INTERNATIONAL STANDARD 3356

INTERNATIONAL ORGANIZATION FOR STANDARDIZATION • МЕЖДУНАРОДНАЯ ОРГАНИЗАЦИЯ ПО СТАНДАРТИЗАЦИИ • ORGANISATION INTERNATIONALE DE NORMALISATION

Milk and dried milk, buttermilk and buttermilk powder, whey and whey powder — Determination of phosphatase activity (Reference method)

Lait et lait sec, babeurre et poudre de babeurre, sérum et poudre de sérum — Détermination de l'activité phosphatasique (Méthode de référence)

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FOREWORD

ISO (the International Organization for Standardization) is a worldwide federation of national standards institutes (ISO Member Bodies). The work of developing International Standards is carried out through ISO Technical Committees. Every Member Body interested in a subject for which a Technical Committee has been set up 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.

Draft International Standards adopted by the Technical Committees are circulated to the Member Bodies for approval before their acceptance as International Standards by the ISO Council.

International Standard ISO 3356 was drawn up by Technical Committee ISO/TC 34, *Agricultural food products*, and circulated to the Member Bodies in April 1974.

It has been approved by the Member Bodies of the following countries :

Austria	Hungary	Poland
Belgium	India	Romania
Bulgaria	Iran	South Africa, Rep. of
Canada	Ireland	Spain
Chile	Israel	Thailand
Czechoslovakia	Italy	Turkey
France	Netherlands	U.S.S.R.
Germany	New Zealand	Yugoslavia

The Member Body of the following country expressed disapproval of the document on technical grounds :

United Kingdom

Printed in Switzerland

Milk and dried milk, buttermilk and buttermilk powder, whey and whey powder — Determination of phosphatase activity (Reference method)

1 SCOPE AND FIELD OF APPLICATION

This International Standard specifies a reference method for the determination of the phosphatase activity in milk and dried milk, buttermilk and buttermilk powder, whey and whey powder.

The method can be applied for the control of proper pasteurization of these products.

2 REFERENCE

ISO/R 707, *Milk and milk products — Sampling.*

3 DEFINITION

For the purposes of this International Standard, the following definition applies:

phosphatase activity: The quantity of active alkaline phosphatase present in the product, expressed as the quantity of phenol, in micrograms, liberated under the specified conditions by 1 ml of the liquid product or, in the case of dried products, by 1 ml of the reconstituted liquid product.

4 PRINCIPLE

Dilution of the liquid product or the reconstituted liquid product with a buffer at pH 10,6 containing disodium phenylphosphate and incubation at 37 °C for 1 h, liberating phenol by reaction with the alkaline phosphatase present in the product. Reaction of the phenol with dibromoquinonechloroimide and photometric measurement of the colour formed.

5 REAGENTS

All reagents shall be of analytical reagent quality and water shall be freshly boiled distilled water, or water of a least equal purity, free from carbon dioxide.

5.1 Barium borate-hydroxide buffer.

5.1.1 Dissolve 50,0 g of barium hydroxide [$Ba(OH)_2 \cdot 8H_2O$], free from carbonate, in water to a volume of 1 000 ml.

5.1.2 Dissolve 22,0 g of boric acid (H_3BO_3) in water to a volume of 1 000 ml.

5.1.3 Warm 500 ml of each solution to 50 °C, mix the solutions, stir, cool rapidly to about 20 °C, adjust the pH if necessary to 10,6 ± 0,1 by addition of solution 5.1.1 or 5.1.2 and filter.

Store the solution in a tightly stoppered container.

Dilute the solution before use with an equal volume of water.

5.2 Colour development buffer.

Dissolve 6,0 g of sodium metaborate ($NaBO_2$) or 12,6 g of $NaBO_2 \cdot 4H_2O$, and 20,0 g of sodium chloride (NaCl) in water to a volume of 1 000 ml.

5.3 Colour dilution buffer.

Dilute 10 ml of the colour development buffer (5.2) to 100 ml with water.

