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Milk and milk products — Determination of alkaline phosphatase activity — Fluorimetric microplate method

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Contents

Forewords

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Milk and milk products — Determination of alkaline phosphatase activity — Fluorimetric microplate method

1 Scope

This document specifies a fluorimetric microplate method for the determination of alkaline phosphatase (ALP, EC 3.1.3.1)~~[5]~~ activity in raw and heat-treated whole milk, semi-skimmed milk, skimmed milk, cream, flavoured milks and cheeses.

This method is applicable to milk and milk-based drinks from cows, sheep and goats. Although the method was not tested in milk from other species, it ~~may~~can also be applicable to milk from other species with a similar composition ~~as~~to cow, sheep~~,~~ or goat milk, such as milk from buffalo and camelids. It is also applicable to milk powder after reconstitution and soft~~-~~, 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).

NOTE This method was adapted from Reference [6].

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 ~~terminological~~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 ~~(ALP)~~ activity
ALP activity
amount of enzyme that is capable of catalysing the ~~alkaline phosphatase present in~~transformation of 1 µmol of substrate per minute under the ~~product, determined by~~conditions of the specified procedure

Note 1 to entry: The ~~alkaline phosphatase~~ALP activity in milk is expressed as milliunits of enzyme activity per litre of sample (mU/l) and as milliunits of enzyme activity per gram of sample (mU/g) in the case of cheese.

~~**3.2**~~
~~**unit of alkaline phosphatase activity**~~
~~amount of alkaline phosphatase enzyme that catalyses the transformation of 1 µmol of substrate per min~~

~~7~~4 Principle

The ~~alkaline phosphatase~~ALP activity of the sample is measured by a continuous fluorimetric direct kinetic assay. A non-fluorescent aromatic monophosphoric substrate, 4-methylumbelliferone phosphate (4-MUP), in the presence of any ~~alkaline phosphatase~~ALP derived from the sample, undergoes hydrolysis of its phosphate, producing the highly fluorescent product 4-methylumbelliferone (4-MU). Fluorimetric measurement of ~~alkaline phosphatase (~~ALP~~)~~ activity is measured at 37 °C, over a 15 ~~-~~min period.

The measured fluorescence is proportional to the concentration of the emitted fluorescent product and is used to calculate the enzyme activity.

~~8~~5 Reagents

Use only reagents of recognized analytical grade.

5.1 Magnesium chloride solution, substance concentration, $c(MgCl_2)$ = 1 mol/l.

Weigh 50,8 g of magnesium chloride hexahydrate ($MgCl_2 \cdot 6\ H_2O$, molecular mass is 203,3 g/mol) in a 50 ml glass beaker (6.17), dissolve with distilled water and transfer in a volumetric flask of 250 ml (6.18). Adjust the volume with water up to the mark.

This solution can be stored in aliquots (e.g. 10 ml) at below −20 °C for one year.

5.2 Tergitol™ 15-S-9 (CAS Registry Number®[1] 84133-50-6)[2] solution, c = 100 g/l.

Weigh 25,0 g of Tergitol™ 15-S-9 in a 150 ml glass beaker (6.17), dissolve, under stirring, with distilled water at 25 °C ± 2 °C and transfer in a volumetric flask of 250 ml (6.18). Adjust the volume with water up to the mark.

This solution can be stored in aliquots (e.g. 15 ml) at a temperature between 2 °C and 8 °C for one year.

5.3 2-amino-2-methyl-1-propanol (AMP) buffer solution, c = 0,11 mol/l, pH = 10,1.

Weigh 9,8 g of AMP, purity > 95 %, molecular mass is 89,14 g/mol, in a 600 ml glass beaker (6.17), add 500 ml of distilled water on a stirrer and adjust pH to 10,1 with a hydrochloric acid solution $c(HCl)$ = 5 mol/l. Transfer in a 1 000 ml volumetric flask (6.18) and adjust with water up to the mark.

This solution can be stored in aliquots (e.g. 30 ml) at below −20 °C for one year.

5.4 Diethanolamine (DEA) buffer solution, c = 2 mol/l, pH = 9,8, $c(Mg^{2+})$ = 0,5 mmol/l.

Weigh 210,3 g of DEA, molecular mass is 105,14 g/mol, in a 1 000 ml glass beaker (6.17), add 700 ml of distilled water while stirring and adjust pH to 9,8 with a hydrochloric acid solution $c(HCl)$ = 5 mol/l HCl 5 mol/l. Add 0,5 ml of $MgCl_2$ solution (5.1). Transfer in a 1 000 ml volumetric flask (6.18) and adjust with water up to the mark.

