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**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

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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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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- the FLM200 instrument (which has been discontinued) has been replaced by the FLM300 version;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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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 System²⁾. 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, $c(\text{DEA}) = 2,4 \text{ mol/l}$, 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[™] controls⁴⁾.

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 $38 \text{ °C} \pm 1 \text{ °C}$ and right-angle optics, allowing excitation at a wavelength of 440 nm and emission between 520 nm and 560 nm [e.g. Fluorophos[®] 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 $38 \text{ °C} \pm 1 \text{ °C}$, suitable for holding cuvettes.

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.

- 6.5 **Plastic paraffin film** (e.g. Parafilm^{®5}) 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).
- 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 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. Examples of preparation of a test sample

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.

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