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Milk and milk products — Determination of the lipase activity of pregastric lipase preparation

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National foreword

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**Milk and milk products — Determination
of the lipase activity of pregastric lipase
preparation**

*Lait et produits laitiers — Détermination de l'activité de lipase de la
préparation de lipase prégastrique*



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Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
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International Dairy Federation
Silver Building • Boulevard Auguste Reyers 70/B • B-1030 Brussels
Tel. + 32 2 733 98 88
Fax + 32 2 733 04 13
E-mail info@fil-idf.org
Web www.fil-idf.org

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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 13082|IDF 218 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 Standing Committees is to prepare International Standards. Draft International Standards adopted by the Standing Committees are circulated to the National Committees for endorsement prior to publication as an International Standard. Publication as an International Standard requires approval by at least 50 % of IDF National Committees casting a vote.

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ISO 13082|IDF 218 was prepared by the International Dairy Federation (IDF) and Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*. It is being published jointly by IDF and ISO.

All work was carried out by the Joint ISO-IDF Project Group *Lipase activity* of the Standing Committee on *Analytical methods for processing aids and indicators* under the aegis of its project leaders, Mrs M. Harboe (DK) and Dr J. Jacobsen (DK).

Introduction

Lipases (EC 3.1.1.3) are the group of esterases that hydrolyse emulsified triacylglycerolesters, which are the main component of milk fat.

Commercial pregastric lipase and some rennet preparations (paste or liquid) contain lipases from calf, kid-goat or lamb sources. These lipase preparations are used particularly in the production of Italian type cheeses, e.g. in Romano, Provolone, and Asiago and in other similar cheese varieties and in enzyme-modified dairy products as described in IDF Bulletin 294^[6]. Lipase is not allowed in Feta, but it is often used in Feta-type cheese.

The method is based on the principle of the FCCIV method for forestomach lipase activity^[7], but in its current form the FCCIV method is not sufficiently developed. As such, it does not provide adequate details in several critical areas, most notably in sample and substrate preparation. However, the FCCIV method served as a useful model for the development of this International Standard.

Milk and milk products — Determination of the lipase activity of pregastric lipase preparation

1 Scope

This International Standard specifies a method for the determination of the lipase activity. It is intended for the preparation of pregastric lipase and rennet paste, both of animal origin.

NOTE No reference method was used to check this method as no stable standard can be found. On the other hand, a reference method can be omitted as the substrate is reproducible and well defined.

2 Terms and definitions

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

2.1

international lipase unit

ILU

amount of lipase activity that releases butanoic acid, also known as butyric acid, at a rate of 1,25 $\mu\text{mol}/\text{min}$ under specified conditions

NOTE 1 Lipase activity is expressed either in international lipase units (ILU) per gram of product or ILU per millilitre of product.

NOTE 2 The definition is based on the direct consumption of titrant while not considering that a small molar fraction of the butyric acid (4 %) is not dissociated and thus cannot be titrated. As such, that creates a small error in the definition.

3 Principle

Triglyceride esters are hydrolysed by lipase. The free fatty acids (as butyric acid) released from the substrate tributyrin are titrated in a pH-stat with sodium hydroxide. The amount of sodium hydroxide consumed within a defined period is used to calculate the activity in ILU per millilitre or ILU per gram.

Due to the non-existence of a reference standard, it is recommended that a control (known) sample be included in the test.

4 Reagents

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and distilled water or demineralized water or water of equivalent purity.

The brand of chemicals can affect the result. Therefore, before using a brand other than the one mentioned, verify whether it gives the same result.

- 4.1 Tributyrin** (glycerintributyrate or glyceryl tributyrate), e.g. Merck No. 1.01958.0100¹⁾ or similar.
- 4.2 Sodium caseinate**, e.g. Sigma C8654¹⁾ or similar.
- 4.3 Lecithin**, from soya bean, e.g. BDH Prod. 29863¹⁾ or similar.
- 4.4 Liquid paraffin**. Use paraffin which is highly liquid (or similar light mineral oil), e.g. Merck No. 7174.1000¹⁾, or similar.
- 4.5 Soda lime granules** [Carbosorb¹⁾], e.g. BDH no 331104¹⁾ or similar.
- 4.6 Sodium hydroxide solution**, $c(\text{NaOH}) = 0,025 \text{ mol/l}$, which can either be purchased or be prepared as follows.

Using a pipette (5.1), add 25,00 ml of 1 mol/l sodium hydroxide with an accurately known titre into a container. Dilute with water to 1 000 ml.

The 0,025 mol/l NaOH solution can be kept in a closed container, protected against carbon dioxide in the air by use of a CO₂ trap with soda lime (4.5) at room temperature for at least 1 month. If necessary, seek advice from the supplier of the equipment or reagent. Change the soda lime at least once a year.