This solution can be stored in aliquots (e.g. 50 ml) at below −20 °C for one year.

5.5 DEA extraction buffer, c = 1,5 mol/l, pH = 9,8, $c(Mg^{2+})$ = 1,5 mmol/l, c(Tergitol™) = 0,1 %.

[1] Chemical Abstracts Service (CAS) Registry Number® is a trademark of the American Chemical Society (ACS). This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

[2] This 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 the product named. Equivalent products may be used if they can be shown to lead to the same results.

Weigh 157,7 g of DEA in a 600 ml glass beaker (6.17), add 500 ml of distilled water on a stirrer and adjust pH to 9,8 with ~~with~~ a hydrochloric acid solution *c*(HCl) = 5 mol/l HCl 5 mol/l. Add 1,5 ml of $MgCl_2$ solution (5.1) and 10 ml of Tergitol™ 15-S-9 solution (5.2). Transfer in a ~~1000~~ 1 000 ml volumetric flask (6.18) and adjust with water up to the mark.

This solution can be stored in aliquots (e.g. 200 ml) at below −20 °C for one year.

5.6 4-methylumbelliferone sodium salt (4-MU) standard stock solution, *c* = 2,5 mmol/l.

Weigh 9,9 mg of 4-MU, purity > 98 %, molecular mass is 198,16 g/mol, in a 25 ml glass beaker (6.17), solubilize and rinse well with the AMP buffer solution (5.3), transfer in a volumetric flask of 20 ml (6.18) and fill up to the mark with the same buffer. Keep solution in the dark.

This solution can be stored in aliquots (e.g. 200 µl) at below −20 °C for one year.

5.7 4-methylumbelliferone phosphate (4-MUP) substrate solution, *c* = 2,5 mmol/l.

Weigh 12,8 mg of 4-MUP, molecular mass is 256,15 g/mol, in a 25 ml glass beaker (6.17), solubilize and rinse well with the DEA buffer solution (5.4), transfer in a volumetric flask of 20 ml (6.18) and fill up to the mark with the same buffer.

Prepare the solution freshly and keep in the dark.

~~9~~6 Apparatus and materials

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

6.1 Fluorescence microplate reader, capable of reading 96-well microplates at an excitation wavelength of 365 nm and emission wavelength of 450 nm. Capable of temperature control at 37 °C ± 1 °C and allowing kinetic measurements (e.g. ~~1~~one reading per ~~min~~minute for 15 min).

6.2 Microplates, black, flat bottom, 96 well.

6.3 Pipette, of capacity 20 µl to 100 µl.

6.4 Pipette, of capacity 100 µl to 1 000 µl.

6.5 Multichannel pipettor, capable of dispensing 20 µl to 100 µl.

6.6 Air displacement pipette, of capacity 5 ml.

A glass pipette can also be used.

6.7 Microtubes, of capacity 2 ml.

6.8 Tubes, of capacity 15 ml and 50 ml.

6.9 Glass test tube, of approximately diameter 12 mm and length 10 cm.

6.10 Water bath, heating block or **incubator** suitable of maintaining a temperature of 37 °C ± 1 °C, 63 °C ± 1 °C and 95 °C ± 1 °C.

6.11 Vortex mixer.

6.12 Parafilm®[3], or other suitable laboratory-grade film.

6.13 Aluminium foil.

6.14 Grinding device.

6.15 ~~Ultra-turrax®[1]~~**ULTRA-TURRAX®**[4]**,** or other homogenizer provided with a stem of diameter of approximately 6 mm to 8 mm.

6.16 Centrifuge, capable of centrifuging at 1 000*g* at 4 °C for 10 ml or 15 ml, and 50 ml tubes.

6.17 Glass beaker, of capacity 5 ml (approximately diameter 20 mm and length 30 mm), 10 ml (of approximately diameter 25 mm and length 30 mm), 25 ml, 50 ml, 150 ml, 600 ml and 1 000 ml.

6.18 One-mark volumetric flasks, of capacity 20 ml, 25 ml, 250 ml and 1 000 ml.

6.19 Analytical balance.

6.20 pH meter.

~~10~~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[1].