5.4 Buffer substrate.

Dissolve 0,5 g of disodium phenylphosphate ($Na_2C_6H_5PO_4 \cdot 2H_2O$) in 4,5 ml of the colour development buffer (5.2), add two drops of the BQC solution (5.6) and let stand at room temperature for 30 min. Extract the colour so formed with 2,5 ml of butan-1-ol and let stand until the butan-1-ol separates. Remove the butan-1-ol and discard. Repeat this extraction if necessary.

The solution may be stored in a refrigerator for a few days; develop the colour and re-extract before use. Prepare the buffer substrate immediately before use by diluting 1 ml of this solution to 100 ml with the barium borate-hydroxide buffer (5.1).

5.5 Zinc-copper precipitant.

Dissolve 3,0 g of zinc sulphate ($ZnSO_4 \cdot 7H_2O$) and 0,6 g of copper(II) sulphate ($CuSO_4 \cdot 5H_2O$) in water to a volume of 100 ml.

5.6 2,6-dibromoquinonechloroimide solution (Gibb's reagent).

Dissolve 40 ± 1 mg of 2,6-dibromoquinonechloroimide (BQC) ($O = C_6H_2Br_2 = NCl$) in 10 ml of 96 % (*V/V*) ethanol.

Store in a dark-coloured bottle in a refrigerator. Reject if discoloured or more than 1 month old.

5.7 Copper(II) sulphate solution.

Dissolve 0,05 g of copper(II) sulphate ($CuSO_4 \cdot 5H_2O$) in water to a volume of 100 ml.

5.8 Sodium hydroxide, 0,5 N solution.

5.9 Phenol standard solutions.

5.9.1 Weigh 200 ± 2 mg of pure anhydrous phenol, transfer to a 100 ml volumetric flask, fill to the mark with water and mix.

This stock solution remains stable for several months in a refrigerator.

5.9.2 Dilute 10 ml of this stock solution to 100 ml with water and mix. 1 ml contains 200 μg of phenol.

6 APPARATUS

NOTES

1 All glassware, stoppers and sampling tools must be carefully cleaned. It is desirable to rinse them with freshly boiled distilled water or to steam them.

2 Certain types of plastics stoppers may cause phenolic contamination and their use shall therefore be excluded.

6.1 Analytical balance.

6.2 Water bath capable of being maintained at 37 ± 1 °C.

6.3 Spectrophotometer, suitable for readings at a wavelength of 610 nm.

6.4 Test tubes, 16 or 18 mm × 150 mm, preferably graduated at 5 and 10 ml.

6.5 Pipettes, graduated 1 and 10 ml (ISO/R 835).

6.6 Glass funnels of convenient size, for example 5 cm diameter.

6.7 Filter paper.[1)]

6.8 Volumetric flasks for the preparation of standard solutions.

6.9 Litmus paper.

7 SAMPLING

See ISO/R 707 (5.3, sampling instructions for bacteriological examination).

8 PROCEDURE

NOTES

1 Avoid the influence of direct sunlight during the determination.

2 Contamination with traces of saliva or perspiration can give false positive results and must be avoided. In this respect special attention shall be devoted to the pipetting.

8.1 Preparation of the test sample

8.1.1 *Milk, buttermilk and whey*

8.1.1.1 Carry out the analysis preferably directly after sampling. Otherwise, keep the sample in a refrigerator, but not for more than 2 days.

8.1.1.2 Mix the sample carefully, if necessary with moderate heating. The temperature of mixing shall under no circumstances exceed 35 °C.

8.1.2 *Dried milk, buttermilk powder and whey powder*

Dissolve 10 g of the product in 90 ml of water. The temperature applied in dissolving shall under no circumstances exceed 35 °C.

8.1.3 *Neutralization of sour products*

Add to a sour product sodium hydroxide solution (5.8) until a drop is neutral to the litmus paper (6.9).

8.2 Test portion

Pipette into each of two test tubes (6.4) 1 ml of the test sample (8.1), using one tube as a control or blank.

