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Dried milk — Assessment of heat treatment intensity — Method using high-performance liquid chromatography

Lait sec — Évaluation de l'intensité du traitement thermique — Méthode par chromatographie en phase liquide à haute performance

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Foreword

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International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 3.

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 International Standard ISO 11814|IDF 162 may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

International Standard ISO 11814|IDF 162 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF), in collaboration with AOAC International. It is being published jointly by ISO and IDF and separately by AOAC International.

Annex A of this International Standard is for information only.

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 and AOAC International 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 National Committees casting a vote.

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All work was carried out by the Joint ISO/IDF/AOAC Action Team *Characterization of milk and milk products according to heat treatment*, of the Standing Committee on *Minor components and characterization of physical properties*, under the aegis of its project leader, Mr M.A.J.S. van Boekel (NL).

Introduction

The limits for (extra) low-heat skim milk powder can be influenced by the origin of the milk used for production, as well as the heat treatment applied during processing of the powder or of the reference sample (see 4.4). Therefore in this International Standard no limits are given for the relative ratio, r (see 9.1) and the relative sample ratio, r_t (see 9.3). It is advisable to have r and r_t standard limits for extra-low-heat powder which depend on the application requirements. The standard limits should be based on the national legislation of the country concerned.

Dried milk — Assessment of heat treatment intensity — Method using high-performance liquid chromatography

1 Scope

This International Standard specifies a high-performance liquid chromatographic (HPLC) method for an assessment of the heat treatment intensity to be applied during the processing of milk powder, in order to differentiate extra-low-heat skim milk powder from low-heat skim milk powder.

2 Term and definition

For the purposes of this International Standard, the following term and definition applies.

2.1
extra-low-heat skim milk powder
skim milk powder with a minimum content of denatured whey proteins, as determined by the procedure specified in this International Standard

3 Principle

A test portion of milk powder is dissolved in water. Casein and denatured whey proteins are precipitated isoelectrically at pH 4,6. The undenatured whey proteins present in the filtrate are determined by high-performance liquid chromatography. The results obtained are interpreted.

4 Reagents

Use only reagents of recognized analytical grade and distilled or demineralized water or water of at least equivalent purity.

4.1 Hydrochloric acid, c(HCl) ≈ 1 mol/l.

Dilute 80 ml of concentrated hydrochloric acid [37 % (mass fraction)] to 1 000 ml with water and mix.

4.2 Eluent, pH 6,0

Dissolve 1,74 g of dipotassium hydrogen phosphate (K_2HPO_4), 12,37 g of potassium dihydrogen phosphate (KH_2PO_4) and 21,41 g of sodium sulfate (Na_2SO_4) in about 700 ml of water. Adjust the pH to 6,0 with phosphoric acid solution (85 %) or potassium hydroxide solution (10 mol/l), if necessary.

Dilute with water to 1 000 ml and mix. Filter the eluent through a 0,45 µm membrane filter prior to use.

4.3 Flushing solvent

Mix 100 ml of acetonitrile (CH_3CN) with 900 ml of water. Filter the mixture through a 0,45 µm membrane filter prior to use.

Other flushing solvents may be used, provided that they inhibit the growth of bacteria and do not affect the separation performance of the column.

4.4 Reference sample

Use extra-low-heat skim milk powder, with a minimum content of denatured whey proteins.

5 Apparatus

Usual laboratory equipment and, in particular, the following.

5.1 Analytical balance, capable of weighing to the nearest 1 mg, with readability to 0,1 mg.

5.2 Beakers, of capacity 50 ml.

5.3 Graduated cylinder, of capacity 50 ml.

5.4 Graduated pipettes, capable of delivering 2 ml.

5.5 Magnetic stirrer.

5.6 Filter paper, medium grade, of diameter about 15 cm.

5.7 Filter funnels, of diameter about 7 cm.

5.8 Conical flasks, of capacity 50 ml.

5.9 HPLC equipment, consisting of the following.

5.9.1 Magnetic stirrer, provided with a heater for keeping the eluent at 85 °C ± 1 °C.

5.9.2 Pump, capable of delivering a flow of 1,0 ml/min.

5.9.3 Injector, hand or automatic, with a 20 µl injection capacity.

5.9.4 Zorbax Bio column, series GF-250 column, length 25 cm and 0,94 cm internal diameter, or an equivalent column in combination with a precolumn, length 3 cm and 0,3 cm internal diameter, packed with protein I-125 (Millipore Waters) or an equivalent packing material.[1)]

