INTERNATIONAL STANDARD

ISO 7208

Second edition
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Skimmed milk, whey and buttermilk — Determination of fat content — Gravimetric method (Reference method)

Lait écrémé, sérum et babeurre — Détermination de la teneur en matière grasse — Méthode gravimétrique (Méthode de référence)

Reference number
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Contents

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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 3.

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.

International Standard ISO 7208 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Subcommittee SC 5, *Milk and milk products*, in collaboration with the International Dairy Federation (IDF) and AOAC International, and will also be published by these organizations.

This second edition cancels and replaces the first edition (ISO 7208:1984), which has been technically revised.

Annexes A and B of this International Standard are for information only.

Skimmed milk, whey and buttermilk — Determination of fat content — Gravimetric method (Reference method)

WARNING — The use of this International Standard may involve hazardous materials, operations and equipment. This standard does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this standard to establish safety and health practices and determine the applicability of regulatory limitations prior to use.

1 Scope

This International Standard specifies the reference method for the determination of the fat content of liquid skimmed milk, whey and buttermilk. It is a particularly accurate gravimetric method especially for the purpose of establishing the operating efficiency of cream separators.

This International Standard also specifies the reference method for establishing correction tables for procedures with skimmed milk butyrometers.

NOTE When a high accuracy of determination is not required, the method specified in ISO 1211 may be used.

2 Normative reference

The following normative document contain provisions which, through reference in this text, constitute provisions of this International Standard. For dated references, subsequent amendments to, or revisions of, this publication do not apply. However, parties to agreement based on this International Standard are encouraged to investigate the possibility of applying the most recent edition of the normative document indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

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

3 Term and definition

For the purposes of this International Standard, the following term and definition apply.

3.1
fat content of skimmed milk, whey and buttermilk
mass fraction of substances determined by the procedure specified in this International Standard

NOTE The fat content is expressed as a mass fraction, in percent [formerly given as % (m/m)].

4 Principle

An ammoniacal ethanolic solution of a test portion is extracted with diethyl ether and light petroleum. The solvents are removed by distillation or evaporation. The mass of the substances extracted is determined.

NOTE This is usually known as the Röse-Gottlieb principle.

5 Reagents

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

The reagents shall leave no appreciable residue when the determination is carried out by the method specified (see 9.2.2).

5.1 Ammonia solution, containing a mass fraction of NH_3 of approximately 25 % (ρ_{20} = 910 g/l).

NOTE If ammonia solution of this concentration is not available, a more concentrated solution of known concentration may be used (see 9.4.2).

5.2 Ethanol (C_2H_5OH), or ethanol denatured by methanol, containing a volume fraction of ethanol of at least 94 %. (See A.5.)

5.3 Congo red solution

Dissolve 1 g of Congo red in water in a 100 ml one-mark volumetric flask (6.14). Dilute to the mark with water.

NOTE The use of this solution, which allows the interface between the solvent and aqueous layers to be seen more clearly, is optional (see 9.4.3). Other aqueous colour solutions may be used provided that they do not affect the result of the determination.

5.4 Diethyl ether ($C_2H_5OC_2H_5$), free from peroxides (see A.3), containing no more than 2 mg/kg of antioxidants, and complying with the requirements for the blank test (see 9.2.2, A.1 and A.4).

NOTE The use of diethyl ether could lead to hazardous situations. Due to expected changes in safety regulations studies are ongoing to replace diethyl ether by another reagent provided that it does not affect the end result of the determination.

5.5 Light petroleum, with any boiling range between 30 °C and 60 °C or, as equivalent, **pentane** ($CH_3[CH_2]_3CH_3$) with a boiling point of 36 °C and complying with the requirements for the blank test (see 9.2.2, A.1 and A.4).

NOTE The use of pentane is recommended because of its higher purity and constant quality.

5.6 Mixed solvent

Mix shortly before use equal volumes of diethyl ether (5.4) and light petroleum (5.5).

6 Apparatus

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

Usual laboratory equipment and, in particular, the following.

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

6.2 Centrifuge, capable of holding the fat-extraction flasks or tubes (6.6) and capable of spinning at a rotational frequency of 500 min^{-1} to 600 min^{-1} to produce a radial acceleration 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 9.4.6).

