II

(Acts whose publication is not obligatory)

COUNCIL

COUNCIL DECISION

of 14 November 1992

laying down methods for the analysis and testing of heat-treated milk for direct human consumption

(92/608/EEC)

THE COUNCIL OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Economic Community,

Having regard to Council Directive 85/397/EEC of 5 August 1985 on health and animal-health problems affecting intra-Community trade in heat-treated milk [1] and in particular Article 11 (6) thereof,

Having regard to the proposal from the Commission,

Whereas, pursuant to Article 11 (6) of Directive 85/397/EEC, the Council is to lay down the detailed arrangements for the checks provided for in Article 11 (2); whereas the purpose of such checks, to be carried out by establishments under the supervision and responsibility of the official department and with periodic inspection by the official department, is to ensure compliance with the requirements of Directive 85/397/EEC and in particular those laid down in Article 3 (A) (3) thereof;

Whereas, the laying-down of procedures for checks includes the determination of the methods to be employed for their implementation;

Whereas methods have to be prescribed for determining the total solids content, the fat content, the solids-non-fat content, the total nitrogen content, the protein content and the density of heat-treated milk intended for direct human consumption;

Whereas, for technical reasons, the initial requirement is to lay down reference methods for analysis and testing to ensure that the standards prescribed in Article 3 (A) (3) of Directive 85/397/EEC are met; whereas it is particularly important to make a study of the conditions under which routine methods for analysis and testing are used; whereas, pending the outcome of this study, it is for the competent authorities to see that appropriate routine methods are used with a view to ensuring that the said standards are met;

Whereas the determination of the aforementioned methods includes in particular the determination of analytical procedures and the fixing of criteria of reliability in order to ensure uniform interpretation of the results,

HAS ADOPTED THIS DECISION:

Article 1

The methods for analysing and testing heat-treated milk shall be the following:

— determination of total solids content,

— determination of fat content,

— determination of total non-fat solids content,

— determination of total nitrogen content,

— determination of protein content,

— determination of specific mass.

[1] OJ No L 226, 24. 8. 1985, p. 13, as last amended by Directive 89/662/EEC (OJ No L 395, 30. 12. 1989, p. 13).

Article 2

The implementation of the reference methods for analysis and testing, the determination of the criteria of reliability and the collection of samples must be carried out according to the rules set out in Annex I.

Article 3

The methods referred to in Article 1 are set out in Annex II.

Article 4

This Decision is addressed to the Member States.

Done at Brussels, 14 November 1992.

For the Council

The President

J. GUMMER

ANNEX I

I. GENERAL PROVISIONS

1. INTRODUCTION

General provisions with respect to reagents, equipment, expression of results, precision and test reports are described. Competent authorities of Member States and enforcement laboratories charged with the sampling and testing of milk must respect these provisions.

2. REAGENTS

2.1. Water

2.1.1. Wherever mention is made to water for solution, dilution or rinsing purposes, distilled water, deionized water or demineralized water of at least equivalent purity, shall be used unless otherwise specified.

2.1.2. Wherever reference is made to 'solution' or 'dilution' without further indication, 'solution in water' or 'dilution with water' is meant.

2.2. Chemicals

All chemicals used shall be of recognized analytical quality unless otherwise specified.

3. EQUIPMENT

3.1. Lists of equipment

The lists of equipment given in the different reference methods contain only those items with a specialized use and items to a particular specification.

3.2. Analytical balance

'Analytical balance' means a balance capable of weighing at 0,1 mg.

4. EXPRESSION OF RESULTS

4.1. Results

Unless otherwise specified, the results stated in the test report (6) shall be the mean arithmetic value obtained from two tests which satisfy the repeatability-criterion (5.1.1.) stated for that method. If the repeatability-criterion is not satisfied, the test must be repeated, if possible, or the result declared invalid.

4.2. Calculation of percentage

Except when otherwise specified, the result shall be calculated as a percentage by mass of the sample.

5. PRECISION CRITERIA: REPEATABILITY AND REPRODUCIBILITY

5.1. The precision criteria given in each method is defined as follows:

5.1.1. *Repeatability (r)* is the value below which the absolute difference between two single test results obtained with the same procedure on identical test material, under the same conditions (same operator, same apparatus, same laboratory, and a short interval of time) lies.

