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Milk and milk-based drinks — Determination of alkaline phosphatase activity — Enzymatic photo-activated system (EPAS) method

Lait et boissons à base de lait — Détermination de l'activité de la phosphatase alcaline — Méthode par un système de photoactivation enzymatique

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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.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

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Foreword

IDF (the International Dairy Federation) is a worldwide federation of the dairy sector with a National Committee in every member country. Every National Committee has 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.

Draft International Standards adopted by the Action Teams and Standing Committees are circulated to the National Committees for voting. Publication as an International Standard requires approval by at least 50 % of the IDF National Committees casting a vote.

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ISO 22160 | IDF 209 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 Action Team on *Heat treatment*, of the Standing Committee on *Minor components & characterization of physical properties*, under the aegis of its project leader, Mr R. Salter (US).

Milk and milk-based drinks — Determination of alkaline phosphatase activity — Enzymatic photo-activated system (EPAS) method

1 Scope

This International Standard specifies a method for the determination of the alkaline phosphatase activity in pasteurized whole milk, semi-skimmed milk, skimmed milk, cream and flavoured milks using a chemiluminescent (EPAS) method.

The method is applicable to milk and milk-based drinks from cows, sheep, buffalo and goats.

The method is also suitable for any liquid-based sample if diluted in such a way that the diluted alkaline phosphatase activity has less than 7 000 milliunits per litre.

NOTE There has been a successful collaborative trial with cow, sheep, buffalo, and goat whole milk, as well as skimmed cow milk (< 0,5 % fat), 20 % fat cream and 2 % fat chocolate milk (all mass fractions).

2 Terms and definitions

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

2.1
alkaline phosphatase activity
ALP
activity of the alkaline phosphatase present in the product, determined according to the procedure described in this International Standard

NOTE The alkaline phosphatase activity is expressed as milliunits of enzyme activity per litre (mU/l) [4], [5].

2.2
unit of alkaline phosphatase activity
amount of alkaline phosphatase enzyme that catalyses the transformation of 1 µmol of stable aromatic substrate per minute

3 Principle

The alkaline phosphatase activity is measured by photo-activation of the hydrolysed product followed by an instrumental measurement of photo-activation. In the presence of alkaline phosphatase, a stable aromatic dioxetane-phosphate substrate is hydrolysed at 35 °C ± 1 °C to produce a photo-activated (chemiluminescent) product. The photo-activation of the product is amplified by a macromolecular enhancing component. The hydrolysis reaction is stopped after a specified incubation time (3 min). The amount of chemiluminescent product thus produced is measured and converted to enzyme units by a luminometer. Luminometer calibration is based on calibration using tablets with known enzyme activity.

4 Reagents

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

4.1 Non-chemiluminescent dioxetane ester substrate [0,2 mol/l 3-(2'-spiroadamantanane)-4-methoxy-4-(3"-phosphate phenyl-1,2 dioxetane disodium salt in DEAE buffer with 1 % fluorosine], is commercially available [e.g. as Charm reagent AP® [1)] liquid].

It is recommended to store the substrate at between 0 °C and 7 °C. If stored in amber plastic vials and at 4 °C, the substrate remains stable for 6 months. If stored at 30 °C, the substrate is stable for 24 h only.

When using the substrate in the assay, keep it at between 0 °C and 7 °C or on ice.

4.2 Stopping solution

Prepare the stopping solution by mixing the same amount of 0,15 mol/l 2-amino-2-methyl-1-propanol and 0,02 % benzalkonium chloride, pH 10,7. The stopping solution shall be at room temperature (18 °C to 24 °C) prior to use. For assay consistency when not using a thermoprobe, record and maintain the temperature to within 0,5 °C of the solution temperature used at calibration.

NOTE The stopping solution is used to stop enzymatic hydrolysis of the dioxetane ester substrate (4.1).

Commercially supplied stopping solution has a shelf life of 1 year when kept at 4 °C, or of 2 months when kept at room temperature. For daily use, room temperature storage is recommended.

4.3 Working calibrators, for example, calibration tablets (dried raw milk with measured phosphatase content in tablet form to rehydrate in milk) with a phosphatase activity of 875 µU/l ± 26 µU/l. Rehydrate the tablets in three different volumes of milk-based drink presenting no phosphatase activity or negative test sample (7.2) to create a standard calibration curve.

Commercially supplied calibration tablets may be stored at 4 °C for 2 years.

4.3.1 Fluid white milk calibrators

In each of three 50 ml test tubes (5.9), marked A_1, B_1 and C_1, respectively, dissolve one calibrator tablet in 100 µl of distilled water.

Add 20 ml to tube A_1, 5 ml to tube B_1 and 2,5 ml to tube C_1 of fluid white milk products (presenting no phosphatase activity) or negative test sample (7.2). This makes calibration standards A_1, B_1 and C_1 with a phosphatase activity of A_1 = 44 mU/l, B_1 = 175 mU/l and C_1 = 350 mU/l, respectively. Cap the tubes and shake their contents vigorously. Allow the tube contents to rehydrate under refrigeration for 10 min. Mix well before use.

