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Milk and milk products — Determination of furosine content — Ion-pair reverse-phase high-performance liquid chromatography method

Lait et produits laitiers — Détermination de la teneur en furosine — Méthode par chromatographie liquide à haute performance en phase inverse par paire d'ions

Reference numbers
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Foreword

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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.

ISO 18329|IDF 193 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.

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 IDF National Committees casting a vote.

International Standard ISO 18329|IDF 193 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.

All work was carried out by the Joint ISO/IDF/AOAC Action Team, *Characterization of heat treatment*, of the Standing Committee on *Minor components and characterization of physical properties,* under the aegis of its project leader, Prof. P. Resmini (IT).

INTERNATIONAL STANDARD

ISO 18329:2004(E)
IDF 193:2004(E)

Milk and milk products — Determination of furosine content — Ion-pair reverse-phase high-performance liquid chromatography method

1 Scope

This International Standard specifies a method for the quantitative determination of furosine (ε-furoylmethyl-lysine) in milk and milk products. The method is particularly applicable to raw or heat-treated milk and to cheese.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 8968-1 | IDF 20-1, *Milk — Determination of nitrogen content — Part 1 — Kjeldahl method*

3 Terms and definitions

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

3.1
furosine content
mass fraction of substance determined by the procedure specified in this International Standard

NOTE Furosine content is expressed in milligrams per 100 g of protein.

4 Principle

The first stable “Maillard Reaction” (MR) product formed in milk and in cheese, *ε*-lactulosyl-lysine, is partially converted by warm acid-hydrolysis into furosine, the determination of which allows the extent of the early stage of MR to be evaluated. The MR extent is related to the type and intensity of heat treatments applied both to raw material and in processing. The determination of furosine is performed by ion-pair reverse-phase (IP-RP) HPLC with UV detection at 280 nm. Quantification of furosine is obtained by reference to a standard sample of furosine.

5 Reagents

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

5.1 Hydrochloric acid (HCl), fuming, with a mass fraction 37 %.

5.2 Hydrochloric acid solution I, $c(\mathrm{HCl}) = 10{,}6$ mol/l.

Mix 8 volumes of hydrochloric acid (5.1) with 1 volume of water to obtain hydrochloric acid solution I.

5.3 Hydrochloric acid solution II, $c(\mathrm{HCl}) = 8{,}0$ mol/l.

Mix 2 volumes of hydrochloric acid (5.1) with 1 volume of water to obtain hydrochloric acid solution II.

5.4 Hydrochloric acid solution III, $c(\mathrm{HCl}) = 3{,}0$ mol/l.

Mix 1 volume of hydrochloric acid (5.1) with 3 volumes of water to obtain hydrochloric acid solution III.

5.5 Methanol (CH_3OH).

5.6 HPLC elution solvents

Prepare HPLC elution solvents by using HPLC-grade reagents.

5.6.1 Water, of HPLC-grade.

5.6.2 Dilute acetic acid (CH_3CO_2H)

Dilute 4 ml of glacial acetic acid with water to 1 000 ml.

5.6.3 Potassium chloride solution, $c(\mathrm{KCl}) = 3$ g/l.

Dissolve 3 g of potassium chloride in 1 000 ml of dilute acetic acid (5.6.2).

5.7 Furosine, (e.g. Neosystem[1] or equivalent).

5.8 Furosine standard solution, $c[\varepsilon\text{-}N\text{-(2-furoylmethyl)-L-lysine}] = 1$ nmol/ml (approx.).

Dissolve 15 mg of furosine (5.7) in 25 ml of hydrochloric acid solution III (5.4) and mix. Dilute 5 ml of this solution with the hydrochloric acid solution III (5.4) to 100 ml. Mix again to obtain dilution 1. Dilute 1 ml of dilution 1 with hydrochloric acid solution III (5.4) to 100 ml to obtain a furosine standard solution of about 1 nmol/ml furosine.

Calculate the exact concentration of furosine in the final furosine standard solution on the basis of the net content declared for the commercial product.

