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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.....	iv
1 Scope	1
2 Terms and definitions.....	1
3 Principle	1
4 Reagents	2
5 Apparatus	3
6 Sampling	3
7 Preparations	3
7.1 Alkaline phosphatase-free milk	3
7.2 Preparation of test sample	4
8 Procedure	4
8.1 Verification of instrument performance	4
8.2 Calibration	4
8.3 Determination	5
8.4 Control tests	5
9 Calculation and expression of results	7
9.1 Calibration ratio	7
9.2 Calculation	7
9.3 Expression of test results	7
10 Precision	8
10.1 Interlaboratory test	8
10.2 Repeatability	8
10.3 Reproducibility	8
11 Test report	8
Annex A (informative) Interlaboratory trial	9
Bibliography	11

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 11816-1|IDF 155-1 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 ISO and IDF.

This edition of ISO 11816-1|IDF 155-1 cancels and replaces ISO 11816-1:1997, 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 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 the 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 Action Team *Characterization to heat treatment*, of the Standing Committee on *Minor components and characterization of physical properties*, under the aegis of its project leader, Mr F. Harding (UK).

This edition of ISO 11816-1|IDF 155-1 cancels and replaces IDF 155A:1999, 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 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 pasteurized whole milk, semi-skimmed milk, skimmed milk and flavoured milks. The method is applicable for milk from cows, sheep and goats, and milk-based drinks.

The method is also suitable for the determination of high alkaline phosphatase activity in raw milk and heat-treated milk with activities of more than 2 000 mU/l after dilution of the sample as specified.

2 Terms and definitions

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

2.1

alkaline phosphatase activity

APL activity

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

NOTE The alkaline phosphatase activity is expressed as milliunits of enzyme activity per litre (mU/l).

2.2

unit of alkaline phosphatase activity

amount of alkaline phosphatase enzyme that catalyses the transformation of 1 μ mol 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 substrate. 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 when stored in unopened bottles at between 2 °C and 8 °C.

4.2 Substrate buffer solution, diethanolamine (DEA) buffer solution, $c(\text{DEA}) = 2,4 \text{ mol/l}$, with pH 10,0, in bottles of 240 ml each.

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

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 Fluorophos® substrate (144 mg) (4.1) and mix well by inversion for 3 min. Use amber glass to protect against light.

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

Use the analog-to-digital test given in 8.4.1.1 to test the suitability of the ready to use working substrate.

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. Do not use the working substrate if a reading above 1 200 is obtained (see 8.4.1.1.5).

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

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

The working calibrator solutions remain stable for 18 months when stored at between 2 °C and 8 °C.

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

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

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

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

1) The reagents specified in 4.1 to 4.5 and the apparatus specified in 5.1 to 5.4 (except 5.3.3) are available as Fluorophos Test System from Advanced Instruments, Inc., Two Technology Way, Norwood, Massachusetts 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. and are examples of suitable products available commercially.

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.

5 Apparatus

Usual laboratory equipment and, in particular, the following.

5.1 Filter fluorimeter, with thermostatically controlled cuvette holder, capable of operating at $38\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ 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⁾]. Measurements should be optimized according to the manufacturers instructions.

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

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.

5.3.3 Pipette, of capacity 2 ml.

5.4 Incubator block, capable of maintaining a temperature of $38\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$, suitable for holding cuvettes.

5.5 Parafilm ²⁾, or other suitable laboratory-grade film.

5.6 Vortex mixer.

5.7 Water bath, capable of maintaining a temperature of $63\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ and $95\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$.

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

6 Sampling

A representative sample should have been sent to the laboratory. It should not have 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|IDF 50.

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 $95\text{ }^{\circ}\text{C}$. Preheat the milk portion to $95\text{ }^{\circ}\text{C}$, 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 $95\text{ }^{\circ}\text{C}$, immediately start the 5-min heating period. Cool the whole rapidly after the heating period.

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

2) Parafilm 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 either ISO or IDF of this product.

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 milks using phosphatase-free milk (7.1) in order to bring their ALP levels within the analytical range of assay ($< 2\,000$ mU/l). Mix the diluted solutions well.

8 Procedure

8.1 Verification of instrument performance

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 by measuring the accuracy of the A/D conversion channel and monitoring the A/D channel for drift over time or temperature, and
- b) the daily instrument control test, using the daily instrument control solution (4.5) to monitor any electronic or optical drift in the fluorimeter.

The use of external positive, negative and normal controls, described in 8.4, are strongly recommended for monitoring daily instrument precision parameters.

8.2 Calibration

Calibration curves are usually stable. However, recalibrate the instrument, which has already been calibrated, when the fluorimeter is initially installed, whenever servicing procedures are likely to affect the stored calibration, when assayed control values show unacceptable results, or every 3 months.

