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

**Part 1:  
Fluorimetric method for milk and  
milk-based drinks**

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

*Partie 1: Méthode fluorimétrique pour le lait et les boissons à base de lait*

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## Foreword

**ISO (the International Organization for Standardization)** is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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. [www.iso.org/directives](http://www.iso.org/directives)

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Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

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 third edition of ISO 11816-1|IDF 155-1 cancels and replaces the second edition (ISO 11816-1:2006), which has been technically revised.

ISO 11816|IDF 155 consists of the following parts, under the general title *Milk and milk products — Determination of alkaline phosphatase activity*:

- *Part 1: Fluorimetric method for milk and milk-based drinks*
- *Part 2: Fluorimetric method for cheese*

## Foreword

**IDF (the International Dairy Federation)** is a non-profit organization representing the dairy sector worldwide. IDF membership comprises National Committees in every member country as well as regional dairy associations having signed a formal agreement on cooperation with IDF. All members of IDF have the right to be represented on the IDF Standing Committees carrying out the technical work. IDF collaborates with ISO in the development of standard methods of analysis and sampling for milk and milk products.

The main task of Standing Committees is to prepare International Standards. Draft International Standards adopted by the Standing Committees are circulated to the National Committees for endorsement prior to publication as an International Standard. Publication as an International Standard requires approval by at least 50 % of IDF National Committees casting a vote.

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ISO 11816-1|IDF 155-1 was prepared by the International Dairy Federation (IDF) and Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*. It is being published jointly by IDF and ISO.

All work was carried out by the Joint ISO-IDF Project Group on *Determination of alkaline phosphatase activity – fluorimetric method*, of the Standing Committee on *Analytical Methods for Processing Aids and Indicators*, under the aegis of its project leader, Ms. Eileen Garry (USA).

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

## Part 1: Fluorimetric method for milk and milk-based drinks

### 1 Scope

This part of ISO 11816|IDF 155 specifies a fluorimetric method for the determination of alkaline phosphatase (ALP, EC 3.1.3.1) activity in raw and heat-treated whole milk, semi-skimmed milk, skimmed milk and flavoured milks. This method is applicable to milk and milk-based drinks from cows, sheep and goats. It is also applicable to milk powder after reconstitution.

The instrument can read activities up to 7 000 milliunits per litre (mU/l). If the activity is higher than 7 000 mU/l, it is diluted with alkaline phosphatase-free milk (7.1) so as to obtain a measurement not higher than 7 000 mU/l.

### 2 Terms and definitions

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

#### 2.1

##### **alkaline phosphatase (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 litre of sample (mU/l).

#### 2.2

##### **unit of alkaline phosphatase activity**

amount of alkaline phosphatase enzyme that catalyses the transformation of 1  $\mu\text{mol}$  of substrate per minute

### 3 Principle

The alkaline phosphatase activity of the sample is measured by a continuous fluorimetric direct kinetic assay. A non-fluorescent aromatic monophosphoric ester substrate, 2'-[2-benzothiazolyl]-6'-hydroxybenzothiazole phosphate, in the presence of any alkaline phosphatase derived from the sample, undergoes hydrolysis of its phosphate radical, producing a highly fluorescent product. Fluorimetric measurement of alkaline phosphatase (ALP) activity is measured at 38 °C over a 3-min period when using Fluorophos®. This includes pre-incubation of substrate and sample, followed by multiple kinetic readings of the reaction rate.

**NOTE** Although this is a 3-min test, the first minute is an equilibration period to ensure that the sample is at 38 °C. Measurements of activity are actually made from the beginning of the second minute to the end of the third minute (i.e. over a 2-min period).

### 4 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled or demineralized water, or water of equivalent purity.

**4.1 Fluorophos® substrate**,<sup>1)</sup> in bottles, each containing 144 mg of Fluorophos® substrate powder, molecular weight 580 grams per mole.

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

**4.2 Substrate buffer solution**, diethanolamine (DEA) buffer solution,  $c(\text{DEA}) = 2,4 \text{ mol/l}$ , with pH 10,0, in bottles of 240 ml each. The substrate buffer solution remains stable for two years from the date of manufacture, provided it is stored in unopened bottles at between 2 °C and 8 °C; protect against light.

#### 4.3 Working substrate

Allow the Fluorophos® substrate (4.1) and the substrate buffer solution (4.2) to come to room temperature. Add the content of one bottle of substrate buffer solution (240 ml) (4.2) to that of one bottle of Fluorophos® substrate (144 mg) (4.1), and mix well by inversion for 3 min to create ~1,0 millimolar (pH 10) solution. 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 A/D (analogue-to-digital) test given in 8.2 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 (8.2).

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

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

**4.4 Calibrator solutions**, Fluoroyellow®(FY) [2-(2-benzothiazolyl)-6'-hydroxybenzothiazole] in substrate buffer solution (4.2).

