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**Milk and milk products — Determination  
of hen's egg white lysozyme by HPLC**

*Lait et produits laitiers — Détermination de lysozyme de blanc d'œufs  
par CLHP*

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## Foreword

**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.

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.

In other circumstances, particularly when there is an urgent market requirement for such documents, a technical committee may decide to publish other types of document:

- an ISO Publicly Available Specification (ISO/PAS) represents an agreement between technical experts in an ISO working group and is accepted for publication if it is approved by more than 50 % of the members of the parent committee casting a vote;
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An ISO/PAS or ISO/TS is reviewed after three years in order to decide whether it will be confirmed for a further three years, revised to become an International Standard, or withdrawn. If the ISO/PAS or ISO/TS is confirmed, it is reviewed again after a further three years, at which time it must either be transformed into an International Standard or be withdrawn.

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/TS 27105|IDF/RM 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 organization representing the dairy sector worldwide. IDF membership comprises National Committees in every member country as well as regional dairy associations having signed a formal agreement on cooperation with IDF. All members of IDF have the right to be represented on the IDF Standing Committees carrying out the technical work. IDF collaborates with ISO in the development of standard methods of analysis and sampling for milk and milk products.

The main task of technical committees is to prepare International Standards. 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 the IDF National Committees casting a vote.

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Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. IDF shall not be held responsible for identifying any or all such patent rights.

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All work was carried out by the Joint ISO-IDF Action Team on *Food additives and vitamins* of the Standing Committee on *Analytical methods for additives and contaminants* under the aegis of its project leaders, Dr. T. Berger (CH) and Prof. L. Pellegrino (IT).

# Milk and milk products — Determination of hen's egg white lysozyme by HPLC

## 1 Scope

This Technical Specification specifies a method for the quantitative determination of hen's egg white lysozyme in milk and milk products.

The method is suitable for measuring low levels of hen's egg white lysozyme with a quantification limit of 5 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 in this Technical Specification

NOTE The lysozyme content is expressed in milligrams per kilogram.

## 3 Principle

Casein and denatured whey proteins from milk and milk products are precipitated isoelectrically at pH 4,3 (cheese and solid milk products) or at pH 2,2 (milk and liquid milk products). 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, $c(\text{NaCl}) = 1 \text{ mol/l}$ .

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

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

Add 4,0 ml of hydrochloric acid with mass fraction 37 % to a 50 ml one-mark volumetric flask (5.9). Make up to the mark with water and mix.

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

Add 2,6 ml of sodium hydroxide with a mass fraction of 50 % to a 50 ml one-mark volumetric flask (5.9). Make up to the mark with water and mix.

**4.1.4 Trifluoroacetic acid** ( $\text{CF}_3\text{COOH}$ ).

**4.1.5 Acetonitrile** ( $\text{CH}_3\text{CN}$ ), HPLC grade.

**4.1.6 Water**, HPLC grade.

**4.2 Lysozyme**

Pure hen's egg white lysozyme<sup>1)</sup>.

NOTE Lysozyme (EC 3.2.1.17, muramidase) is an enzyme widely dispersed in nature, e.g. in hen's egg white (approximately 3 g/100 g to 4 g/100 g), saliva, and 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 cheesemaking to prevent late blowing of semi-hard and hard cheeses.

**5 Apparatus and materials**

Usual laboratory equipment and, in particular, the following.

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**5.1 pH-meter.**

**5.2 Fluted filter**, of diameter 150 mm<sup>2)</sup>. [ISO/TS 27105:2009](https://standards.iteh.ai/catalog/standards/sist/ceeb7ada-855b-4022-bdc1-7c8bdeae6a2f/iso-ts-27105-2009)

**5.3 Membrane filter**, of pore size 0,22 µm<sup>3)</sup>. <https://standards.iteh.ai/catalog/standards/sist/ceeb7ada-855b-4022-bdc1-7c8bdeae6a2f/iso-ts-27105-2009>

**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**, capable of a rotational frequency of 2 500 r/min to 3 000 r/min.

**5.8 HPLC equipment.**

**5.8.1 Elution gradient pumping system**, capable of delivering a flow rate of 1,0 ml/min.

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

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

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1) Sigma L-7651 is an example of a suitable product available commercially. This information is given for the convenience of users of this Technical Specification and does not constitute an endorsement by ISO or IDF of this product.

2) Schleicher & Schuell 595½ is an example of a suitable product available commercially. This information is given for the convenience of users of this Technical Specification and does not constitute an endorsement by ISO or IDF of this product.

3) 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 Technical Specification and does not constitute an endorsement by ISO or IDF of this product.

**5.8.4 Column for reversed phase chromatography**, PLRP-S 300 Å<sup>4</sup>) 5 µm, 250 mm × 4,6 mm.

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

**5.9 One-mark volumetric flasks**, of capacities 10 ml and 50 ml, ISO 1042 [2] class A.

## 6 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 Technical Specification. A recommended sampling method is given in ISO 707 | IDF 50<sup>[1]</sup>.

## 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 (5.9). Make up to the mark with sodium chloride solution (4.1.1) and mix.

Prepare the standard stock solutions on the day of use.

#### 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 (5.9). Make up to the mark with sodium chloride solution (4.1.1) and mix.

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

### 7.2 Test portion

#### 7.2.1 Milk or other liquid milk product

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

#### 7.2.2 Cheese or other solid milk products

Before weighing, grate the test samples of cheese. Weigh 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.

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4) PLRP-S 300 Å is the trade name of a product supplied by Polymer Laboratories, Ltd. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO or IDF of the product named. Equivalent products may be used if they can be shown to lead to the same results.