The furosine standard solution remains stable for 24 months, if stored at −20 °C.

5.9 Nitrogen, gas chromatography purity.

6 Apparatus

Usual laboratory equipment and, in particular, the following.

6.1 C 18 cartridge, or minicolumn (500 mg), used in the solid-phase extraction.

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[1] Furosine is supplied by Neosystem, Rue de Bologne 7, 67100 Strasbourg, France. This is an example of a suitable product 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 this product.

6.2 HPLC equipment, provided with gradient pumping system, metal-free injector with injection loop between 20 µl and 50 µl, and a thermostatic column oven.

6.3 UV-detector, capable of operating at 280 nm wavelength with minimum AUFS of 0,010 or lower.

Under these chromatographic conditions and with an injection of 10 pmol of furosine, the signal-to-noise ratio shall be higher than 10.

6.4 “Furosine dedicated” column, of diameter 4,6 mm and of length 250 mm, 5 µm particle size (e.g. Alltech[2]), or an equivalent column capable of separating the furosine peak on the baseline without interfering with other peaks.

6.5 Integrator, or data-reprocessing software, capable of measuring the peak areas.

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

6.7 Oven, capable of being maintained at 110 °C ± 2 °C.

6.8 Screw-cap Pyrex[3] vials, or other heat-resistant sealing vials.

6.9 Paper filters, of medium porosity.

6.10 Glass syringe, of capacity of 10 ml.

6.11 Kjeldahl apparatus,conforming to ISO 8968-1|IDF 20-1.

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 International Standard. A recommended sampling method is given in ISO 707.

8 Preparation of test portion

8.1 Milk

Pipette 2 ml of test sample into a screw-cap Pyrex-vial (6.8). Add 6 ml of hydrochloric solution I (5.2) and mix.

8.2 Cheese

Weigh an aliquot of test sample corresponding to an amount of 40 mg to 50 mg of protein into a screw-cap vial (6.8). Add 8 ml of hydrochloric solution II (5.3) and mix.

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[2] The “Furosine dedicated” column is supplied by Alltech-Europe, Laarne, Belgium.

[3] Pyrex is an example of a suitable product 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.

8.3 Hydrolysis

8.3.1 Bubble the nitrogen (5.9) through the prepared test portion in the screw-cap vial (8.1 or 8.2) for about 2 min. Close the vial tightly and heat its contents for 23 h in the oven (6.7) set at 110 °C. Gently shake the vial after the first hour of heating.

8.3.2 Cool and filter the obtained hydrolysate (8.3.1) through a paper filter (6.9).

If necessary, the filtered hydrolysate may be kept for 12 months if stored at –20 °C.

8.4 Determination of protein content

Determine the nitrogen content in 2 ml of the filtered hydrolysate (8.3.2) by using the “Kjeldahl” method described in ISO 8968-1|IDF 20-1. Calculate the protein content by multiplying the nitrogen content by 6,38.

9 Procedure

9.1 Solid-phase extraction (SPE) of the filtered hydrolysate

9.1.1 Mount the C18 cartridge (6.1) onto the glass syringe (6.10). Pre-wet the cartridge by eluting 5 ml of methanol (5.5) followed by 10 ml of distilled water. Do not allow the cartridge to go dry.

9.1.2 Take up 0,5 ml of filtered hydrolysate (8.3.2) into the syringe. Slowly inject this into the cartridge, discarding the displaced liquid. Avoid introducing air.

9.1.3 Pipette 3 ml of hydrochloric acid solution III (5.4) into the syringe. Slowly elute until complete drying of the cartridge occurs, then collect the colourless eluate.

The purified hydrolysate remains stable for one week if stored at –20 °C.

9.2 HPLC determination

9.2.1 Chromatographic conditions

9.2.1.1 Elution solvents

Use acetic acid solution (5.6.2) as elution solvent A, and potassium chloride solution (5.6.3) as elution solvent B.

9.2.1.2 Elution gradient

This shall be as given in Table 1.