However, ISO 707 | IDF 50[1] 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~~. (Annex C,~~ (see Figure C.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.

~~11~~8 Preparation

~~11.1~~8.1 Preparation of alkaline phosphatase-free sample

8.1.1 General

The ~~alkaline phosphatase~~ALP-free sample is used as sample blank, for calibration and for sample dilution if necessary.

[3] Parafilm® ~~and Ultra-turrax® are examples~~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 ~~either~~ ISO or IDF of ~~these products~~this product.

[4] 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.

~~11.1.1~~8.1.2 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. Cover the tube or container containing the milk portion and place it in the water bath or in the heating block (6.10) set at 95 °C. Preheat the milk portion to 95 °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 °C, immediately start its 5 -min heating period. Cool the whole portion rapidly after the heating period.

~~11.1.2~~8.1.3 Alkaline phosphatase-free cheese

For each type of cheese to be tested, prepare a phosphatase-free cheese from the supernatant of the cheese (see 9.3.2.6) by heating a portion of the supernatant as described in ~~8.1.1~~8.1.2, replacing the milk by the supernatant.

~~11.2~~8.2 Preparation of test sample

~~11.2.1~~8.2.1 Milk samples

~~11.2.1.1~~8.2.1.1 General

Carefully mix all test samples prior to use.

NOTE It is usually not necessary to prewarm test samples.

~~11.2.1.2~~8.2.1.2 Pasteurized test samples

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

~~11.2.1.3~~8.2.1.3 Dilution of test samples with high ALP values

Prepare dilutions of the test samples of milk using phosphatase-free milk (see ~~8.1.1~~8.1.2) in order to bring their ALP levels within the linearity range of the instrument (see 9.1.2). Mix the diluted solutions well.

~~11.2.2~~8.2.2 Cheese samples

~~11.2.2.1~~8.2.2.1 General

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 Annex C). For large hard cheeses, proceed as described ~~under~~in Clause 7. Grind the test sample by means of a grinding mill or other appropriate device (6.14) and mix thoroughly. Keep the prepared sample in an airtight container.

~~11.2.2.2~~8.2.2.2 Dilution of test samples with high ALP values

Prepare dilutions of the supernatant of the cheese samples (see 9.3.2.6) using phosphatase-free cheese (see ~~8.1.2~~8.1.3) in order to bring their ALP levels within the linearity range of the instrument (see 9.1.2). Mix the diluted solutions well.

~~12~~9 Procedure

~~12.1~~9.1 Instrument

~~12.1.1~~9.1.1 Instrument settings

Use equipment in accordance with the instructions provided by the manufacturer. The instrument (6.1) settings are adjusted according to Table 1.

~~Table — Measurement settings~~

~~Measurement parameter~~	~~Instrument settings~~
~~Plate type~~	~~96 well, black, flat bottom~~
~~Operation mode~~	~~fluorescence type, kinetic 15 min, 1 reading per min~~
~~Excitation wavelength~~	~~365 nm~~
~~Emission wavelength~~	~~450 nm~~
~~Temperature~~	~~37 °C~~
~~Reading action~~	~~shaking 5 s before the first read~~

Set the photo multiplier tube (PMT) and optics according to the instrument manual (e.g. medium sensitivity, ~~6~~six readings per well).

~~NOTE~~ To obtain the best fluorescence results with ~~your~~the instrument, the easiest way is to "scale to high well". A good target for the high well is 80 % of the maximal signal before the detector saturates. After this adjustment, a blank reading should give no more than 10 % of the maximal signal.

Table 1 — Measurement settings

Measurement parameter	Instrument settings
Plate type	96 well, black, flat bottom
Operation mode	fluorescence type, kinetic 15 min, one reading per minute
Excitation wavelength	365 nm
Emission wavelength	450 nm
Temperature	37 °C
Reading action	shaking 5 s before the first read

~~12.1.2~~9.1.2 Test the linearity range of the instrument

It is important to determine the linearity range of the instrument. This can be tested by ensuring that the measured relative fluorescent units (RFU) obtained with the standard curve follow a linear curve with a coefficient $R^2 \geq 0,99$.

~~12.1.3~~9.1.3 Quality control

It is important to check instrument performance for drift, stray light and stability prior to ~~analyzing~~analysing test samples. Follow good laboratory practice principles when operating the filter fluorimeter (6.1) and refer to manufacturer's instructions.