5.1.2. *Reproducibility (R)* is the value below which the absolute difference between two single test results obtained with the same procedure on identical test material, under different conditions (different operators, different apparatus, different laboratories and/or different time) lies.

5.1.3. Unless otherwise specified for each individual method the values for the repeatability- and reproducibility-criteria of each procedure represent the 95 % confidence level intervals as defined by ISO 5725: 2'ed. 1986.

5.1.4. The necessary collaborative trials and studies should be planned and conducted in accordance with international guidelines.

6. TEST REPORT

The test report shall specify the method of analysis used as well as the results obtained. In addition, it shall give any details of procedure used not specified in the method of analysis or which are optional, as well as any other circumstances that may have influenced the results obtained. The test report shall give all the information necessary for the complete identification of the sample.

II. SAMPLING OF HEAT-TREATED MILK

1. SCOPE AND FIELD OF APPLICATION

This procedure specifies the reference method of sampling, transport and storage of samples of heat treated milk.

2. GENERAL

Sampling of heat-treated milk in tanks etc., shall be carried out by a skilled operator who has had suitable training before undertaking the sampling of milk.

If they consider it appropriate, the competent authorities or testing laboratory shall instruct sampling personnel in sampling techniques to ensure that the sample is representative of, and in conformity with, the entire batch.

If they consider it appropriate, the competent authorities or testing laboratory shall instruct sampling personnel on marking the sample to ensure the unambiguous identity of the sample.

3. SAMPLING EQUIPMENT

3.1. General

Sampling equipment shall be made of stainless steel, or other suitable material of adequate strength and of a construction suitable for the intended purpose (mixing, sampling etc.). Plungers and agitators for mixing liquids in containers shall have a sufficient area to produce adequate mixing of the product, but without causing the development of a rancid flavour. Dippers must have a solid handle of sufficient length to enable a sample at any depth of the container to be obtained. The capacity of the dipper shall be not less than 50 ml.

Sample containers and closures should be of glass, suitable metals or plastics.

The materials of which sampling equipment (including containers and closures) is constructed must not cause any change in the sample which could affect the results of the examinations. All surfaces of sampling equipment and sample containers shall be clean and dry, smooth and free from crevices, and corners shall be rounded.

4. SAMPLING TECHNIQUE

4.1. General

Irrespective of the tests to be performed, the milk shall be thoroughly mixed prior to sampling, by either manual or mechanical means.

The sample shall be taken immediately after mixing while the milk is still agitated.

The volume of the sample shall be adequate to the testing requirements. The capacity of the sample containers used shall be such that they are filled almost completely by the sample, thus allowing proper mixing of the contents before testing, but avoiding churning during transport.

4.2. Manual sampling

4.2.1. *Sampling a divided bulk*

Where the quantity of milk to be sampled is in more than one container, take a representative quantity from each container and note the quantity of milk to which each sample relates. Unless the samples from each container are to be tested individually, mix portions of these representative quantities in amounts which are proportional to the quantity in the container from which each sample was taken. Take sample(s) from these bulked proportionate amounts after mixing.

4.2.2. *Sampling from large vessels — Storage, rail and road tanks*

4.2.2.1. Mix the milk by an appropriate procedure, before sampling.

To mix the contents of large vessels or of storage, rail or road tanks, the use of mechanical agitation is advised (4.2.2.2.).

The extent of mixing shall be appropriate to the period of time over which the milk has been at rest. The efficiency of the procedure of mixing applied in any particular circumstances shall be demonstrated as being adequate for the purposes of the analysis envisaged; the criterion of mixing efficiency particularly influences the similarity between analytical results from samples taken either from different parts of the consignments, or from the outlet of the tank at intervals during discharge. A procedure of mixing milk (untreated milk or whole milk) shall be considered efficient if the difference in fat content between two samples, taken under these conditions, is less than 0,1 %.

In a large vessel with a bottom discharge outlet there may be, at the discharge point, a small quantity of milk which is not representative of the whole contents even after mixing. Therefore samples should preferably be taken through a manhole. If samples are taken from the discharge outlet, run off sufficient milk to ensure that the samples are representative of the whole.