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.

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

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

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

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

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 Apparatus

Usual laboratory equipment and, in particular, the following.

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1) The reagents specified in 4.1 to 4.5 and the apparatus specified in 5.1 to 5.4 (except 5.3.3) comprise the Fluorophos Test System, which is the trade name of a product supplied by Advanced Instruments, Inc., Two Technology Way, Norwood, Massachusetts 02062, USA. The manufacturer may 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. Fluorophos and Fluoroyellow are trademarks of Advanced Instruments, Inc. 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.

**5.1 Filter fluorimeter**, with thermostatically controlled cuvette holder, capable of operating at  $38\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$  and right-angle optics, allowing excitation at a wavelength of 440 nm and emission at between 520 nm and 560 nm [e.g. Fluorophos® instrument<sup>1</sup>].

**5.2 Cuvettes**, disposable, of non-fluorescent glass.

**5.3 Pipettes**

**5.3.1 Fixed-volume dispenser**, capable of dispensing 2,0 ml.

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

Follow strict instructions for pipetting technique as this is a critical step in generating accurate results. Ensure that piston of pipette bore is tightly secured prior to use.

**5.3.3 Pipettes**, of capacity 2 ml and 3 ml.

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

**5.5 Suitable laboratory-grade film.**

**5.6 Vortex mixer.**

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

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

## 6 Sampling

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.

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

## 7 Preparations

### 7.1 Alkaline phosphatase-free milk

Prepare phosphatase-free milk of the type to be tested by carefully dispensing the desired portion of milk into a test tube or suitable container, ensuring that no milk touches the rim or sides of the container.

Place the tube or container with the milk portion in the water bath (5.7) set at  $95\text{ }^{\circ}\text{C}$ . Preheat the milk portion to  $95\text{ }^{\circ}\text{C}$ , before starting its 5-min heating period at that temperature. Check the temperature by using a thermometer or thermistor probe placed in the centre of the tube or container. When the milk portion reaches  $95\text{ }^{\circ}\text{C}$ , immediately start its 5-min heating period. Cool the whole portion rapidly after the heating period.

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

## 7.2 Preparation of test sample

### 7.2.1 General

Carefully mix all test samples prior to use.

NOTE It is usually not necessary to prewarm test samples.

### 7.2.2 Pasteurized test samples

Use pasteurized test samples as delivered, in amounts as required.

### 7.2.3 Dilution of test samples with high ALP values

Prepare dilutions of the test samples of milk using phosphatase-free milk (7.1) in order to bring their ALP levels within the analytical range of assay (<7 000 mU/l). Mix the diluted solutions well.

## 8 Procedure

### 8.1 Verification of instrument performance

#### 8.1.1 General

It is important to check instrument performance for drift, stray light and stability prior to analysing test samples. Follow good laboratory practice principles when operating the filter fluorimeter (5.1).

Quality control tests include

- 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 (4.5) to monitor any electronic or optical drift in the fluorimeter, and
- c) the use of external positive, negative and normal controls, described in 8.1.3, which are recommended for monitoring daily instrument precision parameters.

#### 8.1.2 A/D tests

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

**8.1.2.2 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  $302 \pm 4$ . If the reading is outside that range, clean the excitation and emission filters and repeat the A/D test.

**8.1.2.3 Dispense 2,0 ml of daily instrument control solution (4.5) into a labelled cuvette.** Place the cuvette in the incubator block (5.4) 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  $602 \pm 12$ . 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.

### 8.1.3 Controls

Perform positive, negative and PhosphaCheck-N controls<sup>2)</sup> using a powdered milk base with phosphatase and preservative.

The PhosphaCheck® pasteurization controls remain stable for 18 months from the date of manufacture, provided they are stored in unopened and unreconstituted bottles between 2 °C and 8 °C. Once reconstituted, the controls are stable for three days at 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 deionized water at room temperature.

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 prewarmed substrate. Perform the ALP test.

The reading for the negative control shall be < 10 mU/l, the Phosphacheck-N™ normal shall be between 10 and 40 mU/l, and the positive shall be 500 ± 100 mU/l. These controls can be used daily to monitor the precision of the instrument.

## 8.2 Reagent controls to test the suitability of ready-to-use working substrate (4.3)

Dispense 2,0 ml of the working substrate (4.3) into a labelled cuvette. Place the cuvette in the heating block (5.4) set at 38 °C for 20 min. Insert the prewarmed 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.

## 8.3 Calibration

Calibration curves are usually stable. However, recalibrate the instrument, which has already been calibrated, when the fluorimeter is initially installed, whenever servicing procedures (i.e. lamp or filter replacement) are likely to affect the stored calibration, or when assayed control values show unacceptable results.