8.3 Determination

8.3.1 Heat the blank for 2 min in boiling water; cover the tube and the beaker of boiling water with aluminium foil to ensure that the entire tube will be heated. Cool to room temperature.

8.3.2 From this point treat the blank and the test sample alike. Add 10 ml of the buffer substrate (5.4) and mix.

8.3.3 Immediately incubate in the water bath at 37 °C for 60 min, mixing the contents occasionally.

8.3.4 Heat in boiling water for 2 min in the same manner as under 8.3.1. Cool to room temperature.

8.3.5 Add 1 ml of the zinc-copper precipitant (5.5) to each tube and mix thoroughly.

8.3.6 Filter through dry filter paper, discard the first drops, refilter if necessary until the filtrate runs clear, and collect 5 ml in a test tube.

1) Whatman No. 42, S & S 597 or equivalent.

8.3.7 Add 5 ml of the colour development buffer (5.2).

8.3.8 Add 0,1 ml of the BQC solution (5.6), mix, and allow the colour to develop for 30 min at room temperature.

8.3.9 Measure the absorbance against the blank in the spectrophotometer (6.3) at a wavelength of 610 nm.

8.3.10 Repeat the determination with an appropriate dilution of the sample or the reconstituted sample if the absorbance as measured under 8.3.9 exceeds the absorbance of the standard containing 20 μg of phenol per tube as measured under 8.4.4.

Prepare this dilution by mixing 1 volume of the test sample with an appropriate volume of a part of the same test sample heated carefully to boiling in order to inactivate the phosphatase.

8.4 Preparation of the calibration curve

8.4.1 Provide a suitable range of diluted standards, starting from the standard phenol (5.9.2), containing 0 (control or blank), 2, 5, 10 and 20 μg of phenol per millilitre, and pipette respectively 1 ml of water and 1 ml of the four phenol standard solutions into each of five test tubes.

8.4.2 Add to each tube 1 ml of the copper(II) sulphate solution (5.7), 5 ml of the colour dilution buffer (5.3), 3 ml of water and 0,1 ml of the BQC solution (5.6); mix.

8.4.3 Allow the colour to develop for 30 min at room temperature.

8.4.4 Measure the absorbance against the control or blank in the spectrophotometer at a wavelength of 610 nm.

8.4.5 Prepare the calibration curve by plotting the absorbances against the quantities of phenol, in micrograms, indicated under 8.4.1. The standard curve should be a straight line.

9 EXPRESSION OF RESULTS

9.1 Calculation

9.1.1 Convert the absorbance as determined under 8.3.9 to micrograms of phenol by reference to the calibration curve.

9.1.2 Calculate the phosphatase activity, expressed as micrograms of phenol per millilitre of the liquid product or, in the case of dried products, per millilitre of reconstituted liquid product, by means of the following formula :

$$\text{Phosphatase activity} = 2{,}4 \times A \times D$$

where

A is the quantity of phenol in micrograms obtained under 9.1.1;

D is the dilution factor of the dilution according to 8.1.3 and/or 8.3.10. (In the case of no dilution, $D = 1$.)

9.2 Repeatability

The difference between the results of two determinations carried out simultaneously or in rapid succession by the same analyst shall not exceed 2 μg of phenol. If a dilution is applied according to 8.1.3 and/or 8.3.10, this limit is referred to the results obtained on the diluted sample.

10 TEST REPORT

The test report shall show the method used and the result obtained. It shall also mention any operating conditions not specified in this International Standard or regarded as optional, as well as any circumstances that may have influenced the result.

The report shall include all details required for the complete identification of the sample.

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INTERNATIONAL STANDARD ISO 3356-1975 (E)/AMENDMENT 2

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AMENDMENT 2

Foreword (Inside front cover)

Add at the end :

"This International Standard has been developed jointly with the IDF (International Dairy Federation) and the AOAC (Association of Official Analytical Chemists, U.S.A.) on the basis of an IDF draft. The text as approved by the above organizations has also been published by the IDF (IDF Standard No. 63) and by the AOAC (Official Methods of Analysis)."

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