INTERNATIONAL STANDARD

ISO
1735
Second edition
1987-12-15

INTERNATIONAL ORGANIZATION FOR STANDARDIZATION
ORGANISATION INTERNATIONALE DE NORMALISATION
МЕЖДУНАРОДНАЯ ОРГАНИЗАЦИЯ ПО СТАНДАРТИЗАЦИИ

Cheese and processed cheese products — Determination of fat content — Gravimetric method (Reference method)

Fromages et fromages fondus — Détermination de la teneur en matière grasse — Méthode gravimétrique (Méthode de référence)

Reference number
ISO 1735 : 1987 (E)

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 1735 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, in collaboration with the International Dairy Federation (IDF) and the Association of Analytical Chemists (AOAC) and will also be published by these organizations.

This second edition cancels and replaces the first edition (ISO 1735 : 1975), of which it constitutes a technical revision.

Users should note that all International Standards undergo revision from time to time and that any reference made herein to any other International Standard implies its latest edition, unless otherwise stated.

Printed in Switzerland

Cheese and processed cheese products — Determination of fat content — Gravimetric method (Reference method)

0 Introduction

This second edition of ISO 1735 has been prepared within the framework of producing a series of reference methods, which are harmonized to the greatest possible extent, for the gravimetric determination of the fat content of milk, milk products and milk-based foods. These methods are based on either the Röse-Gottlieb (RG), or the Weibull-Berntrop (WB) or the Schmid-Bondzynski-Ratzlaff (SBR) principle.

A method based on the SBR principle, involving digestion with hydrochloric acid, has been chosen because

a) many types of cheese and cheese products do not readily dissolve in ammonia and it is therefore not practical to examine them according to a method based on the RG principle as used for milk and most milk products;

b) ripened cheeses contain, owing to fat splitting, free fatty acids which are not extracted from an ammoniacal solution;

c) all cheeses and cheese products, owing to their low lactose contents (mostly less than 5 % (*m/m*) of the dry matter), can be examined according to the SBR principle with good precision.

Generally speaking, the method is not suitable as a reference method for fresh cheese types, such as cottage cheese and quarg, which have higher lactose contents of up to 25 % (*m/m*) of the non-fat solids. Difficulties may also be expected with some types of fresh cheese, especially cottage cheese, because of their extreme inhomogeneity and the impracticability of attaining homogeneity of the test sample. In such cases, the WB method in ISO 8262-3[1], using a larger mass of test portion, is to be preferred. The same holds for fresh cheeses with added fruit, syrup, "muesli", etc.

1 Scope and field of application

This International Standard specifies the reference method for the determination of the fat content of all types of cheese and processed cheese products having lactose contents below 5 % (*m/m*) of the non-fat solids.

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.*

ISO 5534, *Cheese and processed cheese — Determination of total solids content (Reference method).*

3 Definition

For the purposes of this International Standard, the following definition applies.

fat content of cheese and processed cheese products : All the substances determined by the method specified in this International Standard.

It is expressed as a percentage by mass.

4 Principle

Digestion of a test portion with hydrochloric acid, addition of ethanol and subsequent extraction of the acid-ethanolic solution 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 Schmid-Bondzynski-Ratzlaff 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 (see 10.1). The reagents shall leave no residue greater than 0,5 mg.

1) ISO 8262-3, *Milk products and milk-based foods — Determination of fat content by the Weibull-Berntrop gravimetric method (Reference method) — Part 3 : Special cases.*

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 distil the solvents if they do not meet this requirement.

5.1 Hydrochloric acid solution, $\varrho_{20} \approx 1{,}125$ g/ml.

Dilute 675 ml of concentrated hydrochloric acid ($\varrho_{20} = 1{,}18$ g/ml) to 1 000 ml with water.

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

(See 10.5.)

5.3 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 the introductory paragraphs to clause 5, and also 10.1 and 10.4).

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

5.5 Mixed solvent, prepared shortly before use by mixing equal volumes of the diethyl ether (5.3) and the light petroleum (5.4).

6 Apparatus

WARNING — Since the determination involves the use of volatile flammable solvents, electrical apparatus employed may 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 stoppered fat-extraction flasks or tubes (6.6) can be spun at a rotational frequency of 500 to 600 min^{-1} to produce an acceleration of $80g$ to $90g$ at the outer end of the flasks or tubes.

NOTE — The use of the centrifuge is optional but recommended (see 8.5.7).

