International Standard 8069

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Dried milk — Determination of lactic acid and lactates content — Enzymatic method

Lait sec — Détermination de la teneur en acide lactique et en lactates — Méthode enzymatique

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Foreword

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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. They are approved in accordance with ISO procedures requiring at least 75 % approval by the member bodies voting.

International Standard ISO 8069 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*. It cancels and replaces ISO 3495-1975.

NOTE — The method specified in this International Standard has been developed jointly with the International Dairy Federation (IDF) and the Association of Official Analytical Chemists (AOAC) and will also be published by these organizations.

Users should note that all International Standards undergo revision from time to time and that any reference made herein to any other International Standard implies its latest edition, unless otherwise stated.

Dried milk — Determination of lactic acid and lactates content — Enzymatic method

1 Scope and field of application

This International Standard specifies an enzymatic method for the determination of the lactic acid and lactates content of all types of dried milk.

2 Reference

ISO 707, *Milk and milk products — Methods of sampling.*

3 Definition

lactic acid and lactates content of dried milk: The content of substances determined by the procedure specified in this International Standard and expressed as milligrams of lactic acid per 100 g of non-fat solids.

4 Principle [1)]

Dissolution of the dried milk in warm water. Precipitation of the fat and proteins, followed by filtration. Treatment of the filtrate with the following enzymes and biochemical substances, added simultaneously, but acting in sequence:

— L-lactate dehydrogenase (L-LDH) and D-lactate dehydrogenase (D-LDH) in the presence of nicotinamide adenine dinucleotide (NAD) to oxidize lactate to pyruvate and to convert NAD to its reduced form (NADH);

— glutamate pyruvate transaminase (GPT) in the presence of L-glutamate to transform pyruvate into L-alanine and to convert L-glutamate to α-ketoglutarate.

Spectrometric measurement at a wavelength of 340 nm to determine the amount of NADH produced, which is proportional to the lactic acid and lactates content.

5 Reagents

All reagents shall be of recognized analytical grade. The water used in the preparation of the enzyme solutions shall be of at least doubly glass-distilled purity and the water used for other purposes shall be glass-distilled or of at least equivalent purity.

5.1 Potassium hexacyanoferrate(II) solution.

Dissolve 35,9 g of potassium hexacyanoferrate(II) trihydrate ($K_4[Fe(CN)_6]\cdot 3H_2O$) in water, dilute to 1 000 ml and mix.

5.2 Zinc sulfate solution.

Dissolve 71,8 g of zinc sulfate heptahydrate ($ZnSO_4\cdot 7H_2O$) in water, dilute to 1 000 ml and mix.

5.3 Sodium hydroxide, 0,1 mol/l solution.

Dissolve 4,00 g of sodium hydroxide (NaOH) in water, dilute to 1 000 ml and mix.

5.4 Buffer solution, pH 10.

Dissolve 7,92 g of glycylglycine ($C_4H_8N_2O_3$) and 1,47 g of L-glutamic acid ($C_5H_9NO_4$) in about 80 ml of water. Adjust the pH to 10,0 ± 0,1 at 20 °C with 10 mol/l sodium hydroxide solution, dilute to 100 ml with water and mix.

This solution can be kept for 3 months if stored in a refrigerator at 0 to +5 °C.

5.5 NAD solution.

Dissolve 350 mg of nicotinamide adenine dinucleotide ($C_{22}H_{27}N_7O_{14}P_2$) in 10 ml of water.

This solution can be kept for 4 weeks if stored in a refrigerator at 0 to +5 °C.

When the solution is being used, the vessel should be kept immersed in crushed ice.

1) This method is mainly based on *Methods of Enzymatic Food Analysis: UV-method for the determination of L-lactic acid and D-lactic acid in foodstuffs*, Boehringer Mannheim GmbH.

