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Milk and cheese — Determination of hen's egg white lysozyme content by high performance liquid chromatography

National foreword

This British Standard is the UK implementation of ISO 27105:2016. It supersedes DD ISO/TS 27105:2009 which is withdrawn.

The UK participation in its preparation was entrusted to Technical Committee AW/5, Chemical analysis of milk and milk products.

A list of organizations represented on this committee can be obtained on request to its secretary.

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**IDF
216**

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**Milk and cheese — Determination
of hen's egg white lysozyme
content by high performance liquid
chromatography**

*Lait et fromages — Détermination de la teneur en lysozyme de blanc
d'oeuf par chromatographie liquide haute performance*



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Forewords

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established 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. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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The committee responsible for this document is ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products* and the International Dairy Federation (IDF) and it is being published jointly by ISO and IDF.

This first edition of ISO 27105|IDF 216 cancels and replaces ISO/TS 27105|IDF/RM 216:2009, which has been technically revised.

IDF (the International Dairy Federation) is a non-profit private sector organization representing the interests of various stakeholders in dairying at the global level. IDF members are organized in National Committees, which are national associations composed of representatives of dairy-related national interest groups including dairy farmers, dairy processing industry, dairy suppliers, academics and governments/food control authorities.

ISO and IDF collaborate closely on all matters of standardization relating to methods of analysis and sampling for milk and milk products. Since 2001, ISO and IDF jointly publish their International Standards using the logos and reference numbers of both organizations.

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ISO 27105|IDF 216 was prepared by the IDF Standing Committee on *Analytical Methods for Additives and contaminants* and ISO Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*.

All work was carried out by the ISO/IDF Project Group on *Determination of hen's egg white lysozyme content by high performance liquid chromatography* of the Standing Committee on *Analytical Methods for Additives and Contaminants* under the aegis of the project leaders, T. Berger (CH) and Prof. L. Pellegrino (IT).

This first edition of ISO 27105|IDF 216 cancels and replaces ISO/TS 27105|IDF/RM 216:2009, which has been technically revised.

Introduction

Lysozyme (EC 3.2.1.17, muramidase) is an enzyme widely dispersed in nature; it appears, e.g. in hen's egg white (approximately 3 % to 4 %), saliva and in tear liquid. Lysozyme has a preservative effect because of the lytic activity on the cell wall of some bacteria. Hen's egg white lysozyme is used in cheese making to prevent late blowing of semi-hard and hard cheeses.

Milk and cheese — Determination of hen's egg white lysozyme content by high performance liquid chromatography

1 Scope

This International Standard specifies a method for the quantitative determination of hen's egg white lysozyme content in milk and cheese.

The method is suitable for measuring low levels of hen's egg white lysozyme with a quantification limit of 10 mg/kg.

2 Terms and definitions

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

2.1

hen's egg white lysozyme content

mass fraction of substance determined by the procedure specified

Note 1 to entry: The lysozyme content is expressed as milligram per kilogram.

3 Principle

Casein and denatured whey proteins from milk and cheese are precipitated isoelectrically at pH 4,3 (cheese) or at pH 2,2 (milk). Acid-soluble hen's egg white lysozyme is then determined by reversed-phase high-performance liquid chromatography (HPLC) and fluorescence detection. The lysozyme peak can be verified by LC/MS (see [Annex A](#)).

4 Reagents and reference substances

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

4.1 Reagents and materials

4.1.1 Sodium chloride solution, $c(\text{NaCl}) = 1 \text{ mol/l}$.

Dissolve 58,44 g of sodium chloride in 1 l water.

4.1.2 Hydrochloric acid, $c(\text{HCl}) = 1 \text{ mol/l}$.

Dissolve 4,0 ml of hydrochloric acid with mass fraction 37 % in a 50 ml one-mark volumetric flask. Dilute to the mark with water.

4.1.3 Sodium hydroxide, $c(\text{NaOH}) = 1 \text{ mol/l}$.

Dissolve 2,6 ml of sodium hydroxide with a mass fraction of 50 % in a 50 ml one-mark volumetric flask. Dilute to the mark with water.