6.3 Distillation or **evaporation apparatus,** to enable the solvents and ethanol to be distilled from the fat-collecting flasks or to be evaporated from beakers and dishes (see 8.5.10 and 8.5.13) 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 ± 2 °C throughout the working space. The oven shall be fitted with a suitable thermometer;

or

Vacuum drying oven, capable of being maintained at a temperature of 70 to 75 °C and a pressure less than 66 mbar (50 mmHg).

6.5 Boiling water bath or **hotplate** (see 8.5.2).

6.6 Mojonnier-type fat-extraction flasks, as specified in ISO 3889 (but see the note to 8.5.2).

NOTE — It is also possible to use **fat-extraction tubes** (or **flasks**) with **siphon** or **wash-bottle fittings**, but the procedure is then different and is that specified in the annex. The long inner limb of the fitting may have a hooked end if desired.

The flasks (or tubes, see the note) shall be provided with good quality bark corks or stoppers of other material (for example silicone rubber or PTFE[1]) unaffected by the reagents used. Bark corks shall be washed with the diethyl ether (5.3), kept in water at 60 °C or more (but not boiling) 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 (5.5). 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 beakers of capacity 250 ml, or metal dishes.

If metal dishes are used, they shall preferably be made 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, or glass beads (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.

1) Polytetrafluoroethylene.

6.14 Sheets of cellulose film, unlacquered, soluble in hydrochloric acid, 0,03 to 0,05 mm thick and of dimensions approximately 50 mm × 75 mm. The sheets shall be inert under the test conditions.

6.15 Appropriate grinding or grating device, easy to clean, for preparing the sample.

7 Sampling

See ISO 707.

All laboratory samples shall be kept at a temperature of 0 to 4 °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[1)]

Before the analysis, remove the rind or smear or mouldy surface layer of the cheese in such a way as to obtain a sample representative of the cheese as it is usually consumed.

Prepare the sample using an appropriate device (6.15). Quickly mix the ground or grated mass and, if possible, grind it a second time and again mix thoroughly. Clean the device after preparing each sample. If the sample cannot be ground or grated, mix it thoroughly by intensive kneading, for example with a pestle in a mortar.

Keep the prepared sample in an airtight container until the time of analysis, which should be carried out on the same day. If delay is unavoidable, take every precaution to ensure proper storage of the sample. When refrigerated, ensure that any condensation of moisture on the inside surface of the container is thoroughly and uniformly re-incorporated into the test sample.

8.2 Test portion

Mix the test sample (8.1) by gently stirring and immediately weigh, to the nearest 1 mg, directly or by difference, into a fat-extraction flask (6.6), or a 100 ml beaker or flask, 1 to 3 g of the test sample (3 g for cheeses having fat contents up to 30 % (m/m) and 1 to 3 g for cheeses having higher fat contents yielding 750 to 1 000 mg of fat). The test portion may also be weighed on a sheet of cellulose film (6.14), which is subsequently folded and introduced into the vessel of the type chosen.

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

8.3 Blank test

Carry out a blank test simultaneously with the determination, using the same procedure and same reagents, but omitting the test portion in 8.5.1 (see 10.2).

8.4 Preparation of fat-collecting vessel

Dry a vessel (6.9) containing 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 (6.13) (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 solvent, 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 8 to 10 ml, depending on the shape of the extraction apparatus and the size of the test portion, of the hydrochloric acid solution (5.1) so as to wash the test portion into the small bulb of the extraction flask or onto the bottom of the beaker or flask, and mix.

8.5.2 Heat by gently moving the vessel (to avoid charring) in a boiling water bath or over a flame or on a hotplate, until all the particles are entirely dissolved.

NOTE — Mojonnier-type flasks (6.6) with a spherical lower bulb (forms B and C in ISO 3889) are particularly suitable for direct heating over a flame or on a hotplate.

8.5.3 Allow the vessel to stand for 20 to 30 min in the boiling water bath or keep it gently boiling over the flame or on the hotplate for 10 min. Cool, for example in running water.

8.5.4 If the digestion has been carried out in the extraction apparatus, 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 the digestion has been carried out in a vessel other than the extraction flask, pour the contents of the vessel into the extraction flask. Rinse the vessel successively with 10 ml of the ethanol (5.2), 25 ml of the diethyl ether (5.3) and 25 ml of the light petroleum (5.4), each time pouring the rinsings into the

1) Any specific technique to be used for a particular type of cheese may be indicated in the individual international cheese standards prepared under the FAO/WHO *Code of Principles concerning Milk and Milk Products.*

extraction flask. Mix after the addition of the ethanol as described above and shake the extraction flask, after the addition of diethyl ether and light petroleum, as described in 8.5.5 and 8.5.6 respectively.