5.6 L-LDH from hog muscle, 10 mg/ml solution in 50 % (*V*/*V*) glycerol, pH about 7.

The specific activity of the solution of L-lactate dehydrogenase (L-LDH, EC 1.1.1.27)[1] should be at least 5 500 units/ml[2] (25 °C).

The solution can be kept for 12 months if stored in a refrigerator at 0 to +5 °C.

When the solution is being used, the vessel should be kept immersed in crushed ice.

5.7 D-LDH from *Lactobacillus leichmannii*, 5 mg/ml suspension in 3,2 mol/l ammonium sulfate solution, pH about 6.

The specific activity of the suspension of D-lactate dehydrogenase (D-LDH, EC 1.1.1.28) should be at least 1 500 units/ml (25 °C).

The suspension can be kept for 12 months if stored in a refrigerator at 0 to +5 °C.

When the suspension is being used, the vessel should be kept immersed in crushed ice.

5.8 GPT from pig heart, 20 mg/ml suspension in 3,2 mol/l ammonium sulfate solution, pH about 7.

The specific activity of the suspension of glutamate pyruvate transaminase (GPT, EC 2.6.1.2) should be at least 1 600 units/ml (25 °C).

Centrifuge 2 ml of a suspension containing 10 mg of GPT per millilitre for 10 min at about 4 000*g*, suck off 1,0 ml of the clear supernatant liquid and discard.

The suspension can be kept for 12 months if stored in a refrigerator at 0 to +5 °C.

When the suspension is being used, the vessel should be kept immersed in crushed ice.

5.9 Lithium L-lactate solution.

Dissolve 50 mg of lithium L-lactate ($C_3H_5O_3Li$) in water, dilute to 500 ml and mix.

5.10 Lithium D-lactate solution.

Dissolve 50 mg of lithium D-lactate ($C_3H_5O_3Li$) in water, dilute to 500 ml and mix.

6 Apparatus

Usual laboratory equipment, and in particular

6.1 Analytical balance.

6.2 Glass beaker, of capacity 50 ml.

6.3 Graduated cylinder, of capacity 50 ml.

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

6.5 Pipettes, to deliver 0,02; 0,05; 0,2; 1 and 2 ml.

6.6 Graduated pipettes, of capacity 5 and 10 ml, graduated in 0,1 ml divisions.

6.7 Glass filter funnel, of diameter about 7 cm.

6.8 Filter paper, medium grade, of diameter about 15 cm, free from lactic acid and lactates.

6.9 Glass rod.

6.10 Plastic paddles, suitable for mixing the sample-enzyme mixture in the spectrometric cell.

6.11 Spectrometer, suitable for making measurements at 340 nm, equipped with cells of optical path length 1 cm.

7 Sampling

7.1 See ISO 707.

7.2 Store the sample in such a way that deterioration and change in composition are prevented.

8 Procedure

CAUTION — Avoid contamination, especially with sweat.

8.1 Test to check the activity of reagents

Whenever a new batch of reagents (5.5 to 5.8 inclusive) is brought into use, or when such reagents have been kept in a refrigerator without being used for more than 2 weeks, or when restarting analytical work after a period of analytical inactivity or whenever other conditions may justify it, the following test for the recovery of lactates should be performed.

1) The EC number refers to the Enzymatic Classification number as given by the Nomenclature Committee of the International Union of Biochemistry in *Enzyme Nomenclature Recommendation (1978)*. New York, Academic Press, 1979.

2) This unit (often called the International Unit or Standard Unit) is defined as the amount of enzyme which will catalyse the transformation of 1 µmol of substrate per minute under standard conditions.

8.1.1 Pipette 10 ml of the lithium L-lactate solution (5.9) into each of two 100 ml one-mark volumetric flasks (6.4) and 10 ml of the lithium D-lactate solution (5.10) into each of two other 100 ml one-mark volumetric flasks (6.4) and determine the L-lactic acid and lactates content and the D-lactic acid and lactates content of the solutions in the two pairs of flasks, proceeding as specified in 8.5.2 up to and including 8.6.

