INTERNATIONAL STANDARD

ISO 15163

IDF 110

First edition
2012-05-15

Milk and milk products — Calf rennet and adult bovine rennet — Determination by chromatography of chymosin and bovine pepsin contents

Lait et produits laitiers — Présure de veau et coagulant issu de bovin adulte — Détermination des teneurs en chymosine et en pepsine bovine par chromatographie

Reference numbers
ISO 15163:2012(E)
IDF 110:2012(E)

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
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

Published in Switzerland

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.

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 15163 | IDF 110 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 IDF and ISO.

This first edition of ISO 15163 | IDF 110 cancels and replaces IDF 110B:1997, 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 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.

The main task of Standing Committees is to prepare International Standards. Draft International Standards adopted by the Standing Committees are circulated to the National Committees for endorsement prior to publication as an International Standard. Publication as an International Standard requires approval by at least 50 % of 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 15163 | IDF 110 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 an ISO-IDF Project Group on *Chymosin and bovine pepsin determination,* of the Standing Committee on *Analytical methods for processing aids and indicators,* under the aegis of its project leader, Prof. A. Andrén (SE).

This first edition of ISO 15163 | IDF 110 cancels and replaces IDF 110B:1997, which has been technically revised.

Introduction

Calf rennet and adult bovine rennet preparations contain both chymosin and bovine pepsin in various amounts as main clotting enzymes. The proportion of chymosin decreases relative to pepsin in the abomasum (the fourth “true” stomach) with age and at weaning of the calf.

The ratio of abomasa from young cattle to that of old cattle in the raw material for rennet production thus highly influences the composition of chymosin and pepsin in the final rennet. The higher the abomasa from young milk-fed calves, the higher the proportion of chymosin and vice versa[5][6].

Both chymosin and pepsin have special characteristics relevant to milk-clotting activity and suitability for cheese making. The milk-clotting activity of pepsin is, for example, much more pH-dependent than chymosin and pepsin also has a more general proteolytic activity than chymosin.

Therefore, it is very important to analyse the content of chymosin and pepsin in addition to the strength (total milk-clotting activity) of the rennet[6][7].

Milk and milk products — Calf rennet and adult bovine rennet — Determination by chromatography of chymosin and bovine pepsin contents

1 Scope

This International Standard specifies a reference method for the determination of the amounts of chymosin and bovine pepsin present in a test sample of calf rennet and adult bovine rennet. In addition, it can be used for mixtures of calf/bovine rennet with fermentation-produced bovine chymosin (FPC).

2 Normative references

The following referenced document is 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 11815 | IDF 157:2007, *Milk — Determination of total milk-clotting activity of bovine rennets*

3 Principle

As a first step, the rennet sample is desalted and the enzymes chymosin and bovine pepsin separated on an anion exchange column[8][9]. In a second step, the milk-clotting activity of each of the separated two enzymes is determined by ISO 11815 | IDF 157 (reconstituted milk with pH 6,5). The enzymatic composition of the rennet sample is expressed in percentage chymosin activity and percentage pepsin activity of the sum of the activities in International Milk-Clotting Units (IMCU) of both components, or the results are expressed in milligrams per litre of active chymosin and milligrams per litre of active pepsin.

The total milk-clotting activity of the first batch of calf rennet reference standard powder and the first batch of adult bovine rennet reference standard powder has once and for all been set at 1 000 IMCU/g. Future preparations of reference standards shall be set relative to the previous reference standards (see ISO 11815 | IDF 157).

This International Standard specifies both a manual set-up of the anion exchange chromatography and an alternative automated set-up.

This is a reference method and changes may therefore only be made if confirmed to give the same result, and repeatability and reproducibility at least as high as the original standard method. Any change to what is stated in this International Standard method shall also be mentioned in the test report (see Clause 10).

4 Reagents

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

4.1 Resin, Fractogel® EMD DEAE (M) (Merck cat. no. 1.16883)[1] or Mono Q® 1 ml prepacked column (HR 5/5 or 5/50 GL from GE Healthcare)[2] or equivalent resin.

NOTE 1 Fractogel® EMD DEAE (M) is a suitable resin for manual chromatography and Mono Q® is suitable for the automated chromatography.

NOTE 2 If the Fractogel® or Mono Q resins are substituted by another resin, it will most likely be necessary to change the buffers in 4.12, resulting in a need to re-evaluate the method.

4.2 Piperazine hexahydrate ($C_4H_{10}N_2 \cdot 6H_2O$).

4.3 Sodium chloride (NaCl).

4.4 Thymol, optional preservative.

4.5 Sodium hydroxide (NaOH).

4.6 Hydrochloric acid solution, c(HCl) = 1 mol/l.

4.7 Ethanol (C_2H_5OH), with a volume fraction of at least 96 %.

4.8 Ethanol (C_2H_5OH), with an approximate volume fraction of at least 20 %.

Add 105 ml ethanol 96 % (4.7) to 400 ml water and mix. If a sterile filtration is desired, filter the water before mixing it with the ethanol.

