International Standard 1211

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Milk — Determination of fat content — Gravimetric method (Reference method)

Lait — Détermination de la teneur en matière grasse — Méthode gravimétrique (Méthode de référence)

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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.

Draft International Standards adopted by the technical committees are circulated to the member bodies for approval before their acceptance as International Standards by the ISO Council. They are approved in accordance with ISO procedures requiring at least 75 % approval by the member bodies voting.

International Standard ISO 1211 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*.

It cancels and replaces ISO Recommendation R 1211-1970, of which it constitutes a technical revision.

NOTE — The method specified in this International Standard has been developed jointly with the IDF (International Dairy Federation) and the AOAC (Association of Official Analytical Chemists, USA). The text as approved by the above organizations will also be published by FAO/WHO (Code of principles concerning milk and milk products and associated standards), by the IDF and by the AOAC (Official Methods of Analysis).

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0 Introduction

This International Standard constitutes a revision of ISO/R 1211. Attempts to produce a single International Standard specifying the use of the Röse-Gottlieb method applicable to all milk products have been found impracticable for the time being. Therefore, it has been decided to revise and harmonize the existing standardized methods for individual products or groups of products and to standardize similar methods for those products for which methods had not been prepared previously.

The main modifications as compared to ISO/R 1211 are :

a) preference is given to the use of Mojonnier-type fat-extraction flasks and the use of a centrifuge to separate the solvent layers;

b) addition of ethanol before the second extraction;

c) emphasis is placed on the necessity of cooling the fat-collecting vessels to ambient temperature before performing weighings.

The use of a centrifuge enables rapid and complete separation of the solvent layers and minimizes the need for redissolution of the extracted fat or a repetition of the determination.

The addition of ethanol before the second extraction has been specified to reduce the risk of formation of a viscous or gelified aqueous layer, especially in the case of products containing sucrose (for example sweetened condensed milk, edible ices and, to a lesser extent, milk powder). It has been found that this addition distinctly improves the precision of the method, also for milk.

Emphasis has been placed on the necessity of cooling fat-collecting vessels to ambient temperature before weighing, as errors from this source of the order of 0,01 % fat per degree Celsius have been reported for liquid milk. The use of a desiccator is therefore not recommended. The use of an empty control vessel will compensate for these errors to some extent. It has been found, however, that the use of such a vessel together with a blank test on 10 ml of water carried out with the determination is complicated and produces no improvement in precision.

An empty control vessel is, however, necessary, and is therefore specified when performing the blank test to check the reagents in order to avoid a false impression of the presence or absence of non-volatile matter.

1 Scope and field of application

This International Standard specifies the reference method for the determination of the fat content of raw and processed liquid milk, partly skimmed milk and skimmed milk in which no appreciable separation or splitting of fat has occurred (see the note to 8.1).

NOTE — When greater accuracy is required for skimmed milk, for instance to establish the operating efficiency of cream separators, the special method for skimmed products, specified in ISO 7208, *Skimmed milk, whey and buttermilk — Determination of fat content — Gravimetric method (Reference method)*, should be used.

2 References

ISO 707, *Milk and milk products — Methods of sampling.*

ISO 3889, *Milk and milk products — Determination of fat content — Mojonnier-type fat extraction flasks.*

3 Definition

fat content of milk : All the substances determined by the method specified in this International Standard.

It is expressed as a percentage by mass.

4 Principle

Extraction of an ammoniacal ethanolic solution of a test portion with diethyl ether and light petroleum, removal of the solvents by distillation or evaporation, and determination of the mass of the substances extracted which are soluble in light petroleum. (This is usually known as the Röse-Gottlieb principle.)

5 Reagents

All reagents shall be of recognized analytical grade and shall leave no appreciable residue when the determination is carried out by the method specified. The water used shall be distilled water or water of at least equivalent purity.

To test the quality of the reagents, carry out a blank test as specified in 8.3. Use an empty fat-collecting vessel, prepared as specified in 8.4, for mass control purposes. The reagents shall leave no residue greater than 0,5 mg (see 10.1).