8.1.2 Calculate the lithium lactate content, expressed in milligrams per litre, using the formulae:

a) for the L-lactate solution

$341 \times A$

b) for the D-lactate solution

$346 \times A$

where A is the absorbance at 340 nm, calculated in accordance with 8.6.4.

8.1.3 Taking into account the purity of the lithium L-lactate and lithium D-lactate preparation, the recovery of lithium L- or D-lactate from any of the flasks shall be within the range 100 ± 5 %. If the recoveries are not within this range, the reagents, the operating technique, the accuracy of the pipettes and the condition of the spectrometer should be checked and the required action should be taken to obtain appropriate results. The test shall be repeated until satisfactory results are obtained.

8.2 Preparation of the test sample

Transfer the sample into a container, of capacity about twice the volume of the sample, provided with an airtight lid. Close the container immediately and mix the sample thoroughly by repeatedly shaking and inverting the container.

During preparation, avoid exposure of the sample to the atmosphere as far as possible, in order to minimize adsorption of water.

8.3 Test portion

Weigh, to the nearest 1 mg, 1 g of the test sample into the glass beaker (6.2).

8.4 Blank test

Carry out a blank test, proceeding as specified in 8.5 and 8.6, using all the reagents but omitting the test portion.

8.5 Preparation of solution and deproteination

8.5.1 Dissolve the test portion (8.3) in about 20 ml of warm water (40 to 50 °C), while stirring with the glass rod (6.9). Transfer the contents of the glass beaker quantitatively into a 100 ml one-mark volumetric flask (6.4) by rinsing with water. Cool the contents of the flask to about 20 °C.

8.5.2 Add to the solution (8.5.1), in the following order, 5,0 ml of the potassium hexacyanoferrate(II) solution (5.1), 5,0 ml of the zinc sulfate solution (5.2) and 10,0 ml of the sodium hydroxide solution (5.3), swirling thoroughly after each addition. Dilute to 100 ml with water, mix thoroughly and let the mixture stand for 30 min.

8.5.3 Filter through a filter paper (6.8), discarding the first fraction of the filtrate.

8.6 Determination

8.6.1 Transfer, by means of a pipette, into a spectrometric cell (see 6.11)

1,0 ml of the filtrate (8.5.3);

1,0 ml of the buffer solution (5.4);

0,20 ml of the NAD solution (5.5);

0,02 ml of the GPT suspension (5.8).

Mix the contents of the cell using the plastic paddle (6.10).

8.6.2 Measure, 5 min after mixing the contents of the cell, the absorbance of the test solution against water at a wavelength of 340 nm.

Add to the cell 0,02 ml of the L-LDH suspension (5.6) and 0,05 ml of the D-LDH suspension (5.7). Mix again using the plastic paddle and leave at room temperature.

NOTE — The L- or D-lactic acid and lactates content can be determined separately by adding either L-LDH or D-LDH.

8.6.3 Measure, 45 min and 60 min after the addition of the LDH suspension(s), the absorbance of the test solution against water.

NOTE — When only L-lactic acid and lactates are measured, the absorbance can be determined after 30 min and 45 min, respectively.

8.6.4 Calculate the absorbance A to be used for the calculation (9.1) by means of the formula

$$[(A_{s60} - A_{s0}) - 4(A_{s60} - A_{s45})] - [(A_{b60} - A_{b0}) - 4(A_{b60} - A_{b45})]$$

where

A_{s60} is the absorbance of the test solution measured after 60 min in 8.6.3;

A_{s0} is the absorbance of the test solution measured in 8.6.2;

A_{s45} is the absorbance of the test solution measured after 45 min in 8.6.3;

A_{b60} is the absorbance of the blank test solution measured after 60 min in 8.6.3;

A_{b0} is the absorbance of the blank test solution measured in 8.6.2;

A_{b45} is the absorbance of the blank test solution measured after 45 min in 8.6.3.