When changing the sodium hydroxide batch, check the actual stability of the titre by comparing the old and new titrant, e.g. using a control sample.

For samples with low activity and manual titrations, use a 0,010 mol/l NaOH instead of a 0,025 mol/l NaOH solution. As such, the 0,010 mol/l NaOH solution gives a higher and more useful consumption of titrant. Prepare the 0,010 mol/l NaOH solution freshly before use (unless the titre has been checked) as it is unstable. If using the 0,010 mol/l NaOH solution, correct the calculation according to the formulae in 8.1.

4.7 Lecithin solution, with a mass per volume fraction of 10 %. Weigh 10,0 g of lecithin in a suitable bottle. Use magnetic stirring to dissolve it in approx. 95 ml of liquid paraffin, which may take between 1 day and 2 days of mixing. When the lecithin is completely dissolved, make it up to a total volume of 100 ml with the liquid paraffin.

When stored in a refrigerator, the lecithin solution is stable for 1 year.

4.8 Control sample. Include a control sample of known activity in each series of test for lipase samples. Collect the results and use them for the evaluation of the variation of the test.

The control sample can be the last sample analysed or another well-known sample.

When carrying out the method for the very first time, use a control sample obtained from another laboratory or the first sample analysed being kept as control sample for the next series of analyses. If needed, store the control sample(s) in a freezer.

NOTE It can be difficult to get a suitable control sample for rennet paste.

5 Apparatus

Usual laboratory equipment and, in particular, the following.

The laboratory equipment can be substituted by other equipment verified as giving similar results.

5.1 Micropipette or any other pipette, of capacities 1 ml and 10 ml with a repeatability of 0,5 % or higher.

1) Example of a suitable product available commercially. This information is given for the benefit of users of this document and does not constitute an endorsement by ISO of this product.

5.2 One-mark volumetric flasks, of required capabilities, ISO 1042^[3] class A.

5.3 Water bath, capable of circulating the water externally and of maintaining a constant temperature in the reaction vessel of $42\text{ °C} \pm 0,5\text{ °C}$.

5.4 Blender, Warren¹⁾, Ultraturax¹⁾ or any equivalent apparatus.

5.5 pH stated equipment, including the following components:

- a) a thermostated reaction vessel capable of stirring effectively, e.g. mechanical or magnetic stirring;
- b) a burette for titration;
- c) a recorder, printer or computer.

A Metrohm 718 Stat Titrimo¹⁾ is suitable for the purpose. A manual titration set-up may also be used but that can reduce the precision of the method.

For control purposes, therefore, mention the equipment used in the test report.

5.6 Stomacher and stomacher bags, for dissolving rennet paste, e.g. standard bags BA 6041 from Seward¹⁾ or equivalent.

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 International Standard. A recommended sampling method is given in ISO 707 | IDF 50^[2].

Test samples may be stored at a temperature of 5 °C or lower for 2 months. In case of a long storage period, store the test samples frozen, e.g. at -18 °C , as that will significantly improve the stability of the lipase powder.

7 Procedure

7.1 Substrate

Disperse 600 mg of sodium caseinate (4.2) in 95 g water in the blender vessel. Add 0,5 ml lecithin solution (4.3) and 1,0 ml tributyrin (4.1). Blend for 60 s at low speed. Pour the substrate into a flask or beaker and keep it at room temperature on a magnetic stirrer using slow speed.

Use the substrate within 4 h.

7.2 Preparation of lipase test solution

7.2.1 Liquid lipase sample

Accurately pipette the required amount of the liquid lipase sample or control into a 100 ml one-mark volumetric flask (5.2) to obtain a 100 ml lipase solution with a concentration of $(4 \pm 1)\text{ ILU/ml}$. Make up to the mark with water.

NOTE Volumetric flasks of different capacities can be used or the sample can be analysed undiluted if the lipase activity is 5 ILU or below.

7.2.2 Powder lipase sample

Lipase powder may be inhomogeneous. Therefore, mix the powder gently in order to take a representative sample. Weigh the required amount of each powder lipase sample or control into a beaker to obtain a 100 ml lipase solution with a concentration of (4 ± 1) ILU/ml.

Dissolve or suspend the lipase test sample or the control sample in approximately 90 ml water with constant and efficient stirring. Check the pH and adjust, if needed, to $8,50 \pm 0,1$ at suitable intervals with a sodium hydroxide solution of appropriate concentration, i.e. 0,1 mol/l NaOH solution. After a total dissolution time of 20 min, transfer the content to a 100 ml one-mark volumetric flask (5.2). Make up to the mark with water.

Transfer the lipase solution back into a dry beaker and stir continuously. Analyse the solution as soon as possible, but no later than 2 h after preparation of the lipase sample. Note the dilution factor d (= total volume in millilitres per gram or millilitres per millilitre sample).