If the residue of the complete reagent blank test is greater than 0,5 mg, determine the residue of the solvents separately by distilling 100 ml of the diethyl ether and light petroleum respectively. Use an empty control vessel to obtain the real mass of residue which shall not exceed 0,5 mg.

Replace unsatisfactory reagents or solvents, or redistil solvents.

5.1 Ammonia solution, containing approximately 25 % (m/m) of NH_3, $\varrho_{20} \approx 910$ g/l.

NOTE — If ammonia solution of this concentration is not available, a more concentrated solution of known concentration may be used (see 8.5.1).

5.2 Ethanol, or ethanol denatured by methanol, at least 94 % (V/V).

(See 10.5.)

5.3 Congo-red solution.

Dissolve 1 g of Congo-red in water and dilute to 100 ml.

NOTE — The use of this solution, which allows the interface between the solvent and aqueous layers to be seen more clearly, is optional (see 8.5.2). Other aqueous colour solutions may be used provided that they do not affect the result of the determination.

5.4 Diethyl ether, free from peroxides (see 10.3) and containing no or not more than 2 mg/kg of antioxidants and complying with the requirements for the blank test (see clause 5, and also 10.1 and 10.4).

5.5 Light petroleum, having any boiling range between 30 and 60 °C.

5.6 Mixed solvent, prepared shortly before use by mixing equal volumes of the diethyl ether (5.4) and the light petroleum (5.5).

6 Apparatus

WARNING — Since the determination involves the use of volatile flammable solvents, electrical apparatus employed shall be required to comply with legislation relating to the hazards in using such solvents.

Usual laboratory equipment, and in particular

6.1 Analytical balance.

6.2 Centrifuge, in which the fat-extraction flasks or tubes (6.6) can be spun at a rotational frequency of 500 to 600 min^{-1} to produce a gravitational field of 80 g to 90 g at the outer end of the flasks or tubes.

NOTE — The use of the centrifuge is optional but recommended (see 8.5.5).

6.3 Distillation or evaporation apparatus, to enable the solvents and ethanol to be distilled from the flasks or to be evaporated from beakers and dishes (see 8.5.12) at a temperature not exceeding 100 °C.

6.4 Drying oven, electrically heated, with ventilation port(s) fully open, capable of being maintained at a temperature of 102 ± 2 °C throughout the working space. The oven shall be fitted with a suitable thermometer.

6.5 Water-bath, capable of being maintained at 35 to 40 °C.

6.6 Mojonnier-type fat-extraction flasks, as specified in ISO 3889.

NOTE — It is also possible to use **fat-extraction tubes**, with **siphon or wash-bottle fittings**, but the procedure is then different and is specified in the annex.

The flasks (or tubes, see the note) shall be provided with good quality bark corks or stoppers of other material (for example silicone rubber) unaffected by the reagents used. Bark corks shall be extracted with the diethyl ether (5.4), kept in water at 60 °C or more for at least 15 min, and shall then be allowed to cool in the water so that they are saturated when used.

6.7 Rack, to hold the fat-extraction flasks (or tubes) (see 6.6).

6.8 Wash bottle, suitable for use with the mixed solvent (5.6). A plastic wash bottle shall not be used.

6.9 Fat-collecting vessels, for example boiling flasks (flat-bottomed), of capacity 125 to 250 ml, conical flasks, of capacity 250 ml, or metal dishes. If metal dishes are used, they shall preferably be of stainless steel, shall be flat-bottomed, preferably with a spout, and shall have a diameter of 80 to 100 mm and a height of approximately 50 mm.

6.10 Boiling aids, fat-free, of non-porous porcelain or silicon carbide (optional in the case of metal dishes).

6.11 Measuring cylinders, of capacities 5 and 25 ml.

6.12 Pipettes, graduated, of capacity 10 ml.

6.13 Tongs, made of metal, suitable for holding flasks, beakers or dishes.

7 Sampling

See ISO 707.

All laboratory samples shall be kept at a temperature of 3 to 6 °C from the time of sampling to the time of commencing the procedure.

8 Procedure

NOTE — The alternative procedure using fat-extraction tubes with siphon or wash-bottle fittings (see the note to 6.6) is described in the annex.

