powder, while avoiding foam formation. Dilute to the mark with the buffer solution and mix well again.

9.2.2 Microbial coagulant reference working solution

In order to obtain a proper clotting time, pipette 3 ml of the microbial coagulant reference standard solution (9.2.1) into another 50 ml one-mark volumetric flask. Dilute to the mark with the buffer solution (5.1) and mix well.

NOTE The final obtained dilution factor is 333,33 times. The expected clotting time for the reference working solution should be in the range of (350 to 550) s.

Keep the microbial coagulant reference working solution at room temperature during the day of its preparation. It may be stored at between 0 °C and 5 °C for 2 days.

9.3 Preparation of microbial coagulant test solution

Take an appropriate test portion of approx. 3 ml from a liquid microbial coagulant (8.1) or of between 3 g and 5 g from a powdered microbial coagulant (8.2). Dilute the test portion with the buffer solution (5.1) until a microbial coagulant test solution is obtained with a clotting time that is similar to the microbial coagulant reference working solution (9.2.2) with a tolerance of ± 40 s. Note the final dilution factor of the test solution to be used in the calculation (10.1).

9.4 Clotting

9.4.1 Add using a pipette (6.2), 25 ml \pm 0,1 ml of the substrate (9.1) to a dry flask or test tube (6.8). Pre-heat the substrate, while rotating the flask or test tube, for at least 12 min but no longer than 20 min in the water bath (6.9) set at 32 °C. Then quickly add, using the micropipette (6.1), 0,5 ml of the microbial coagulant reference working solution (9.2.2) to the substrate. Activate the stopwatch (6.7) at the same time. Mix by swirling, while avoiding foam formation, and immediately attach the flask or test tube to the rotating spindle.

Read the clotting time from the stopwatch when the first flocculation is observed in the substrate film on the wall of the flask or test tube.

9.4.2 Repeat the procedure of 9.4.1 without delay but replacing the microbial coagulant reference working solution (9.2.2) by the microbial coagulant test solution (9.3).

9.4.3 Repeat operations 9.4.1 and 9.4.2 without delay to obtain duplicate values. Calculate the mean of the clotting times for the microbial coagulant reference working solution and the microbial coagulant test solution, respectively.

9.4.4 Instead of 25 ml of substrate and 0,5 ml of microbial coagulant reference working solution in 9.4.1, 10 ml of substrate and 0,2 ml of working solution or 50 ml substrate and 1,0 ml working solution may be used. However, it is important that the ratio between the substrate and the working solution be 50:1.

10 Calculation and expression of results

10.1 Calculation

Calculate the total milk-clotting activity of the test sample, a_t , expressed in International Milk-Clotting Units (IMCU) per gram or per millilitre, by using the following equation:

$$a_t = \frac{t_r \times w_r \times V_1 \times d \times a_r}{t_t \times V_2 \times V_3}$$

where

a_t is the total milk-clotting activity of the test sample compared to the microbial coagulant reference standard powder;

a_r is the milk-clotting activity (strength) of the microbial coagulant reference standard powder (5.5), in IMCU/g; this value is labelled on the glass ampoule of the reference powder;

- d is the noted final value of the dilution factor obtained with the test solution (9.3);
- t_t is the mean clotting time obtained with the microbial coagulant test solution (9.4.2 and 9.4.3), in seconds;
- t_r is the mean clotting time obtained with the microbial coagulant reference working solution (9.4.1 and 9.4.3), in seconds;
- V_1 is the volume taken in 9.2 from the microbial coagulant reference standard solution, in millilitres ($V_1 = 3$ ml);
- V_2 is the final volume in 9.2.1 of the microbial coagulant reference standard solution, in millilitres ($V_2 = 50$ ml);
- V_3 is the final volume in 9.2.2 of the microbial coagulant reference working solution, in millilitres ($V_3 = 50$ ml);
- w_r is the mass of the microbial coagulant reference standard weighed in 9.2, in grams;

NOTE The equation may be simplified by introducing the numerical values as follows: $w_r = 2,500$ g; $V_1 = 3$ ml; $V_2 = 50$ ml; $V_3 = 50$ ml:

$$a_t = \frac{t_r \times 0,003 \times d \times a_r}{t_t}$$

10.2 Expression of results

Express the results in International Milk-Clotting Units (IMCU) per gram or per millilitre to the nearest integer.

11 Precision

11.1 Interlaboratory test

Details of the interlaboratory test on the precision of the method will be published in the IDF Bulletin.

The values for repeatability and reproducibility derived from this interlaboratory test were determined in accordance with ISO 5725-1 and ISO 5725-2. The values obtained may not be applicable to concentration ranges and matrices other than those given. For example, due to some differences in solubility and a certain degree of inhomogeneity of coagulant powders, the figures for the precision parameters, repeatability (11.2) and reproducibility (11.3), may be somewhat higher when analysing coagulant powders. If significantly less than 95 % of the cases are within the values given in 11.2 and 11.3, it is recommended to work on improving the execution of the method.

NOTE 1 IDF 135 provides specific guidance for interlaboratory tests on methods of analysis and milk products. It is based on ISO 5725.

NOTE 2 The values for repeatability and reproducibility are derived from the standard deviations which are estimates of the true standard deviation of the method. Each value given for the repeatability and reproducibility is the maximum difference between two test results, which is expected in 95 % of the cases when two results are compared.

NOTE 3 The interlaboratory test on the precision of the method has been performed using specific microbial coagulant reference standards for each type of microbial coagulant (*Rhizomucor miehei*, *Rhizomucor pusillus* and *Cryphonectria parasitica* respectively). For practical reasons it was decided later that one microbial coagulant reference standard would be sufficient for the purpose of the method, but no interlaboratory test has been performed using only one microbial coagulant reference standard. It is however expected that the precision of the method will be at least as good as presented in this method. An adapted interlaboratory test using only the microbial coagulant reference standard derived from *Rhizomucor miehei* will be performed at the next revision of this method.

11.2 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 % of the arithmetic mean of the two results.