4.3.2 Cream and flavoured-milk calibrators

In each of three 50 ml test tubes (5.9), marked A_2, B_2 and C_2, respectively, dissolve one calibrator tablet in 100 µl of distilled water.

Add 10 ml to tube A_2, 5 ml to tube B_2 and 2,5 ml to tube C_2 of cream or flavoured-milk products (presenting no phosphatase activity) or negative test sample (7.2), making calibration standards A_2, B_2, C_2 with phosphatase activities of A_2 = 88 mU/l, B_2 = 175 mU/l and C_2 = 350 mU/l, respectively. Cap the tubes and shake their contents vigorously. Allow the tube contents to rehydrate under refrigeration for 10 min. Mix well before use.

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1) The reagents specified in Clause 4 and the apparatus specified in 5.1 are available from Charm Sciences Inc., 659 Andover St., Lawrence, MA 01843, USA. These are examples of suitable products 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. Equivalent products may be used if they can be shown to produce the same results.

4.4 Positive control

As positive control, use freeze-dried alkaline phosphatase in a 15 ml amber bottle. Rehydrate the positive control with 10 ml of a representative dairy drink presenting no phosphatase activity, or with a prepared negative test sample (7.2). Rehydrated positive control contains 450 mU/l of phosphatase enzyme.

Allow the control to stand for 10 min to rehydrate. Vigorously shake before use.

The rehydrated positive control is stable when stored at between 0 °C and 7 °C for 48 h. Positive control rehydrated with fluid milk is stable frozen at or below −15 °C for 2 months. Thaw the positive control in water at room temperature. Vigorously shake the control to homogenize before use. Do not re-freeze.

5 Apparatus

Usual laboratory equipment and, in particular, the following.

5.1 Luminometer, capable of operating at a wavelength of 540 nm, with linear outputs being converted by internal software into enzyme activity [e.g. Charm Luminometer® models NovaLum, Luminator K or T [1)]]. A vial adapter is needed with NovaLum and Lum-T model. NovaLum and Lum-T should be used in the propped up position. A temperature probe supported by NovaLum should be used to measure the stopping solution (4.2) temperature.

Measurements should be optimized according to the manufacturer's recommendations for the apparatus used.

5.2 Mini-vials, disposable, made of non-luminescent plastic, with caps, and of capacity 2 ml.

5.3 Fixed volume pipette, of capacity 100 µl.

5.4 Fixed-volume dispenser, capable of dispensing 1,0 ml. Check that the volume of water dispensed is accurate to 1,00 g ± 0,05 g before use.

5.5 One-mark volumetric flasks, of capacity 100 ml.

5.6 Analytical balance, capable of weighing to the nearest 1 mg.

5.7 Incubator block or **dry well bath**, capable of operating at 35 °C ± 1 °C and 63 °C ± 0,2 °C with wells of mini-vial dimensions.

5.8 Water bath, adjustable, capable of operating at 63 °C ± 0,2 °C and 95 °C ± 2 °C.

5.9 Test tubes, of capacity 50 ml, of diameter 13 mm and length 100 mm, with leakproof caps.

5.10 Printer, with connection cables for printout of results.

6 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 International Standard. A recommended sampling method is given in ISO 707 | IDF 50.

7 Preparations

7.1 Test samples

7.1.1 General

Carefully mix all test samples prior to use. The ambient temperature of the room should be between 18 °C and 24°C.

Samples should be tested at refrigeration temperature (0 to 7) °C or on ice during assay.

7.1.2 Pasteurized samples

Use the test samples as obtained in amounts as required.

7.1.3 Raw milk

Pipette 1 ml (or an appropriate amount such that the activity after dilution is less than 7 000 mU/l) of test sample into a 100 ml one-mark volumetric flask (5.5). Dilute to the mark with alkaline phosphatase-free milk (7.2). Mix carefully.

7.1.4 Flavoured-milk products and cream products

Use the test samples as obtained in amounts as required. Test samples of viscous products may require a pipetting accuracy determination using mass (100 µl = 100 mg). Wipe the pipette tips after drawing the sample. Possibly, modification of the pipette tip (cutting wider opening) might be required for accurate dispensing.

7.2 Alkaline phosphatase-free milk

Prepare alkaline phosphatase-free milk (negative test sample) by heating 35 ml or necessary volume of a test portion (7.1.2, 7.1.3 or 7.1.4) in a tube in a water bath (5.8) set at 95 °C. Allow the test portion to reach 95 °C and keep it at this temperature for 1 min. Then cool rapidly.

The negative sample is used as the 0 (zero) calibrator and shall have a mean value of less than 5 mU/l (or less than 15 mU/l with flavoured-milk products and cream products) in the appropriately calibrated luminometer channel. If stored at 4 °C, the negative sample may be kept for 48 h.

