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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 fluorométrique pour le fromage

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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 part of ISO 11816|IDF 155 may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 11816-2 | IDF 155-2 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.

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

— *Part 1: Milk and milk-based drinks*

— *Part 2: Fluorometric method for cheese*

— *Part 3: Enzyme photo-activated system (EPAS) method for milk and milk-based products.*

The present title of part 1 of ISO 11816 (IDF 155) is *Milk and milk products — Determination of alkaline phosphatase activity using a fluorometric method — Part 1: Milk and milk-based drinks*. This title will be changed to the title listed above when this part is revised.

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 IDF National Committees casting a vote.

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All work was carried out by the Joint ISO/IDF/AOAC Action Team, *Characterization of milk and milk products according to heat treatment*, of the Standing Committee on *Minor components and characterization of physical properties*, under the aegis of its project leader, Mrs E. Lechner (DE).

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

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

— *Part 2: Fluorometric method for cheese*

— *Part 3: Enzyme photo-activated system (EPAS) method for milk and milk-based products*

The present title of part 1 of ISO 11816 (IDF 155) is *Milk and milk products — Determination of alkaline phosphatase activity using a fluorometric method — Part 1: Milk and milk-based drinks.* This title will be changed to the title listed above when this part is revised.

Milk and milk products — Determination of alkaline phosphatase activity —

Part 2:
Fluorometric method for cheese

1 Scope

This part of ISO 11816 | IDF 155 specifies a fluorometric method for the determination of alkaline phosphatase activity in cheese.

This method is also applicable to soft cheeses and semi-hard cheeses for distinguishing raw milk cheeses from cheese produced with pasteurized milk, provided that the mould is only on the surface and not also in the inner part (e.g. blue-veined cheeses). This method can also be used to check the proper pasteurization of cheese or its raw material.

In large hard cheeses where the whey curd mixture is scalded at temperatures above 50 °C, high temperatures remain for a relatively long time. This is especially the case in the centre of these cheeses, thus promoting phosphatase inactivation. To distinguish, therefore, between hard cheeses from raw milk and hard cheeses from pasteurized milk using this method requires a specific sampling technique for the cheese (see Clause 7).

2 Normative references

The following referenced documents are 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 11816-1, *Milk and milk products — Determination of alkaline phosphatase activity using a fluorometric method — Part 1: Milk and milk-based drinks*

3 Terms and definitions

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

3.1
alkaline phosphatase activity
ALP
activity of the alkaline phosphatase present in the product, determined according to the procedure described in this part of ISO 11816 | IDF 155

NOTE The alkaline phosphatase activity is expressed as milliunits of enzyme activity per gram.

3.2
unit of alkaline phosphatase activity
amount of alkaline phosphatase enzyme that catalyses the transformation of 1 µmol of substrate per minute per gram of sample

4 Principle

In the presence of alkaline phosphatase, a non-fluorescent monophosphoric ester substrate (Fluorophos®) is hydrolysed at 38 °C ± 2 °C for 3 min to form a highly fluorescent product (Fluoroyellow®). The amount of Fluoroyellow is measured with a calibrated fluorometer and the activity of the alkaline phosphatase is calculated.

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).

5 Reagents

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

5.1 Fluorophos substrate[1], crystallized, M = 580 g/mol.

NOTE Fluorophos substrate is a water-soluble, non-fluorescent aromatic monophosphoric ester.

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

5.3 Working substrate

Add a volume of the substrate buffer solution (5.2) to the Fluorophos substrate (5.1) to obtain a concentration of Fluorophos substrate of c = 1,044 mmol/l. Mix well by inversion.

Use amber glass to protect against light.

If using the Fluorophos test system, add the content of 1 bottle of the substrate buffer solution (5.2) to one bottle of the Fluorophos substrate (5.1) and mix well by inversion.

5.4 Working calibrators, Fluoroyellow in DEA buffer.

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

5.4.2 Calibrator solution B, containing 17,24 × 10^{-3} µmol/l of Fluoroyellow.

5.4.3 Calibrator solution C, containing 34,48 × 10^{-3} µmol/l of Fluoroyellow.

5.5 Milk, preheated to 95 °C for 1 min, then cooled to room temperature.

6 Apparatus

Usual laboratory equipment and, in particular, the following.