8.5.5 Add 25 ml of the diethyl ether (5.3), close the flask with a cork (see 6.6) saturated with water or with a stopper 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 (5.5), using the wash bottle (6.8), so that the rinsings run into the flask or the prepared fat-collecting vessel (see 8.4).

8.5.6 Add 25 ml of the light petroleum (5.4), 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 8.5.5.

8.5.7 Centrifuge the closed flask for 1 to 5 min at a rotational frequency of 500 to 600 min^{-1} (see 6.2). 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.

8.5.8 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 or the fat-collecting vessel.

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 is shown, but this does not imply any preference over the other types (however, see also the note to 8.5.2).

8.5.9 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).

8.5.10 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.

If desired, the solvent or part of the solvent may be removed from the vessel by distillation or evaporation as described in 8.5.13.

8.5.11 Carry out a second extraction (without the addition of ethanol) by repeating the operations described in 8.5.5 to 8.5.10 inclusive, but using only 15 ml of the diethyl ether (5.3) and 15 ml of the light petroleum (5.4); use the ether to rinse the inside of the neck of the extraction flask.

If necessary, raise the interface to slightly above 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).

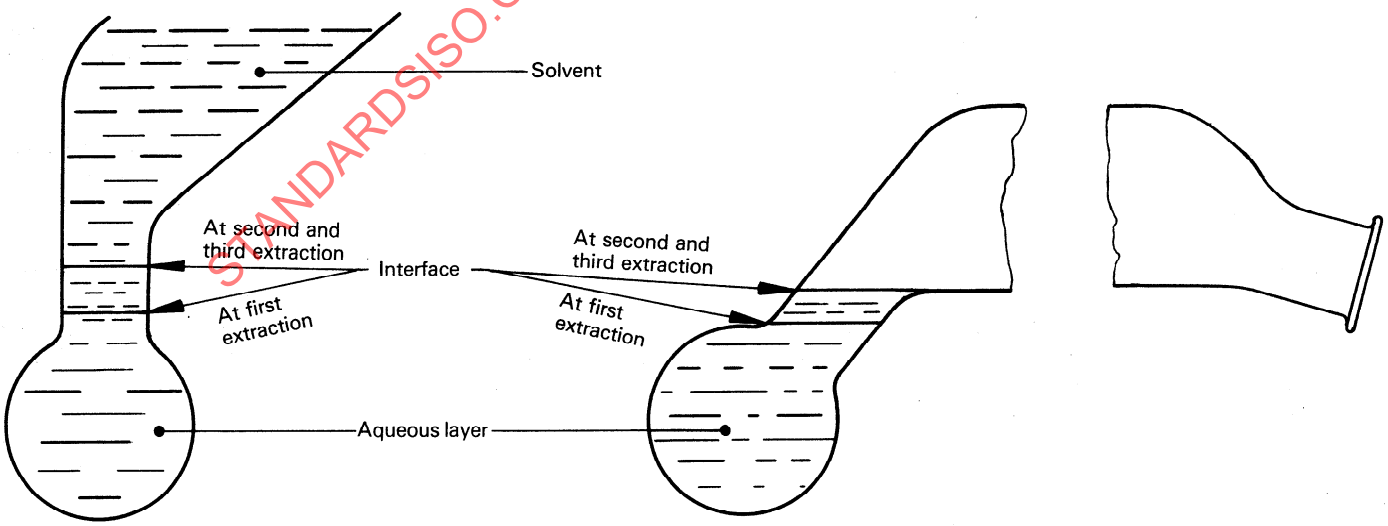

Figure 1 — Before decantation
(8.5.8, 8.5.11, 8.5.12)

Figure 2 — After decantation
(8.5.9, 8.5.11, 8.5.12)

8.5.12 Carry out a third extraction (without the addition of ethanol) by again repeating the operations described in 8.5.5 to 8.5.9 inclusive, but using only 15 ml of the diethyl ether (5.3) and 15 ml of the light petroleum (5.4); use the ether to rinse the inside of the neck of the extraction flask.

If necessary, raise the interface to slightly above 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 products having fat contents of 3 % (m/m) or less.

8.5.13 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 (5.5) before commencing the distillation.

8.5.14 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).

8.5.15 Repeat the operations described in 8.5.14 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.

8.5.16 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 8.5.15) and its initial mass (see 8.4).

8.5.17 If the extracted matter is not wholly soluble in the light petroleum, or in case of doubt, extract the fat completely from the vessel by repeatedly washing with warm light petroleum.