Fluid white milks may be kept for up to 6 months if stored frozen at or below −15 °C. Thaw the negative sample in water at room temperature. Vigorously shake the sample to make it homogeneous before use. Do not re-freeze.

It should be noted that some milk products, such as sheep milk, will precipitate and separate when the negative test sample is prepared at 95 C for 1 min. In this case, a lower temperature for a longer time is required, e.g. 63 °C for 30 min.

8 Procedure (see Annex A)

8.1 Calibration

8.1.1 Establish a calibration curve for each type of product to be tested. Begin by setting the luminometer background (B_g) to 100 and the correction (C_r) to 100.

Consult the luminometer manual for instructions on making adjustments. Calibration curves are stable and need to be run when new batch lots/lot numbers of the dioxetane ester substrate (4.1) and stopping buffer (4.2) are used. A flowchart for the calibration is given in Annex A.

NOTE The calibration mode on some luminometers automatically guides users through steps 8.1.1 to 8.2.2. (The calibration menu is item 8 of the main menu.) Select alkaline phosphatase calibration. Select channel to calibrate. Select calibrate. Select test portion to calibrate, for example milk, cream or chocolate (flavoured-milk product) or other. The luminometer prompts the user for the next steps.

8.1.2 Limit calibration assays to three tubes per assay. Use the fixed-volume pipette (5.3), provided with a clean tip, to add 100 µl of the dioxetane ester substrate (4.1) to the bottom of three mini-vials (5.2).

8.1.3 For the test portions obtained in 7.1.2 to 7.1.4, add, with the fixed-volume pipette (5.3) provided with a clean tip, 100 µl of negative test sample (7.2) to each vial. Deliver the contents of the sample to the bottom of the tube to ensure all of the sample contacts the substrate. Mix the contents of the vials in the vial rack with a back and forth motion for about 10 times in 5 s.

8.1.4 Place the mini-vials (8.1.3) in the incubator block (5.7) set at 35 °C for 3 min. At the end of incubation, add, with the fixed-volume dispenser (5.4), within 15 s, 1,0 ml of stopping solution (4.2) to the contents of all mini-vials.

8.1.5 Remove the mini-vials from the incubator. Cap each vial and shake each vigorously for 5 s. If necessary unscrew the cap and attach the vial to the luminometer adapter. Insert the vial into the luminometer. Read and record the readings of the luminometer (5.1) at count completion (beep). Repeat with each vial. Do not touch the liquid solution with the adapter to avoid contaminating the next vial. If the solution does contact the vial, rinse it with water and dry before re-use. Calculate the mean negative value (N). Repeat the determination specified in 8.1.2 to 8.1.5 if any one value varies by more than 30 % of the mean.

8.1.6 For the test portions obtained in 7.1.2 to 7.1.3, repeat steps 8.1.2 through 8.1.5 with calibrator solution C_1 (4.3.1), each in triplicate. For the test portion obtained in 7.1.4, repeat steps 8.1.2 through 8.1.5 with calibrator solution C_2 (4.3.2), each in triplicate. Calculate the mean calibrator C_1 or C_2 value (C_x). Repeat this determination if any one value varies by more than 30 % of the mean.

8.1.7 Calculate the correction value, C_r, using the mean calibration value, C_x (see 8.1.6), and the mean negative value, N (see 8.1.5) in Equation (1). Enter C_r in the luminometer. Consult the luminometer manual for instructions on how to change the luminometer correction to the new correction value number C_r:

$$C_r = (C_x - N) \times 0{,}286 \qquad (1)$$

NOTE Automated luminometers will print N and C_x and automatically calculate and adjust the values of C_r and B_g.

8.1.8 Calculate the background value using C_r and the mean negative value N in Equation (2), and enter the value B_g in the luminometer. Consult the luminometer manual for instructions on how to change the luminometer background to the new background value number B_g:

$$B_g = [N/(C_r/100)] + 100 \qquad (2)$$

NOTE Automated luminometers will print N and C_x and automatically calculate, adjust and print C_r and B_g.

8.1.9 Repeat steps 8.1.2 to 8.1.5 with the negative test sample (7.2), in triplicate. Verify whether the mean value with milk (in mU/l) is less than 5 mU/l, or in the case of flavoured-milk products and creams, less than 15 mU/l. If the mean value is out of range, go to step 8.1.1.

8.1.10 Repeat step 8.1.6 with calibrator C_1 or C_2. The mean reading target range is between 320 mU/l and 400 mU/l for calibrator C (either C_1 or C_2). Repeat this determination if any one value varies by more than 30 % of the mean. If that value is out of range, go to step 8.1.1.

8.1.11 Perform the calibration assay (8.1.2 to 8.1.5) substituting in step 8.1.3 working calibrator A (A_1 or A_2 depending the test portion mentioned in 4.3) in place of the negative sample. Determine the mean value for calibrator A_1 or A_2. Repeat this determination if any one value varies by more than 40 % of the mean value.