

INTERNATIONAL
STANDARD

ISO
11816-2

IDF
155-2

Second edition
2016-08-15

**Milk and milk products —
Determination of alkaline
phosphatase activity —**

**Part 2:
Fluorimetric method for cheese**

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

Partie 2: Méthode fluorimétrique pour le fromage



Reference numbers
ISO 11816-2:2016(E)
IDF 155-2:2016(E)

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Forewords

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.

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 (see www.iso.org/directives).

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For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT) see the following URL: [Foreword - Supplementary information](#)

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 second edition of ISO 11816-2|IDF 155-2 cancels and replaces the 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*

IDF (the International Dairy Federation) is a non-profit private sector organization representing the interests of various stakeholders in dairying at the global level. IDF members are organized in National Committees, which are national associations composed of representatives of dairy-related national interest groups including dairy farmers, dairy processing industry, dairy suppliers, academics and governments/food control authorities.

ISO and IDF collaborate closely on all matters of standardization relating to methods of analysis and sampling for milk and milk products. Since 2001, ISO and IDF jointly publish their International Standards using the logos and reference numbers of both organizations.

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ISO 11816|IDF 155 was prepared by the IDF Standing Committee on *Analytical Methods for Processing Aids and Indicators* and ISO Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*.

The work was carried out by the IDF/ISO 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 ISO/IDF International Standard cancels and replaces 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). For large hard cheeses, specific conditions of sampling apply (see [Clause 7](#)).

The instrument can read activities in the supernatant up to 7 000 milliunits per litre (mU/l).

2 Normative reference

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 5725-1, *Accuracy (trueness and precision) of measurement methods and results — Part 1: General principles and definitions*

ISO 5725-2, *Accuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method*

3 Terms and definitions

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

3.1 alkaline phosphatase activity

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 gram of sample (mU/g).

3.2 unit of alkaline phosphatase activity

amount of alkaline phosphatase enzyme that catalyses the transformation of 1 μmol of substrate per minute

4 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®¹⁾. 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).

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®substrate²⁾ in bottles, each containing 144 mg of Fluorophos® substrate powder, molar mass 580 grams per mole.

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

This substrate remains stable for 2 years from the date of manufacture, provided it is stored in unopened bottles at a temperature between 2 °C and 8 °C. Protect against light.

5.2 Substrate buffer solution, diethanolamine (DEA) buffer solution, $c(\text{DEA}) = 2,4 \text{ mol/l}$, with pH 10,0.

The substrate buffer solution remains stable for 2 years from the date of manufacture, provided it is stored in unopened bottles at a temperature between 2 °C and 8 °C. Protect against light.

5.3 Working substrate.

Allow the Fluorophos® substrate (5.1) and the substrate buffer solution (5.2) to come to room temperature. Add the content of one bottle substrate buffer solution (240 ml) (5.2) to that of one bottle Fluorophos® substrate (144 mg) (5.1) and mix well by inversion for 3 min. 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 analog-to-digital (A/D) test given in 9.2 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.

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

5.4 Working calibrator solutions, Fluoroyellow® (FY) [2'-(2-benzothiazolyl)-6'-hydroxybenzothiazole] in substrate buffer solution (5.2).

The working calibrator solutions remain stable for 18 months from manufacturing date when stored in unopened bottles at a temperature between 2 °C and 8 °C.

1) Fluorophos is a registered trademark. 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. Equivalent products may be used if they can be shown to lead to the same results.

2) The reagents specified in 5.1 to 5.6 and the apparatus specified in 6.1 to 6.4 (except 6.3.1) are available 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. 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.

Mix gently prior to use to ensure optimal results.

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

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

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

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

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

Mix gently prior to use to ensure optimal results.

5.6 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 a temperature between 2 °C and 8 °C.

5.7 Positive, negative and Phosphacheck-N™ controls.

6 Apparatus

Usual laboratory equipment and, in particular, the following.