4.2.2.2. Mixing of the contents of large vessels or of storage, rail or road tanks can be carried out:

— by a mechanical agitator built into the tanks and driven by an electric motor;

— by a propellor or agitator driven by an electric motor and placed on the manhole with the agitator suspended in the milk;

— in the case of rail or road tankers by recirculation of the milk through the transfer hose attached to the tanker unloading pumps and inserted through the manhole;

— by clean filtered compressed air. In this case, minimal air pressure and volume should be used to prevent the development of rancid flavour.

4.3. **Sampling of heat-treated milk for direct consumption in retail-packings**

Samples of heat-treated milk for direct consumption in retail packages are to be the complete sealed package. If possible, the samples must be taken from the packaging machine or cold room in the treatment establishment as soon as possible after processing (for pasteurized milk on the same day as processing).

The samples are taken from each type of heat-treated milk (pasteurized, UHT-treated and sterilized) in numbers corresponding to the examinations which will be made and in accordance with instructions laid down by the testing laboratory or other competent authority.

5. IDENTIFICATIONS OF THE SAMPLE

The sample shall be marked with an identification code so that it can be readily identified using instructions given by the testing laboratory or competent authority.

6. PRESERVATION, TRANSPORT AND STORAGE OF SAMPLES

In accordance with the competent national authority, instructions concerning the conditions of preservation (chemical, temperature), transport, storage and time between sampling and analysis of milk shall be prepared by the testing laboratory according to the type of milk and the procedure of analysis to be used.

In the instruction the following points shall be included:

— During transport and storage, precautions shall be taken to prevent exposure to contaminating odours and to direct sunlight. If the container used for samples is transparent, it shall be stored in a dark place.

ANEX II

I. DETERMINATION OF TOTAL SOLIDS CONTENT

1. SCOPE AND FIELD OF APPLICATION

This procedure specifies the reference method for the determination of the total solids content of milk.

2. DEFINITION

Total solids content: The mass remaining after completion of the specified drying procedure and expressed as a percentage by mass.

3. PRINCIPLE

Evaporation of the water from a test portion at a temperature of 102 ± 2 °C in a drying oven.

4. APPARATUS AND GLASSWARE

Usual laboratory equipment and, in particular:

4.1. **Analytical balance**

4.2. **Desiccator,** provided with an efficient desiccant (for example freshly dried silica gel with a water content indicator).

4.3. **Drying oven,** ventilated, thermostatically controlled at 102 ± 2 °C throughout the total working space.

4.4. **Flat-bottom dishes,** of height 20 to 25 mm, diameter 50 to 75 mm, and of appropriate material provided with well-fitting, readily removable lids.

4.5. **Boiling water bath**

4.6. **Homogenizer**

5. PREPARATION OF THE TEST SAMPLE

Bring the sample of milk to a temperature of 20 to 25 °C. Thoroughly mix to ensure a homogeneous distribution of the fat throughout the sample. Avoid agitating so vigorously as to cause frothing of the milk or churning of the fat. If it is found difficult to disperse the cream layer, warm slowly to between 25 and 40 °C and with careful mixing, incorporate any cream adhering to the container. Cool the sample quickly to 20—25 °C.

If desired, a homogenizer may be used to assist the dispersion of the fat.

Correct results cannot be expected if the sample contains separated liquid fat or visible separate irregularly shaped white particles adhering to the walls of the container.

6. PROCEDURE

6.1. **Preparation of the dish**

Heat a dish (4.4.) with its lid placed next to it in the oven (4.3.), controlled at 102 ± 2 °C, for at least 30 minutes. Place the lid on the dish and immediately transfer to the desiccator (4.2.), allow to cool to room temperature (i.e. for at least 30 minutes) and weigh to the nearest 0,1 mg.

6.2. **Test portion**

Immediately weigh, to the nearest 0,1 mg, 3 to 5 g of the prepared test sample (5.) into the prepared dish (6.1.).

6.3. **Determination**

6.3.1. Pre-dry the dish for 30 minutes by heating it on the boiling water bath (4.5).

6.3.2. Heat the dish, with its lid alongside, in the oven (4.3), controlled at 102 ± 2 °C, for two hours. Place the lid on the dish and remove from the oven.

