INTERNATIONAL STANDARD

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Milk and milk products — Determination of alkaline phosphatase activity using a fluorimetric method —

Part 1:
Milk and milk-based drinks

Lait et produits laitiers — Détermination de l'activité de la phosphatase alcaline à l'aide de la méthode fluorimétrique —

Partie 1: Lait et boissons à base de lait

Reference number
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Foreword

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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.

International Standard ISO 11816-1 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Subcommittee SC 5, *Milk and milk products*, in collaboration with the International Dairy Federation (IDF) and AOAC INTERNATIONAL, and will also be published by these organizations.

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

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

— *Part 2: Cheeses*

Annex A of this part of ISO 11816 is for information only.

International Organization for Standardization
Case postale 56 • CH-1211 Genève 20 • Switzerland
Internet central@iso.ch
X.400 c=ch; a=400net; p=iso; o=isocs; s=central

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Milk and milk products — Determination of alkaline phosphatase activity using a fluorimetric method —

Part 1:
Milk and milk-based drinks

1 Scope

This part of ISO 11816 specifies a fluorimetric method for the determination of alkaline phosphatase activity in pasteurized whole milk, semi-skimmed milk, skimmed milk and flavoured milks.

The method is also suitable for the determination of high alkaline phosphatase activity in raw milk and heat-treated milk having activities of more than 7 000 milliunits per litre.

2 Definitions

For the purposes of this part of ISO 11816, the following definitions apply.

2.1 alkaline phosphatase activity (APL): Activity of the alkaline phosphatase present in a product, determined in accordance with the procedure specified in this part of ISO 11816. It is expressed as milliunits per litre.

NOTE 1 See reference [1] in annex A.

2.2 unit of alkaline phosphatase activity: Amount of alkaline phosphatase enzyme that catalyses the transformation of 1 μmol of substrate per minute per litre of sample.

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, 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.

NOTE 2 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 Substrate, for example Fluorophos substrate[1], crystallized.

NOTE 3 Fluorophos substrate is a water-soluble, non-fluorescent aromatic monophosphoric ester substrate, which is stable for 1 year when crystallized and stored in glass vials at 4 °C.

4.2 Substrate diluent[1]: diethanolamine (DEA) buffer (pH 10,0), 2,4 mol/l solution.

NOTE 4 The buffer solution is stable for 1 year at 4 °C.

4.3 Working substrate[1]

Add a volume of the substrate diluent (4.2) to the substrate (4.1) to give a concentration of 1,044 mmol/l and mix well by inversion. Use amber glass to protect against light.

If using the Fluorophos Test System[1], add the content of 1 vial of the substrate diluent (4.2) to one vial of the substrate (4.1) and mix well by inversion.

NOTE 5 This solution is stable when stored in the dark for 8 weeks at 4 °C and 8 h at 38 °C. It is sufficient for 115 tests.

4.4 Working calibrators[1], for example Fluoroyellow in DEA buffer.

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.

NOTES

6 Calibrator solutions are stable for 1 year when stored at 4 °C.

7 The volumes of reagents supplied for the Fluorophos Test System may be changed by the manufacturer. The user should refer to the manufacturer's instructions for preparing the reagents if the volumes supplied are different from those specified in 4.1 to 4.4.

5 Apparatus

Usual laboratory equipment and, in particular, the following.

5.1 Filter fluorimeter[1], with thermostatted cuvette holder maintained at 38 °C ± 1 °C, and right-angle optics, allowing excitation at a wavelength of 440 nm and emission at 560 nm.

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

5.3 Fixed-volume dispenser, to dispense 2,0 ml.

5.4 Positive-displacement pipette, of capacity 0,075 ml.

5.5 Pipettes, of capacities 1 ml and 2 ml.

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[1] The reagents specified in 4.1 to 4.4 and the apparatus specified in 5.1 to 5.6 (except 5.5) are available as the Fluorophos Test System from Advanced Instruments Inc., Two Technology Way, Norwood, MA 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 and does not constitute an endorsement by ISO of these products.