6.1 Filter fluorimeter, with thermostatically controlled cuvette holder, capable of operating at $38 \text{ °C} \pm 1 \text{ °C}$ and right-angle optics, allowing excitation at a wavelength of 440 nm and emission between 520 nm and 560 nm [e.g. Fluorophos® instrument¹⁾].

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

6.3 Pipettes.

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

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

6.4 Heating block, capable of maintaining a temperature of $38 \text{ °C} \pm 1 \text{ °C}$, suitable for holding cuvettes.

6.5 Plastic paraffin film (e.g. Parafilm®³⁾) or other suitable laboratory-grade film.

6.6 Vortex mixer.

6.7 Grinding device.

6.8 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).

3) Parafilm® and Ultra turrax® are examples of a suitable product 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.

6.9 High-speed homogenizer (e.g. Ultra turrax^{®3}) provided with the stem of diameter of approximately 6 mm to 8 mm.

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

6.11 Centrifuge, capable of centrifuging at 1 000g at 4 °C.

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

6.13 Glass Pasteur pipette, an air-displacement pipette can also be used.

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

7 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.^[1]

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 homogeneously 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 (see [Figure B.1](#)).

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.

8 Preparation of test sample

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 (see [Figure B.2](#)). For large hard cheeses, proceed as described under [Clause 7](#). Grind the test sample by means of a grinding mill or other appropriate device ([6.7](#)) and mix thoroughly. Keep the prepared sample in an airtight container.

9 Procedure

9.1 Verification of instrument performance

9.1.1 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 ([6.1](#)).

Quality control tests includes the following:

- 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 ([5.5](#)) to monitor any electronic or optical drift in the fluorimeter.

- c) The use of external positive, negative and normal controls, described in [9.1.3](#), is recommended for monitoring daily instrument precision parameters.

9.1.2 Daily instrument tests

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

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.

9.1.2.2 Dispense 2,0 ml of the daily instrument control solution ([5.5](#)) into a labelled cuvette, using the pipette ([6.3.1](#)). Place the cuvette in the heating block ([6.4](#)) 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.

9.1.3 Controls

Perform positive, negative and Phosphacheck-N™ controls⁴⁾ using a powdered milk base, with phosphatase and preservative ([5.7](#)).

The PhosphaCheck® pasteurization controls remain stable for 18 months from manufacturing date when stored in unopened and unreconstituted bottles at a temperature between 2 °C and 8 °C. Once reconstituted, the controls are stable for 3 days (72 h) at a temperature between 2 °C and 8 °C. Do not freeze.

Allow the controls to come to room temperature. Reconstitute the PhosphaCheck® pasteurization controls before use. Remove the metal and rubber stopper. Add 3,0 ml of deionised water at room temperature, using the pipette ([6.3.1](#)). Replace the stopper and mix gently by inversion for 1 min and then let stand for 15 min. Do not shake the controls or allow them to foam. Mix gently before each use to ensure optimal results.

After calibrating an unused channel with the negative control, analyse the three control solutions (i.e. positive, negative and PhosphaCheck-N™) by adding 0,075 ml of each control solution to 2 ml of pre-warmed substrate. Perform the ALP test.

The reading for the negative control shall be <10 mU/l, the PhosphaCheck-N™ control shall be between 10 mU/l and 40 mU/l and the positive control shall be $500 \text{ mU/l} \pm 100 \text{ mU/l}$.

9.2 Reagent controls to test the suitability of ready to use working substrate ([5.3](#))

Dispense 2,0 ml of the working substrate ([5.3](#)) into a labelled cuvette, using the pipette ([6.3.1](#)). Place the cuvette in the heating block ([6.4](#)) set at 38 °C for 20 min. Insert the pre-warmed cuvette with the working substrate into the cuvette holder. Close the lid. When the display is stable, record the displayed value.

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.

4) 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 document and does not constitute an endorsement by either ISO or IDF of these products.

9.3 Calibration

9.3.1 General

Calibration curves are usually stable. However, recalibrate the instrument when the fluorimeter is initially installed, whenever servicing procedures are performed (i.e. lamp or filter replaced), when assayed control values show unacceptable results leading to adjustments to bring A/D mode into specification.

