International Standard

ISO 11816-2

IDF 155-2

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

Third edition
2024-01

Reference numbers
ISO 11816-2:2024(en)
IDF 155-2:2024(en)

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Published in Switzerland

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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 document 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 of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document 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 the European Committee for Standardization (CEN) Technical Committee CEN/TC 302, *Milk and milk products — Methods of sampling and analysis*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement). It is being published jointly by ISO and IDF.

This third edition cancels and replaces the second edition (ISO 11816-2 | IDF 155-2:2016), which has been technically revised.

The main changes are as follows:

— the FLM200 instrument (which has been discontinued) has been replaced by the FLM300 version;

— the instructions for use of the instrument and the flow of those instructions have been revised in accordance with FLM300, which has an upgraded user interface and electronics (there has been no change to the assay or the test procedure with the changes to the interface and software);

— the instrument now includes the heater block which was a separate item previously.

A list of all parts in the ISO 11816 | IDF 155 series can be found on the ISO website.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

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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This document 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.* It is being published jointly by ISO and IDF.

The work was carried out by the IDF/ISO Action Team P19 of the *Standing Committee on Analytical Methods for Processing Aids and Indicators* under the aegis of its project leader Mr Rick Zampa (US).

Milk and milk products — Determination of alkaline phosphatase activity —

Part 2:
Fluorimetric method for cheese

1 Scope

This document 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 used for the determination of ALP can read activities in the supernatant up to 7 000 milliunits per litre (mU/kg).

2 Normative references

There are no normative references in this document.

3 Terms and definitions

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

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
alkaline phosphatase activity
ALP activity
activity of the enzyme present in the product, determined by the specified procedure

Note 1 to entry: The ALP activity is expressed as milliunits of enzyme activity per gram of sample (mU/kg).

4 Principle

The ALP 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 ALP derived from the sample, undergoes hydrolysis of its phosphate radical, producing a highly fluorescent product. Fluorimetric measurement of ALP activity is measured at

38 °C over a 3 min period when using a 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.

The reagents specified in 5.1 to 5.7 and the apparatus specified in 6.1 to 6.4 (except 6.3.1) comprise the Fluorophos® Test System[2]. The manufacturer can 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.

5.1 Fluorophos® substrate, in bottles, each containing 144 mg of Fluorophos® substrate powder, molar mass of 580 g/mol.

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

This 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.

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

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.

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 of substrate buffer solution (240 ml) (5.2) to that of one bottle of 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 analogue-to-digital (A/D) test given in 9.1.3 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 between 2 °C and 8 °C, or for 6 h when stored at 38 °C.

1) Fluorophos® is the registered trademark of a product supplied by Advanced Instruments, LLC. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of the product named. Equivalent products may be used if they can be shown to lead to the same results.

2) The Fluorophos® Test System is the trade name of a product supplied by Advanced Instruments, LLC. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of the product named. Equivalent products may be used if they can be shown to lead to the same results.

5.4 Calibrator solutions, Fluoroyellow®[3] (FY) [2′-(2-benzothiazolyl)-6′-hydroxybenzothiazole] in substrate buffer solution (5.2).

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.

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 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.

5.6 Fluorophos® cheese extraction buffer, diethanolamine (DEA) buffer, pH-value 8,0 with magnesium and Triton X-100.

The cheese extraction buffer remains stable for three years from the date of manufacture, provided it is stored in unopened bottles at between 2 °C and 8 °C.

5.7 Positive, negative and PhosphaCheck-N™ controls[4].

6 Apparatus

Usual laboratory equipment and, in particular, the following shall be used.

6.1 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 between 520 nm and 560 nm [e.g. Fluorophos® instrument].

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 °C ± 1 °C, suitable for holding cuvettes.

6.5 Plastic paraffin film (e.g. Parafilm®[5]) or other suitable laboratory-grade film.

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3) Fluoroyellow® is the registered trademark of a product supplied by Advanced Instruments, LLC. 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.

4) The controls and instrument performance check instructions are products supplied by Advanced Instruments, LLC. 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) Parafilm® is an example 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 ISO or IDF of this product.

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).

6.9 High-speed homogenizer (e.g. ULTRA-TURRAX®[6]) 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 1 000 *g* 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 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 document. A recommended sampling method is given in ISO 707 | IDF 50.

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 homogenously 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. Examples of preparation of a test sample of a large hard cheese (Figure B.1), a soft cheese (Figure B.2) and a semi-hard cheese (Figure B.3) are given in Annex B.

6) ULTRA-TURRAX® is an example 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 ISO or IDF of this product.

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 include the following:

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 (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, which are recommended for monitoring daily instrument precision parameters.

9.1.2 Daily instrument tests

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

9.1.3 Using FLM200

9.1.3.1 Access the A/D test according to the instrument's user guide through "SETUP" menu. Press "SETUP" key, then select menu items "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 FLU ± 4 FLU. If the reading is outside that range, clean the excitation and emission filters, according to manufacturer's recommendations and repeat the A/D test.

9.1.3.2 Dispense 2,0 ml of daily instrument control solution (5.5) into a labelled cuvette. Place the cuvette in the heating block (6.4) 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 FLU ± 12 FLU. 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 FLU. Allow the numbers to equilibrate for 15 min.

9.1.4 Using FLM300

9.1.4.1 Access the A/D test according to the instrument's user guide through "SETUP" menu. With nothing in the cuvette holder, press "SETUP". Select "Diag.", by moving the [*] with the [<or >] keys and press "ENTER". "A/D Test" will be displayed. Press "START". Allow the figures appearing on the display screen to stabilize. The display should read 302 FLU ± 4 FLU. If the reading is outside that range, clean the excitation and emission filters, according to manufacturer's recommendations and repeat the A/D test.

