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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 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](http://www.iso.org/foreword)

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*

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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* 6-2-2016

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 1 000*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 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.