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Milk and milk products — Determination of alkaline phosphatase activity —

Part 2: Fluorometric method for cheese

Lait et produits laitiers — Détermination de l'activité de la phosphatase alcaline —

Partie 2: Méthode fluorimétrique pour le fromage

ICS: 67.100.30

ISO/CEN PARALLEL PROCESSING

This draft has been developed within the International Organization for Standardization (ISO), and processed under the **ISO lead** mode of collaboration as defined in the Vienna Agreement.

This draft is hereby submitted to the ISO member bodies and to the CEN member bodies for a parallel five month enquiry.

Should this draft be accepted, a final draft, established on the basis of comments received, will be submitted to a parallel two-month approval vote in ISO and formal vote in CEN.

To expedite distribution, this document is circulated as received from the committee secretariat. ISO Central Secretariat work of editing and text composition will be undertaken at publication stage.

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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 11816-2 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*.

This second/third/... edition cancels and replaces the first/second/... edition (ISO 11816-2 | IDF 155-2 :2003), [clause(s) / subclause(s) / table(s) / figure(s) / annex(es)] of which [has / have] been technically revised.

ISO 11816 consists of the following parts, under the general title *Milk and milk products — Determination of alkaline phosphatase activity*:

— *Part 1: Fluorimetric method for milk and milk-based drinks*

— *Part 2: Fluorimetric method for cheese*

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 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 11816-2 | IDF 155-2 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 *Alkaline phosphatase activity in cheese (P06)*, of the Standing Committee on *Analytical Methods for Processing Aids and Indicators*, under the aegis of its project leader, Mrs. M. Nicolas (FR).

This edition of ISO 11816-2 | IDF 155-2 cancels and replaces its first edition (ISO 11816-2 | IDF 155-2:2003), which has been technically revised.

ISO 11816 | IDF 155 consists of the following parts, under the general title *Milk and milk products – Determination of alkaline phosphatase activity:*

— *Part 1: Fluorimetric method for milk and milk-based drinks*

— *Part 2: Fluorimetric method for cheese*

Milk and milk products — Determination of alkaline phosphatase activity — Part 2: Fluorimetric method for cheese

1 Scope

This part of ISO 11816 | IDF 155 specifies a fluorimetric method for the determination of alkaline phosphatase (ALP, EC 3.1.3.1) activity in cheese.

This method is applicable to soft cheeses, semi-hard and hard cheeses provided that the mould is only on the surface of the cheese and not also in the inner part (e.g. blue veined cheeses).

The instrument can read activities in the supernatant as high as 7 000 milliunits per litre (mU/l)

NOTE In large hard cheeses where the whey curd mixture is scalded at temperatures above 50 °C, high temperatures remain for a relatively long time, especially in the centre of those cheeses, promoting phosphatase inactivation. To determine the alkaline phosphatase activity in this kind of cheese a specific cheese sampling shall be applied (see 6).

2 Terms and definitions

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

2.1
alkaline phosphatase activity
ALP activity
activity of the alkaline phosphatase present in the product, determined by the procedure specified in this part of ISO 11816| IDF 155

NOTE 1 to entry: The alkaline phosphatase activity is expressed as milliunits of enzyme activity per gram of sample (mU/g).

2.2
unit of alkaline phosphatase activity
amount of alkaline phosphatase enzyme that catalyses the transformation of 1 µmol of substrate per minute.

3 Principle

The alkaline phosphatase activity of the sample is measured by a continuous fluorimetric direct kinetic assay. A non-fluorescent aromatic monophosphoric ester substrate, 2'-[2-benzothiazolyl]-6'-hydroxybenzothiazole phosphate, in the presence of any alkaline phosphatase derived from the sample, undergoes hydrolysis of its phosphate radical, producing a highly fluorescent product. Fluorimetric measurement of alkaline phosphatase (ALP) activity is measured at 38 °C over a 3-min period when using the Fluorophos®. This includes pre-incubation of substrate and sample, followed by multiple kinetic readings of the reaction rate.