If there are changes in the calibration curve, recalibrate the instrument using a new set of calibrator solutions A, B and C ([5.4.1](#), [5.4.2](#) and [5.4.3](#)). Establish a calibration curve for each type of product to be tested unless they have same fat content.

Calibration curves for cheese are established using a pasteurized cheese of the same family of the cheese to test. When the calibration ratios for different cheese types are within 5 % of each other, these products may be run on the same channel.

9.3.2 Mix calibrator solutions A, B and C by gentle inversion prior to use. Transfer, using the pipette ([6.3.1](#)), 2,0 ml of calibrator solution A, calibrator solution B and calibrator solution C ([5.4.1](#), [5.4.2](#) and [5.4.3](#)) respectively, each in duplicate, to six pre-labelled cuvettes ([6.2](#)). Place the cuvettes in the heating block ([6.4](#)) maintained at 38 °C and preheat for 20 min.

9.3.3 Add, to six cuvettes using the positive displacement or air-displacement pipette ([6.3.2](#)), 0,075 ml of the supernatant ([9.4.6](#)) of a pasteurised cheese of the same family as the sample to test. Cover the cuvettes with film ([6.5](#)). Mix their contents using the vortex mixer ([6.6](#)) for 5 s or by gently inverting the cuvettes. Return the cuvettes to the heating block ([6.4](#)). Complete the calibration within 10 min after the addition of the test sample to the calibrator.

9.3.4 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 fluorimeter ([6.1](#)). 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 ([5.4.1](#)), 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 ([5.4.2](#)) and C ([5.4.3](#)) 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.

9.4 Determination

9.4.1 Weigh, to the nearest 1 mg, 0,3 g to 0,5 g of the prepared test sample ([Clause 8](#)) into a 10 ml glass beaker ([6.8](#)) or into a glass test tube ([6.12](#)).

9.4.2 Add a first portion of 5 ml of cheese extraction buffer ([5.6](#)). Let the cheese extraction buffer in contact with the cheese between approximately 5 min and 10 min for soft and semi-hard cheese and between 15 min and 20 min for hard cheese. Homogenize the buffer/cheese mixture using the ultra turrax® ([6.9](#)) until complete dissolution of the test sample (approximately 35 s for soft and semi-hard cheeses and 50 s for hard cheese). Transfer this first buffer/cheese mixture quantitatively into a 25 ml one-mark volumetric flask ([6.10](#)).

9.4.3 Add a second portion of 5 ml of cheese extraction buffer ([5.6](#)) while rinsing the ultra turrax® stem and mix using the ultra turrax® ([6.9](#)) approximately for 30 s. Transfer the buffer/cheese mixture quantitatively into the 25 ml one-mark volumetric flask ([6.10](#)).

9.4.4 Rinse the ultra turrax® stem and the beaker with a new portion of 5 ml of cheese extraction buffer (5.6) and transfer to the 25 ml volumetric flask. Dilute to the 25 ml mark with cheese extraction buffer (5.6) and shake gently.

9.4.5 Introduce 5 ml of the final buffer/cheese mixture in a glass test tube for centrifuge (6.12). Centrifuge at 1 000 *g* at 4 °C during 10 min to remove the fat.

After centrifugation, three more or less distinct layers are obtained: a pellet, a supernatant and the surface fat.

9.4.6 Incline the glass test tube (6.12) and introduce the pipette Pasteur (6.13) into the supernatant and pipette a volume of the supernatant corresponding to the capacity of the pipette Pasteur. Transfer in a beaker of 5 ml (6.8).

From this point on, measurements carried out (9.4.7 to 9.4.9) are done in the same way as for the determination of alkaline phosphatase activity in milk described in ISO 11816-1|IDF 155-1, replacing the milk sample by the supernatant.

9.4.7 Dispense, using a pipette (6.3.1), 2,0 ml of working substrate (5.3) into a labelled cuvette. Place the cuvette in the heating block (6.4) set at 38 °C and heat for 20 min.

