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Milk and milk products — Determination of alkaline phosphatase activity —

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

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

Partie 1: Méthode fluorimétrique pour le lait et les boissons à base de lait

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Contents

Page

Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2. www.iso.org/directives

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Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

The committee responsible for this document is 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 third edition of ISO 11816-1|IDF 155-1 cancels and replaces the second edition (ISO 11816-1:2006), 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*

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.

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ISO 11816-1|IDF 155-1 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 *Determination of alkaline phosphatase activity – fluorimetric method*, of the Standing Committee on *Analytical Methods for Processing Aids and Indicators*, under the aegis of its project leader, Ms. Eileen Garry (USA).

This third edition of ISO 11816-1|IDF 155-1 cancels and replaces IDF 155-1:2006, 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*:

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Milk and milk products — Determination of alkaline phosphatase activity —

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

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 raw and heat-treated whole milk, semi-skimmed milk, skimmed milk and flavoured milks. This method is applicable to milk and milk-based drinks from cows, sheep and goats. It is also applicable to milk powder after reconstitution.

The instrument can read activities up to 7 000 milliunits per litre (mU/l). If the activity is higher than 7 000 mU/l, it is diluted with alkaline phosphatase-free milk (7.1) so as to obtain a measurement not higher than 7 000 mU/l.

2 Terms and definitions

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

2.1
alkaline phosphatase (ALP) activity
activity of the alkaline phosphatase present in the product, determined by the specified procedure

Note 1 to entry: The alkaline phosphatase activity is expressed as milliunits of enzyme activity per litre of sample (mU/l).

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 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, molecular weight 580 grams per mole.

This is a non-fluorescent aromatic monophosphoric ester substrate, 2'-[2-benzothiazolyl]-6'-hydroxybenzothiazole phosphate (Fluorophos®). The Fluorophos® substrate remains stable for two years from the date of manufacture, provided it is stored in unopened bottles at between 2 °C and 8 °C; protect against light.

4.2 Substrate buffer solution, diethanolamine (DEA) buffer solution, c(DEA) = 2,4 mol/l, with pH 10,0, in bottles of 240 ml each. The substrate buffer solution remains stable for two years from the date of manufacture, provided it is stored in unopened bottles at between 2 °C and 8 °C; protect against light.

4.3 Working substrate

Allow the Fluorophos® substrate (4.1) and the substrate buffer solution (4.2) to come to room temperature. Add the content of one bottle of substrate buffer solution (240 ml) (4.2) to that of one bottle of Fluorophos® substrate (144 mg) (4.1), and mix well by inversion for 3 min to create ~1,0 millimolar (pH 10) solution. Use amber glass to protect against light.

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

Use the A/D (analogue-to-digital) test given in 8.2 to test the suitability of the ready-to-use working substrate. Do not use the working substrate if a reading above 1 200 FLU (fluorescence units) is obtained (8.2).

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

NOTE The volume of the working substrate (240 ml) obtained is sufficient for approximately 115 tests.

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

The calibrator solutions remain stable for 18 months from the date of manufacture, provided they are stored in unopened bottles at between 2 °C and 8 °C. Mix gently prior to use to ensure optimal results.

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

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

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

4.5 Daily instrument control solution, containing 34,48 x10^{-3} µmol/l of Fluoroyellow®.

The daily instrument control solution remains stable for 18 months from the date of manufacture, provided it is stored in unopened bottles at between 2 °C and 8 °C. Mix gently prior to use to ensure optimal results.

5 Apparatus

Usual laboratory equipment and, in particular, the following.

1) The reagents specified in 4.1 to 4.5 and the apparatus specified in 5.1 to 5.4 (except 5.3.3) comprise the Fluorophos Test System, which is the trade name of a product supplied by Advanced Instruments, Inc., Two Technology Way, Norwood, Massachusetts 02062, USA. The manufacturer may change the packaging configurations supplied with 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 trademarks of Advanced Instruments, Inc. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of the products named. Equivalent products may be used if they can be shown to lead to the same results.

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 at between 520 nm and 560 nm [e.g. Fluorophos® instrument[1)]].

5.2 Cuvettes, disposable, of non-fluorescent glass.

5.3 Pipettes

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

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

Follow strict instructions for pipetting technique as this is a critical step in generating accurate results. Ensure that piston of pipette bore is tightly secured prior to use.

5.3.3 Pipettes, of capacity 2 ml and 3 ml.

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

5.5 Suitable laboratory-grade film.

5.6 Vortex mixer.

5.7 Water bath, capable of maintaining a temperature of 63 °C ± 1 °C and 95 °C ± 1 °C.

5.8 One-mark volumetric flasks, of capacity 100 ml.

6 Sampling

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.

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

7 Preparations

7.1 Alkaline phosphatase-free milk

Prepare phosphatase-free milk of the type to be tested by carefully dispensing the desired portion of milk into a test tube or suitable container, ensuring that no milk touches the rim or sides of the container.

Place the tube or container with the milk portion in the water bath (5.7) set at 95 °C. Preheat the milk portion to 95 °C, before starting its 5-min heating period at that temperature. Check the temperature by using a thermometer or thermistor probe placed in the centre of the tube or container. When the milk portion reaches 95 °C, immediately start its 5-min heating period. Cool the whole portion rapidly after the heating period.

Test the thus-treated milk portion to ensure that its ALP activity is less than 10 mU/l.

7.2 Preparation of test sample

7.2.1 General

Carefully mix all test samples prior to use.

NOTE It is usually not necessary to prewarm test samples.

7.2.2 Pasteurized test samples

Use pasteurized test samples as delivered, in amounts as required.

7.2.3 Dilution of test samples with high ALP values

Prepare dilutions of the test samples of milk using phosphatase-free milk (7.1) in order to bring their ALP levels within the analytical range of assay (<7 000 mU/l). Mix the diluted solutions well.

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 principles when operating the filter fluorimeter (5.1).

Quality control tests include

a) the daily A/D 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, and

c) the use of external positive, negative and normal controls, described in 8.1.3, which are recommended for monitoring daily instrument precision parameters.

8.1.2 A/D tests

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

8.1.2.2 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.

8.1.2.3 Dispense 2,0 ml of daily instrument control solution (4.5) into a labelled cuvette. Place the cuvette in the incubator block (5.4) set at 38°C for 20 min. Insert the prewarmed 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.