6.3 Distillation or evaporation apparatus, for distilling the solvents and ethanol from the boiling or conical flasks, or evaporating from beakers and dishes (see 9.4.13) 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 °C ± 2 °C throughout its working space.

The oven shall be fitted with a suitable thermometer.

6.5 Water bath, capable of being maintained at a temperature of between 35 °C and 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 then the procedure is different. The alternative procedure is given in annex B.

The fat-extraction flasks shall be provided with good quality bark corks or stoppers of other material [e.g. silicone rubber or polytetrafluoroethylene (PTFE)] unaffected by the reagents used. Bark corks shall be extracted with the diethyl ether (5.4), kept in water at a temperature of 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, for holding the fat-extraction flasks (or tubes) (6.6).

6.8 Wash bottle, suitable for use with the mixed solvent (5.6).

A plastics wash bottle shall not be used.

6.9 Fat-collecting vessels, such as boiling flasks (flat-bottomed), of capacities 125 ml to 250 ml, conical flasks, of capacity 250 ml, or metal dishes.

If metal dishes are used, they shall be of stainless steel, flat-bottomed with a diameter of 80 mm to 100 mm and a height of approximately 50 mm.

6.10 Boiling aids, fat-free, of non-porous porcelain or silicon carbide (optional when metal dishes are used).

6.11 Measuring cylinders, of capacities 5 ml and 25 ml, or any other apparatus suitable for the product concerned.

6.12 Pipettes, graduated, of capacity 10 ml.

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

6.14 Volumetric flask, one-mark, of capacity 100 ml.

7 Sampling

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 707.

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

Store the samples at a temperature of between 2 °C and 6 °C from the time of sampling.

8 Preparation of test sample

Warm the test sample to a temperature of between 35 °C and 40 °C by means of the water bath (6.5), if necessary. Gently mix the test sample thoroughly by repeatedly inverting the sample bottle without causing frothing or churning. Cool the test sample quickly to approximately 20 °C.

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 test sample, white particles are visible on the walls of the sample bottle or fat droplets float on the surface of the sample.

9 Procedure

NOTE 1 In the determination, two test portions (9.1) are extracted in two Mojonnier-type fat-extraction flasks (6.6). The extracts of the two flasks are poured into one prepared fat-collecting vessel (9.3).

NOTE 2 If it is required to check whether the repeatability limit (11.2) is met, carry out two single determinations in accordance with 9.1 to 9.4.

NOTE 3 An alternative procedure using fat-extraction tubes with siphon or wash-bottle fittings (see note in 6.6) is given in annex B.

9.1 Test portion

Mix the test sample (clause 8) by gently inverting the bottle three or four times. Immediately weigh, to the nearest 1 mg, 10 g to 11 g of the test sample, directly or by difference, in each of two fat-extraction flask (6.6).

Transfer the test portion as completely as possible into the lower (small) bulb of the fat-extraction flask.

9.2 Blank tests

9.2.1 Blank test for method

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 A.2).

If the value obtained in the blank test regularly exceeds 1,0 mg, check the reagents if this has not been recently done (9.2.2). Corrections of more than 2,5 mg should be mentioned in the test report.

9.2.2 Blank test for reagents

To test the quality of the reagents, carry out a blank test as specified in 9.2.1. Additionally use an empty fat-collecting vessel, prepared as specified in 9.3, for mass control purposes. The reagents shall leave no residue greater than 1,0 mg (see A.1).

If the residue of the complete reagent blank test is greater than 1,0 mg, determine the residue of the solvents separately by distilling 100 ml of the diethyl ether and light petroleum, respectively. Use an empty fat-collecting vessel, prepared for control purposes as described above, to obtain the real mass of residue which shall not exceed 1,0 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-collecting 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 the redistillation.

Replace unsatisfactory reagents and solvents, or redistil solvents.

9.3 Preparation of fat-collecting vessel

Dry a fat-collecting vessel (6.9) with a few boiling aids (6.10) in the oven (6.4) set at 102 °C for 1 h.

NOTE 1 Boiling aids are desirable to promote gentle boiling during the subsequent removal of solvents, especially when using glass fat-collecting vessels; their use is optional with metal dishes.