9.4.8 Using a pipette (6.3.2), add 0,075 ml of the supernatant (9.4.6) to the substrate. Cover the cuvette with film (6.5). Immediately mix its contents using the vortex mixer (6.6) 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 (6.1). Start the test within 20 s after the addition of the test portion to the substrate.

9.4.9 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 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.

If the activity of the supernatant is higher than 7 000 mU/l, then dilute the supernatant with the cheese extraction buffer (5.6) so as to obtain a measurement not higher than 7 000 mU/l.

The instrument may display and print out the message “Error: Unstable Reading, Repeat Test”. 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.

9.5 Test sample related controls

9.5.1 Recommended negative and positive control tests

9.5.1.1 Negative control test

A cheese can be included made from pasteurized milk as negative control test with each batch of test samples. Value of the alkaline phosphatase activity of the negative test sample, expressed in milliunits of enzyme activity per gram, shall be <10.

9.5.1.2 Positive control test

One or more positive controls can be included with each batch of test samples.

9.5.2 Interfering substance test

Where higher than expected ALP values are obtained, add, using a pipette (6.3.2), 0,075 ml of the supernatant (9.4.6) to a cuvette with 2,0 ml of calibrator solution A (5.4.1), which was previously pre-warmed in the heating block (6.4) set at 38 °C for 20 min, and mix.

Place the cuvette in the Fluorophos® instrument (6.1) and test as specified in (9.4.9). 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.

9.5.3 Heat-stable microbial alkaline phosphatase control test

The control test for microbial alkaline phosphatase is recommended.

If in cheeses produced with pasteurized milk the determination (9.4) produces a positive result, then the microbial alkaline phosphatase test is compulsory.

Introduce another test portion of the supernatant (9.4.6) into a tube. Place a thermometer or thermistor probe into the tube and put the whole in the water bath (6.14) maintained 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 9.4.7 to 9.4.9, using 0,075 ml of the supernatant heated at 63 °C for 30 min. Any residual activity is due to the presence of heat-stable microbial alkaline phosphatase.

10 Calculation and expression of results

10.1 Calibration ratio

Results are calculated automatically by the Fluorophos® instrument by means of the algorithm built into the filter fluorimeter (6.1). If the results are to be calculated manually, proceed as follows.

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 fluorimeter (6.1).

Calculate the calibration ratio, K , using Formula (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 (5.4.3) against calibrator solution A (5.4.1) set at zero fluorescence (see 9.3);

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 9.3).

10.2 Calculation

10.2.1 Supernatant

Calculate the alkaline phosphatase activity of the supernatant, ALP₁ (9.4), using [Formula \(2\)](#):

$$ALP_1 = \frac{F_{av} \times c_B}{K \times V} \times f_1 \quad (2)$$

where

ALP₁ is the numerical value of the alkaline phosphatase activity of the supernatant (9.4), expressed in milliunits of enzyme activity per litre;

F_{av} is the numerical value of the average amount of fluorescence produced per minute in the test portion (9.4), measured against calibrator solution A (see 9.3) 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 (5.4.2), in micromoles per 2 ml of calibrator;

f_1 is the numerical value of the conversion factor from units per millilitre to milliunits per litre;

$$f_1 = 1 \times 10^6$$

V is the numerical value of the volume of the test portion, in millilitres.

10.2.2 Cheese

Calculate the alkaline phosphatase activity of the test sample, ALP, using [Formula \(3\)](#):

$$ALP = \frac{ALP_1 \times 25 \times f_2}{1\,000 \times m} \quad (3)$$

where

ALP is the numerical value of the alkaline phosphatase activity of the test sample, expressed in milliunits of enzyme activity per gram;

ALP₁ is the numerical value of the alkaline phosphatase activity of the supernatant (9.4), expressed in milliunits of enzyme activity per litre;

f_2 is the numerical value of the dilution factor, corresponding to the secondary dilution, if any, of the initial supernatant to obtain an activity detection value of not more than 7 000 mU per litre in the test portion;

m is the mass, in grams, of the test portion in the 25 ml one-mark volumetric flask (9.4).

