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

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National foreword

This British Standard is the UK implementation of ISO 15174:2012.

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A list of organizations represented on this committee can be obtained on request to its secretary.

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Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
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International Dairy Federation
Silver Building • Boulevard Auguste Reyers 70/B • 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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Contents

Page

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.

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Attention is drawn to the possibility that some of the elements of this International Standard may be the subject of patent rights. IDF 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). It is being published jointly by ISO and IDF.

This second edition of ISO 15174 | IDF 176 cancels and replaces the first edition (ISO 15174 | IDF 176:2002), which has been technically revised.

Foreword

IDF (the International Dairy Federation) is a non-profit organization representing the dairy sector worldwide. IDF membership comprises National Committees in every member country as well as regional dairy associations having signed a formal agreement on cooperation with IDF. All members of IDF have the right to be represented at 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.

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ISO 15174 | IDF 176 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 Project Group on *Microbial coagulants* of the Standing Committee on *Analytical methods for processing aids and indicators* under the aegis of its project leader, Mrs. M. Harboe (DK) and Prof. A. Andrén (SE).

This second edition of ISO 15174 | IDF 176 cancels and replaces the first edition (ISO 15174 | IDF 176:2002), which has been technically revised.

Introduction

Microbial coagulants are derived from various microbial sources, the most common sources being *Rhizomucor miehei* (EC 3.4.23.23), *R. pusillus* (EC 3.4.23.23) and *Cryphonectria parasitica*, formerly named *Endothia parasitica* (EC 3.4.23.22).

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 effect on the rheology of the milk-gel formed.

For practical and economic reasons, therefore, it is very important to have an international method for determination of the total milk-clotting activity of microbial coagulants relative to an international recognized reference standard. Also for practical reasons, it was decided to use the *R. 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 described in ISO 11815 | IDF 157.

A qualitative determination of the microbial coagulants in a sample can be performed according to ISO 15163 | IDF 110:—[7], Annex A. For mixtures of different clotting enzymes, no correct determination of the total milk-clotting activity for the sample can be obtained.

INTERNATIONAL STANDARD

ISO 15174:2012(E)
IDF 176:2012(E)

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

1 Scope

This International Standard specifies a method for comparison of 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 of concentration 0,5 g/l (pH ~6,5).

2 Principle

The time needed for a visible flocculation of a standard milk substrate prepared with a 0,5 g/l calcium chloride solution (pH ~6,5) is determined. The clotting time of a microbial coagulant sample is compared under identical chemical and physical conditions to that of the microbial coagulant reference standard with known milk-clotting activity.

3 Reagents and materials

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

3.1 Buffer solution, pH 5,5. Add, using a pipette (4.1), 10,0 ml of 1 mol/l acetic acid (CH_3COOH) to 10,0 g of sodium acetate trihydrate ($CH_3COONa \cdot 3H_2O$) and mix. Dilute with water to 1 000 ml. If necessary, adjust the pH to 5,5 with 1 mol/l acetic acid or with 1 mol/l sodium acetate solution.

3.2 Calcium chloride stock solution, $\rho(CaCl_2) = 500$ g/l. Calcium chloride solutions with the required accurate calcium chloride concentration of 500 g/l and the actual density stated are commercially available.[1)] Store the solution according to the manufacturer's instructions.

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.

3.3 Calcium chloride working solution, $\rho(CaCl_2) = 0,5$ g/l. Use the density of the calcium chloride stock solution (3.2) to calculate the mass needed to get a final calcium chloride concentration of 0,5 g/l in the 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 $\rho(CaCl_2) = 500$ g/l, in which case the solution mass is ~2,70 g.

Weighing of the calcium chloride stock solution (3.2) is recommended to be able to prepare the calcium chloride working solution, as the viscous solution is difficult to pipette.

1) Chr. Hansen's A/S, Hvidovre, Denmark is an example of a suitable supplier. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of this supplier.

Weigh, to the nearest 0,01 g, about 2,70 g of the calcium chloride stock solution (3.2) of exact known concentration at room temperature (18 °C to 22 °C) in a 2 000 ml one-mark volumetric flask. Dilute with water to the 2 000 ml mark and mix. The calcium chloride solution shall be freshly prepared on the day of its use.