8.1 Preparation of the test sample

Adjust the temperature of the laboratory sample (clause 7) to 35 to 40 °C, by means of the water-bath (6.5) if necessary. Mix the sample thoroughly, but gently, by repeatedly inverting the sample bottle without causing frothing or churning, and cool quickly to approximately 20 °C.

Churned milk should not be cooled as it has to be weighed at 30 to 40 °C in 8.2.

NOTE — A reliable value for the fat content cannot be expected :

a) if the milk is churned;

b) when a distinct smell of free fatty acids is perceptible;

c) if during, or after, preparation of the sample, white particles are visible on the walls of the sample bottle or fat droplets float on the surface of the sample.

8.2 Test portion

Mix the test sample (8.1) by gently inverting the bottle three or four times and immediately weigh, to the nearest 1 mg, 10 to 11 g of the test sample, directly or by difference, into one of the extraction flasks (6.6).

The test portion shall be delivered as completely as possible into the lower (small) bulb of the extraction flask.

8.3 Blank test

Carry out a blank test simultaneously with the determination, using the same procedure and same reagents, but replacing the test portion by 10 ml of water (see 10.2).

8.4 Preparation of fat-collecting vessel

Dry a vessel (6.9) with a few boiling aids (6.10) in the oven (6.4) for 1 h. (See note 1.)

Allow the vessel to cool (protected from dust) to the temperature of the weighing room (glass vessel for at least 1 h, metal dish for at least 0,5 h). (See note 2.)

Using tongs (to avoid, in particular, temperature variations), place the vessel on the balance and weigh to the nearest 0,1 mg.

NOTES

1 Boiling aids are desirable to promote gentle boiling during the subsequent removal of solvents, especially in the case of glass vessels; their use is optional in the case of metal dishes.

2 The vessel should not be placed in a desiccator, to avoid insufficient cooling or unduly long cooling times.

8.5 Determination

8.5.1 Add 2 ml of the ammonia solution (5.1), or an equivalent volume of a more concentrated ammonia solution (see the note to 5.1), and mix thoroughly with the test portion in the small bulb of the flask. After the addition of the ammonia, carry out the determination without delay.

8.5.2 Add 10 ml of the ethanol (5.2) and mix gently but thoroughly by allowing the contents of the flask to flow backward and forward between the two bulbs; avoid bringing the liquid too near to the neck of the flask. If desired, add 2 drops of the Congo-red solution (5.3).

8.5.3 Add 25 ml of the diethyl ether (5.4), close the flask with a cork (see 6.6) saturated with water or with a stopper of other material (see 6.6) wetted with water, and shake the flask vigorously, but not excessively (in order to avoid the formation of persistent emulsions), for 1 min with the flask in a horizontal position and the small bulb extending upwards, periodically allowing the liquid in the large bulb to run into the small bulb. If necessary, cool the flask in running water, then carefully remove the cork or stopper and rinse it and the neck of the flask with a little of the mixed solvent (5.6) using the wash bottle (6.8) so that the rinsings run into the flask.

8.5.4 Add 25 ml of the light petroleum (5.5), close the flask with the rewetted cork or rewetted stopper (by dipping in water), and shake the flask gently for 30 s as described in 8.5.3.

8.5.5 Centrifuge the closed flask for 1 to 5 min at a rotational frequency of 500 to 600 min^{-1}. If a centrifuge is not available, allow the closed flask to stand in the rack (6.7) for at least 30 min until the supernatant layer is clear and distinctly separated from the aqueous layer. If necessary, cool the flask in running water.

8.5.6 Carefully remove the cork or stopper and rinse it and the inside of the neck of the flask with a little of the mixed solvent so that the rinsings run into the flask.

If the interface is below the bottom of the stem of the flask, raise it slightly above this level by gently adding water down the side of the flask (see figure 1) to facilitate the decantation of solvent.

NOTE — In figures 1 and 2, one of the three types of flasks as specified in ISO 3889 has been chosen, but this does not imply any preference over the other types.

8.5.7 Holding the extraction flask by the small bulb, carefully decant as much as possible of the supernatant layer into the prepared fat-collecting vessel (see 8.4) containing a few boiling aids (6.10) in the case of flasks (optional with metal dishes), avoiding decantation of any of the aqueous layer (see figure 2).