4.9 Urea, $c(N_2H_4CO)$ = 8 mol/l.

Dissolve 48 g urea in water and fill to a total volume of 100 ml.

4.10 Dialysis tubing, of diameter approximately 1 cm (Union Carbide)[3] or equivalent (optional).

NOTE The quality of the dialysis tubing is not critical.

4.11 Desalting columns, Bio-Rad – Econopac 10DG (cat. no. 732-2010)[4] or equivalent (optional).

Use either the dialysis tubing (4.10) or the desalting columns for desalting the rennet.

4.12 Buffer solutions

4.12.1 Buffer solution I, piperazine [$(CH_2)_4$ $(NH)_2$], c[$(CH_2)_4$ $(NH)_2$] = 0,025 mol/l.

1) Fractogel® EMD DEAE (M) is an example of a suitable product available commercially. This information is given for the convenience of the users of this document and does not constitute an endorsement of the product by ISO or IDF.

2) Mono Q® 1 ml prepacked column is an example of a suitable product available commercially. This information is given for the convenience of the users of this document and does not constitute an endorsement of the product by ISO or IDF.

3) Union Carbide is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement of the product by ISO and IDF.

4) Bio–RAD - Econopac 10DG is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement of the product by ISO or IDF.

Weigh 4,85 g of piperazine (4.2) and 42,8 g of hydrochloric acid solution (4.6) in a beaker and mix. Transfer quantitatively the contents of beaker to a 1 000 ml one-mark volumetric flask (5.5), dilute with water to the 1 000 ml mark, and mix. The pH shall be 5,30 ± 0,05. If not, adjust with piperazine or hydrochloric acid. Before use, degas and preserve the buffer solution as described in 4.12.5.

4.12.2 Buffer solution II, c(NaCl) = 0,25 mol/l.

Weigh 14,6 g of NaCl into a 1 000 ml one-mark volumetric flask (5.5). Add the buffer solution I (4.12.1) to the 1 000 ml mark and mix. Do not adjust the pH. Buffer solution II is used only for the manual method. Before use, degas and preserve the buffer solution as described in 4.12.5.

4.12.3 Buffer solution III, c(NaCl) = 0,50 mol/l.

Weigh 29,2 g of NaCl into a 1 000 ml one-mark volumetric flask (5.5). Add buffer solution I (4.12.1) to the 1 000 ml mark and mix. Do not adjust the pH. Buffer solution III is used only for the manual version. Before use, degas and preserve the buffer solution as described in 4.12.5.

4.12.4 Buffer solution IV, c(NaCl) = 1,0 mol/l.

Weigh 58,4 g of NaCl into a 1 000 ml one-mark volumetric flask (5.5). Add buffer solution I (4.12.1) to the 1 000 ml mark and mix. Do not adjust the pH. Before use, degas and preserve the buffer solution as described in 4.12.5.

4.12.5 Degassing and preservation

Before use, degas the buffer solutions I to IV (4.12.1 to 4.12.4) under vacuum or by use of an ultrasound water bath. Preserve buffer solutions I to IV for use in the manual method by adding a few thymol crystals and in the automated method by sterile filtering using a filter of 0,2 µm.

Buffer solutions I to IV can be kept for at least 5 days at room temperature or for 2 months in a refrigerator.

5 Apparatus

Usual laboratory apparatus and, in particular, the following.

5.1 Multiway peristaltic pump or **other suitable pump** (for manual set-up only).

5.2 pH-meter, of sensitivity ±0,01 pH unit.

5.3 Chromatographic column, of diameter ~1,0 cm and length 10 cm, with one flow adaptor or equivalent column suitable for a gel bed height of ~5 cm (for manual set-up only).

5.4 Magnetic stirrer.

5.5 One-mark volumetric flasks, of the capacities required, ISO 1042[2].

5.6 FPLC®[5], **ÄKTA®**[6] or **HPLC equipment**, suitable for the purpose, used for the automatic set-up only.

5.7 Laboratory equipment, for the determination of the clotting time (see ISO 11815 | IDF 157).

5) FPLC® is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement of the product by ISO or IDF.

6) ÄKTA® is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement of the product by ISO or IDF.

6 Sampling

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 707 | IDF 50[1].

NOTE 1 Sampling of liquid rennet is given in ISO 707 | IDF 50:2008, Clause 9, and of powdered rennet in ISO 707 | IDF 50:2008, Clause 13.

A representative sample should be sent to the laboratory. It should not be damaged or changed during transport or storage.