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

3.4 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 Microbial coagulant reference standard powder (*Rhizomucor miehei*), in glass ampoules. The exact total milk-clotting activity is labelled on the ampoules.

Store the microbial coagulant reference standard powder in the dark at −18 °C, protected against moisture. For short periods, e.g. during transport, the powder may be kept at ambient temperatures.

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 total milk-clotting activity of the international microbial coagulant reference standard powder *(R. miehei*) is the amount of activity set relative to the first batch of international calf rennet reference standard powder, which was defined to contain 1 000 IMCU/g (see ISO 11815 | IDF 157[6]).

NOTE 1 The total milk-clotting activity is expressed as a percentage relative to the arithmetic mean of test results.

The total milk clotting activity of the microbial coagulant reference standard powder is labelled on the glass ampoules and/or stated on the certificate provided. There is a requirement for future preparations of microbial reference standards to be set relative to the previous batch of the microbial reference standard.

NOTE 2 The total proteolytic (milk-clotting) activity of the microbial coagulant reference standard powder is checked every second year by an alternative method, e.g. on a synthetic hexapeptide substrate of NIZO[3)].

The international microbial coagulant reference standard powder is commercially available from DSM Food Specialties[4)].

4 Apparatus

Usual laboratory equipment and, in particular, the following.

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

4.2 One-mark pipettes, for delivering appropriate volumes, ISO 648[1], class A.

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.

2) Cecalait, Poligny, France, is an example of a suitable supplier. This information is given for the convenience of users of this document Standard and does not constitute an endorsement by ISO or IDF of this supplier.

3) NIZO Food Research BV, Ede, Netherlands is an example of a suitable supplier. This information is given for the convenience of the user of this document and does not constitute an endorsement by ISO or IDF of this supplier.

4) DSM Food Specialities, Dairy Ingredients Group, Delft, Netherlands is an example of a suitable supplier. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of this supplier.

4.3 **One-mark volumetric flasks**, of required capacities, ISO 1042[3], class A.

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

4.5 **pH meter**, capable of being read to the nearest 0,01 pH unit.

4.6 **Analytical balance**, capable of being read to the nearest 1 mg.

4.7 **Stopwatch**, capable of being read to the nearest second.

4.8 **Flasks** or **test-tubes**, for milk-clotting testing, with suitable capacity (see 7.4).

4.9 **Water bath**, capable of maintaining a temperature of 32 °C ± 1 °C, but also capable of maintaining the temperature constant to within ±0,2 °C throughout the bath. The water bath should be equipped with the following attachments.

4.9.1 **Electric motor**, provided with a rotating spindle to which the flask or test tubes (4.8) can be attached, capable of rotating at a suitable angle of about 30° with the water surface of the water bath.

NOTE The rotational frequency is not important for this International Standard. A rotational frequency of 2 r/min to 4 r/min is suitable.

4.9.2 **Electric lamp**, placed so as to illuminate the flask or test-tube (4.8) effectively.

NOTE A screen with a dark background, placed in the water bath, can be used to improve observation of the milk-clotting in the flask or test-tube.

5 Sampling

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given for liquid microbial coagulant (6.1) in ISO 707 | IDF 50:2008[2], Clause 9 and for powdered microbial coagulant (6.2) in ISO 707 | IDF 50:2008[2], Clause 13.

It is important the laboratory receive a truly representative sample whichhas not been damaged or changed during transport or storage.

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

6 Preparation of test sample

6.1 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 (7.3).

Liquid coagulant is rather viscous. When pipetting the sample, use the correct technique. Alternatively, accurate and precise dilutions, especially for high strength coagulants, can be made. This is done by weighing the liquid samples on an analytical balance and by calculating its volume, in millilitres, by dividing its mass by the density of the coagulant used.

6.2 Powdered microbial coagulant

Mix the test sample thoroughly to obtain a homogeneous powder. Bring the sample to room temperature (18 °C to 22 °C) before preparing the coagulant test solution (7.3).

NOTE Powdered products can rapidly separate.