8.5.8 Rinse the outside of the neck of the extraction flask with a little of the mixed solvent, collecting the rinsings in the fat-collecting vessel and taking care that the mixed solvent does not spread over the outside of the extraction flask.

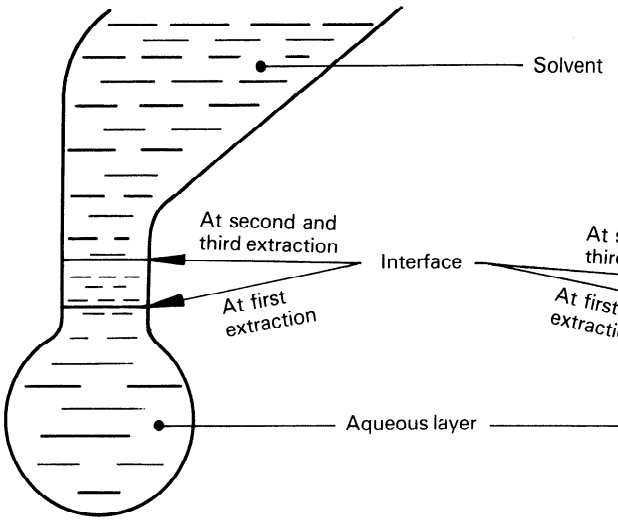

Figure 1 — Before decantation
(8.5.6, 8.5.10, 8.5.11)

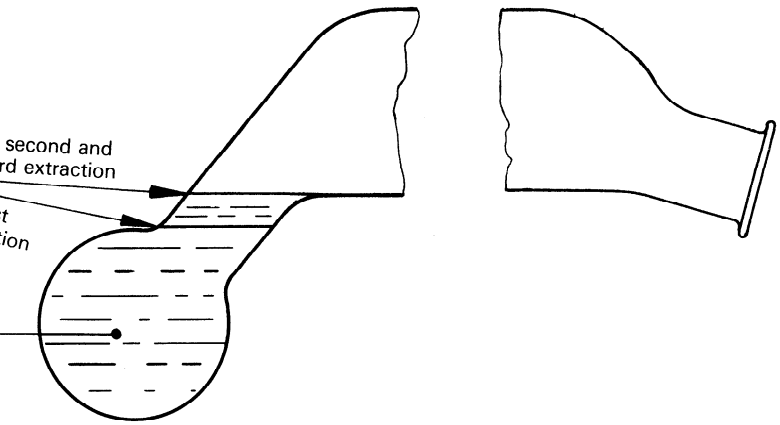

Figure 2 — After decantation
(8.5.7, 8.5.10, 8.5.11)

If desired, the solvent or part of the solvent may be removed from the vessel by distillation or evaporation as described in 8.5.12.

8.5.9 Add 5 ml of the ethanol (5.2) to the contents of the extraction flask, using the ethanol to rinse the inside of the neck of the flask and mix as described in 8.5.2.

8.5.10 Carry out a second extraction by repeating the operations described in 8.5.3 to 8.5.7 inclusive, but using only 15 ml of the diethyl ether (5.4) and 15 ml of the light petroleum (5.5); use the ether to rinse the inside of the neck of the extraction flask.

If necessary, raise the interface to the middle of the stem of the flask (see figure 1) to enable the final decantation of solvent to be as complete as possible (see figure 2).

8.5.11 Carry out a third extraction without addition of ethanol by again repeating the operations described in 8.5.3 to 8.5.7 inclusive, but using only 15 ml of the diethyl ether (5.4) and 15 ml of the light petroleum (5.5); use the ether to rinse the inside of the neck of the extraction flask.

If necessary, raise the interface to the middle of the stem of the flask (see figure 1) to enable the final decantation of solvent to be as complete as possible (see figure 2).

NOTE — The third extraction should be omitted for milk with a fat content of less than 0,5 % (m/m).

8.5.12 Remove the solvents (including ethanol) as completely as possible from the flask by distillation, or from the beaker or dish by evaporation (see 6.3), rinsing the inside of the neck of the flask with a little of the mixed solvent (5.6) before commencing the distillation.