9.1.4.2 Dispense 2,0 ml of daily instrument control solution (5.5) into a labelled cuvette. Place the cuvette in the heating block (6.4) set at 38 °C for 20 min. Press "SETUP". Select "SETUP" by moving the [*] with the [< or >] keys and press "ENTER". "Daily Instrument Control Adj." will be scrolling across the screen. Press "START" to select it. Insert the cuvette labelled C1 into the instrument chamber, close the door, and press "ENTER". C1 shall read 602 FLU ± 12 FLU. If it does not meet this specification, move the [*] with the [< or >] keys to the right of "YES" to adjust it into the specification. If it does meet this specification, move the [*] with the [< or >] keys to the left of "NO" and press "ENTER".

9.1.5 Controls

Perform positive, negative and PhosphaCheck-N™ controls using a powdered milk base, with phosphatase and preservative (5.7).

The PhosphaCheck-N™ pasteurization controls remain stable for 18 months from the date of manufacture, provided they are stored in unopened and unreconstituted bottles at between 2 °C and 8 °C. Once reconstituted, the controls are stable for three days (72 h) at between 2 °C and 8 °C. Do not freeze.

Allow the controls to come to room temperature. Reconstitute the PhosphaCheck-N™ pasteurization controls before use. Remove the metal and rubber stopper. Add 3,0 ml of deionized 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 75 µl 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 mU/l ± 100 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.

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), or when assayed control values show unacceptable results leading to adjustments to bring the 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.

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, of calibrator solution B and of 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.

Add, to six cuvettes using the positive displacement or air-displacement pipette (6.3.2), 0,075 ml of the supernatant (see 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.

9.3.2 Using FLM200

9.3.2.1 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). When using the FLM200 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 into the fluorimeter and press "START". When the measurement is finished, measure the second A calibrator solution.

9.3.2.2 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.

9.3.3 Using FLM300

9.3.3.1 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). When using the FLM300 instrument, press "CALIBRATE". Select "Cheeses or Other (mU/kg)" with the [< or >] keys and press "ENTER". Using the [< or >] keys, select the channel to be calibrated and press "ENTER". Beginning with calibrator solution A (5.4.1), insert this solution into the fluorimeter and press "START". When the measurement is finished, measure the second A calibrator solution.

9.3.3.2 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 (see 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 the cheese extraction buffer (5.6). Let the cheese extraction buffer be in contact with the cheese for 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 the 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 the cheese extraction buffer (5.6) and transfer to the 25 ml volumetric flask. Dilute to the 25 ml mark with the 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 (6.12) for centrifuge. 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), 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 into a beaker of 5 ml (6.8).

From this point on, measurements carried out (see 9.4.7 to 9.4.9) are done in the same way as for the determination of ALP 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 Using FLM200

9.4.9.1 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.

9.4.10 Using FLM300

Press the "TEST" key. Select "Cheeses or Other (mU/kg)" with the [< or >] keys and press ENTER. Using the [< or >] keys, select the product to be analysed and press "ENTER". Enter an ID number and press "ENTER". Press "START" 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 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/kg, then dilute the supernatant with the cheese extraction buffer (5.6) so as to obtain a measurement not higher than 7 000 mU/kg.

It is possible that the instrument displays and prints 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 a negative control test with each batch of test samples. The value of the ALP 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 (see 9.4.6) into 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/kg, an interfering substance is shown to be present. In that case, repeat the test using a fresh sample.

9.5.3 Heat-stable microbial ALP control test

The control test for microbial ALP is recommended.

If in cheeses produced with pasteurized milk the determination (see 9.4) produces a positive result, then the microbial ALP 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 tube with the new test portion 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 ALP.

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 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_c is the area in square meters of the measurement surface (in the case of this procedure, S is a sphere with a radius of 1 m, i.e. $S = 4\pi$);

F_B 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 ALP activity of the supernatant, ALP_1 (see 9.4), using Formula (2):

$$A_{p1} = \frac{F_{av} \times c_B}{K \times V} \times f_1 \quad (2)$$

where

Ap_1 is the numerical value of the ALP activity of the supernatant (see 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 (see 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 ALP activity of the test sample, APL, using Formula (3):

$$A_p = \frac{A_{p1} \times 25 \times f_2}{1\,000 \times m} \quad (3)$$

where

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

A_{p1} is the numerical value of the ALP activity of the supernatant (see 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 (see 9.4).

If A_{P1} reads < 10 mU/kg, report ALP (value of the ALP activity of the test sample) as 1 mU/kg.

10.3 Expression of test results

Express the test results to the nearest whole unit of a milliunit. Results rounded to 1 mU/kg are not required at all levels since once the result is above 200 mU/kg the product has failed to pass the test and further accuracy is not required.

11 Precision

11.1 Interlaboratory study

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

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 at least 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 a reference to this document, i.e. ISO 11816-2 | IDF 155-2;

d) all operating details not specified in this document, or regarded as optional, together with details of any incidents which can have influenced the test result(s);

e) the test result(s) obtained, or, if the repeatability has been checked, the final quoted result obtained;

f) the date of the test.

Annex A
(informative)

Interlaboratory study

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 ALP 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 [8].

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 coefficient of variation, $C_{V,r}$, %	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 coefficient of variation, $C_{V,R}$ %	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.**