Consider the mass of the test portion(s) to be taken from the test sample. Often test portions of 3 g to 5 g are sufficient. However, when testing inhomogeneous test samples and accurate test results are desired, larger test portions are necessary.

7 Procedure

7.1 Preparation of substrate

Fill a 1 000 ml one-mark volumetric flask (4.3) up to the mark with calcium chloride working solution (3.3).

Weigh, to the nearest 0,1 g, 110 g of the low-heat, low-fat, spray-dried milk powder (3.4) 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 content of the beaker allowing the 1 000 ml one-mark-volumetric flask to drain. Stir the substrate thus obtained with a magnetic stirrer for 30 min while taking care to avoid the formation of foam.

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

NOTE The pH of the prepared substrate is approximately 6,50. The pH is not critical and does not require adjustment.

7.2 Preparation of the microbial coagulant reference solution

7.2.1 Microbial coagulant reference standard solution

The microbial coagulant reference standard powder (3.5) is dissolved according to the following procedure.

Ensure that the glass ampoule with the microbial coagulant reference standard powder is at room temperature (18 °C to 22 °C) before opening it, to avoid moisture getting into the powder.

Open the ampoule and weigh, to the nearest milligram, the amount of microbial coagulant reference standard powder, which contains a total of 2 500 IMCU into a 50 ml one-mark volumetric flask (4.3). Add 15 ml to 20 ml of the buffer solution (3.1) and mix by swirling, while avoiding foam formation, to dissolve the powder. Dilute to the 50 ml mark with the buffer solution (3.1) and mix well again.

NOTE ISO 15174 | IDF 176:2001 and ISO 11815 | IDF 157[6] use reference standard powder (3.5) of approximately 1 000 IMCU/g and specify the weighing out of 2,500 g powder, which contains a total of approximately 2 500 IMCU. On producing the second batch, it was not possible to reach the stipulated 1 000 IMCU/g. Thus, it has been decided to maintain the figure of 2 500 IMCU and to adjust the mass to be weighed out depending on the total activity of the reference standard powder. For example, if a reference standard has a total activity of 2 200 IMCU/g, then weigh out 1,136 g (2500/2200) of the powder.

7.2.2 Microbial coagulant reference working solution

In order to obtain a proper clotting time, pipette 3 ml of the microbial coagulant reference standard solution (7.2.1) into another 50 ml one-mark volumetric flask. Dilute to the 50 ml mark with the buffer solution (3.1) and mix well.

NOTE The expected clotting time for the reference working solution is in the range 350 to 550 s.

Keep the microbial coagulant reference working solution between 0 °C and 5 °C (on ice) and use on the day of its preparation.

7.3 Preparation of microbial coagulant test solution

Take an appropriate test portion (for powder between 3 g and 5 g) from the prepared test sample (6.1 or 6.2). Dilute the test portion with the buffer (3.1) until a microbial coagulant test solution is obtained with a clotting time that is similar to the microbial coagulant reference working solution (7.2.2) with a tolerance of ±40 s. Note the final dilution factor of the test solution to be used in the calculation (8.1).

7.4 Clotting

7.4.1 Add, using a pipette (4.2), 25 ml ± 0,1 ml of the substrate (7.1) to a dry flask or test tube (4.8). Pre-heat the substrate, while rotating the flask or test-tube in the water bath (4.9) for at least 12 min, but no longer than 20 min. Then quickly add, using the micropipette (4.1), 0,5 ml of the microbial coagulant reference working solution (7.2.2) to the substrate. Activate the stopwatch (4.7) 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.

Always place test portions as close as possible to the reference samples in the bath, in order to obtain, as far as possible, identical conditions. The method being a relative analysis, maintaining the same (clotting) temperature for the test portions and the reference samples is of vital importance. To fulfil the aforementioned requirement, check the temperature of the water bath by measuring the temperature of milk samples at different positions in the bath. If the maximum allowed variation of ±0,2 °C (see 4.9) cannot be reached, then the design of the water bath or its water-circulating system requires improvement.

7.4.2 Repeat the procedure in 7.4.1 without delay by replacing the microbial coagulant reference working solution (7.2.2) by the microbial coagulant test solution (7.3).