8.5.13 Heat the fat-collecting vessel (flask placed on its side to allow solvent vapour to escape) for 1 h in the drying oven (6.4), controlled at 102 ± 2 °C. Remove the fat-collecting vessel from the oven, allow to cool (not in a desiccator, but protected from dust) to the temperature of the weighing room (glass vessel for at least 1 h, metal dish for at least 0,5 h) and weigh to the nearest 0,1 mg.

Do not wipe the vessel immediately before weighing. Place the vessel on the balance using tongs (to avoid, in particular, temperature variations).

8.5.14 Repeat the operations described in 8.5.13 until the mass of the fat-collecting vessel decreases by 0,5 mg or less, or increases, between two successive weighings. Record the minimum mass as the mass of the fat-collecting vessel and extracted matter.

8.5.15 Add 25 ml of the light petroleum to the fat-collecting vessel in order to verify whether or not the extracted matter is wholly soluble. Warm gently and swirl the solvent until all the fat is dissolved.

If the extracted matter is wholly soluble in the light petroleum, take the mass of fat as the difference between the final mass of the vessel containing the extracted matter (see 8.5.14) and its initial mass (see 8.4).

8.5.16 If the extracted matter is not wholly soluble in the light petroleum, or in case of doubt and always for regulatory purposes or in case of dispute, extract the fat completely from the vessel by repeatedly washing with warm light petroleum.

Allow any trace of insoluble material to settle and carefully decant the light petroleum without removing any insoluble material. Repeat this operation three more times, using the light petroleum to rinse the inside of the neck of the vessel.

Finally, rinse the outside of the top of the vessel with mixed solvent so that the solvent does not spread over the outside of the vessel. Remove light petroleum vapour from the vessel by heating the vessel for 1 h in the drying oven (6.4), controlled at 102 ± 2 °C, allow to cool and weigh, as described in 8.5.13 and 8.5.14.

Take the mass of fat as the difference between the mass determined in 8.5.14 and this final mass.

9 Expression of results

9.1 Method of calculation and formula

The fat content, expressed as a percentage by mass, is equal to

$$\frac{(m_1 - m_2) - (m_3 - m_4)}{m_0} \times 100$$

where

m_0 is the mass, in grams, of the test portion (8.2);

m_1 is the mass, in grams, of the fat-collecting vessel and extracted matter determined in 8.5.14;

m_2 is the mass, in grams, of the prepared fat-collecting vessel (see 8.4), or, in the case of undissolved material, of the fat-collecting vessel and insoluble residue determined in 8.5.16;

m_3 is the mass, in grams, of the fat-collecting vessel used in the blank test (8.3) and any extracted matter determined in 8.5.14;

m_4 is the mass, in grams, of the fat-collecting vessel (see 8.4) used in the blank test (8.3), or, in the case of undissolved material, of the fat-collecting vessel and insoluble residue determined in 8.5.16.

Report the result to the nearest 0,01 % (m/m).

9.2 Precision

NOTE — The values for repeatability and reproducibility are expressed for the 95 % probability level and were derived from the results of an inter-laboratory trial in accordance with ISO 5725, *Precision of test methods — Determination of repeatability and reproducibility by inter-laboratory tests.*

9.2.1 Repeatability

The difference between two single results found on identical test material by one analyst within a short time interval should not exceed the following values :

— for raw and processed liquid milk : 0,02 g of fat per 100 g of product;

— for milk having a fat content from 0,5 to 2 % (m/m) : 0,02 g of fat per 100 g of product;

— for milk having a fat content ⩽ 0,5 % (m/m) : 0,01 g of fat per 100 g of product.

9.2.2 Reproducibility

The difference between two single and independent results found by two operators working in different laboratories on identical test material should not exceed the following values :

— for raw and processed liquid milk : 0,04 g of fat per 100 g of product;

— for milk having a fat content from 0,5 to 2 % (m/m) : 0,03 g of fat per 100 g of product;

— for milk having a fat content ⩽ 0,5 % (m/m) : 0,025 g of fat per 100 g of product.

