International Standard ISO 5543

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

Caseins and caseinates — Determination of fat content — Gravimetric method (Reference method)

Caséines et caséinates — Détermination de la teneur en matière grasse — Méthode gravimétrique (Méthode de référence)

First edition — 1986-04-15
Corrected and reprinted — 1987-09-01

UDC 637.147.2 : 543.85 **Ref. No. ISO 5543-1986 (E)**

Descriptors : agricultural products, dairy products, caseins, chemical analysis, determination of content, fats, gravimetric analysis.

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 5543 was prepared by Technical Committee ISO/TC 34, *Agricultural food products.*

NOTE — The method specified in this International Standard has been developed jointly with the International Dairy Federation (IDF) and the Association of Official Analytical Chemists (AOAC) and will also be published by these organizations.

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

Caseins and caseinates — Determination of fat content — Gravimetric method (Reference method)

0 Introduction

This International Standard has been prepared within the framework of producing a series of methods, which are harmonized to the greatest possible extent, for the gravimetric determination of the fat content of milk, milk products and milk-based food.

A method based on the principle of Schmid-Bondzynski-Ratzlaff (SBR), involving digestion with hydrochloric acid, has been chosen, because :

a) many caseins do not readily dissolve in ammonia, either because they contain or consist of hard lumps, or because they are not soluble, or are only poorly soluble, as such (for example rennet casein), and therefore cannot be examined according to the method based on the Röse-Gottlieb (RG) principle as used for milk and most milk products;

b) all caseins and caseinates, due to their low lactose content [less than 5 % (m/m) of the dry matter], can be examined according to the SBR principle. This has the advantage over the Weibull method in that the method can be carried out using the same apparatus as that specified for the RG method and, at the same time, is less time consuming;

c) methods based on the SBR principle have already found wide application in many countries as official or standardized methods for the examination of all caseins and caseinates.

1 Scope and field of application

This International Standard specifies the reference method for the determination of the fat content of all types of caseins and caseinates.

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 5550, *Caseins and caseinates — Determination of water content (Reference method).*

3 Definition

fat content of caseins and caseinates : 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.

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 Hydrochloric acid solution, $\varrho_{20} \approx 1{,}125$ g/ml. (See also note to 8.5.1.)

Dilute 675 ml of hydrochloric acid ($\varrho_{20} = 1{,}18$ g/ml) to 1000 ml with water.

5.2 Ethanol, or **ethanol denatured 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).

5.6 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 8.5.4) and only useful with products giving colourless or only slightly coloured digests. Other aqueous colour solutions may be used provided that they do not affect the result of the determination.

6 Apparatus and materials

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 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 8.5.7).

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 8.5.12) at a temperature not exceeding 100 °C.

6.4 Drying oven, electrically heated, with ventilation port(s) fully open, capable of being maintained at 102 ± 2 °C throughout the working space and fitted with a suitable thermometer; or **vacuum drying oven**, capable of being maintained at 70 to 75 °C, at 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 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 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 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.

6.14 Grinding device, for grinding the laboratory sample if necessary. This device should be such that no undue heat will be developed and no loss of moisture occurs. (A hammer mill shall not be used.)

6.15 Test sieve, of woven wire cloth, diameter 200 mm, nominal size of openings 500 µm, with receiver, complying with the requirements of ISO 565.

6.16 Container with lid, airtight, of capacity such that the test sample can be mixed by shaking.

1) polytetrafluoroethylene

7 Sampling

See ISO 707.

The laboratory sample shall be stored in a securely closed airtight container.

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

8.1.1 Thoroughly mix the laboratory sample (clause 7), if necessary after transferring all of it to an airtight container of suitable capacity, by repeatedly shaking and inverting the container.

8.1.2 Transfer 50 g of the laboratory sample to the test sieve (6.15). If it does not pass completely through the sieve, use the grinding device to achieve this condition. Immediately transfer all the sieved sample to the container (6.16) and mix thoroughly in the closed container. During these operations, take precautions to avoid any change in the water content of the product.

8.1.3 After the test sample has been prepared, proceed with the determination (8.5) as soon as possible.

If the 50 g portion directly passes, or nearly completely passes, the sieve, use the prepared test sample (see 8.1.1) for the determination.

8.2 Test portion

Mix the test sample (8.1) by gently stirring, or by rotating and inverting the container. Immediately weigh, to the nearest 1 mg, directly or by difference, into a fat-extraction flask (6.6), or into a 100 ml beaker or flask, 2 to 3 g of the test sample.

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 omitting the test portion as 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 7,5 to 10 ml, depending on the shape of the extraction apparatus, of the hydrochloric acid (5.1) so as to wash the test portion into the small bulb of the extraction flask or on to the bottom of the beaker or flask, and mix.

NOTE — When adding 7,5 to 8,5 ml of hydrochloric acid, some laboratories prefer to use 1,15 g/ml hydrochloric acid.