7.4.3 Repeat operations 7.4.1 and 7.4.2 to obtain two values. Calculate the mean of the clotting times for the microbial coagulant reference working solution and the microbial coagulant test solution, respectively.

NOTE Instead of 25 ml of the substrate and 0,5 ml of the microbial coagulant reference working solution in 7.4.1, 10 ml substrate and 0,2 ml working solution or 50 ml substrate and 1,0 ml working solution can be used. In either case, the ratio between the substrate and the working solution should be 50 to 1.

8 Calculation and expression of results

8.1 Calculation

Calculate the total milk-clotting activity of the test sample compared to the microbial coagulant reference standard powder, a_t, expressed in international milk-clotting units (IMCU) per gram or IMCU per millilitre, by using Equation (1):

$$a_t = \frac{t_r m_r V_1 d a_r}{t_t V_2 V_3} \quad (1)$$

where

t_r is the numerical value of the mean clotting time, in seconds, obtained with the microbial coagulant reference working solution (7.4.1 and 7.4.3);

m_r is the mass, in grams, of the microbial coagulant reference standard weighed in 7.2;

V_1 is the volume, in millilitres, taken in 7.2 from the microbial coagulant reference standard solution (V_1 = 3 ml);

d is the recorded final value of the dilution factor obtained with the test solution (7.3);

a_r is the numerical value of the milk-clotting activity (strength), in IMCU per gram, of the microbial coagulant reference standard powder (3.5); this value appears on the label of the glass ampoule of the reference powder;

t_t is the numerical value of the mean clotting time, in seconds, obtained with the microbial coagulant test solution (7.4.2 and 7.4.3);

V_2 is the final volume, in millilitres, in 7.2.1 of the microbial coagulant reference standard solution ($V_2 = 50$ ml);

V_3 is the final volume, in millilitres, in 7.2.2 of the microbial coagulant reference working solution ($V_3 = 50$ ml);

Equation (1) can be simplified to Equation (2) by introducing the following values: $V_1 = 3$ ml; $V_2 = 50$ ml; $V_3 = 50$ ml.

$$a_t = \frac{t_r m_r \times 0{,}001\ 2 \times d a_r}{t_t} \quad (2)$$

8.2 Expression of results

Express the results in international milk-clotting units (IMCU) per gram or IMCU per millilitre to the nearest whole number.

9 Precision

9.1 Interlaboratory test

The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given.

The values for repeatability and reproducibility are derived from the standard deviations, s_d, which are estimates of the true standard deviation of the method. If, in the long run, significantly less than 95 % of the cases are within the values given in 9.2 and 9.3, work on improving the execution of the analysis is recommended.

Due to some differences in solubility and a certain degree of inhomogenity of powders, the percentage value for the precision parameters, repeatability and reproducibility, mentioned below, can be somewhat higher when analysing coagulant powders.

An interlaboratory test on the precision of the method has been performed using specific microbial coagulant reference standards for each type of microbial coagulant (*R. miehei, R. pusillus* and *Cryphonectria parasitica*, respectively).

For practical reasons, it was later decided 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.

9.2 Repeatability

The coefficient of variation of repeatability, $C_{V,r}$, as a percentage, which expresses the variability of independent analytical results obtained by the same operator, using the same apparatus under the same conditions on the same test sample and in a short interval of time will in not more than 5 % of cases be greater than 2,0 % relative to the arithmetic mean of the test results.

If two determinations are obtained under these conditions, the absolute difference, $r_{(rel)}$, as a percentage, between the two results , should not exceed 5,5 % relative to the arithmetic mean of the test results.

9.3 Reproducibility

The coefficient of variation of reproducibility, $C_{V,R}$, as a percentage, which expresses the variability of independent analytical results by operators in different laboratories, using different apparatus under different conditions for the analysis on the same test sample will in not more than 5% of cases be greater than 5,6 % relative to the arithmetic mean of the test results.

If two determinations are obtained under these conditions, the absolute difference, $R_{(rel)}$, as a percentage, between the two results should not exceed 15,7 % relative to the arithmetic mean of the test results.