10 Notes on procedure

10.1 Blank test to check the reagents

In this blank test, a vessel for mass control purposes has to be used in order that changes in the atmospheric condition of the balance room or temperature effects of the fat-collecting vessel will not falsely suggest the presence or absence of non-volatile matter in the extract of the reagent. This vessel may be used as a counterweight vessel in the case of a two-pan balance. Otherwise, deviations of the apparent mass ($m_3 - m_4$ in the formula in 9.1) of the control vessel shall be considered when checking the mass of the fat-collecting vessel used for the blank test. Hence, the change in apparent mass of the fat-collecting vessel, corrected for the apparent change in mass of the control vessel, shall show no increase in mass greater than 0,5 mg.

Very occasionally, the solvents may contain volatile matter which is strongly retained in fat. If there are indications of the presence of such substances, carry out blank tests on all the reagents and for each solvent using a fat vessel with about 1 g of anhydrous butterfat. If necessary, redistil solvents in the presence of 1 g of anhydrous butterfat per 100 ml of solvent. Use the solvents only shortly after redistillation.

10.2 Blank test carried out simultaneously with the determination

The value obtained in the blank test, carried out simultaneously with the determination, enables the apparent mass of substances extracted from a test portion ($m_1 - m_2$) to be corrected for the presence of any non-volatile matter derived from the reagents and also for any change of atmospheric conditions of the balance room and some temperature difference between the fat-collecting vessel and the balance room at the two weighings (8.5.14 and 8.4 or 8.5.16).

Under favourable conditions (low value in the blank test on reagents, equable temperature of the balance room, sufficient cooling time for fat vessel), the value will usually be less than 0,5 mg and can then be neglected in the calculation in the case of routine determinations. Slightly higher values (positive and negative) up to 2,5 mg are also often encountered. After correction for these values, the results will still be accurate. When corrections for a value of more than 2,5 mg are applied, it should be mentioned in the test report (clause 11).

If the value obtained in this blank test regularly exceeds 0,5 mg, the reagents should be checked if this has not been recently done. Any impure reagent or reagents traced should be replaced or purified (see the note to clause 5 and 10.1).

10.3 Test for peroxides

To test for peroxides, add 1 ml of a freshly prepared 100 g/l potassium iodide solution to 10 ml of the diethyl ether in a small glass-stoppered cylinder which has been previously rinsed with the ether. Shake the cylinder and allow to stand for 1 min. No yellow colour should be observed in either layer.

Other suitable methods of testing for peroxides may be used.

To ensure that the diethyl ether is free, and is maintained free, from peroxides, treat the ether as follows at least 3 days before it is to be used.

Cut zinc foil into strips that will reach at least half-way up the bottle containing the ether, using approximately 80 cm^2 of foil per litre of ether.

Before use, completely immerse the strips of foil for 1 min in a solution containing 10 g of copper(II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) and 2 ml of concentrated [98 % (m/m)] sulfuric acid per litre. Wash the strips gently but thoroughly with water, place the wet copper-plated strips in the bottle containing the ether, and leave the strips in the bottle.

Other methods may be used provided that they do not affect the result of the determination.

10.4 Diethyl ether containing antioxidants

Diethyl ether containing about 1 mg of antioxidants per kilogram is available in some countries, especially for fat determinations. This content does not exclude its use for reference purposes.

In other countries, diethyl ether having higher antioxidant contents, for example up to 7 mg per kilogram, is available. Such ether should only be used for routine determinations with an obligatory blank test carried out simultaneously with the determination(s) to correct for systematic errors due to the antioxidant residue. For reference purposes, such ether shall always be distilled before use.

10.5 Ethanol

Ethanol denatured otherwise may be used provided that the denaturant does not affect the result of the determination.

11 Test report

The test report shall show the method used and the results obtained. It shall also mention all operating conditions not specified in this International Standard, or regarded as optional, together with details of any incidents likely to have influenced the results. The blank value ($m_3 - m_4$, see 9.1) shall be reported if it exceeds 2,5 mg.

The test report shall include all the information necessary for the complete identification of the sample.

Annex

Alternative procedure using fat-extraction tubes with siphon or wash-bottle fittings (see figure 3 as an example)

A.0 Introduction

If fat-extraction tubes with siphon or wash-bottle fittings are to be used, use the procedure specified in this annex.