Lipase powder often has a poor solubility, which varies with pH. The high pH facilitates the dissolution and it is critical for the reproducibility that the same pH is always used during dissolving of lipase powder.

NOTE Volumetric flasks of different capacities can be used, if necessary.

7.3 Rennet paste samples

Mix the rennet paste to obtain a homogeneous paste. Dissolve $15 \text{ g} \pm 1 \text{ g}$ of rennet paste in a stomacher bag (5.6) in 40 ml of water. Adjust the pH of the solution obtained to $8,5 \pm 0,1$ using a 0,1 mol/l NaOH solution. Using a stomacher (5.6), homogenize the solution at a recommended speed of 230 r/min for 60 s. Readjust its pH to 8,5 and analyse the rennet paste sample obtained as soon as possible but not later than 2 h after its preparation.

Alternatively, the paste dissolution in the plastic bag can also be done manually in the stomacher bag during 60 s.

Record the exact amount of sample taken and the total amount of diluted sample, in grams, to three significant digits.

Set the dilution factor d (8.1) of the rennet paste to: the total mass of the diluted sample (including the mass of the paste) divided by the mass of paste.

Typical rennet paste has a low activity. If the required activity of (4 ± 1) ILU/ml cannot be reached in the test solution, analyse the rennet paste test solution without further dilution. In that case mention also in the test report that the activity of the test solution was below the required activity range.

7.4 Test procedure

7.4.1 Preparation of equipment

7.4.1.1 Prepare the equipment for the analysis as in 7.4.1.2 to 7.4.1.7.

7.4.1.2 Preheat the water bath (5.3) in order to obtain a temperature of $42,0 \text{ }^\circ\text{C}$ throughout in the reaction vessel. If needed, adjust the water bath temperature slightly so as to obtain a temperature of $42,0 \text{ }^\circ\text{C} \pm 0,5 \text{ }^\circ\text{C}$ in the reaction vessel.

7.4.1.3 Fill the burette (5.5) with sodium hydroxide (4.6).

7.4.1.4 Calibrate the pH electrode.

7.4.1.5 Set the titration pH to 6,20 or select the user-defined lipase titration program.

7.4.1.6 Place the reaction vessel on the titrator and start the stirrer.

7.4.1.7 Mount the pH electrode and thermometer, ready for the analysis

IMPORTANT — Effective stirring allows the reaction to proceed optimally.

7.4.2 Test

Start the test as follows.

- a) Using a pipette (5.1), add 10 ml of substrate (7.1) to the reaction vessel.
- b) Using a pipette (5.1), add 1 ml of lipase test solution (7.2) or rennet paste test solution (7.3) to the substrate.
- c) Start the titration and run it for 15 min. The first 5 min is used to adjust pH to 6,20 and the sodium hydroxide consumption is not used for the calculation. Make sure that the pH reaches a stable level of pH 6,20 during the first 4 min of titration. Record the sodium hydroxide consumption during the last 10 min (*c* in 8.1).

If the pH does not reach a stable level of 6,2 during the first 2 min of titration, then adjust the pH as needed by manual addition of drops of hydrochloric acid (HCl) of 0,1 mol/l or 1 mol/l to obtain a pH of $6,20 \pm 0,02$ after 4 min of titration. Repeat the test if needed.

The rate of sodium hydroxide consumption shall be linear. If the consumption is not linear, this could be due to insufficient stirring, which is critical. Ensure effective stirring by observing that there is movement on the surface of the liquid in the reaction vessel. Check the linearity of the titration curve, e.g. by recording the consumption of sodium hydroxide solution for two consecutive periods of 5 min, instead of at least 10 min, and compare the consumption during each period to demonstrate linearity.

Normally, it is not necessary to determine the blank value. However, if the lipase activity in the test sample is so low that the stipulated activity level of (4 ± 1) ILU cannot be achieved, then analyse the blank value of the water sample which should then be subtracted. Record this action in the test report indicating that the activity was below the normal value permitted for the method.

Perform the determination of the blank before analysing the samples. If the last sample being analysed is positive, measure a second blank value as a final test for getting assurance that the positive result was not due to contamination of the substrate with traces of lipase.

If a reaction vessel of 10 ml is not available, the analysis can be done on a larger scale, e.g. by adding 2 ml sample to 20 ml substrate. In that case, the result obtained should be divided by two.

NOTE 1 The consumption of 0,025 mol/l sodium hydroxide (*c* in 8.1) is typically between 1,5 ml and 2,5 ml.

NOTE 2 The limit of quantification is typically 0,04 ILU/ml corresponding to a 0,025 mol/l sodium hydroxide consumption of 0,020 ml.

NOTE 3 Preferably, the pH should already be stable during the test after 4 min in order to be sure that the pH is fully stable at 6,20 when the actual titration starts after 5 min.