Protect the fat-collecting vessel from dust and allow it to cool to the temperature of the weighing room (glass fat-collecting vessel for at least 1 h, metal dish for at least 30 min).

NOTE 2 To avoid insufficient cooling or unduly long cooling times, the fat-collecting vessel should not be placed in a desiccator.

Use tongs to place the fat-collecting vessel on the balance. Weigh the fat-collecting vessel to the nearest 1,0 mg.

NOTE 3 Tongs should preferably be used to avoid, in particular, temperature variations.

9.4 Determination

9.4.1 Carry out the determination without delay.

Carry out the operations described in 9.4.2 to 9.4.13 on both pre-treated test portions (9.1).

9.4.2 Add 2 ml of ammonia solution (5.1) to the two test portions in the fat-extraction flasks (9.1), or an equivalent volume of a more concentrated ammonia solution (see note in 5.1). Mix thoroughly with the test portion in the small bulb of each of the two fat-extraction flasks.

9.4.3 Add 10 ml of ethanol (5.2). Mix gently but thoroughly by allowing the contents of the two fat-extraction flasks to flow backwards and forwards between the small and large bulb. Avoid bringing the liquid too near to the necks of the flasks. If desired, add 2 drops of the Congo red solution (5.3).

9.4.4 Add 25 ml of diethyl ether (5.4). Close both fat-extraction flasks with a cork saturated with water or with a stopper of other material wetted with water (6.6). Shake the flasks vigorously, but not excessively, for 1 min to avoid the formation of persistent emulsions.

While shaking, keep both fat-extraction flasks in a horizontal position with their small bulbs extending upwards, periodically allowing the liquid to run from the large bulb into the small bulb. If necessary, cool the flasks in running water to about room temperature. Carefully remove the corks or stoppers and rinse them and the necks of both flasks with a little mixed solvent (5.6). Use the wash bottle (6.8) so that the rinsings run into the flask concerned.

9.4.5 Add 25 ml of the light petroleum (5.5). Close both fat-extraction flasks with the rewetted (by dipping into water) corks or stoppers. Shake the flasks gently again for 30 s as described in 9.4.3. Proceed with shaking as described in 9.4.4.

9.4.6 Centrifuge the two closed fat-extraction flasks for between 1 min and 5 min at a radial acceleration of 80 g to 90 g. If a centrifuge is not available, allow the closed flasks 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 both flasks in running water to room temperature.

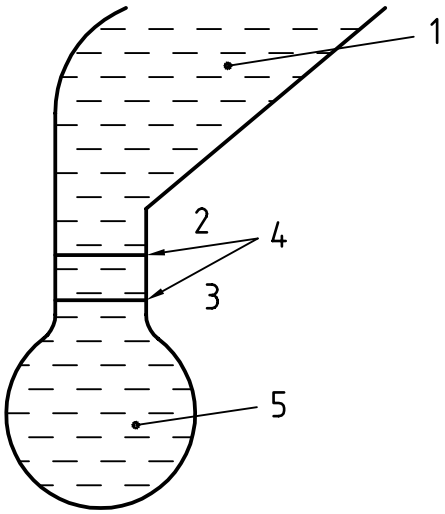
9.4.7 Carefully remove the corks or stoppers and rinse them and the inside of the necks of the two fat-extraction flasks with a little mixed solvent (5.6). Use the wash bottle (6.8) so that the rinsings run into the flask concerned. If the interface is below the bottom of the stem of one or both flasks, raise it slightly above this level by gently adding water down the side of the flask concerned (see Figure 1) to facilitate the decanting of solvent.

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

9.4.8 Hold both fat-extraction flasks by the small bulb and carefully decant as much as possible of the supernatant layer of the flasks into one and the same prepared fat-collecting vessel (see 9.3) containing a few boiling aids (6.10) in the case of a boiling or conical flask (optional with metal dishes). Avoid decanting any of the aqueous layer (see Figure 2).

9.4.9 Rinse the outside of the necks of both fat-extraction flasks with a little mixed solvent (5.6). Collect both rinsings in the same fat-collecting vessel as mentioned in 9.4.8. Take care that the mixed solvent does not spread over the outside of the fat-extraction flasks. If desired, remove the solvent or a part of it from the fat-collecting vessel by distillation or evaporation as described in 9.4.13.