A.1 Procedure

A.1.1 Preparation of the test sample

See 8.1.

A.1.2 Test portion

Proceed as specified in 8.2 but using the fat-extraction tubes (see 6.6).

The test portion shall be delivered as completely as possible on to the bottom of the extraction tube.

A.1.3 Blank test

See 8.3 and 10.2.

A.1.4 Preparation of fat-collecting vessel

See 8.4.

A.1.5 Determination

A.1.5.1 Add 2 ml of the ammonia solution (5.1), or an equivalent volume of a more concentrated ammonia solution (see the note to 5.1), and mix thoroughly with the pre-treated test portion at the bottom of the tube. After the addition of the ammonia, carry out the determination without delay.

A.1.5.2 Add 10 ml of the ethanol (5.2) and mix gently but thoroughly at the bottom of the tube. If desired, add 2 drops of the Congo-red solution (5.3).

A.1.5.3 Add 25 ml of the diethyl ether (5.4), close the tube with a cork (see 6.6) saturated with water or with a stopper of other material (see 6.6) wetted with water, and shake the tube vigorously, but not excessively (in order to avoid the formation of persistent emulsions), with repeated inversions for 1 min. If necessary, cool the tube in running water, then carefully remove the cork or stopper and rinse it and the neck of the tube with a little of the mixed solvent (5.6) using the wash bottle (6.8) so that the rinsings run into the tube.

A.1.5.4 Add 25 ml of the light petroleum (5.5), close the tube with the rewetted cork or rewetted stopper (by dipping in water), and shake the tube gently for 30 s, as described in A.1.5.3.

A.1.5.5 Centrifuge the closed tube for 1 to 5 min at a rotational frequency of 500 to 600 min^{-1}. If a centrifuge is not available, allow the closed tube to stand in the rack (6.7) for at least 30 min until the supernatant layer is clear and distinctly separated from the aqueous layer. If necessary, cool the tube in running water.

A.1.5.6 Carefully remove the cork or stopper and rinse it and the neck of the tube with a little of the mixed solvent so that the rinsings run into the tube.

A.1.5.7 Insert a siphon fitting or a wash-bottle fitting into the tube and push down the long inner limb of the fitting until the inlet is approximately 4 mm above the interface between the layers. The inner limb of the fitting shall be parallel to the axis of the extraction tube.

Carefully transfer the supernatant layer out of the tube into the fat-collecting vessel (see 8.4) containing a few boiling aids (6.10) in the case of flasks (optional with metal dishes), avoiding the transfer of any of the aqueous layer. Rinse the outlet of the fitting with a little of the mixed solvent, collecting the rinsings in the fat-collecting vessel.

A.1.5.8 Loosen the fitting from the neck of the tube, slightly raise the fitting and rinse the lower part of its long inner limb with a little of the mixed solvent. Lower and re-insert the fitting and transfer the rinsings to the fat-collecting vessel.

Rinse the outlet of the fitting with a little of the mixed solvent, collecting the rinsings in the vessel. If desired, the solvent or part of the solvent may be removed from the vessel by distillation or evaporation as described in 8.5.12.

A.1.5.9 Again loosen the fitting from the neck, slightly raise the fitting and add 5 ml of the ethanol to the contents of the tube, using the ethanol to rinse the long inner limb of the fitting; mix as described in A.1.5.2.

A.1.5.10 Carry out a second extraction by repeating the operations described in A.1.5.3 to A.1.5.8, but using only 15 ml of the diethyl ether (5.4) and 15 ml of the light petroleum (5.5). Use the ether to rinse the long inner limb of the fitting during the removal of the fitting from the tube after the previous extraction.

A.1.5.11 Carry out a third extraction without the addition of ethanol by again repeating the operations described in A.1.5.3 to A.1.5.8 using 15 ml of the diethyl ether and 15 ml of the light petroleum and rinsing the long inner limb of the fitting as described in A.1.5.10.

NOTE — The third extraction should be omitted for milk with a fat content of less than 0,5 % (m/m).

A.1.5.12 Proceed as described in 8.5.12 to 8.5.16.