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

Mix calibrator solutions A, B and C by gentle inversion prior to use. Transfer, using the pipette (5.3.3), 2,0 ml of calibrator solution A, of calibrator solution B and of calibrator solution C (4.4.1, 4.4.2 and 4.4.3) respectively, each in duplicate, to six prelabelled cuvettes (5.2). Place the cuvettes in the incubator block (5.4) set at 38 °C and preheat for 20 min.

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

Starting with calibrator solution A, perform the following calibration routine. Wipe the outside of each cuvette with soft tissue before placing the cuvette in the filter fluorimeter (5.1). When using the Fluorophos® instrument, press "CALIB" and select the "ALP Dairy" menu. Scroll through the menu and

2) The controls and instrument performance check instructions are products supplied by Advanced Instruments, Inc., Two Technology Way, Norwood, Massachusetts 02062, USA. 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.

press "ENTER" when the product to be calibrated is displayed. Beginning with calibrator solution A (4.4.1), insert this solution in the fluorimeter and press "START". When the measurement is finished, measure the second A calibrator solution.

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

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

## **8.4 Determination**

Dispense, using the fixed volume dispenser (5.3.1) or pipette, 2,0 ml of working substrate (4.3) into a labelled cuvette. Place the cuvette in the incubator block (5.4) set at 38 °C and heat for 20 min.

Add, using the pipette (5.3.2), 0,075 ml of the well-mixed test portion (7.2.2 or 7.2.3) to the substrate. Cover the cuvette with suitable laboratory-grade film (5.5). Immediately mix its contents using the vortex mixer (5.6) for 5 s or by gently inverting the cuvette. Wipe the outside of the cuvette with soft tissue and place it into the filter fluorimeter (5.1). The test must be started within 20 s after the addition of the test portion to the substrate.

When using the Fluorophos® instrument, press the "TEST" key, "ALP Dairy" appears, then press "ENTER". Scroll through the menu and press "ENTER" when the product to be analysed is displayed. Then press the "START" key to begin the test. The display will count down 60 s while the substrate and sample are being warmed to 38 °C. After 60 s, the fluorimeter starts measuring, displaying a fluorescence of the sample in fluorescence units (FLU). The display starts at around 200 FLU and slowly increases over the next 2 min. At the end of the 3-min period, the Fluorophos® automatically performs 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.

If results are to be calculated manually (9.2), divide the difference of 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 is higher than 7 000 mU/l, then dilute with the alkaline phosphatase-free milk (7.1) so as to obtain a measurement not higher than 7 000 mU/l.

The Fluorophos® instrument might 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.

## **8.5 Test sample-related controls**

### **8.5.1 Recommended negative and positive control tests**

#### **8.5.1.1 Negative control test**

Include a negative control test with each batch of test samples. Heat a test sample as described in 7.1. The instrument reading shall be less than 10 mU/l.

#### **8.5.1.2 Positive control test**

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

### 8.5.2 Interfering substance test

Where higher than expected ALP values are obtained, add, using a pipette (5.3.2), 0,075 ml of test portion (7.2.2 or 7.2.3) into a cuvette with 2,0 ml of calibrator solution A (4.4.1), which was previously prewarmed in the incubator block (5.4) set at 38 °C for 20 min, and mix.

Place the cuvette containing this mixture in the instrument (5.1) and test as in 8.4. If the obtained value exceeds 20 mU/l, an interfering substance is shown to be present. In that case, repeat the test using a fresh sample.

### 8.5.3 Heat-stable microbial alkaline phosphatase control test

If the determination (8.4) produces a higher result than the one expected, proceed as follows: Add another test portion (7.2.2 or 7.2.3) to a tube. Place a thermometer or thermistor probe into the tube and put the whole portion in the water bath (5.7) set at 63 °C. When the test portion reaches 63 °C, keep it at that temperature for 30 min, then cool rapidly. Determine any residual phosphatase activity according to 8.4. Any residual activity is due to the presence of heat-stable microbial alkaline phosphatase.

## 9 Calculation and expression of results

### 9.1 Calibration ratio

Results are calculated automatically by the Fluorophos® instrument by means of the algorithm built into the filter fluorimeter (5.1). If results are to be calculated manually, proceed as follows.

Record the fluorescence values of calibrator solution B (4.4.2) and calibrator solution C (4.4.3) read against calibrator solution A (4.4.1) set to zero fluorescence on the filter fluorimeter (5.1).

Calculate the calibration ratio,  $K$ , using 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 (4.4.3) against calibrator solution A (4.4.1) set at zero fluorescence (see 8.3);

$F_B$  is the numerical value of the fluorescence obtained by measuring calibrator solution B (4.4.2) against calibrator solution A (4.4.1) set at zero fluorescence (see 8.3).