Table 1 — Elution gradient

Time min	Solvent A[a] %	Solvent B[a] %
0	100	0
12,5	100	0
19,5	50	50
22,0	50	50
24,0	100	0
32,0	100	0

[a] Equivalent elution gradients may be used according to the adopted column.

9.2.1.3 Flow rate

Set the flow rate of the elution solvent at 1,2 ml/min.

9.2.1.4 Column temperature

The column temperature shall be kept at constant value ranging between 30 °C and 35 °C for the “Furosine dedicated” column, or according to the type of column adopted.

9.2.1.5 Equilibration

Equilibrate the chromatographic system by flushing the column for a few minutes with a mixture of solvent A and B in the ratio 50:50 and a flow rate of 1,2 ml/min. Then set the initial condition (9.2.1.2) until a stable baseline is observed.

9.2.1.6 Determination

Perform a blank run by injecting between 20 µl and 50 µl of hydrochloric acid solution III (5.4) in order to check the purity of the solvents. After the final equilibration step, the detector response shall stabilize to the initial value.

Submit both the purified hydrolysate (9.1.3) and the furosine standard solution (5.8) to chromatographic separation by injecting between 20 µl and 50 µl of each. The furosine peak shall be resolved on the baseline with a retention time ranging from 20 min to 24 min.

Determine the furosine peak area for both purified hydrolysate (9.1.3) and furosine standard solution (5.8) by using baseline integration (see examples of HPLC patterns in Annex A).

NOTE The furosine signal at 280 nm has proven to be linear over the range (1 to 1 000) pmol injected, while the range of interest is between (5 and 500) pmol. Therefore, it is not necessary to perform a multi-point calibration.

10 Calculation and expression of results

10.1 Calculation

Calculate the furosine content of the test sample, w, using the following equation:

$$w = A_t \times \frac{c}{A_f} \times \frac{1}{V} \times \frac{d}{F} \times \frac{M}{10} \times \frac{8}{m \times 4}$$

where

w is the furosine content of the sample, expressed in milligrams per 100 g of protein;

A_t is the numerical value of the peak area of furosine obtained for the test sample (9.2.1.6);

c is the absolute amount of furosine in the injected volume of standard solution (5.8) (between 20 µl and 50 µl), in picomoles;

A_f is the numerical value of the peak area of furosine obtained for the furosine standard solution (9.2.1.6);

V is the numerical value of the injected volume, in microlitres (V is between 20 µl and 50 µl);

d is the numerical value of the dilution factor involved in the SPE (d = 6);

F is the numerical value of the recovery factor of the SPE (F = 0,95);

M is the numerical value of the molecular mass of the furosine (M = 254);

m is the numerical value of the mass of the protein content in 2 ml of hydrolysate (8.4), in milligrams.

10.2 Expression of results

Express the results to 1 decimal place.

11 Precision

11.1 Interlaboratory test

Details of an interlaboratory test on the precision of the method are summarized in Annex B. The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given.

11.2 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 6 % of the arithmetic mean of the results.

11.3 Reproducibility

The absolute difference between two 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 15 % of the arithmetic mean of the results.

12 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;