NOTE Although this is a 3 min test, the first minute is an equilibration period to ensure that the sample is at 38 °C. Measurements of activity are actually made from the beginning of the second minute to the end of the third minute (i.e. over a 2 min period).

4 Reagents

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

4.1 Fluorophos® substrate[1)] in bottles, each containing 144 mg of Fluorophos® substrate powder.

This is a non-fluorescent aromatic monophosphoric ester substrate, 2'-[2-benzothiazolyl]-6'-hydroxybenzothiazole phosphate (Fluorophos®).

The Fluorophos® substrate remains stable for 2 years from manufacturing date when stored in unopened bottles at between 2 °C and 8 °C.

4.2 Substrate buffer solution, diethanolamine (DEA) buffer solution, *c*(DEA) = 2,4 mol/l, with pH 10,0.

The substrate buffer solution remains stable for 2 years from manufacturing date when stored in unopened bottles at between 2 °C and 8 °C.

4.3 Working substrate

Allow one bottle of Fluorophos® substrate (4.1) and one bottle of substrate buffer solution (4.2) to come to room temperature. Add the content of one bottle substrate buffer solution (240 ml) (4.2) to that of one bottle Fluorophos® substrate (144 mg) (4.1) and mix well by gentle inversion for 3 min.

Allow the obtained solution to stand at room temperature for at least 30 min prior to use.

Use the Analog-to-Digital (A/D) test given in 8.2.1 to test the suitability of the ready to use working substrate. Do not use the working substrate if a reading above 1 200 FLU is obtained.

The working substrate remains stable for 60 days when protected from light and stored at between 2 °C and 8 °C, or for 6 h at 38 °C.

4.4 Working calibrator solutions, Fluoroyellow® (FY) [2'-(2-benzothiazolyl)-6'-hydroxybenzothiazole] in substrate buffer solution (4.2).

The working calibrator solutions remain stable for 18 months from manufacturing date when stored in unopened bottles at between 2 °C and 8 °C.
Mix gently prior to use to assure optimal results.

4.4.1 Calibrator solution A, containing 0 µmol/l of Fluoroyellow®.

4.4.2 Calibrator solution B, containing 17,24 x 10^{-3} µmol/l of Fluoroyellow®.

4.4.3 Calibrator solution C, containing 34,48 x 10^{-3} µmol/l of Fluoroyellow®.

4.5 Daily instrument control solution, containing 34,48 x 10^{-3} µmol/l of Fluoroyellow®.

The D.I control solution remains stable for 18 months from manufacturing date when stored in unopened bottles at between 2 °C and 8 °C.

Mix gently prior to use to assure optimal results.

1) The reagents specified in 4.1 to 4.6 and the apparatus specified in 5.1 to 5.4 (except 5.3.1) are available from Advanced Instruments, Inc., Two Technology Way, Norwood, Massachussetts 02062, USA. The manufacturer may change packaging configurations supplied with the Fluorophos Test System. The user should refer to the manufacturer's instructions for preparing reagents if different from those specified herein. Fluorophos and Fluoroyellow are registered trademarks of Advanced Instruments Inc. This information is given for the convenience of users of this document and does not constitute an endorsement by either ISO or IDF of these products.

4.6 Fluorophos® Cheese extraction buffer, diethanolamine (DEA) buffer, pH 8,0 with magnesium and Triton X-100.

The cheese extraction buffer remains stable for 3 years from manufacturing date when stored in unopened bottles at between 2 °C and 8 °C.

5 Apparatus

Usual laboratory equipment and, in particular, the following.

5.1 Filter fluorimeter, with thermostatically controlled cuvette holder, capable of operating at 38 °C ± 1 °C and right-angle optics, allowing excitation at a wavelength of 440 nm and emission between 520 nm and 560 nm [e.g. Fluorophos® instrument [1)]].