## 9.2 Calculation

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

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

where

$A_p$  is the numerical value of the alkaline phosphatase activity of the test sample (7.2.2 or 7.2.3), in milliunits of enzyme activity per litre;

$F_{av}$  is the numerical value of the average amount of fluorescence produced per minute by the test portion (8.4), measured against calibrator solution A (see 8.3) 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 (4.4.2), in micromoles per 2 ml of calibrator;

$f$  is the numerical value of the conversion factor from units per millilitre to milliunits per litre;  $f = 1 \times 10^6$ ; in the case of samples diluted to obtain activities of not more than 7 000 mU/l,  $f = (\text{dilution factor of the test sample}) \times 10^6$ ;

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

## 9.3 Expression of test results

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

## 10 Precision

### 10.1 Collaborative test

Details of three collaborative tests on the precision of the method are reported in [Annex A](#). The values for repeatability and reproducibility limit are expressed for the 95 % probability level and might not be applicable to concentration ranges and matrices other than those given.

### 10.2 Repeatability

For values less than 125 mU/l, the absolute difference between two independent single test results, obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5 % of cases be greater than 14 mU/l.

For values 125 mU/l or higher and less than 620 mU/l, the absolute difference between two independent single test results, obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5 % of cases be greater than 12 % of the mean of the two determinations.

### 10.3 Reproducibility

For values less than 125 mU/l, the absolute difference between two single test results, obtained with the same method on identical test material in different laboratories with different operators using different equipment, will in not more than 5 % of cases be greater than 23 mU/l.

For values 125 mU/l or higher and less than 620 mU/l, the absolute difference between two single test results, obtained with the same method on identical test material in different laboratories with different

operators using different equipment, will in not more than 5 % of cases be greater than 24 % of the mean of the two determinations.

## 11 Test report

The test report shall specify:

- 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;
- 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 might have influenced the test result(s);
- e) the test result(s) obtained, or, if the repeatability has been checked, the final quoted result obtained.

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## Annex A (informative)

### Collaborative trials

A collaborative trial, organized by QuadraChem Laboratories and Frank Harding, involving 13 laboratories from seven countries (USA, UK, France, Norway, Italy, Netherlands and Switzerland) was carried out, in accordance with ISO 5725-1 and ISO 5725-2, on four types of cow's milk (whole, semi-skimmed, skimmed, flavoured) and on whole sheep's and whole goat's milk. The trial was completed in March 2004.

NOTE 1 Only flavoured, whole sheep's and whole goat's milk results are reported in this annex for the March 2004 trial. Results from cow's milk (whole, semi-skimmed, and skimmed) are reported from subsequent trials (see below) completed in January 2008.

Another collaborative trial, organized by ANSES and Marina Nicolas, involving 19 laboratories from 18 countries (Northern Ireland, France, Austria, Switzerland, Hungary, Ireland, Norway, Germany, Finland, Belgium, Spain, Cyprus, Bulgaria, Portugal, Poland, Netherlands, Czech Republic and Greece) was carried out in accordance with ISO 5725-1 and ISO 5725-2 on cow's milk (whole, semi-skimmed and skimmed). The trial was completed in January 2008. Data from this trial replaces data from 2004 trial.

Another collaborative trial, organized by ANSES and Marina Nicolas, was carried out in accordance with ISO 5725-1 and ISO 5725-2 on UHT semi-skimmed goat's milk. The trial was completed in December 2010. Data from this trial are reported below.

The results obtained were subjected to statistical analysis in accordance with ISO 5725-1 and ISO 5725-2 to give the precision determinations of Clause 10. The actual average enzyme levels of the study sample are reported in Table A.1. Tables A.2 and A.3 report repeatability and reproducibility limits. Tables A.4 and A.5 report the coefficients of variation of repeatability and reproducibility, respectively.

NOTE 2 Reference can be made to data from Tables A2, A3, A4 and A5 to monitor laboratory performance.

The overall report of the initial study was published in Reference<sup>[5]</sup> and the report of the additional trial was published by the European Union Reference Laboratory for Milk and Milk Products, ANSES (EX-AFSSA), French Food Safety Agency.

**Table A.1 — Enzyme mean values (mU/l) for each studied level in each matrix**

Type of milk	Target enzyme level				
	20 (mU/l)	40 (mU/l)	100 (mU/l)	350 (mU/l)	500 (mU/l)
Whole cow's milk	24	40	120	350	488
Semi-skimmed cow's milk	27	40	124	345	479
Skimmed cow's milk	31	42	96	349	449
Flavoured cow's milk <sup>a</sup>	–	54	108	436	618
Whole sheep's milk	31	47	110	428	608
Whole goat's milk	22	47	125	407	570
Semi-skimmed goat's milk	29	57	110	317	474

<sup>a</sup> The flavoured milk tested in this trial was strawberry.