6.3.3. Allow to cool in the desiccator (4.2.) to room temperature (i.e. for at least 30 minutes) and weigh to the nearest 0,1 mg.

6.3.4. Heat the dish again, with its lid alongside, in the oven for one hour. Place the lid on the dish and remove from the oven. Allow to cool for approximately 30 minutes in the desiccator and weigh to the nearest 0,1 mg.

6.3.5. Repeat the operations described in 6.3.4. until the difference in mass between two consecutive weighings does not exceed 0,5 mg. Record the lowest mass.

7. EXPRESSION OF RESULTS

7.1. Calculation and formula

Calculate the total solids content as a parcentage by mass from:

$$W_T = \frac{m_2 - m_0}{m_1 - m_0} \times 100$$

where

W_T = the total solids content in g per 100 g,

m_0 = the mass, in grams, of the dish and lid (see 6.1.),

m_1 = the mass, in grams, of the dish, lid and test portion (see 6.2.),

m_2 = the mass, in grams, of the dish, lid and dried test portion (see 6.3.5.).

Round the value obtained to the nearest 0,01 % (percentage by mass).

7.2. Precision

7.2.1. *Repeatability (r):* 0,10 g of total solids per 100 g of product.

7.2.2. *Reproducibility (R):* 0,20 g of total solids per 100 g of product.

II. DETERMINATION OF FAT CONTENT

1. SCOPE AND FIELD OF APPLICATION

This procedure specifies the reference method for the determination of the fat content of raw milk and of whole milk, partly skimmed milk and skimmed milk.

2. DEFINITION

Fat content of milk: all the material as determined by the specified method. It is expressed as a percentage by mass.

3. PRINCIPLE

An ammoniacal ethanolic solution of a test portion is extracted with diethyl ether and light petroleum, the solvents removed by distillation or evaporation, and the mass of the extracted material soluble in light petroleum, is determined. (The procedure is usually known as the Röse-Gottlieb method).

4. REAGENTS

All reagents shall be of recognized analytical quality and shall leave no appreciable residue when taken through a blank test.

To test the quality of the reagents, carry out a determination as specified in 6.3. Use an empty flask, beaker or metal dish (5.8.) for weighing, prepared as specified in 6.4. as a tare (to allow correction for the effects on the weighing result, caused by changes in the atmospheric conditions). If the residue, corrected for the apparent change of the mass of the tare is larger than 2,5 mg, determine the residue or the solvents separately by evaporating 100 ml diethyl ether (4.4.) and 100 ml light petroleum (4.5.) respectively. Also use a tare for the weighing. When the residue is larger than 2,5 mg, cleanse the solvent by means of distillation or replace the solvent.

4.1. **Ammonia solution,** containing approximately 25 % (m/m) of NH_3. A more concentrated ammonia solution may also be used (see 6.5.1. and A.1.5.1.).

4.2. **Ethanol,** at least 94 % (v/v). Ethanol denatured by methanol may be used provided it is certain that the results of the determination are not affected.

4.3. **Congo red or Cresol red solution**

Dissolve 1 g of Congo red or Cresol 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 6.5.2.). Other aqueous colour solutions may be used provided that they do not affect the result of the determination.

4.4. **Diethylether,** free from peroxides not containing more than 2 mg/kg of antioxidants, and meeting the requirements of the blank test (6.3.).

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

4.6 **Mixed solvent,** prepared shortly before use by mixing equal volumes of diethyl ether (4.4.) and light petroleum (4.5.).

5. APPARATUS AND GLASSWARE

Warning: Since the determination involves the use of volatile flammable solvents, any electrical apparatus employed shall comply with legislation relating to the use of such solvents.

Usual laboratory equipment and, in particular:

5.1. **Analytical balance**

5.2. **Centrifuge,** in which the fat-extraction flasks or tubes (5.6.) can be spun at a rotational frequency of 500 to 600 rev min^{-1} to produce a gravitational field of 80 to 90 g at the outer end of the flasks or tubes.

Note: The use of a centrifuge is optional (6.5.5.).