NOTE 2 Powdered products can separate rapidly.

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

7 Procedure

7.1 Check

Prior to determination, check the rennet for absence of main milk-clotting enzymes of non-bovine origin by using a suitable method (see Annex A). However, checking can be skipped if the rennet is known to contain chymosin and pepsin of bovine origin only.

7.2 Preparation of a fresh column with Fractogel

After degassing under vacuum, pour a suspension of the ready-to-use Fractogel resin (4.1) directly from the supplier's bottle, or by use of an ultrasound water bath, into the column (5.3), fixed in a vertical position, with the outlet open, until the layer of settled Fractogel resin is 4,5 cm to 5,5 cm high. The gel bed shall not run dry during the whole operation.

Close the outlet tube. Immerse the end of the intake tube of the peristaltic pump into a beaker containing buffer solution I (4.12.1). Connect the tube of the adaptor to the exit tube of the pump. Adjust the flow rate to (1,3 ± 0,1) ml/min. Fill the tube of the adaptor with buffer solution I (4.12.1), not exceeding a total internal tubing volume of 1,5 ml.

Close the column with the adaptor, as described by the supplier of the column. Compress the gel bed a few millimetres with the adaptor in order to avoid free space above the gel bed. Avoid any air bubble entering the column. Rinse the column with buffer solution I (4.12.1) for 5 min at a flow rate of 1,3 ml/min.

7.3 Regeneration and equilibration of the Fractogel resin in the column

After the preparation of a new column and after each run, regenerate and equilibrate the Fractogel resin (4.1) in the column by the following procedure.

Regenerate the Fractogel resin in the column at a flow rate of 1,3 ml/min using at least 15 ml of buffer solution IV (4.12.4) (~11,5 min). Equilibrate the column with at least 40 ml of buffer solution I (4.12.1) (~30 min). The column is now ready for loading the test sample.

The same column can be used over 20 times. Regenerate the column more extensively after frequent use by rinsing with 0,1 mol/l NaOH for 10 min followed by water for 10 min. Then follow the normal regeneration and equilibration procedure as described in the preceding.

7.4 Storage of the column with Fractogel

If the column has to be stored for more than 1 week, rinse it with at least 15 ml of 20 % ethanol (4.8) with the help of the peristaltic pump (5.1). Store the column with the inlet and the outlet tubes tightly closed.

The column can be stored for several months at room temperature avoiding direct sunlight.

7.5 Preparation of test sample

7.5.1 General

Liquid samples may be used as they are; just proceed to 7.5.2.

A powder rennet sample is prepared as follows. Dissolve a powder rennet sample so as to give, for example, 200 IMCU/ml in a suitable amount of buffer solution I (4.12.1) or by using a buffer solution with pH 5,5 (see ISO 11815|IDF 157) before desalting.

Determine the clotting time of the dissolved rennet sample in accordance with ISO 11815|IDF 157 before desalting. Make a rough estimate of the strength of the sample, in IMCU/ml, by measuring it relative to the calf rennet reference standard in order to determine the amount of sample to apply on to the column (7.6.1 or 7.6.2).

If the results are expressed in milligrams per litre, determine the clotting time of the rennet sample at least twice in accordance with ISO 11815|IDF 157. In this case, measure the clotting times simultaneously or in rapid succession “before” and "after" desalting.

Before applying the test sample on to the column, desalt it by dialysis (7.5.2) or by gel filtration (7.5.3).

7.5.2 Desalting by dialysis

Immerse the dialysis tubing (4.10) in boiling water for about 5 min and rinse the inside and the outside of the tubing with water.

Dialyse 5 ml of the prepared test sample (7.5) under examination (~900 IMCU) against 500 ml of buffer solution I (4.12.1) at 4 °C for at least 5 h but no longer than 20 h. Compress the dialysis tubing very firmly by hand on closing it in order to reduce the dilution, which occurs during dialysis. Stir the buffer with a magnetic stirrer (5.4) during the dialysis.

If the results are expressed in milligrams per litre, determine the clotting time of the dialysed rennet sample at least twice in accordance with ISO 11815|IDF 157.

7.5.3 Desalting by gel filtration

Follow the instructions of the supplier.

Equilibrate the desalting column (4.11) with buffer solution I (4.12.1). Apply 3,3 ml of the prepared test sample (7.5) on to the column. Elute the sample with 4,0 ml of buffer solution I (4.12.1).

For test samples with a low amount of either chymosin or pepsin, it is sometimes necessary to run the aforementioned procedure twice in order to have enough activity to be able to determine the low component adequately.

If the results are expressed in milligrams per litre, determine the clotting time of the gel-filtered rennet sample at least twice in accordance with ISO 11815|IDF 157.