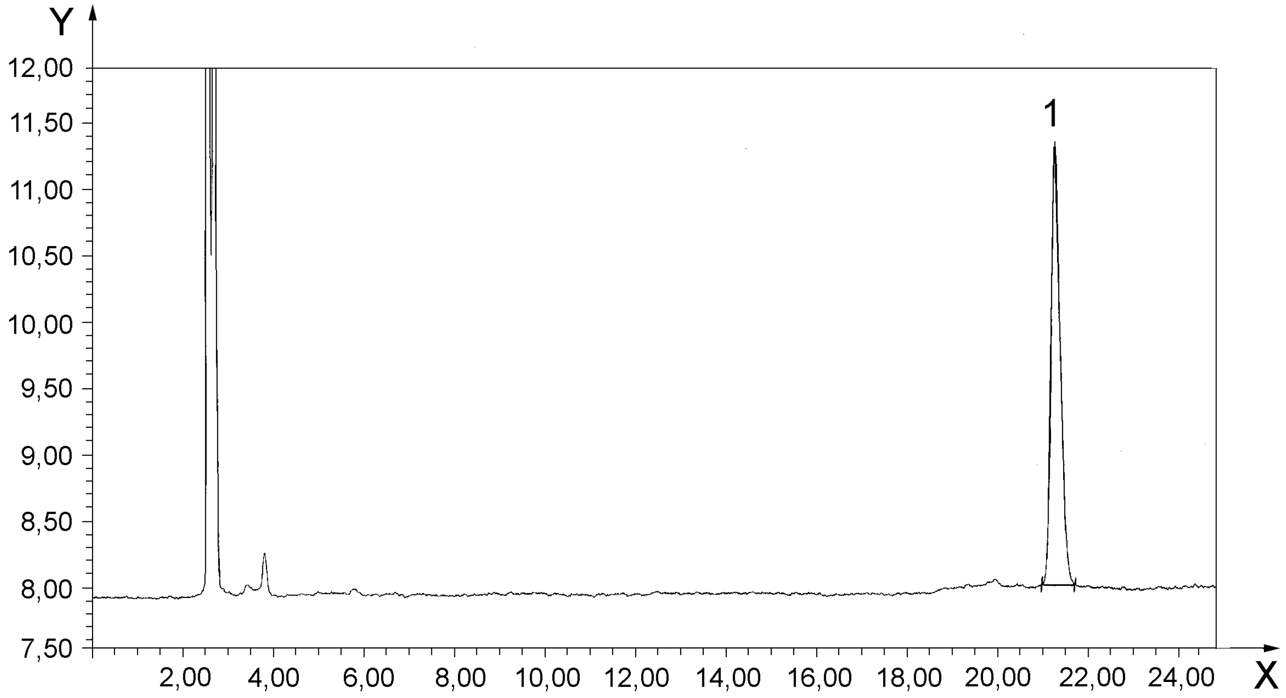
c) the method used, with reference to this International Standard;

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

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

Annex A
(informative)