5.3. **Distillation or evaporation apparatus,** to permit the solvents and ethanol to be distilled from the flasks or to be evaporated from beakers and dishes (sec 6.5.12. and 6.5.15.) at a temperature not exceeding 100 °C.

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

5.5. **Water bath,** capable of being maintained at a temperature of 35 – 40 °C.

5.6. **Mojonnier-type fat extraction flasks**

Note: It is also possible to use fat-extraction tubes with siphon or wash-bottle fittings, but the procedure is then different and is specified in the Appendix.

The flasks (or tubes) shall be provided with ground-glass or good quality bark corks or other stoppers unaffected by the reagents used. Rark corks shall be extracted with the diethyl ether (4.4.) kept in water at 60 °C or more for at least 15 minutes, and shall then be allowed to cool in water so that they are saturated when used.

5.7. **Rack,** to hold the fat-extraction flasks (or tubes) (see 5.6.).

5.8. **Wash bottle,** suitable for use with the mixed solvent (4.6.). A plastic wash bottle shall not be used.

5.9. **Fat-collecting vessels,** for example boiling flasks (flat-bottom), or Erlenmeyer flasks of capacity 125—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.

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

5.11. **Measuring cylinders,** of capacities 5 and 25 ml.

5.12. **Pipettes,** graduated, of capacity 10 ml.

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

6. PROCEDURE

Note: The alternative procedure using fat-extraction tubes with siphon or wash-bottle fittings (see the note to 5.6.) is described in the Appendix.

6.1. **Preparations of the test sample**

Adjust the temperature of the laboratory sample to approximately 35—40 °C for 15 minutes, by means of a water bath is necessary. Mix the sample thoroughly, but gently, by repeatedly inverting the sample bottle without causing frothing or churning, and cool quickly to approximately 20 °C.

6.2. **Test portion**

Mix the test sample (6.1.) by gently inverting the bottle three or four times and immediately weigh, to the nearest 1 mg, 10 to 11 g of the test sample, directly or by difference, into one of the extraction flasks (5.6.).

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

6.3. **Blank test**

Carry out the blank test simultaneously with the determination using the same procedure and same reagents, but replacing the test portion by 10 to 11 ml of water.

The change in apparent mass of the fat collecting vessel, corrected for apparent change in mass of the control vessel, should not be greater than 2,5 mg.

6.4. **Preparation of fat-collecting vessel**

Dry a vessel (5.9.) together with a few boiling aids (5.10.) to promote gentle boiling during the subsequent removal of solvent in the oven (5.4.) for one hour. Allow the vessel to cool (not in a desiccater but protected from dust) to the temperature of the weighing room (for glass vessels allow at least one hour, for metal dishes allow at least 30 minutes). Taking care to avoid temperature variations, use tongs to place the vessel on the balance and weigh to the nearest 0,1 mg.

6.5. **Determination**

6.5.1. Add 2 ml of the ammonia solution (4.1.) or an equivalent volume of a more concentrated ammonia solution and mix thoroughly with the test portion in the small bulb of the flask. After the addition of the ammonia, carry out the determination without delay.

6.5.2. Add 10 ml of the ethanol (4.2.) and mix gently but thoroughly by allowing the contents of the flask to flow backwards and forwards between the two bulbs; avoid bringing the liquid too near to the neck of the flask. If desired, add two drops of the Congo red or Cresol red solution (4.3.).

6.5.3. Add 25 ml of diethyl ether (4.4.), close the flask with a cork saturated with water or with a stopper wetted with water (see 5.6.), and shake the flask vigorously, but not excessively (in order to avoid the formation of persistant emulsions), for one minute with the flask in a horizontal position and the small bulb extending upwards. Periodically allow 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 (4.6.) using the wash bottle (5.8.) so that the rinsings run into the flask.

6.5.4. Add 25 ml of light petroleum (4.5.), close the flask with the rewetted cork or stopper (rewet by dipping in water), and shake the flask gently for 30 seconds as described in 6.5.3.

6.5.5. Centrifuge the closed flask for one to five minutes at a rotational frequency of 500 to 600 rev min^{-1} (5.2.). If a centrifuge is not available (see note to 5.2.) allow the closed flask to stand in the rack (5.7.) for at least 30 minutes until the supernatant layer is clear and distinctly separated from the aqueous layer. If necessary, cool the flask in running water.