NOTE Typical retention times obtained by the procedure specified in this International Standard and using the GF-250 column are (see Figures A.1 and A.2 for examples):

— immunoglobulin fraction (Ig): 8,2 min
— bovine serum albumin fraction (BSA): 8,8 min
— β-lactoglobulin A and B fraction (β-Lg): 9,7 min
— α-lactalbumin (α-La): 10,6 min

5.9.5 Thermostatic column oven, capable of maintaining a temperature of 30 °C ± 1 °C.

5.9.6 UV-detector, capable of operating at 280 nm.

1) Zorbax Bio column and Millipore Waters packing material are examples of suitable products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO or IDF of these products.

5.9.7 **Integrator**, capable of measuring peak area.

Chose the integration control parameters in such a way that

a) the baseline is allocated at the beginning and at the end of the chromatogram (see Figure A.2),

b) the peak areas of the whey proteins are measured by the perpendicular drop method (see Figure A.2), and

c) any late peaks from the last sample do not interfere with the integration of the next sample.

6 Sampling

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 707.

It is important that the laboratory receive a sample which is truly representative and has not damaged or changed during transport or storage.

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

7 Preparation of test sample

Transfer the test sample to a container of capacity about twice the volume of the sample, provided with an airtight lid. Close the container immediately. Mix the milk powder thoroughly by repeatedly shaking and inverting the container.

8 Procedure

8.1 Test portion

Weigh, to the nearest 1 mg, 2 g of the prepared test sample (clause 7) into a beaker (5.2).

8.2 Test solution

8.2.1 Add 40 ml of water preheated to 40 °C. Dissolve the test portion by stirring for 30 min using a magnetic stirrer (5.5).

8.2.2 Adjust the test solution to pH 4,6 by a dropwise addition of the hydrochloric acid (4.1) using a graduated pipette (5.4). Stir during the pH adjustment with the aid of the magnetic stirrer (5.5). Allow the mixture stand for 15 min at room temperature. Check the pH of the mixture and, if necessary, re-adjust the pH to 4,6.

8.2.3 Filter the mixture through a filter paper (5.6) into a conical flask (5.8), discarding the first fraction of the filtrate.

8.3 Reference solution

Weigh, to the nearest 1 mg, 2 g of the reference sample (4.4) into a beaker (5.2) and proceed as in 8.2.

8.4 Determination by HPLC

8.4.1 During HPLC analysis, maintain the eluent reservoir at a temperature of 85 °C in order to keep the eluent degassed and to prevent bacterial growth.

8.4.2 Prior to use, condition the column by repeatedly injecting 20 µl of the standard sample solution until constant peak areas and retention times are obtained. Typical retention times obtained by the procedure specified in this International Standard are given in 5.9.4 (see also Figure A.2).

8.4.3 Inject 20 µl of the reference solution (8.3) and the test solution (8.2.3) respectively into the HPLC equipment operating at a flow rate of 1,0 ml/min of eluent (4.2).

8.4.4 The integrator (5.9.7) automatically calculates the peak area of the whey proteins. The baseline location shall be checked in every chromatogram. For the test solution and the reference solution, equal baseline sets shall be obtained. The analysis or the integration shall be repeated if the baseline is improperly located. During the reintegration procedure, the use of forced baseline-set times is allowed only if no baseline drift in the chromatograms of the test solution or the reference solution is observed.

8.4.5 In the case of a series of analyses, perform repeated injection of the reference solution after every five test solutions. If a slight drift is noticed for the results of the reference solution, correct the peak area of the individual whey proteins of the test solution. If a large drift is noticed, check that the apparatus is functioning properly and/or recondition the column (see 8.4.2) and repeat the analyses.