4.1.4 Trifluoroacetic acid (CF_3COOH), analytical grade.

4.1.5 Acetonitrile (CH₃CN), HPLC grade.

4.1.6 Water, HPLC grade.

4.2 Lysozyme

Pure hen's egg white lysozyme¹⁾. Sufficiently pure and characterized lysozyme is difficult to obtain. A control on lot-to-lot variability is needed.

5 Apparatus

Usual laboratory equipment and, in particular, the following.

5.1 pH-meter, accurate to 0,1 unit.

5.2 Fluted filter, diameter 15 cm²⁾.

5.3 Membrane filter, pore size 0,22 µm³⁾.

5.4 Balance, capable of weighing to the nearest 100 mg, with a readability of 10 mg.

5.5 Analytical balance, capable of weighing to the nearest 0,1 mg, with a readability of 0,01 mg.

5.6 Magnetic stirrer.

5.7 Homogenizer ⁴⁾, capable of spinning at a rotational frequency of 3 000 rpm to 3 500 rpm.

5.8 HPLC equipment.

5.8.1 Gradient pumping system, capable of operating with a speed of 1,0 ml/min.

5.8.2 Manual or automatic injector, capable of injecting amounts of 50 µl.

5.8.3 Column heater, capable of maintaining a column temperature of 45 °C ± 2 °C.

5.8.4 Column, reversed phase⁵⁾, 5 µm, 250 mm × 4,6 mm.

1) Lysozyme SIGMA L-6876 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 this product.

2) Fluted filter Schleicher&Schuell 595 ½ 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 this product.

3) Millipore Millex-GV PVDF 0,22 µm 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 this product. Equivalent products may be used if they can be shown to lead to the same results.

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5.8.5 Fluorescence detector, capable of operating at 280 nm excitation and at 340 nm emission.

6 Sampling

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 707|IDF 50.^[1]

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

7 Procedure

7.1 Preparation of lysozyme standard solution

7.1.1 Lysozyme standard stock solution

Weigh to the nearest 0,01 mg, 10 mg of lysozyme (4.2) into a 10 ml one-mark volumetric flask and wait for complete dissolution. Dilute to the mark with sodium chloride solution (4.1.1).

Prepare fresh the standard stock solutions daily.

7.1.2 Lysozyme standard working solution

Pipette 80 µl of the lysozyme standard stock solution (see 7.1.1) into a 10 ml one-mark volumetric flask. Dilute to the mark with sodium chloride solution (4.1.1).

The obtained lysozyme standard working solution contains 8,0 mg of lysozyme per litre.

7.2 Test portion

7.2.1 Milk

Weight to the nearest 0,01 g, 10,00 g of test sample into a 100 ml beaker.

7.2.2 Cheese

Before weighing, grate the test samples of cheese. Weight to the nearest 0,01 g, 2,00 g of test sample into a 100 ml beaker.

NOTE Soft cheese can be grated after freezing.

7.2.3 Preparation of test solution

Add 20 ml of sodium chloride solution (4.1.1) to the test portion (see 7.2.1 or 7.2.2) and mix. Adjust the pH of the obtained solution drop wise with sodium hydroxide solution (4.1.3) to pH 6,0.

Homogenize the test solution for 30 s by using the homogenizer (5.7) at a speed rate of 2 500 rpm to 3 000 rpm. Rinse the homogenizer in a separate 100 ml beaker using 10 ml of sodium chloride solution (4.1.1). Add the rinsing to the test solution.

Stir the beaker containing the test solution on a magnetic stirrer at room temperature for 1 h. Adjust the pH of the test solution obtained from the test portion of milk (see 7.2.1) to pH 2,2 or that obtained from the test portion of cheese to pH 4,3 by using hydrochloric acid (4.1.2).

Transfer the test solution from the 100 ml beaker to a 50 ml one-mark volumetric flask. Use sodium chloride solution (4.1.1) to rinse the 100 ml beaker and add the rinsing to the test solution. Dilute to the mark with the sodium chloride solution (4.1.1) and mix.

Allow the test solution to stand at room temperature for at least 15 min.

