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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 du blanc d'oeuf par chromatographie liquide à haute performance

ICS: 67.100.01

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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 2.

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.

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ISO 27105 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*.

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

ISO 27105 | IDF 216 was prepared by Technical Committee ISO/TC 34, Food products, Subcommittee SC 5, Milk and milk products and the International Dairy Federation (IDF). It is being published jointly by ISO and IDF.

Foreword

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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This International Standard was prepared by the IDF Standing Committee on Analytical Methods for Dairy Microorganisms and the ISO Technical Committee 34 on Food products, Subcommittee 5 on Milk and milk products (ISO/TC 34/SC 5).

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).

Milk and cheese– Determination of hen's egg white lysozyme by HPLC

1 Scope

This 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 per kilogram.

NOTE 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 % - 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.

2 Terms and definitions

For the purpose of this document, the following term and definition applies.

2.1 Hen's egg white lysozyme content

Mass fraction of substance determined by the procedure specified in this standard.

NOTE 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 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

4.1.1 Sodium chloride solution, c(NaCl) = 1 mol/l.

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

4.1.2 Hydrochloric acid, c(HCl) = 1 mol/l.

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

4.1.3 Sodium hydroxide, c(NaOH) = 1 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 50 ml mark with water.

4.1.4 Trifluoracetic acid (CF_3COOH), analytical grade.

4.1.5 Acetonitrile (CH_3CN), HPLC grade.

4.1.6 Water, HPLC grade.

4.2 Lysozyme

Pure hen's egg white lysozyme, e.g. Lysozyme, SIGMA L-6876[1)]. It has to be noted that sufficient 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, e.g. Schleicher&Schuell 595½, Ø 15 cm [1)].

5.3 Membrane filter, pore size 0,22 μm, e.g. Millipore Millex-GV PVDF 0,22 μm[1)]. Filters of other producers need to be tested for applicability.

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, Ultra turrax®[1)] or another giving equivalent results and capable of spinning at a rotational frequency of 3000 to 3500 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 phase, e.g. PLRP-S 300 Å[1)], 5 µm, 250 mm x 4,6 mm

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

6 Sampling

1) SIGMA L-6876, Schleicher&Schuell 595½, Millipore Millex-GV PVDF 0,22 μm, Ultra Turrax®, PLRP-S 300 Å (Polymer Laboratories, UK) are products available commercially. This information is given for the convenience of users of this standard and does not constitute an endorsement by either ISO or IDF of these products.

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 10 ml 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 (7.1.1) into a 10 ml one-mark volumetric flask. Dilute to the 10 ml mark with sodium chloride solution (4.1.1).

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

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 portion

Add 20 ml of sodium chloride solution (4.1.1) to the test portion (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.

Disperse the test portion for 30 s by using the homogenizer (5.7) at a speed rate of 2500 rpm to 3000 rpm. Rinse the homogenizer in a separate 100 ml beaker by shortly dispersing in 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 portion obtained from milk (7.2.1) to pH 2,2 and that obtained from cheese to pH 4,3 by using hydrochloric acid (4.1.2).

Transfer the test solution to a 50 ml one-mark volumetric flask. Use sodium chloride solution (4.1.1) to rinse the 100 ml beaker. Dilute to the 50 ml 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:

1. Stock solution I: 1 ml of trifluoracetic acid (4.1.4) in 1 l of water (4.1.6).
2. Stock solution II: 1 ml of trifluoracetic 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. Isocratic elution in the range of the lysozyme peak, as used in this 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.

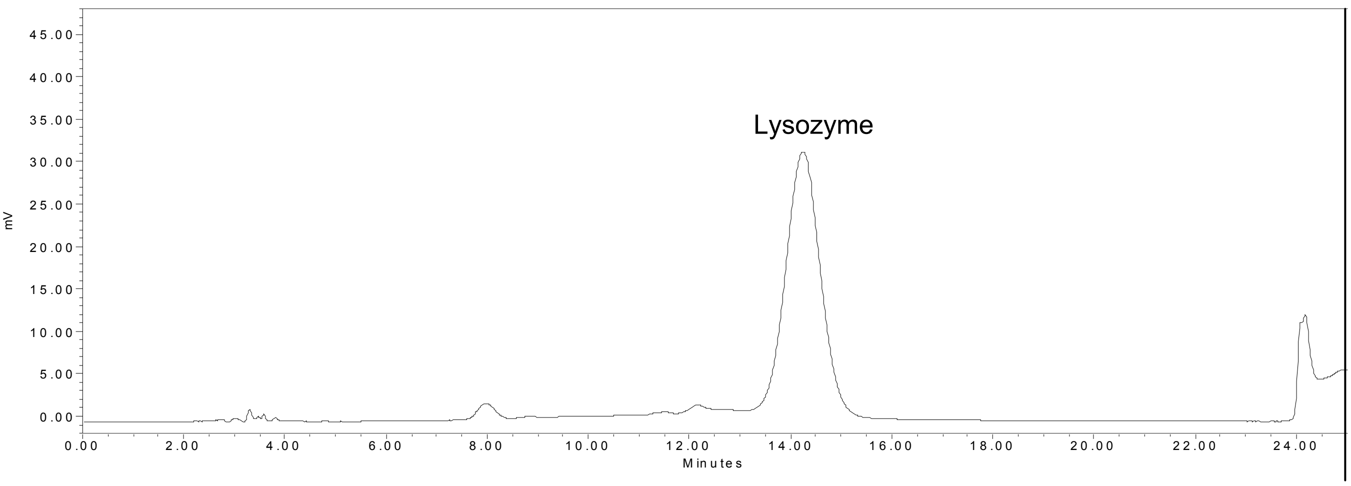

Figure 1 – HPLC fluorescence signal from the standard working solution (7.1.2)