5.2 Cuvettes, disposable, non-fluorescent glass, of diameter 12 mm and of length 75 mm.

5.3 Pipettes.

5.3.1 Pipette, of capacity 2,0 ml and 3,0 ml.

5.3.2 Positive-displacement or **air-displacement pipette**, of capacity 0,075 ml.

5.4 Heating block, capable of maintaining a temperature of 38 °C ± 1 °C, suitable for holding cuvettes.

5.5 Parafilm®[2)] or other suitable laboratory-grade film.

5.6 Vortex mixer.

5.7 Grinding device.

5.8 Glass Beaker, of capacity 5 ml (of approximately diameter 20 mm and length 30 mm), and 10 ml (of approximately diameter 25 mm and length 30 mm).

5.9 Ultra turrax®[2)] provided with the stem of diameter of approximately 6 mm to 8 mm.

5.10 One-mark volumetric flasks, of capacity 25 ml.

5.11 Centrifuge, capable of centrifuging at 1 000 *g* at 4 °C

5.12 Glass test tube, of approximately diameter 12 mm and length 10 cm.

5.13 Glass Pasteur pipette; an air-displacement pipette can also be used.

5.14 Water bath, capable of maintaining a temperature of 63 °C ± 1 °C.

6 Sampling

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

Sampling is not part of the method specified in this part of ISO 11816 | IDF 155. A recommended sampling method is given in ISO 707 | IDF 50, see [1].

2) Parafilm® and Ultra turrax® are examples of a suitable product available commercially. This information is given for the convenience of users of ISO 11816-2 | IDF 155-2 and does not constitute an endorsement by either ISO or IDF of these products.

However, ISO 707 | IDF 50 is not suitable for large hard cheeses where the whey curd mixture has been scalded at temperatures above 50 °C. If the cheese is made from raw milk, the ALP activity is not homogenously distributed within these cheeses. The activity is high in the outer layer of the cheese wheel, between 0 cm to 4 cm below the rind of the round side, but very low or even undetectable in the core.
Samples of large hard cheeses, therefore, shall be sampled by taking a portion of 1 cm, taken at 0,5 cm below the rind of the round side. (Annex B).

NOTE In case of doubt regarding the type of cheese, between a hard and a semi-hard cheese, proceed to the sampling as described for large hard cheeses.

7 Preparation of test sample

7.1 Preparation

Remove the rind or the surface from the test sample with a clean knife. Ensure that the test sample is not contaminated with surface microflora during its preparation. Especially for soft cheese with moulded surface, remove all the rind but in a layer as thin as possible, so as to avoid eliminating the fat layer under the mould surface (Annex B). For large hard cheeses proceed as described under 6. Grind the test sample by means of a grinding mill or other appropriate device (5.7) and mix thoroughly. Keep the prepared sample in an airtight container.

8 Procedure

8.1 Verification of instrument performance

8.1.1 General

It is important to check instrument performance for drift, stray light and stability prior to analysing test samples. Follow Good Laboratory Practice standards when operating the filter fluorimeter (5.1).

Quality control tests include:

a) The daily A/D (Analog-to-Digital) test, used to check the proper functioning of the equipment.

b) The daily instrument control test, using the daily instrument control solution (4.5) to monitor any electronic or optical drift in the fluorimeter.

c) The use of external positive, negative and normal controls, described in 8.1.2, is recommended for monitoring daily instrument precision parameters.

8.1.2 A/D Tests

When using the Fluorophos® instrument, perform the A/D test daily before testing commences.

8.1.2.1 Access the A/D test through the ‘SETUP menu. Press ‘SETUP’ key, then select menu item ‘A/D Test’ by pressing < or >. With nothing in the cuvette holder, press START. Allow the figures appearing on the display screen to stabilize. The display should read 302 ± 4. If the reading is outside that range, clean the excitation and emission filters and repeat the A/D test.

8.1.2.2 Dispense 2,0 ml of the daily instrument control solution (4.5) into a labelled cuvette, using the pipette (5.3.1). Place the cuvette in the heating block (5.4) set at 38 °C for 20 min. Insert the pre-warmed cuvette into the cuvette holder. Close the lid. When the display is stable, record the displayed value, which should be 602 ± 12. If outside that range, use the small screwdriver supplied to slowly turn the potentiometer screw on the left-hand side of the instrument clockwise or anticlockwise, as necessary, until the display reads 602. Allow the numbers to equilibrate for 15 min.

8.1.3 Controls