If there are changes in the calibration curve, recalibrate the instrument using a new set of calibrator solutions A, B and C (4.4.1, 4.4.2 and 4.4.3). Establish a calibration curve for each type of product to be tested.

Mix calibrator solutions A, B and C by gentle inversion prior to use. Transfer, using the pipette (5.3.3), 2,0 ml of calibrator solution A, of calibrator solution B and of calibrator solution C (4.4.1, 4.4.2 and 4.4.3) respectively, each in duplicate, to six prelabelled cuvettes (5.2). Place the cuvettes in the incubator block (5.4) set at 38 °C and preheat for 10 min.

Add, using the positive displacement or air-displacement pipette (5.3.2), 0,075 ml of alkaline phosphatase-free milk (7.1) to all six cuvettes. Cover the cuvettes with parafilm (5.5). Mix their contents using the vortex mixer (5.6) for 5 s or by gently inverting the cuvettes. Return the cuvettes to the incubator block (5.4). Complete the calibration within 10 min after the addition of the test sample to the calibrator.

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 fluorimeter (5.1). When using the Fluorophos[®] instrument, press "CALIB" and select the "ALP Dairy" menu. Scroll through the menu and press "ENTER" when the product to be calibrated is displayed. Beginning with calibrator solution A (4.4.1), insert this solution in the fluorimeter and press "START". When the measurement is finished, measure the second A calibrator solution.

Follow the same procedure for the B (4.4.2) and C (4.4.3) calibrator solutions until the procedure is completed. The Fluorophos[®] instrument automatically calculates the amount of fluorescence obtained with calibrator solution B and C against calibrator solution A to set the calibration ratio within the instrument.

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

8.3 Determination

Dispense, using the fixed volume dispenser (5.3.1), 2,0 ml of working substrate (4.3) into a labelled cuvette. Place the cuvette in the incubator block (5.4) set at 38 °C and heat for 15 min.

Add, using the pipette (5.3.2), 0,075 ml of the well-mixed test portion (7.2.2 or 7.2.3) to the substrate. Cover the cuvette with parafilm (5.5). Immediately mix its contents using the vortex mixer (5.6) for 5 s or by gently inverting the cuvette. Wipe the outside of the cuvette with soft tissue and place it in the filter fluorimeter (5.1).

Press the "TEST" key, "ALP Dairy" appears, then press "ENTER". Scroll through the menu and press "ENTER" when the product to be analysed is displayed. Then press the "START" key to begin the test. The display will count down 60 s while the substrate and sample are being warmed to 38 °C. After 60 s the fluorimeter starts measuring, displaying a fluorescence of the sample in fluorescence units (FLU). The display starts at around 200 FLU and slowly increases over the next 2 min. At the end of the 3-min period, the Fluorophos instrument performs automatically the necessary calculations and displays the sample identification number, the ALP activity in milliunits per litre, and the average increase in fluorescence, if previously selected. This information will then be printed.

Divide the difference between the two fluorescence readings by the interval period (recorded in minutes) to obtain the average increase of fluorescence produced per minute (F/min). Use the F/min value to calculate the ALP activity of the test sample.

The instrument may display and print out the message "Error: Unstable Reading, Repeat Test". In the case of very high results, dilute the test sample with heat-treated phosphatase-free milk (7.1) and perform another determination.

For very low results (normally below 6 FLU/min), where the unstable readings are more common, leave the sample cuvette in the Fluorophos[®] chamber and perform another determination. A valid result is then usually obtained. If, however, an unstable reading error is obtained again, repeat the entire determination with a new test sample.

8.4 Control tests

8.4.1 System and reagent controls

8.4.1.1 A/D test

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

8.4.1.1.2 Dispense 2,0 ml of the daily instrument control solution (4.5) into a labelled cuvette. Place the cuvette in the incubator block (5.4) set at 38 °C for 10 min.

8.4.1.1.3 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.

8.4.1.1.4 Insert the prewarmed cuvette with the control solution (8.4.1.1.2) 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.

8.4.1.1.5 The A/D test can also be used to test the suitability of ready-to-use working substrate (4.3). Freshly made substrate alone in the A/D mode usually gives a display reading of about 650 FLU which increases over time.

Do not use the working substrate when a display reading of above 1 200 FLU is obtained.

8.4.1.2 Positive, negative and PhosphaCheck-N™ controls ³⁾

After calibrating the channel used for cow milk, analyse the three control solutions (i.e. positive, negative and PhosphaCheck-N™) by adding 75 µl of each control solution to 2 ml of prewarmed substrate. Perform the ALP test.

The reading for the negative control shall be < 10 and that for the PhosphaCheck-N™ control shall be < 40 .

8.4.2 Test sample and instrument-related controls

8.4.2.1 Negative control test

Include a negative control test with each batch of test samples. Heat a test sample as described in 7.1. The instrument reading shall be less than 10 mU/l, indicating no fluorescence activity is detected. If the value exceeds 10 mU/l, repeat step 8.4.1.2.