If ALP₁ reads <10 mU/l, report ALP (value of the alkaline phosphatase activity of the test sample) as 1 mU/g.

10.3 Expression of results

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

11 Precision

11.1 Interlaboratory test

The values for repeatability and reproducibility limits were derived from the results of interlaboratory tests carried out in accordance with ISO 5725-1 and ISO 5725-2. The values are expressed for the 95 % probability level and may not be applicable to concentration ranges and matrices other than those given.

11.2 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, will in not more than 5 % of the cases be greater than 12 % of the mean of the two determinations.

11.3 Reproducibility

The absolute difference between two independent single test results, obtained using the same method on identical test material in different laboratories by different operators using different equipment, will in not more than 5 % of the cases be greater than 29 % of the mean of the two determinations.

12 Test report

The test report shall specify the following information:

- a) all information necessary for the complete identification of the sample;
- b) the sampling method used, if known;
- c) the test method used, together with reference to this part of ISO 11816|IDF 155, i.e. ISO 11816-2|IDF 155-2:2016;
- d) 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);
- e) the test result(s) obtained, or, if the repeatability has been checked, the final quoted result obtained.

Annex A (informative)

Interlaboratory trial

The data given in [Table A.1](#) were obtained in two interlaboratory studies organized by ANSES and conducted in accordance with ISO 5725-1 and ISO 5725-2 for collaborative study procedures to validate characteristics of a method of analysis.

A first collaborative trial, involving 14 laboratories from 13 countries (Austria, Belgium, Finland, France, Germany, Ireland, Italy, United Kingdom, Netherlands, Poland, Slovenia, Spain and Switzerland) was carried out in November 2013 on hard and semi-hard cheeses from cow milk submitted to different heat treatments. Two laboratories did not respect the repeatability conditions and one showed significant differences compared to the others for two of the samples. They were therefore excluded from the statistical evaluation.

NOTE One hard cheese sample from pasteurized milk was also submitted to the validation trial and all participants gave their AP content as 1 mU/g. No statistical parameters can be calculated for this (pasteurized) sample.

A second collaborative trial, involving 15 laboratories from 12 countries (Austria, Belgium, France, Germany, Ireland, Italy, United Kingdom, Netherlands, Poland, Portugal, Slovenia and Switzerland) was organized in March 2014 on soft and semi-hard cheeses having undergone different heat treatments. Three laboratories showed significant differences compared to the others depending on the samples and were therefore excluded from the statistical evaluation for the relevant samples. The detailed results of these collaborative trials are reported in Reference [5].

Table A.1 — Summary of collaborative trial statistical parameters

Sample	1 ^a	2 ^b	3 ^c	4 ^d	5 ^e	6 ^f	7 ^g
Year of interlaboratory test	2013	2013	2013	2014	2014	2014	2014
Number of participating laboratories	14	14	14	15	15	15	15
Number of outliers	0	0	0	0	1	0	1
Number of accepted results	11	11	12	12	14	15	14
Mean value, \bar{x} , mU/g	994	1 964	136	4 408	2 608	104	973
Repeatability standard deviation s_r , mU/g	41	69	5	262	133	4	28
Repeatability relative standard deviation, RSD_r , %	4	4	4	6	5	4	3
Repeatability limit r [$r = 2,8 \times s_r$], mU/g	115	194	14	732	373	12	78
Repeatability relative, %	12	10	10	17	14	11	8
Reproducibility standard deviation s_R , mU/g	105	234	11	570	249	11	83
^a Hard cheese sample 1, Grana Padano (grinded). ^b Hard cheese sample 2, Comté. ^c Semi-hard cheese sample 1, Raclette. ^d Semi-hard cheese sample 2, Raclette. ^e Soft cheese sample 1, Brie (mixed). ^f Soft cheese sample 2, Brie. ^g Soft cheese sample 3, Brie.							