8.5.2 Heat by gently moving the vessel 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 60 min in the boiling water bath, shaking occasionally during the initial 15 min, or keep it gently boiling over the flame or on the hotplate for 10 min. Cool, for example in running water.

NOTE — If, at a later stage of the procedure, difficulties are encountered due to a viscous aqueous phase, repeat the determination with a smaller test portion and a longer heating or boiling time.

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 desired, add 2 drops of the Congo-red solution (see 5.6).

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 successively with 10 ml of ethanol (5.2), 25 ml of diethyl ether (5.3) and 25 ml of light petroleum (5.4), each time pouring the solvent into the 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 has been chosen, but this does not imply any preference over the other types (see, however, 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 8.5.12.

8.5.11 Carry out a second extraction by 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).

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 (5.5) 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), maintained 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.

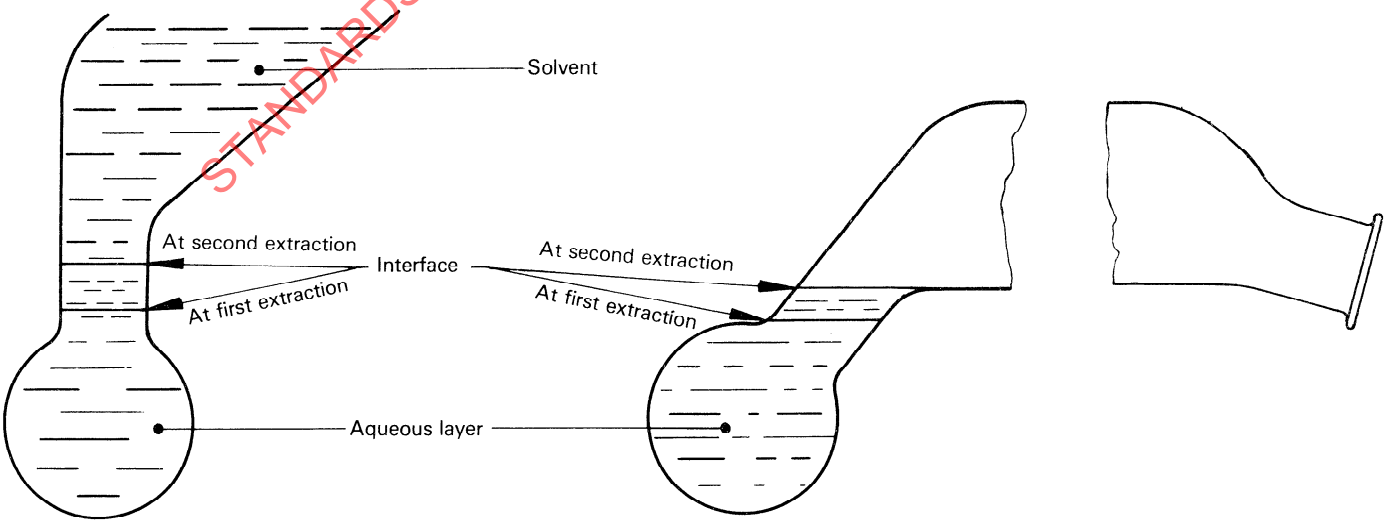

Figure 1 — Before decantation (8.5.8, 8.5.11)

Figure 2 — After decantation (8.5.9, 8.5.11)

8.5.15 Add 25 ml of the light petroleum to the fat-collecting vessel in order to verify whether 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), maintained 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 8.5.15 and this final mass.

9 Expression of results

9.1 Method of calculation and formula

9.1.1 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 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 8.5.16.

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

$$w_f \times \frac{100}{100 - w_w}$$

where

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

w_w is the water content of the sample, determined in accordance with ISO 5550.

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 in accordance with ISO 5725, *Precision of test methods — Determination of repeatability and reproducibility by interlaboratory 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 0,1 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 0,2 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 conditions 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 not be 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-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 (8.5.14 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 the 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 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 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 direct use for reference purposes.

In other countries, only 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 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, together with details of 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.

Annex

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

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 on to the bottom of the extraction tube, 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 to 15 ml of the hydrochloric acid (5.1) so as to wash the test portion on to the bottom of the tube, beaker or flask, and mix.

A.1.5.2 Heat by gently moving the vessel 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 60 min in the boiling water bath, shaking occasionally during the initial 15 min, or keep it gently boiling over the flame or on the hotplate for 10 min. Cool, for example in running water.

NOTE — If, at a later stage of the procedure, difficulties are encountered due to a viscous aqueous phase, repeat the determination with a smaller test portion and a longer heating or boiling time.

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 desired, add 2 drops of the Congo-red solution (5.6).

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 successively with 10 ml of ethanol (5.2), 25 ml of diethyl ether (5.3) and 25 ml of light petroleum (5.4), each time pouring the solvent 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.