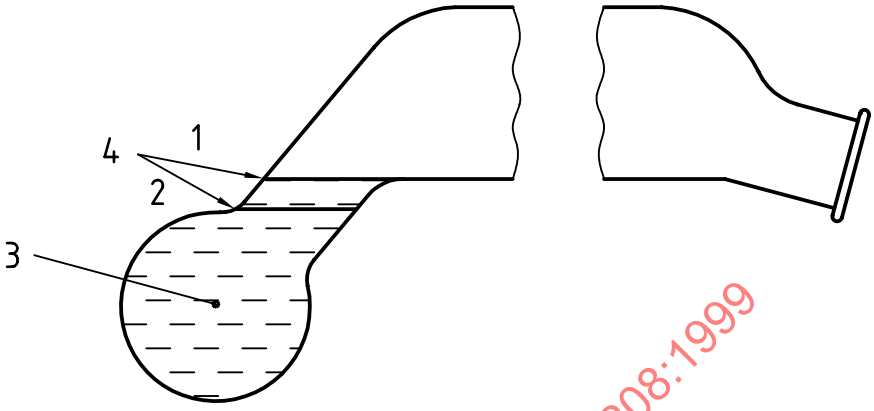
9.4.10 Add 5 ml of ethanol (5.2) to the contents of both fat-extraction flasks. Using the ethanol, rinse the inside of the necks of the flasks and mix as described in 9.4.3.



Key

1 Solvent
2 At second and third extraction
3 At first extraction
4 Interface
5 Aqueous layer

Figure 1 — Before decanting



Key

1 At second and third extraction
2 At first extraction
3 Aqueous layer
4 Interface

Figure 2 — After decanting

9.4.11 Carry out a second extraction by repeating the operations described in 9.4.4 to 9.4.9 inclusive. Instead of 25 ml, use only 15 ml of diethyl ether (5.4) and 15 ml of light petroleum (5.5). Using the diethyl ether, rinse the inside of the necks of both fat-extraction flasks too.

If necessary, raise the interface slightly to the middle of the stem of one or both flasks by gently adding water down the side of the flask concerned (see Figure 1) to enable the final decanting of solvent to be as complete as possible (see Figure 2).

9.4.12 Carry out a third extraction without addition of ethanol by again repeating the operations described in 9.4.4 to 9.4.9 inclusive. Again, use only 15 ml of diethyl ether (5.4) and 15 ml of light petroleum (5.5). Using the diethyl ether, rinse the inside of the necks of both fat-extraction flasks again.

If necessary, raise the interface slightly to the middle of the stem of one or both flasks by gently adding water down the side of the flask concerned (see Figure 1) to enable the final decanting of solvent to be as complete as possible (see Figure 2).

9.4.13 Remove the solvents (including the ethanol) as completely as possible from the fat-collecting vessel, by distillation if using a boiling or conical flask, or by evaporation if using a beaker or dish (6.3). Rinse the inside of the neck of the boiling or conical flask with a little mixed solvent (5.6) before commencing the distillation.

9.4.14 Heat the fat-collecting vessel, with the boiling or conical flask placed on its side to allow solvent vapour to escape, for 1 h in the drying oven (6.4) set at 102 °C. Remove the fat-collecting vessel from the oven and immediately verify whether or not the fat is clear. If the fat is not clear, fatty extraneous matter is presumed to be present and the whole procedure shall be repeated. If the fat is clear, protect the fat-collecting vessel from dust and allow the fat-collecting vessel to cool (preferably not in a desiccator) to the temperature of the weighing room (a glass fat-collecting vessel for at least 1 h, a metal dish for at least 30 min).

Do not wipe the fat-collecting vessel immediately before weighing. Use tongs to place the fat-collecting vessel on the balance. Weigh the fat-collecting vessel to the nearest 1,0 mg.

9.4.15 Heat the fat-collecting vessel, with the boiling or conical flask placed on its side to allow solvent vapour to escape, for 30 min in the drying oven (6.4) set at 102 °C. Cool and reweigh as described in 9.4.14. If necessary, repeat the heating and weighing procedures until the mass of the fat-collecting vessel decreases by 1,0 mg or less, or increases between two successive weighings. Record the minimum mass as the mass of the fat-collecting vessel and extracted matter.