6.1 Filter fluorometer[1], with thermostatted cuvette holder, capable of operating at 38 °C ± 1 °C and of allowing excitation at a wavelength of 440 nm and emission at 560 nm.

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1) The reagents specified in 5.1 to 5.4 and the apparatus specified in 6.1 to 6.4 (except for 6.3.3) are available as Fluorophos Test System from Advanced Instruments, Two Technology Way, Norwood, Massachussetts 02062, USA. Fluorophos and Fluoroyellow are registered trademarks of Advanced Instruments Inc. and are examples of suitable products available commercially. This information is given for the convenience of users of this part of ISO 11816 | IDF 155 and does not constitute an endorsement by ISO or IDF of these products.

6.2 **Cuvettes**, of non-fluorescent glass, of diameter 12 mm and length 75 mm.

6.3 **Pipettes**

6.3.1 **Fixed-volume dispenser**, capable of dispensing 2,0 ml.

6.3.2 **Positive-displacement pipette**, of capacity 0,075 ml.

6.3.3 **Pipettes**, of capacities 1 ml and 2 ml (for calibrators).

6.4 **Incubator block**, capable of operating at 38 °C ± 1 °C, suitable for holding cuvettes.

6.5 **Parafilm**[2] or other suitable laboratory-grade film.

6.6 **One-mark volumetric flasks**, of capacities 10 ml and 25 ml.

6.7 **Water bath**, capable of operating at 63 °C ± 2 °C.

6.8 **Pottering tube** or **Ultra Turrax**®[3].

7 Sampling

It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed 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.

However, ISO 707 is not suitable for large hard cheeses produced with raw milk because the ALP activity is differently distributed within these cheeses. The activity may be high in the outer layers of the cheese wheel, which is between 0 cm to 4 cm below the rind of the round side, but very low to zero in the core. Samples of hard cheeses, therefore, shall be taken between 2 cm and 3 cm below the rind on the round side.

8 Preparation of test sample

8.1 General

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. Grind the test sample by means of a grinding mill or other appropriate device and mix thoroughly. Keep the thus-prepared sample in an airtight container. Test the sample on the same day or store the sample in a freezer.

8.2 Cheese from pasteurized milk

8.2.1 Weigh, to the nearest 1 mg, an amount of between 0,3 g and 0,5 g of the prepared test sample (8.1) into a 25 ml glass beaker. Add 1 ml to 2 ml of the prepared milk (5.5) and stir intensively with a glass pestle to form a paste. Hard cheeses shall be left to soak or pretreated using the alternative method given in 8.2.2.

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2) Parafilm is an example of a suitable product available commercially.

3) Ultra Turrax is an example of a suitable product available commercially.

This information is given for the convenience of users of this part of ISO 11816 | IDF 155 and does not constitute an endorsement by ISO or IDF of these products.

Add more milk (5.5) in portions of 1 ml and stir the mixture intensively after each addition. Transfer the milk/cheese mixture quantitatively, using the milk, into a 25 ml volumetric flask (6.6). Dilute to the mark with milk and shake thoroughly. Remove any separated fat with a spatula. Before any test portion is taken, mix the contents of the flask by inverting it several times.

8.2.2 Alternatively, especially for hard cheeses, it is recommended to use a pottering tube or an Ultra Turrax® (6.8) for the preparation of the milk/cheese mixture. If the pottering method is used, mix the former weighed amount (between 0,3 g and 0,5 g) of the prepared test sample intensively with 10 ml of the milk (5.5).

8.2.3 The measurements shall be carried out in the same way as for the determination of alkaline phosphatase activity in whole milk as described in ISO 11816-1 | IDF 155-1.

8.3 Cheese from raw milk

Prepare the test sample as specified in 8.1 or 8.2. Dilute the test sample a further 1:10 with the milk (5.5) when the activity of the milk/cheese mixture is more than 7 000 mU/l.

9 Procedure

9.1 Calibration

Calibration curves are usually stable. The instrument shall be recalibrated every two to three months. Check the instrument if there are changes of more than 10 % in the calibration ratio, using a new lot of calibrators. Preheat the calibrator solutions A, B and C prior to use. Using the pipette (6.3.3), transfer 2,0 ml of the preheated calibrator solution A, solution B and solution C (5.4.1, 5.4.2 and 5.4.3 respectively), each in duplicate, to the labelled cuvettes (6.2). Place the cuvettes in the incubator block (6.4) and preheat to 38 °C for 5 min. Add with the positive displacement pipette (6.3.2), 0,075 ml of milk (5.5) to all six cuvettes. Cover the cuvettes with parafilm (6.5).