8 Calculation and expression of results

8.1 Calculation

Calculate the lipase activity of the test sample, a_t , in international lipase units (ILU) per gram or in ILU per millilitre, by using the equation:

$$a_t = \frac{V c f_1 d}{t f_2}$$

where

V is the volume, in millilitres, of the consumption of sodium hydroxide solution (7.4.2) consumed;

c is the concentration, in moles per litre, of the titrant sodium hydroxide titrant;

f_1 is a factor, to convert milligrams of butyric acid to micrograms;

f_2 is a factor to convert the activity per 1,25 $\mu\text{mol}/\text{min}$ according to definition;

d is the dilution factor for the sample;

t is the time, in minutes, for which the sodium hydroxide consumption is noted.

The equation can be simplified by introducing the known values as follows for: $c = 0,025$; $f_1 = 1\ 000$; $f_2 = 1,25$; $t = 10$, giving:

$$a_t = V \times 2,00 \times d$$

8.2 Expression of results

Express the results to three significant figures.

9 Precision

9.1 Interlaboratory test

The values for repeatability and reproducibility derived from this interlaboratory test were determined in accordance with ISO 5725-1^[4] and ISO 5725-2^[5]. Details of the interlaboratory test on the precision of the method are shown in Annex A.

The values are expressed for the 95 % probability level and may not be applicable to concentration ranges and matrices other than those given.

If, in the long run, significantly less than 95 % of the cases are within the values given in 9.2 and 9.3, improvement in execution of the method is recommended.

9.2 Repeatability

The coefficient of variation of repeatability, $C_{V,r}$, as a percentage, which expresses the variability of independent analytical results obtained by the same operator, using the same apparatus under the same conditions on the same test sample and in a short interval of time, will in not more than 5 % of cases be greater than 9,9 % relative to the arithmetic mean of the test results.

If two determinations are obtained under these conditions, the absolute difference, r_{rel} , as a percentage, between the two results should not exceed 27,7 % relative to the arithmetic mean of the test results.

9.3 Reproducibility

The coefficient of variation of reproducibility, $C_{V,R}$, as a percentage, which expresses the variability of independent analytical results by operators in different laboratories, using different apparatus under different conditions for the analysis on the same test sample, will in not more than 5 % of cases be greater than 24,5 % relative to the arithmetic mean of the test results.

If two determinations are obtained under these conditions, the absolute difference, R_{rel} , as a percentage, between the two results should not exceed 68,7 % relative to the arithmetic mean of the test results.

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 method used, with reference to this International Standard (ISO 13082|IDF 218:2011);
- 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;
- f) if the repeatability has been checked, the final quoted result obtained.

Annex A (normative)

Summary of results of interlaboratory test

A.1 General

An international collaborative study involving 12 laboratories from seven countries was carried out on pregastric lipase powder. The test was organized by M. Harboe, Denmark. The test results were subjected to statistical analysis by O. Leray (Fr), according to ISO 5725-1^[4] and ISO 5725-2^[5].

A.2 Samples and results

The international collaborative study was carried out on six different batches of commercial lipase powder, each having low, medium or high level of activity. The samples contained calf, lamb or kid-goat pregastric lipase alone or in mixtures. These six samples were divided into 12 blind duplicated samples.

The results shown in Table A.1 are from an interlaboratory study carried out in 2009. The results in Table A.1 exclude those of laboratory No. 8 for samples 2/5 (Cochran), laboratory No. 1 for samples 3/4 (Cochran) and laboratory No. 12 for samples 8/9 and 7/11 (Grubbs).

Table A.1 — Results of interlaboratory study

Parameter	Lipase sample						Means
	2/5 High	3/4 High	1/6 Medium	10/12 Medium	7/11 Low	8/9 Low	
Number of labs retained after eliminating outliers	12	12	12	11	10	10	
Mean value, ILU/g	88,9	80,8	51,1	45,3	9,3	21,2	
Standard deviation of repeatability, s_r	10,5	7,2	5,5	2,3	1,4	1,7	
Coefficient of variation of repeatability, $C_{V,r}$, %	11,8	8,9	10,8	5,2	14,6	8,1	9,9
Repeatability limit, r	29,3	20,1	15,5	6,5	3,8	4,8	
Absolute difference of repeatability, r_{rel} , %	33	24,8	30,3	14,5	40,9	22,6	27,7
Standard deviation of reproducibility, s_R	15,5	17,3	12,7	12,5	2,2	6,7	
Coefficient of variation of reproducibility, $C_{V,R}$, %	17,4	21,5	24,8	27,6	24,2	31,8	24,5
Reproducibility limit, R	43,3	48,5	35,5	35	6,3	18,9	
Absolute difference of reproducibility, R_{rel} , %	48,7	60,1	69,3	77,3	67,7	89	68,7

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