10 Calculation and expression of results

10.1 Calculation

Calculate the fat content of the sample using the following equation:

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

where

w_f is the mass fraction of fat in the sample, in percent;

m_0 is the sum of the mass of the two test portions (9.1), in grams;

m_1 is the mass of the fat-collecting vessel and extracted matter, determined in 9.4.15, in grams;

m_2 is the mass of the prepared fat-collecting vessel (9.3), in grams;

m_3 is the mass of the fat-collecting vessel used in the blank test (9.2) and any extracted matter determined in 9.4.15, in grams;

m_4 is the mass of the fat-collecting vessel (9.3) used in the blank test (9.2), in grams.

10.2 Expression of results

Round the result to three decimal places.

11 Precision

11.1 Interlaboratory test

Details of an interlaboratory test in accordance with ISO 5725[1)] on the precision of the method have been published (see reference [6]).

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

11.2 Repeatability

The absolute difference between two independent single test results, obtained using 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, will in not more than 5 % of cases be greater than a mass fraction of 0,005 %.

11.3 Reproducibility

The absolute difference between two independent single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will in not more than 5 % of cases be greater than a mass fraction of 0,015 %.

1) ISO 5725:1986 (now withdrawn) was used to obtain the precision data.

12 Test report

The test report shall specify:

— all information necessary for the complete identification of the sample;

— the sampling method used, if known;

— the test method used, together with reference to this International Standard;

— 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);

— the corrections made, if a value of more than 2,5 mg is obtained in the blank test for the method;

— the test result(s) obtained; or

— if the repeatability has been checked, the final quoted result obtained.

Annex A
(informative)

Notes on procedures

A.1 Blank test to check the reagents (see 9.2.2)

In this blank test, a fat-collecting 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 reagents. This fat-collecting 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 10.1) of the fat-collecting vessel for control purposes should 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 fat-collecting vessel for control purposes, shall show no increase in mass greater than 1,0 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-collecting 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.

A.2 Blank test carried out simultaneously with the determination (see 9.2.1)

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 in the balance room and some temperature difference between the fat-collecting vessel and the balance room at the two weighings (9.4.15 and 9.3).

Under favourable conditions (low value in the blank test on reagents, equable temperature of the balance room, sufficient cooling time for fat-collecting 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 of more than 2,5 mg are applied, this should be mentioned in the test report (clause 12).

If the value obtained in this blank test regularly exceeds 1,0 mg, the reagents should be checked, if no recent check has been made. Any impure reagent or reagents traced should be replaced or purified (see 9.2.2 and A.1).

A.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 the diethyl ether 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 diethyl ether at least 3 days before it is to be used as follows.

Cut zinc foil into strips that will reach at least half-way up the bottle containing the diethyl ether, using approximately 80 cm^2 of foil per litre of diethyl 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/l of concentrated [98 % (mass fraction)] sulfuric acid.

Wash the strips gently but thoroughly with water, place the wet copper-plated strips in the bottle containing the diethyl ether, and leave the strips in the bottle.

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

A.4 Diethyl ether containing antioxidants

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

In other countries, diethyl ether with higher antioxidant contents, for example up to 7 mg/kg, 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 diethyl ether shall always be distilled before use.

A.5 Ethanol

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

Annex B
(informative)

Alternative procedure using fat-extraction tubes with siphon or wash-bottle fittings

B.1 General

If fat-extraction tubes with siphon or wash-bottle fittings are to be used, use the procedure specified in this annex. The tubes shall be provided with good quality bark corks or stoppers as specified for the flasks in 6.6 (see Figure B.1 as an example).

B.2 Procedure

B.2.1 Preparation of test sample

See clause 8.

B.2.2 Test portion

Proceed as specified in 9.1 but using the fat-extraction tubes (see note in 6.6 and Figure B.1). The two test portions shall be delivered as completely as possible to the bottom of each of the two fat-extraction tubes.

B.2.3 Blank test

See 9.2 and A.2.