NOTE — National legislation may mandatorily prescribe such an extraction, either in general or in particular cases.

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 top 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 8.5.14 and 8.5.15.

Take the mass of fat as the difference between the mass determined in 8.5.15 and this final mass.

9 Expression of results

9.1 Method of calculation and formula

9.1.1 The fat content w_f, 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 8.5.15;

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 8.5.17;

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 8.5.15;

m_4 is the mass, in grams, of the prepared 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 8.5.17.

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

9.1.2 The fat content of the dry matter, expressed as a percentage by mass, is equal to

$$w_f \times \frac{100}{w_d}$$

where

w_f is the fat content of the sample, calculated in 9.1.1;

w_d is the dry matter content of the sample, determined in accordance with ISO 5534.

9.2 Precision

NOTE — The values for repeatability and reproducibility are expressed at the 95 % probability level and were derived from the results of an interlaboratory trial carried out in accordance with ISO 5725[1].

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 0,2 g of fat per 100 g of product.

1) ISO 5725, *Precision of test methods — Determination of repeatability and reproducibility for a standard test method by inter-laboratory tests.*

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 0,3 g[1] 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 9.1.1) 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 not exceed 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-collecting vessel with about 1 g of fresh anhydrous butterfat. If necessary, distil solvents in the presence of 1 g of anhydrous butterfat per 100 ml of solvent. Solvents treated in this way should only be stored for short periods following distillation.

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 any temperature difference between the fat-collecting vessel and the balance room at the two weighings (8.4 and 8.5.15 or 8.5.17).

Under favourable conditions (low value in the blank test on reagents, equable temperature of the balance room, sufficient cooling time for the fat-collecting vessel), the value will usually be less than 0,5 mg and can 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, this fact 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 introductory paragraphs to clause 5, and also 10.1).

10.3 Test for peroxides in diethyl ether

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 diethyl ether (without antioxidants) is free, and is maintained free, from peroxides, treat the ether as follows 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 direct use for reference purposes.

In other countries, only diethyl ether with a higher antioxidant content, 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 than by methanol 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 result obtained. It shall also mention all operating conditions not specified in this International Standard, or regarded as optional, as well as any incidents likely to have influenced the results. The blank value ($m_3 - m_4$, see 9.1.1) 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.

1) This value is tentative.

Annex

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

(for examples, see figure 3)

(This annex forms an integral part of the standard.)

A.0 Introduction

If fat-extraction tubes with siphon or wash-bottle fittings are to be used (see the note to 6.6), 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 the note to 6.6), or use a 100 ml beaker or flask.

The test portion shall be delivered as completely as possible onto the bottom of the extraction tube or beaker or flask.

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 10 ml of the hydrochloric acid solution (5.1) so as to wash the test portion onto the bottom of the tube, beaker or flask, and mix.

A.1.5.2 Heat by gently moving the vessel (to avoid charring) in a boiling water bath or over a flame or on a hotplate, until all the particles are entirely dissolved.

NOTE — Fat-extraction flasks having a foot are not suitable for direct heating over a flame or on a hotplate.

A.1.5.3 Allow the vessel to stand for 20 to 30 min in the boiling water bath or keep it gently boiling over the flame or on the hotplate for 10 min. Cool, for example in running water.

A.1.5.4 If the digestion has been carried out in the extraction tube, add 10 ml of the ethanol (5.2) and mix gently but thoroughly at the bottom of the tube.

If the digestion has been carried out in a vessel other than the extraction tube, pour the contents of the vessel into the extraction tube. Rinse the vessel successively with 10 ml of the ethanol (5.2), 25 ml of the diethyl ether (5.3) and 25 ml of the light petroleum (5.4), each time pouring the rinsings into the extraction tube. Mix after the addition of the ethanol as described above and shake the extraction tube, after the addition of diethyl ether and light petroleum, as described in A.1.5.5 and A.1.5.6 respectively.

A.1.5.5 Add 25 ml of the diethyl ether (5.3), close the tube with a cork (see 6.6) saturated with water or with a stopper 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 (5.5), using the wash bottle (6.8), so that the rinsings run into the tube.

A.1.5.6 Add 25 ml of the light petroleum (5.4), 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.1.5.5.

A.1.5.7 Centrifuge the closed tube for 1 to 5 min at a rotational frequency of 500 to 600 min^{-1} (see 6.2). 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.1.5.8 Carefully remove the cork or stopper and rinse it and the inside of the neck of the tube with a little of the mixed solvent so that the rinsings run into the tube.

A.1.5.9 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 prepared 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.1.5.10 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.