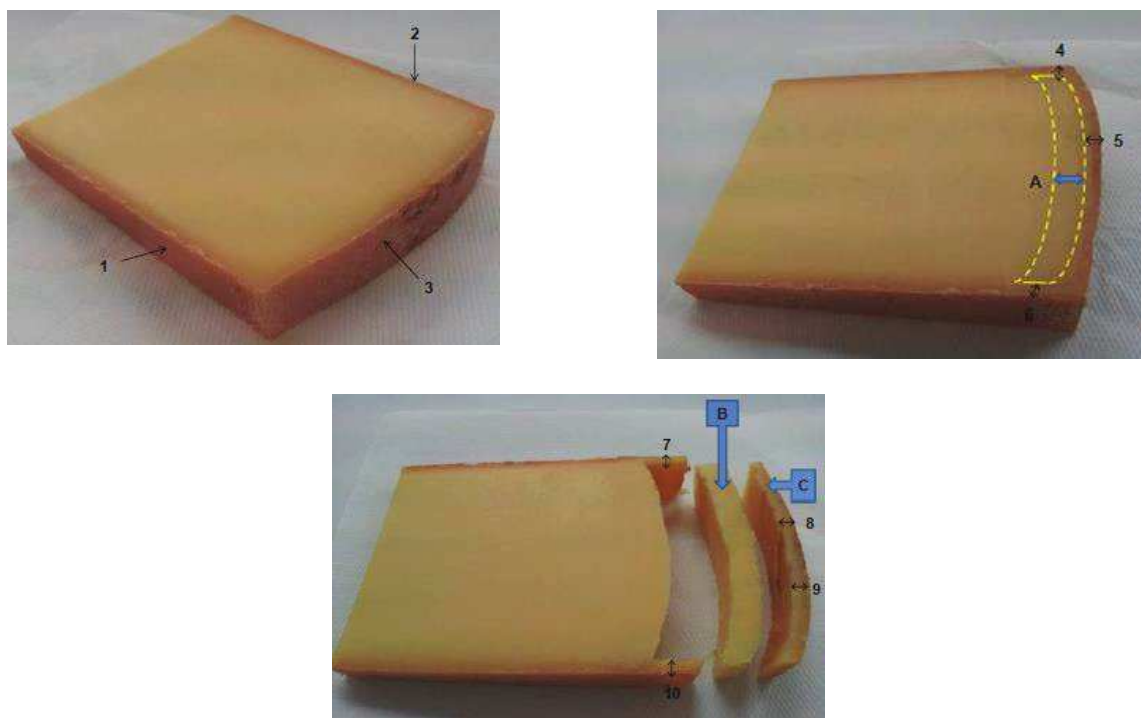
Table A.1 (continued)

Sample	1 ^a	2 ^b	3 ^c	4 ^d	5 ^e	6 ^f	7 ^g
Reproducibility relative standard deviation, RSD_R , %	11	12	8	13	10	11	9
Reproducibility limit R [$R = 2,8 \times s_R$], mU/g	295	654	32	1 595	696	31	232
Reproducibility relative, %	30	33	23	36	27	30	24
^a Hard cheese sample 1, Grana Padano (grinded). ^b Hard cheese sample 2, Comté. ^c Semi-hard cheese sample 1, Raclette. ^d Semi-hard cheese sample 2, Raclette. ^e Soft cheese sample 1, Brie (mixed). ^f Soft cheese sample 2, Brie. ^g Soft cheese sample 3, Brie.							

Annex B (informative)

Examples of preparation of a test sample

This annex gives some examples of preparation of test samples for the determination of alkaline phosphatase activity in large hard cheese, soft cheese and semi-hard cheese.



Key

1	rind	8	0,5 cm
2	rind	9	0,5 cm
3	rind	10	0,5 cm
4	0,5 cm	A	test portion 1 cm
5	0,5 cm	B	test portion 1 cm
6	0,5 cm	C	rind
7	0,5 cm		

Figure B.1 — Preparation of the test sample of a large hard cheese



Figure B.2 — Preparation of the test sample of a soft cheese



Figure B.3 — Preparation of the test sample of a semi-hard cheese

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ISO 11816-2:2016(E)
IDF 155-2:2016(E)

ICS 67.100.30

Price based on 15 pages

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