When the column is not being used for more than 1 day after finishing the analyses, wash it with the flushing solvent (4.3) at a flow rate of 0,2 ml/min for at least 3 h. Never store the column in the eluent.

9 Calculation and expression of results

9.1 Calculation of the relative ratio

Calculate the relative ratio, r, of the sum of the peak areas of immunoglobulin (Ig) and bovine serum albumin (BSA) by using the following equation:

$$r = \frac{A_{\mathrm{it}} + A_{\mathrm{bt}}}{A_{\mathrm{ir}} + A_{\mathrm{br}}} \times f_1 \times 100$$

where

A_{it} is the numerical value of the peak area of the immunoglobulin (Ig) fraction of the test sample solution, determined as in 8.4.4;

A_{bt} is the numerical value of the peak area of the bovine serum albumin (BSA) fraction of the test sample solution;

A_{ir} is the mean numerical value of the peak area of the immunoglobulin (Ig) fraction of the reference sample solutions used before and after the test sample solution;

A_{br} is the mean numerical value of the peak area of the bovine serum albumin (BSA) fraction of the reference sample solutions used before and after the test sample solution;

f_1 is the factor to correct for a different reference sample or batch of NILAC powder (see Note).

NOTE The original values for r and r_{t} (9.3) have been derived using the NILAC reference sample (4.4). When another reference sample or an NILAC powder of another charge is applied, these values should be adapted with a correction factor.

9.2 Calculation of peak areas

Calculate the relative ratio of the peak areas for both the test sample solution, A_{t}, by using equation (1) and that of the reference solution, A_{r}, by using equation (2):

$$A_t = \frac{A_{it} + A_{bt}}{C_{\beta t}} \quad (1)$$

$$A_r = \frac{A_{ir} + A_{br}}{C_{\beta r}} \quad (2)$$

where

$C_{\beta t}$ is the numerical mean value of the peak area of the β-lactoglobulin A and B fraction of the test sample solution;

$C_{\beta r}$ is the mean numerical value of the peak area of the β-lactoglobulin A and B fraction of the reference sample solutions used before and after the test sample solution.

9.3 Calculation of the relative sample ratio

Calculate the relative ratio of the test sample, r_t, by using the following equation:

$$r_t = \frac{A_t}{A_r} \times f_1 \times 100$$

where

A_t is the numerical value of the relative ratio of the peak area of the test sample solution, obtained in 9.2 [equation (1)];

A_r is the numerical value of the relative ratio of the peak area of the reference sample solution, obtained in 9.2 [equation (2)].

9.4 Determination of the heat class of the test sample

If the value of r is below the limit specified by national legislation of the country in which the International Standard is used, the sample does not belong to the extra-low-heat class.

If the value of r_t is equal to or above the limit specified by national legislation of the country in which the International Standard is used, the sample belongs to the extra-low-heat class.

If the value of r_t is below the limit specified by national legislation of the country in which the International Standard is used, the sample belongs to the low-heat class. (See also the Introduction.)

9.5 Expression

Express the results of the calculations for r and r_t to one decimal place and those of A_t and A_r to three decimal places.

10 Precision

10.1 Interlaboratory test

The values for the repeatability and reproducibility were derived from the results of an interlaboratory test carried out in accordance with ISO 5725-1 and ISO 5725-2. Details of the interlaboratory test of the method are summarized in reference [5]. The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given.

NOTE IDF 135 provides specific guidance for interlaboratory tests on methods of analysis and milk products. It is based on ISO 5725-1 and ISO 5725-2.

10.2 Repeatability

The absolute difference between two independent single test results, obtained using 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 5 for the relative sample ratio (r_t).

10.3 Reproducibility

The absolute difference between two independent single test results, obtained using 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 17 for the relative sample ratio (r_t).

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, with reference to this International Standard;

d) all operational details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the test results;

e) the test results obtained or, if the repeatability has been checked, the final quoted result obtained.