Gently invert all cuvettes to mix the contents and replace the cuvettes in the incubator block (6.4).

Starting with calibrator solution A, perform the following calibration routine. Wipe the outside of each cuvette with soft tissue before placing the cuvette in the filter fluorometer (6.1). Use calibrator solution A (5.4.1) to set the fluorometer (6.1) to zero fluorescence. Then read and record the amount of fluorescence obtained with calibrator solution B (5.4.2) and solution C (5.4.3) against calibrator solution A (5.4.1).

Once calibration is completed, proceed to analyse the test samples.

9.2 Determination

Use the fixed-volume dispenser (6.3.1) to dispense 2,0 ml of working substrate (5.3) into a labelled cuvette. Place the cuvette in the incubator block (6.4) and preheat to 38 °C for 5 min to 10 min. Add 0,075 ml of the test portion (8.2 or 8.3) to the substrate. Cover the cuvette with parafilm (6.5) and immediately mix the contents of the cuvette by gentle inversion.

Wipe the outside of the cuvette with soft tissue and place the cuvette in the filter fluorometer (6.1). Allow the cuvette to stand for 1 min for temperature equilibration. Then record the fluorescence, at the beginning of the second minute and at the end of the third minute. Divide by two the difference between the two fluorescence readings to obtain the average amount of fluorescence produced per minute.

Record the average increase in fluorescence per minute for each milk/cheese mixture and use that value to calculate the alkaline phosphatase activity in milliunits per litre of the test portion. Use the alkaline phosphatase activity of the test portion to calculate the activity of the sample.

Results may be calculated automatically by using the programmable calculator which forms an integral part of the fluorometer, or they may be calculated manually according to Clause 10.

9.3 Control tests

9.3.1 Negative control test

Include a negative control test with each batch of samples by using the prepared milk (5.5). The instrument reading shall be less than 10 mU/l to indicate no fluorescence activity detected.

9.3.2 Positive control test

Include a positive control test with a phosphatase activity level of or close to the decision level with each batch of samples. For example, add 0,1 ml of fresh mixed raw milk to 100 ml of the prepared milk (5.5).

9.3.3 Interfering substance control test

Perform this test on the product being examined by adding 0,075 ml of the test portion (8.2 or 8.3) to 2,0 ml of the calibrator solution A (5.4.1). Place the cuvette with this mixture into the filter fluorometer and allow it to attain 38 °C in 5 min. Then record the rate of any increase in fluorescence over the next 2 min. No alkaline phosphatase activity should be observed during the 2-min measurement period.

9.3.4 Microbial alkaline phosphatase control

If in cheeses produced with pasteurized milk, the determination (9.2) produces a positive result, proceed as follows. Take another test portion (8.2 or 8.3). Heat it for 30 min in the water bath (6.7) set at 63 °C. Then cool it rapidly. Determine any residual phosphatase activity according to 9.2. Any residual activity is due to the presence of microbial alkaline phosphatase.

10 Calculation and expression of results

10.1 Calibration ratio

Record the fluorescence values of calibrator solution B (5.4.2) and calibrator solution C (5.4.3) read against calibrator solution A (5.4.1) set to zero fluorescence on the filter fluorometer. Calculate the calibration ratio, r_{cal}, of the established calibration curve using Equation (1):

$$r_{cal} = \frac{F_C + 2F_B}{4} \quad (1)$$

where

F_C is the numerical value of the fluorescence obtained by measuring calibrator solution C (5.4.3) against calibrator solution A (5.4.1) set at zero fluorescence (see 9.1);

F_B is the numerical value of the fluorescence obtained by measuring calibrator solution B (5.4.2) against calibrator solution A (5.4.1) set at zero fluorescence (see 9.1).

10.2 Calculation

10.2.1 Milk/cheese mixture

Calculate the alkaline phosphatase activity, A_{P1}, of the milk/cheese mixture (8.2 or 8.3), in milliunits of enzyme activity per litre, using Equation (2):

$$A_{P1} = \frac{F_{av} \cdot c_B}{r_{cal} \cdot V} \cdot f_1 \quad (2)$$