Firstly, filter the test solution through a fluted filter (5.2) and then through the membrane filter (5.3) directly into a HPLC vial.

7.3 HPLC determination

7.3.1 Chromatographic conditions

Prepare the following stock solutions:

- stock solution I: 1 ml of trifluoroacetic acid (4.1.4) in 1 l of water (4.1.6);
- stock solution II: 1 ml of trifluoroacetic acid (4.1.4) in 1 l of acetonitrile (4.1.5).

Use the following elution solvents for HPLC:

- elution solvent A contains: stock solution I:stock solution II = 72,25:27,75 (w/w);
- elution solvent B contains: stock solution II.

Table 1 — Suggested gradient elution

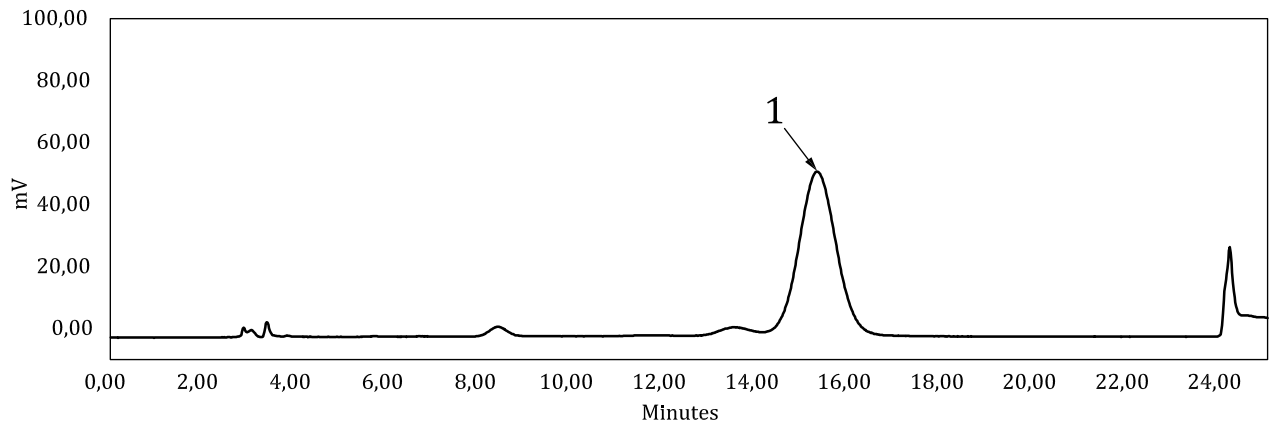
Time (min)	Elution solvent A ^a (%)	Elution solvent B ^a (%)
0,0	100	0
20,0	100	0
21,0	50	50
22,0	50	50
23,0	100	0
35,0	100	0

^a The gradient elution might require slight modification in order to achieve the resolution shown in Figure 1 and Figure 2. Isocratic elution in the range of the lysozyme peak, as used in this International Standard, leads to a cleaner chromatogram but to larger peak width. The use of a gradient may lead to smaller peak widths and more stable retention times but also to more peaks in the lysozyme range with a risk of misinterpretation.

Set the flow rate of the gradient elution pumping system of the HPLC equipment at 1,0 ml/min.

Set the temperature of the column heater at 45 °C. Determine the equilibration time by monitoring the column elution. The detector response at the end of the run (baseline) should be equal to its initial value. An isocratic flushing of 15 min is usually sufficient.