5.6 Incubator block, suitable to hold the cuvettes and capable of being maintained at a temperature of 38 °C ± 1 °C.

5.7 Parafilm or other suitable laboratory grade film.

5.8 Vortex shaker

5.9 Water bath, capable of being maintained at 95 °C ± 1 °C.

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

6 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. A recommended sampling method is given in ISO 707 [2].

7 Preparation of test sample

Carefully mix the laboratory sample. It is usually not necessary to prewarm the test sample.

8 Procedure

8.1 Test portion

8.1.1 Pasteurized samples

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

8.1.2 Raw milk and heat-treated milk

Pipette (5.5) 1 ml of the test sample of raw milk into a volumetric flask (5.10). Dilute to 100 ml with phosphatase-free milk (8.4.1). Mix carefully. Dilute test samples of heat-treated milk with phosphatase-free milk (8.4.1) so as to obtain an alkaline phosphatase activity in the test portion of less than 7 000 milliunits per litre (mU/L).

8.2 Calibration

Establish a calibration curve for each type of product to be tested. Calibration curves are usually stable but the instrument shall be recalibrated every 2 to 3 months. Check the instrument if there are changes in the calibration curve.

Using the pipette (5.5), transfer 2,0 ml of the calibrator solutions A, B and C (4.4.1, 4.4.2 and 4.4.3 respectively), each in duplicate, into the labelled cuvettes (5.2). Place the cuvettes in the incubator block (5.6) and prewarm to 38 °C for 5 min. Add, by means of the positive-displacement pipette (5.4), 0,075 ml of the prepared test portion (8.1) to all six cuvettes. Cover the cuvettes with the parafilm (5.7).

Vortex (5.8) for 5 s, or gently invert all six cuvettes to mix the contents and then return the cuvettes to the incubator block. Starting with calibrator solution A (4.4.1), perform the following calibration routine. Set the fluorimeter (5.1) to zero fluorescence using calibrator solution A (4.4.1) and then read and record the amount of fluorescence obtained with calibrator solution B and solution C against calibrator solution A. Wipe the outside of each cuvette with soft tissue before placing it in the fluorimeter. Once calibration is completed, proceed with the analysis of the samples.

8.3 Determination

Using the fixed-volume dispenser (5.3), transfer 2,0 ml of the working substrate (4.3) to a labelled cuvette. Place the cuvette in the incubator block (5.6) and prewarm to 38 °C for 5 min.

Add 0,075 ml of the test portion (8.1) to the substrate. Cover and immediately mix the contents of the cuvette by vortexing for 5 s or by gentle inversion. Wipe the outside of the cuvette with soft tissue and place the cuvette in the fluorimeter. Allow 1 min for temperature equilibration, then record the amount of fluorescence at the beginning of the second minute and at the end of the third minute. Divide the difference between the amount of fluorescence at the beginning of the second minute and that at the end of the third minute by 2 to obtain the average amount of fluorescence produced per minute.

Record the average amount of fluorescence per minute for each test portion. Use that value to calculate the alkaline phosphatase activity, in milliunits per litre.

NOTE 8 Results may be calculated automatically by using the calculator which forms an integral part of the advanced fluorimeter, or may be calculated manually according to clause 9.

8.4 Control tests

8.4.1 Negative control test

Include a negative control with each batch of samples produced by heating 5 ml of the product to be tested to 95 °C in the water bath (5.9) and holding it at this temperature for 1 min, then cooling it rapidly.

NOTE 9 An alkaline phosphatase activity of less than 10 mU/L indicates that no fluorescence activity has been detected.

8.4.2 Positive control test

Include a positive control with a phosphatase activity level at or close to the decision level with each batch of samples.

NOTE 10 For example, add 0,2 ml of fresh, mixed-herd, raw milk to 100 ml of a sample that has previously been heated to 95 °C for 1 min and rapidly cooled (8.4.1).