B.2.4 Preparation of fat-collecting vessel

See 9.3.

B.2.5 Determination

B.2.5.1 Carry out the determination without delay.

Carry out the operations described in B.2.5.2 to B.2.5.13 on both pre-treated test portions (B.2.2).

B.2.5.2 Add 2 ml of ammonia solution (5.1) to both test portions in the fat-extraction tubes (B.2.2), or an equivalent volume of a more concentrated ammonia solution (see note in 5.1). Mix thoroughly with the pretreated test portions at the bottom of the fat-extraction tubes.

B.2.5.3 Add 10 ml of ethanol (5.2). Mix gently but thoroughly at the bottom of both fat-extraction tubes. If desired, add 2 drops of the Congo red solution (5.3).

B.2.5.4 Add 25 ml of diethyl ether (5.4). Close both fat-extraction tubes with corks saturated with water or with stoppers of other material wetted with water (6.6). Shake both tubes vigorously, but not excessively, with repeated inversions for 1 min, to avoid the formation of persistent emulsions. If necessary, cool the tubes in running water. Carefully remove the corks or stoppers and rinse them and the necks of the two tubes with a little mixed solvent (5.6). Use the wash bottle (6.8) so that the rinsings run into the tube concerned.

B.2.5.5 Add 25 ml of the light petroleum (5.5). Close both fat-extraction tubes with the rewetted (by dipping in water) corks or stoppers. Shake the tubes gently for 30 s, as described in B.2.5.4.

B.2.5.6 Centrifuge the two closed fat-extraction tubes for 1 min to 5 min at a radial acceleration of 80 g to 90 g. If a centrifuge is not available, allow both closed tubes 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 tubes in running water to room temperature.

B.2.5.7 Carefully remove the corks or stoppers and rinse them and the necks of the two fat-extraction tubes with a little mixed solvent (5.6). Use the wash bottle (6.8) so that the rinsings run into the tube concerned.

B.2.5.8 Insert the siphon fittings or the wash-bottle fittings into the two fat-extraction tubes. Push down the long inner limb of each fitting until the inlet is approximately 4 mm above the interface between the layers. The inner limb of the fittings shall be parallel to the axis of the fat-extraction tubes.

Carefully transfer the supernatant layer out of both fat-extraction tubes into one and the same fat-collecting vessel (see 9.3) containing a few boiling aids (6.10) in the case of boiling or conical flasks (optional with metal dishes). Avoid the transfer of any of the aqueous layer. Rinse the outlet of the fittings with a little mixed solvent, collecting both rinsings in the same fat-collecting vessel.

NOTE The supernatant layer can be transferred out of the fat extraction-tube by using, for example, a rubber bulb attached to the short stem to apply pressure.

B.2.5.9 Loosen the fittings from the neck of both fat-extraction tubes. Slightly raise the fittings and rinse the lower part of their long inner limbs with a little mixed solvent (5.6). Lower and re-insert the fittings and transfer both rinsings to the same fat-collecting vessel again.

Rinse the outlet of the fittings with a little mixed solvent again, collecting the rinsings in the same fat-collecting vessel. If desired, remove the solvent or a part of it from the fat-collecting vessel by distillation or evaporation as described in 9.4.13.

B.2.5.10 Again loosen the fittings from the necks. Slightly raise the fittings and add 5 ml of ethanol to the contents of both fat-extraction tubes. Use the ethanol to rinse the long inner limbs of the fittings. Mix as described in B.2.5.3.

B.2.5.11 Carry out a second extraction by repeating the operations described in B.2.5.4 to B.2.5.9. Instead of 25 ml, use only 15 ml of diethyl ether (5.4) and 15 ml of light petroleum (5.5). Use the diethyl ether to rinse the long inner limbs of both fittings during the removal of the fittings from the two fat-extraction tubes after the previous extraction.

B.2.5.12 Carry out a third extraction without the addition of ethanol by again repeating the operations described in B.2.5.4 to B.2.5.9. Again, use only 15 ml of diethyl ether and 15 ml of light petroleum. Using the diethyl ether, rinse the long inner limbs of both fittings as described in B.2.5.11.

B.2.5.13 Proceed as described in 9.4.13 to 9.4.15.