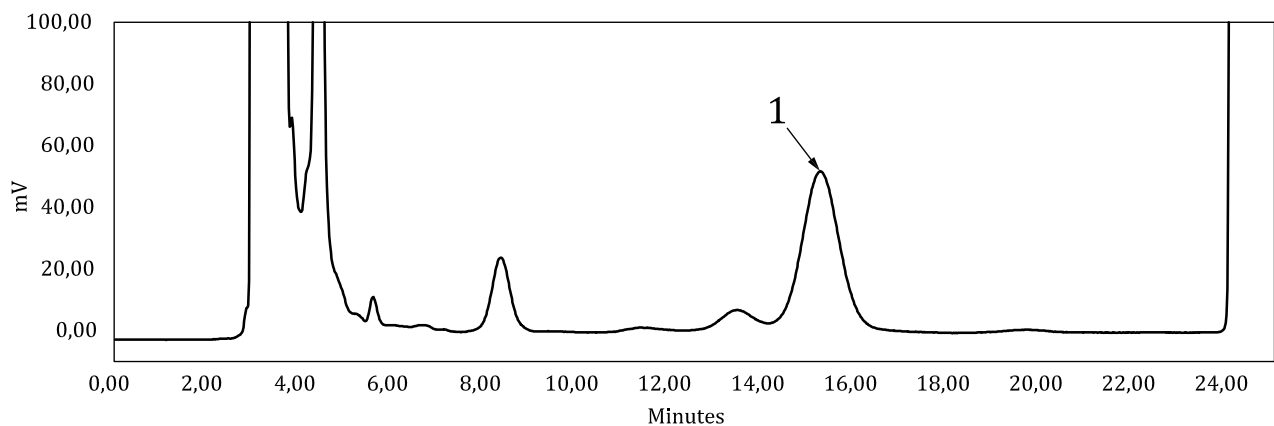
Injection: Use a manual or automatic injector to inject 50 µl of the solutions into the column.



Key

1 lysozyme

Figure 1 — HPLC fluorescence signal from the standard working solution (7.1.2)



Key

1 lysozyme

Figure 2 — HPLC fluorescence signal from a hard cheese sample

8 Calculation and expression of results

8.1 Single-point calibration

Calculate the lysozyme content of the test sample, w , expressed in milligrams per kilogram, by using Formula (1):

$$w = \frac{H_t \times c_s \times V_t}{H_s \times m_t} \quad (1)$$

where

c_s is the concentration, in milligrams per litre, of the standard working solution (see [7.1.2](#));

H_t is the numerical value of the peak height or area of the test solution (see [7.2](#)), in counts;

H_s is the numerical value of the peak height or area of the standard working solution (see [7.1.2](#)), in counts;

m_t is the mass, in grams, of the test portion (see [7.2.1](#) or [7.2.2](#));

V_t is the volume, in millilitres, of the test solution (see [7.2.3](#)).

Check equipment linearity and reagent blank regularly. This may be needed in case of lysozyme contents of more than 100 mg/kg of cheese which can lead to a non-linear response. In such cases, the use of a multiple point calibration is necessary.

8.2 Expression of results

Express the results to one decimal place. Express test results of below 10 mg/kg as: “less than 10 mg/kg”, or equivalent expression.

9 Precision

9.1 Interlaboratory test

The values for repeatability and reproducibility derived from an interlaboratory test^[7] were determined in accordance with ISO 5725-1^[2] and ISO 5725-2^[3]

The values for the repeatability and the reproducibility limit are expressed for the 95 % probability level and may not be applicable to concentration ranges and matrices other than those given. Details of the interlaboratory test on the precision of the method are given in [Annex B](#).

9.2 Repeatability

The absolute difference between two individual single test results, obtained with 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, shall in not more than 5 % of the cases be greater than 7,7 % (95 % confidence intervals 6,2 % to 8,5 %).

9.3 Reproducibility

The absolute difference between two individual single test results, obtained with the same method on identical test material in different laboratories with different operators using different equipment, shall in not more than 5 % of the cases be greater than 33,8 % (95 % confidence intervals 20,3 % to 57,5 %).

Running the chromatographic system on another temperature, using a different column type, or other differing conditions do not result automatically in false or less sensitive results but need thoroughly to be validated.

10 Test report

The test report shall contain at least the following information:

- 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, i.e. ISO 27105|IDF 216;

- d) all operational details not specified in this International Standard, or regarded as optional, together with details of any incident which may have influenced the test result(s);
- e) the test result(s) obtained, and, if the repeatability has been checked, the final quoted results obtained.

Annex A (informative)

Verification by LC/MS

A.1 General

In case of lysozyme peaks in the test solutions, findings can be verified using liquid chromatography/mass spectrometry LC/MS. MS parameters for lysozyme have to be optimized and tuned according to the system manual of the instrument (e.g. needle voltage 3,0 kV, probe 550 °C, cone voltage 130 V).