8.4.3 Interfering-substance control test

Perform this test on the product being examined by adding 0,075 ml of the test portion (8.1) to 2,0 ml of the calibrator solution A (4.4.1) which has been prewarmed in the incubator block (5.6) to 38 °C for 5 min. Place the cuvette containing this mixture in the fluorimeter (5.1). Allow it, in 1 min, to attain 38 °C ± 1 °C and then record the rate of any increase in fluorescence at the beginning of the second minute and at the end of the third minute. No alkaline phosphatase activity should be observed during the 2 min of measurement period.

8.4.4 Microbial alkaline phosphatase control

If the determination (8.3) produces a positive result, then proceed as follows.

Take another test portion (8.1), heat it to 62,8 °C, hold it at this temperature for 30 min and then cool it rapidly. Determine any residual phosphatase activity as specified in 8.3. Any residual activity is due to the presence of microbial alkaline phosphatase.

9 Calculation and expression of results

Results may be calculated automatically by means of the calculator built into the filter fluorimeter (5.1).

If results are to be calculated manually, proceed as follows.

Record the fluorescence values of calibrator solution B (4.4.2) and solution C (4.4.3) read against calibrator solution A (4.4.1) set to zero fluorescence on the fluorimeter (5.1).

Calculate the calibration ratio, R, of the established calibration curve using the following equation:

$$R = \frac{C_A + 2B_A}{4}$$

where

B_A is the numerical value of the fluorescence obtained by measuring calibrator solution B against solution A set at zero fluorescence;

C_A is the numerical value of the fluorescence obtained by measuring calibrator solution C against solution A set at zero fluorescence.

Record the average amount of fluorescence produced per minute (8.3) by the test portion.

Calculate the alkaline phosphatase activity (ALP), expressed as milliunits per litre, using the following equation:

$$\text{ALP} = \frac{F}{R} \times \frac{c}{V} \times f$$

where

F is the average amount of fluorescence produced per minute (8.3) by the test portion, measured against the calibrator solution A at the beginning of the second minute and at the end of the third minute;

c is the concentration of Fluoroyellow in calibrator solution B (4.4.2), in micromoles per 2 ml of calibrator;

f is the dilution factor (1×10^6) in the case of pasteurized samples (8.1.1); in the case of test samples of raw milk (8.1.2), f is equal to 1×10^8; in the case of test samples of heat-treated milk (8.1.2), multiply $f = 1 \times 10^6$ with the dilution factor f_t of the test sample ($f = f_t \times 10^6$);

V is the volume, in millilitres, of the test portion (8.3).

Round the result to the nearest whole unit of a milliunit.

10 Precision

The repeatability and reproducibility values quoted in 10.1 and 10.2 apply at a level of phosphatase activity of around 500 mU/L, which corresponds to a phosphatase activity of 0,1 % of added raw milk in the properly pasteurized product.

These values are expressed at the 95 % probability level and were derived from the results of an interlaboratory test carried out in accordance with ISO 5725 [3].

10.1 Repeatability

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, should not exceed:

— for whole milk: 62 mU/L

— for skimmed milk: 55 mU/L

— for flavoured (chocolate) milk: 79 mU/L

10.2 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, should not exceed:

— for whole milk: 98 mU/L

— for skimmed milk: 89 mU/L

— for flavoured (chocolate) milk: 130 mU/L

11 Test report

The test report shall specify

— the method in accordance with which sampling was carried out, if known;

— the method used;

— the test result(s) obtained; and

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

It shall also mention all operating details not specified in this part of ISO 11816, or regarded as optional, together with details of any incidents that may have influenced the test result(s).

The test report shall include all information required for the complete identification of the sample.

Annex A
(informative)

Bibliography

[1] International Union of Biochemistry Nomenclature. *J. Am. Med. Assoc.*, **260**, 1988, p. 73.

[2] ISO 707:— [2], *Milk and milk products — Methods of sampling.*

[3] ISO 5725:1986, *Precision of test methods — Determination of repeatability and reproducibility for a standard test method by inter-laboratory test* (now withdrawn), was used to obtain the precision data.

2) To be published. (Revision of ISO 707:1985)