### 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 solution obtained dropwise 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 frequency of 2 500 r/min to 3 000 r/min. Rinse the homogenizer in a separate 100 ml beaker using 10 ml of sodium chloride solution (4.1.1). Add the rinsings 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 and liquid milk products (7.2.1) to pH 2,2 and that obtained from cheese and solid milk products to pH 4,3 by using hydrochloric acid (4.1.2).

Transfer the test solution to a 50 ml one-mark volumetric flask (5.9). Use sodium chloride solution (4.1.1) to rinse the 100 ml beaker. Dilute to the mark with the sodium chloride solution (4.1.1) and mix.

Allow the test solution to stand at room temperature for 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:

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

Use the following for HPLC:

- 1) elution solvent A containing: Stock solutions I and II with a mass fraction ratio I to II of 100:38,4.
- 2) elution solvent B containing: Stock solution II.

A suggested elution gradient is given in Table 1.

Table 1 — Suggested elution gradient

Time min	Elution solvent A <sup>a</sup> %	Elution solvent B <sup>a</sup> %
0,0	100	0
20,0	100	0
21,0	50	50
22,0	50	50
23,0	100	0
35,0	100	0

<sup>a</sup> The elution gradient might require slight modification in order to achieve the resolution shown in Figure 1.

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

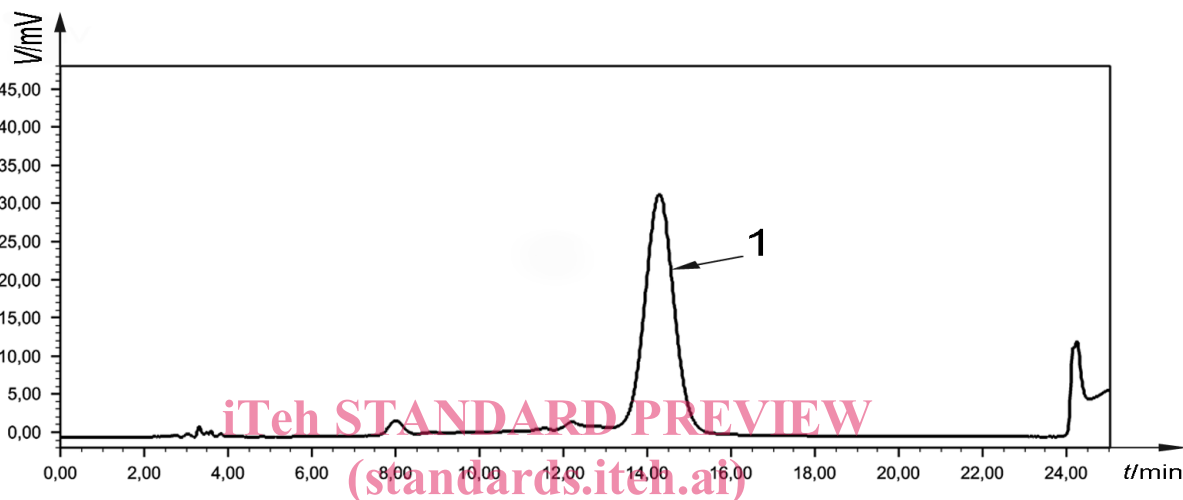


Set the temperature of the column heater at 45 °C. Determine the equilibration time by monitoring the column elution.

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 (7.2.3) into the column.

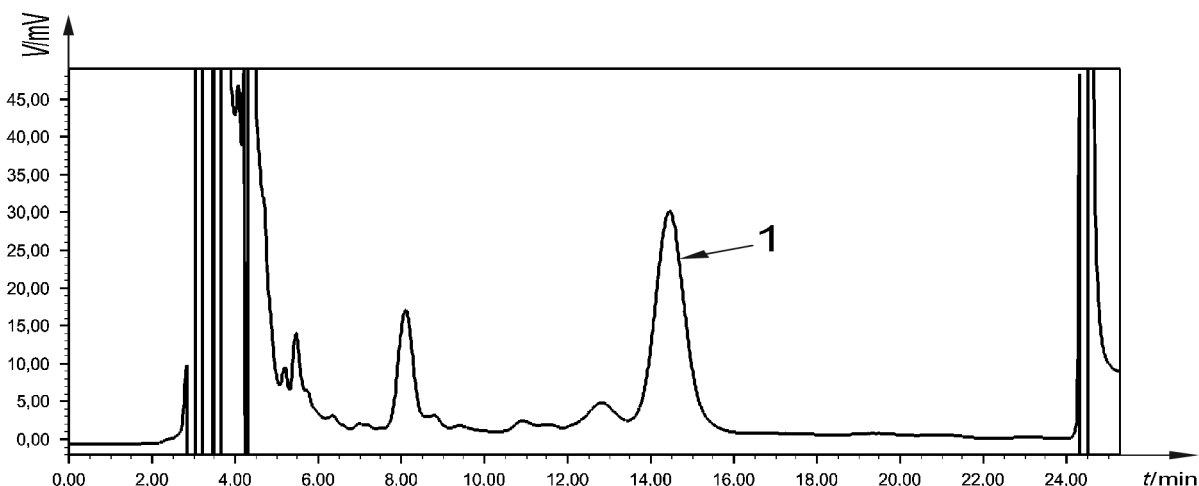
HPLC fluorescence signals from the standard working solution (7.1.2) and a hard cheese sample (7.2.3) are given in Figures 1 and 2.



**Key**

- $t$  time
- $V$  peak response <https://standards.iteh.ai/catalog/standards/sist/ceeb7ada-855b-4022-bdc1-7c8bdeae6a2f/iso-ts-27105-2009>
- 1 lysozyme

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



**Key**

- $t$  time
- $V$  peak response
- 1 lysozyme

**Figure 2 — HPLC fluorescence signal from a hard cheese sample**