8.4.2.2 Positive control test ³⁾

Include one or more positive controls with each batch of test samples. Prepare samples at or near decision levels using raw milk samples diluted with the phosphatase-free milk (7.1).

8.4.3 Interfering substance test

Where higher than expected ALP values are obtained, add, using a pipette (5.3.2), 0,075 ml of test portion (7.2.2 or 7.2.3) to a cuvette with 2,0 ml of calibrator solution A (4.4.1), which was previously prewarmed in the incubator block (5.4) set at 38 °C for 10 min, and mix.

Place the cuvette containing this mixture in the Fluorophos® instrument (5.1) and test as in 8.2. If the obtained value exceeds 20 mU/l, an interfering substance is shown to be present. In that case, repeat the test using a fresh sample.

8.4.4 Heat-stable microbial alkaline phosphatase control test

Add another test portion (7.2.2 or 7.2.3) to a tube. Place a thermometer or thermistor probe into the tube and put the whole in the water bath (5.7) set at 63 °C. When the test portion reaches 63 °C, keep it at that temperature for 30 min, then cool rapidly. Determine any residual phosphatase activity according to 8.3. Any residual activity is due to the presence of heat-stable microbial alkaline phosphatase.

3) The controls specified and instrument performance check instructions are available from Advanced Instruments Inc., Two Technology Way, Norwood, MA 02062, USA.

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

9 Calculation and expression of results

9.1 Calibration ratio

Results are calculated automatically by the Fluorophos® instrument by means of the algorithm 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 calibrator solution C (4.4.3), read against calibrator solution A (4.4.1) set to zero fluorescence on the filter fluorimeter (5.1).

Calculate the calibration ratio, K , using Equation (1):

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

where

- K is the numerical value of the calibration ratio of the established calibration curve;
- F_C is the numerical value of the fluorescence obtained by measuring calibrator solution C (4.4.3) against calibrator solution A (4.4.1) set at zero fluorescence (see 8.2);
- F_B is the numerical value of the fluorescence obtained by measuring calibrator solution B (4.4.2) against calibrator solution A (4.4.1) set at zero fluorescence (see 8.2).

9.2 Calculation

Calculate the alkaline phosphatase activity, A_p , using Equation (2):

$$A_p = \frac{F_{av} \times c_B}{K \times V} \times f \quad (2)$$

where

- A_p is the numerical value of the alkaline phosphatase activity of the test sample (7.2.2 or 7.2.3), in milliunits of enzyme activity per litre;
- F_{av} is the numerical value of the average amount of fluorescence produced per minute by the test portion (8.3), measured against calibrator solution A (see 8.2) currently from the beginning of the second minute to the end of the third minute;
- c_B is the concentration of the 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 (7.2.2); in the case of test samples of raw milk (7.2.3), f is equal to 1×10^8 ; in the case of test samples of heat-treated milk (7.2.3), multiply $f = 1 \times 10^6$ by the dilution factor f_t of the test sample ($f = f_t \times 10^6$);
- V is the numerical value of the volume of the test portion, in millilitres.

9.3 Expression of test results

Express the test results to the nearest whole unit of a milliunit.

10 Precision

10.1 Interlaboratory test

Details of an interlaboratory test on the precision of the method are summarized in Annex A. The values derived from this test may not be applicable to concentration ranges and matrices other than those given.

10.2 Repeatability

The absolute difference between two independent single test results, obtained with 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, will in not more than 5 % of cases be greater than the values for r given in Table 1.

Table 1 — Repeatability limit, r , values

Product	Alkaline phosphatase activity level mU/l				
	20	40	100	350	500
Cow milk	—	21,50	22,10	89,60	93,30
Sheep milk	10,43	16,26	33,67	96,82	99,76
Goat milk	8,63	7,98	26,20	42,83	28,56

10.3 Reproducibility

The absolute difference between two single test results, obtained with the same method on identical test material in different laboratories with different operators using different equipment, will in not more than 5 % of cases be greater than the values for R given in Table 2.

Table 2 — Reproducibility limit, R , values

Product	Alkaline phosphatase activity level mU/l				
	20	40	100	350	500
Cow milk	—	31,80	51,00	136,40	211,10
Sheep milk	16,63	20,34	46,63	170,24	233,10
Goat milk	10,69	20,55	28,71	127,89	87,51

11 Test report

The test report shall specify:

- all information necessary for the complete identification of the sample;
- the sampling method used, if known;
- the test method used, together with reference to this part of ISO 11816|IDF 155;
- all operating details not specified in this part of ISO 11816|IDF 155, or regarded as optional, together with details of any incidents which may have influenced the test result(s);
- the test result(s) obtained, or, if the repeatability has been checked, the final quoted result obtained.