NOTES

1 A slowly proceeding side reaction may occasionally occur. The contribution to the absorbance caused by this side reaction can be eliminated by extrapolation to the absorbance at time zero.

2 When the absorbance is measured after 30 min and 45 min, respectively, the formula should be

$$[(A_{s45} - A_{s0}) - 3(A_{s45} - A_{s30})] - [(A_{b45} - A_{b0}) - 3(A_{b45} - A_{b30})]$$

where

A_{s30} is the absorbance of the test solution measured after 30 min in 8.6.3;

A_{b30} is the absorbance of the blank test solution measured after 30 min in 8.6.3.

8.6.5 If the increase in absorbance calculated according to 8.6.4 exceeds 0,500, repeat the procedures specified in 8.6.1 up to and including 8.6.4, using an appropriate aqueous dilution of the filtrate from both the test portion (8.5.3) and the blank test solution (8.4).

9 Expression of results

9.1 Method of calculation and formula

The lactic acid and lactates content, expressed as milligrams of lactic acid per 100 g of non-fat solids, is given by the formula

$$\frac{AM_r}{\kappa l m} \times \frac{V_1 V_4 V_5}{V_2 V_3} \times \frac{100}{W_{nfs}} \times 10^5$$

where

A is the absorbance at 340 nm, calculated in accordance with 8.6.4;

M_r is the relative molecular mass of lactic acid (90,1);

κ is the molar absorption coefficient of NADH at 340 nm, i.e. $6{,}3 \times 10^6$ cm^2/mol;

l is the optical path length, in centimetres, of the spectrometric cells (1 cm);

m is the mass, in grams, of the test portion (8.3);

V_1 is the total volume, in millilitres, of liquid in the spectrometric cell (see 8.6.2);

NOTE — V_1 = 2,29 ml, when both L- and D-lactic acid and lactates are determined;

V_1 = 2,24 ml, when only L-lactic acid and lactates are determined;

V_1 = 2,27 ml, when only D-lactic acid and lactates are determined.

V_2 is the volume, in millilitres, of the filtrate (see 8.5.3) in the spectrometric cell (see 8.6.4);

V_3 is the volume, in millilitres, of the filtrate (see 8.5.3) taken for dilution (see 8.6.5), if appropriate;

V_4 is the volume, in millilitres, of the solution in 8.5.2 (i.e. 100 ml);

V_5 is the volume, in millilitres, to which the test solution was diluted (see 8.6.5), if appropriate;

W_{nfs} is the non-fat solids content, expressed as a percentage by mass, of the sample.

Report the lactic acid and lactates content to the nearest 1 mg/100 g of non-fat solids.

9.2 Precision

NOTE — The values for repeatability and reproducibility have been derived from the results of an interlaboratory test in accordance with ISO 5725.

9.2.1 Repeatability

The difference between the results of two determinations carried out simultaneously or in rapid succession by the same analyst using the same apparatus shall not exceed 10 mg/100 g of non-fat solids if the arithmetic mean of the lactic acid and lactates content is up to and including 60 mg/100 g of non-fat solids and shall not exceed 15 % of the arithmetic mean of the results if the lactic acid and lactates content exceeds 60 mg/100 g of non-fat solids.

9.2.2 Reproducibility

The difference between two single and independent results, obtained by two analysts working in different laboratories on identical test material shall not exceed 15 mg/100 g of non-fat solids if the arithmetic mean of the lactic acid and lactates content is up to and including 100 mg/100 g of non-fat solids and shall not exceed 20 % of the arithmetic mean of the results if the lactic acid and lactates content exceeds 100 mg/100 g of non-fat solids.

10 Test report

The test report shall show the method used and the results obtained. It shall also mention any operating details not specified in this International Standard, or regarded as optional, together with details of any incidents likely to have influenced the results.

The test report shall include all the information necessary for the complete identification of the sample.

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