NOTE The values for precision parameters are valid when considering a broad range of laboratories. Experience has shown that highly trained laboratories are able to perform the analysis with reproducibility between laboratories of 2 %.

10 Test report

The test report shall contain at least the following information:

a) all information necessary for the complete identification of the sample;

b) the sampling method used, if known;

c) the method used, with reference to this International Standard (ISO 15174 | IDF 176:2012);

d) all operating 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;

f) if the repeatability has been checked, the final quoted result obtained.

Annex A
(informative)

Interlaboratory trial

A.1 General

An international collaborative test involving 15 laboratories from nine countries was carried out on microbial coagulants. The test was organized by A. Andrén, Sweden. The test results were subjected to statistical analysis [5)] by Professor Seppo Niemelä, Finland, and re-analysed and updated in 2010 according to ISO 5725-1:1994[4] and ISO 5725-2:1994[5].

A.2 Samples and results

The interlaboratory study was carried out on three different liquid coagulant preparations of *R. miehei* (Rm, thermolabile), *R. pusillus* (Rp) or *C. parasitica* (Cp), respectively. Each preparation was divided into two parts and one of them were diluted to 85 % (Rm), 75 % (Rp) or 65 % (Cp) of the original strength. The six samples thus obtained were divided into two, resulting in 12 blind duplicated samples.

NOTE 1 *R. miehei* was at the time of the interlaboratory study named *Mucor miehei* and abbreviated Mm.

NOTE 2 *C. parasitica* was at the time of the interlaboratory study named *Endothia parasitica* and abbreviated Ep.

The interlaboratory study was performed using a reference standard for each of the three types of microbial coagulants, whereas this edition of this International Standard uses only one reference standard. This gives only an insignificant difference in result and, most importantly, all laboratories used the same method and reference standard.

The results shown in Table A.1 are from the interlaboratory study carried out in 1993 [6)]. The results in Table A.1 exclude those of laboratory No. 10 for samples 7/11 (Cochran) and 9/12 (Cochran) and laboratory No. 14 for samples 1/5 (Cochran and Grubbs) and 3/6 (Grubbs).

Table A.1 — Results of interlaboratory trial

Sample No.	Coagulant	Mean IMCU/ml	$C_{V,r}$ %	r	$r_{(rel)}$ %	$C_{V,R}$ %	R	$R_{(rel)}$ %	Outliers
8/10	Rm 100 %	208,6	3,1	17,9	8,6	3,6	20,9	10,0	
1/5	Rm 85 %	178,7	1,4	7,2	4,0	4,0	20,1	11,3	1 (Cochran, Grubbs)
2/4	Rp 100 %	422,3	1,9	21,9	5,2	8,2	97,3	23,0	
9/12	Rp 75 %	312,8	2,3	20,0	6,4	9,2	80,8	25,8	1 Cochran
7/11	Cp 100 %	232,1	1,5	9,8	4,2	5,3	34,6	14,9	1 Cochran
3/6	Cp 65 %	154,9	1,7	7,4	4,8	3,2	13,9	9,0	1 Grubbs
Mean			2,0		5,5	5,6		15,7	

5) Results obtained originaly using ISO 5725:1986 (superseded).

6) Results obtained from an interlaboratory study according to ISO 5725:1986 (superseded), but statistically analysed according to ISO 5725-1:1994 and ISO 5725-2:1994.

Bibliography

[1] ISO 648, *Laboratory glassware — Single-volume pipettes*

[2] ISO 707 | IDF 50:2008, *Milk and milk products — Guidance on sampling*

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

[4] ISO 5725-1:1994, *Accuracy (trueness and precision) of measurement methods and results — Part 1: General principles and definitions*

[5] ISO 5725-2:1994, *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*

[6] ISO 11815 | IDF 157, *Milk — Determination of total milk-clotting activity of bovine rennets*

[7] ISO 15163 | IDF 110, *Milk and milk products — Calf rennet and adult bovine rennet — Determination by chromatography of chymosin and bovine pepsin contents*

[8] International Collaborative Study on Microbial Coagulants — Determination of total milk-clotting activity. *Bull. IDF* (in press)

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