Examples of HPLC patterns



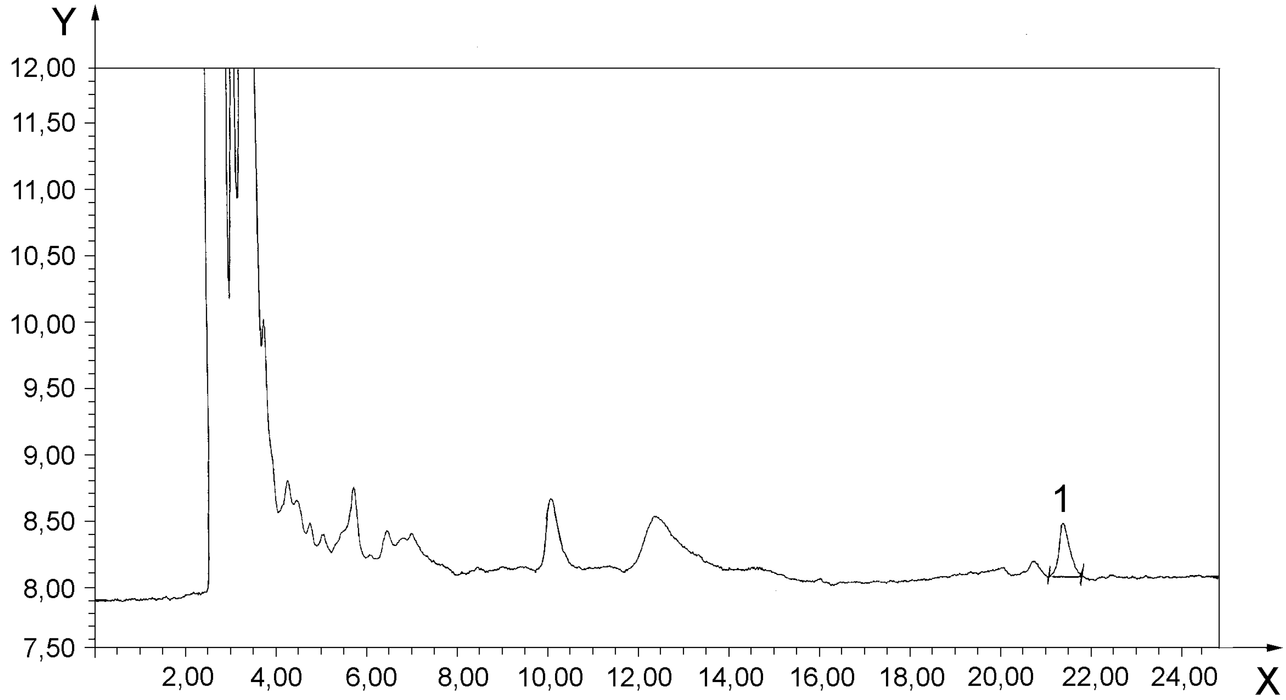
Key

X retention time, in minutes

Y voltage, in millivolts

1 furosine peak

Figure A.1 — HPLC pattern of a furosine standard solution (53 pmol of furosine in the peak)



NOTE Key as in Figure A.1.

Figure A.2 — HPLC pattern of a peroxidase-positive pasteurized milk (8 pmol of furosine in the peak)

Annex B
(informative)

Results of interlaboratory trials

The results obtained were subjected to statistical analysis in accordance with ISO 5725[4)] to give the precision data shown in Table B.1.

An international collaborative test was carried out involving eight laboratories, using test samples of six types of milk. The test was organized within the Dairy Expert Group of the EU Commission.

Table B.1 — Results of interlaboratory test

	Pasteurized milk A	**Pasteurized milk B**	**HT pasteurized milk A**	**HT pasteurized milk B**	**UHT milk A**	**UHT milk B**
Participating laboratories	8	8	8	8	8	8
Mean value[a]	8,68	9,31	11,73	19,99	46,07	80,45
Repeatability standard deviation, s_r[a]	0,18	0,17	0,24	0,43	0,83	1,46
Repeatability limit, r (= 2,8 s_r)[a]	0,51	0,48	0,66	1,21	2,33	4,08
Reproducibility standard deviation, s_R,[a]	0,61	0,70	0,73	0,85	2,34	2,33
Reproducibility limit, R (= 2,8 s_R)[a]	1,72	1,95	2,05	2,39	6,55	6,52

[a] Values expressed as milligrams of furosine per 100 g of protein.

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4) ISO 5725:1986, *Precision of test methods — Determination of repeatability and reproducibility for a standard test method by inter-laboratory tests* (now withdrawn) was used to obtain the precision data.

Bibliography

[1] ISO 707, *Milk and milk products — Guidance on sampling*[5]

[2] ISO 5725-1, *Accuracy (trueness and precision) of measurement methods and results — Part 1: General principles and definitions*

[3] ISO 5725-2, *Accuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method*

[4] RESMINI, P., PELLEGRINO, L. and BATTELLI, G. Accurate quantification of furosine in milk and dairy products by direct HPLC method. *It. J. Food Sci.*, 3, 1990, pp.173-183

[5] RESMINI, P., PELLEGRINO, L., MASOTTI, F., TIRELLI, A. and PRATI, F. Determinazione del latte in polvere ricostituito nel latte crudo ed in quello pastorizzato, mediante HPLC della furosina. *Sci. Tecn. Latt-Cas.*, **43**, 1992, pp. 169-186

[6] RESMINI, P., PELLEGRINO, L. and MASOTTI, F. *Evaluation of the Maillard reaction extent for quality control of low-heat treated dairy products.* IDF Special Issue 9303, *Protein and fat globule modifications by heat-treatment, homogenisation and other technological means for high quality dairy products*, 1993, pp. 153-164

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[5] Equivalent to IDF 50.

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