Use an LC/MS equipment capable of running the conditions described in [A.2](#) and [A.3](#) [different from HPLC (see [7.3](#)) because of non-useful trifluoroacetic acid] and measure the mass signals $m/z = 1\ 431 [M+H_{10}]^{10+}$; $1\ 590 [M+H_9]^{9+}$ and $1\ 788 [M+H_8]^{8+}$, retention time is approx. 12,5 min.

Verify the presence of lysozyme by homogeneous distribution of these masses all over the presumed lysozyme peak.

A.2 LC/MS equipment

A.2.1 Gradient pumping system, capable of operating at 0,8 ml/min.

A.2.2 Manual or automatic injector, capable of injecting amounts of 5 µl.

A.2.3 Column heater, capable of maintaining the column temperature at $40\text{ °C} \pm 2\text{ °C}$.

A.2.4 Column, reversed phase⁶⁾, 5 µm, 250 mm × 4,6 mm, conditioned for several hours without trifluoroacetic acid.

A.2.5 Mass spectrometer detector, capable of operating in ion mode ESI+ at m/z 1 431; 1 590 and 1 788.

A.3 Chromatographic conditions

Use the following elution solvents for LC/MS:

- elution solvent A containing 5 ml of formic acid (analytical grade) in 1 l of water ([4.1.6](#));
- elution solvent B containing 5 ml of formic acid in 1 l of acetonitrile ([4.1.5](#)).

Set the flow rate of the pumping system of the HPLC equipment at 0,80 ml/min. The flow split should be: 0,5 ml/min MS; 0,3 ml/min waste (when 25 % B), motor valve 0,0 min eluent MS; 0,5 min eluent waste; 7,0 min eluent MS.

Using a manual or automatic injector inject 5 µl.

6) PLRP-S 300Å (Polymer Laboratories, UK) 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 this product.

Table A.1 — Suggested gradient elution

Time (min)	Elution solvent A^a (%)	Elution solvent B^a (%)
0,0	70	30
9,0	30	70
9,1	0	100
12,0	0	100
12,1	70	30
16,0	70	30

^a The gradient elution might require slight modification in order to achieve a resolution as accomplished under HPLC conditions shown in [Table 1](#) and [Figure 1](#).

Annex B (informative)

Interlaboratory test

An international collaborative test according to ISO/TS 27105|IDF/RM 216:2009 involving 20 laboratories was carried out on seven samples of lysozyme in milk and cheese from Italy and Switzerland. Two laboratories did not send results and three did not respect the ISO/TS 27105|IDF/RM 216:2009. Four of the samples contained no measurable amount of analyte. They were correctly identified by the laboratories as such and used to calculate the detection limit and selectivity. One laboratory showed a significant bias and was excluded from the statistical evaluation. Observed homogeneity in individual precision values allowed the calculation of precision values r and R for the concentration ranges in [Table B.1](#), which are recommended for use.

[Table B.1](#) shows the results for different samples and concentration ranges. The values were calculated on the basis of the mean values of the single samples reported by the laboratories.

Table B.1 — Results of an interlaboratory test with different samples and concentration ranges

Parameter	Sample 1, milk 10 mg/kg to 100 mg/kg	Sample 2, cheese 100 mg/kg to 200 mg/kg	Sample 3, cheese 200 mg/kg to 300 mg/kg
Number of laboratories after eliminating outliers	14	14	15
Mean value, mg/kg	15,4	143,7	238,9
Repeatability standard deviation, s_r , mg/kg	0,5	3,5	8,7
Coefficient of variation of repeatability, CV_r %	8,4	6,9	10,2
Repeatability limit, $r = 2,8 \cdot s_r$, mg/kg	1,3	9,9	24,3
Reproducibility standard deviation, s_R , mg/kg	2,5	14,4	26,8
Coefficient of variation of reproducibility, CV_R %	44,8	28,0	31,4
Reproducibility limit, $R = 2,8 \cdot s_R$, mg/kg	6,9	40,2	74,9

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