6.5.6. Carefully remove the cork or stopper and rinse it and the inside of the neck of the flask with a little of the mixed solvent (4.6.) so that the rinsings run into the flask.

If the interface is below the bottom of the neck of the flask, raise it slightly above this level by gently adding water down the side of the flask to facilitate decantation of solvent.

6.5.7. Holding the extraction flask by the small bulb, carefully decant as much as possible of the supernatant layer into the prepared fat-collecting vessel (6.4.) containing a few boiling aids (5.10.) in the case of flasks (optional with metal dishes), avoiding decantation of any of the aqueous layer.

6.5.8. Rinse the outside of the neck of the extraction flask with a little of the mixed solvent (4.6.), 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 6.5.12.

6.5.9. Add 5 ml of the ethanol (4.2.) to the contents of the extraction flask, using the ethanol to rinse the inside of the neck of the flask and mix as decribed in 6.5.2.

6.5.10. Carry out a second extraction by repeating the operations described in 6.5.3. to 6.5.8. inclusive, but using only 15 ml of diethyl ether (4.4.) and 15 ml of light petroleum (4.5.); use the ether to rinse the inside of the neck of the extraction flask. If necessary, raise the interface to the middle of the stem of the flask to enable the final decantation of solvent to be as complete as possible.

6.5.11. Carry out a third extraction by further repeating the operations described in 6.5.3. to 6.5.8. inclusive, but using only 15 ml of diethyl ether (4.4.) and 15 ml of light petroleum (4.5.); use the ether to rinse the inside of the neck of the extraction flask. If necessary, raise the interface to the middle of the neck of the flask to enable the final decantation of solvent to be as complete as possible.

The third extraction can be omitted for skimmed milk.

6.5.12. Remove the solvents (including ethanol) as completely as possible from the flask by distillation, or from the beaker or dish by evaporation (5.3.), rinsing the inside of the neck of the flask with a little of the mixed solvent (4.6.) before commencing the distillation.

6.5.13. Heat the fat-collecting vessel (with the flask placed on its side to allow solvent vapour to escape) for one hour in the oven (5.4.). 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 (for glass vessels allow at least one hour, for metal dishes allow at least 30 minutes) and weigh to the nearest 0,1 mg.

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

6.5.14. Repeat the operations described in 6.5.13. 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 observed as the mass of the fat-collecting vessel and extracted matter.

6.5.15. Add 25 ml of 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 (6.5.14.) and its initial mass (6.4.).

6.5.16. 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.

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 one hour in the oven, allow to cool and weigh, as described in 6.5.13. and 6.5.14.

Take the mass of fat as the difference between the mass determined in 6.5.14. and this final mass.

7. EXPRESSION OF RESULTS

7.1. Calculation and formula

Calculate the fat content as a percentage by mass by:

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

where

F = the fat content,

m_0 = the mass, in grams, of the test portion (6.2.),

m_1 = the mass, in grams, of the fat-collecting vessel and extracted matter determined in 6.5.14.,

m_2 = the mass, in grams, of the prepared fat-collecting vessel or, in the case of undissolved material, of the fat-collecting vessel and insoluble residue determined in 6.5.16.,

m_3 = the mass, in grams, of the fat-collecting vessel used in the blank test (6.3.) and any extracted matter determined in 6.5.14.,

m_4 = the mass, in grams, of the prepared fat-collecting vessel (see 6.4.) used in the blank test (6.3.), or in the case of undissolved material, of the fat-collecting vessel and insoluble residue determined in 6.5.16.

Report the result to the nearest 0,01 %.

7.2. **Precision**

7.2.1. *Repeatability (r):*

— for whole milk and partly skimmed milk: 0,02 g of fat per 100 g of product,

— for skimmed milk: 0,01 g of fat per 100 g of product.

7.2.2. *Reproducibility (R):*

— for whole milk: 0,04 g of fat per 100 g of product,

— for partly skimmed milk: 0,03 g of fat per 100 g of product,

— for skimmed milk: 0,025 g of fat per 100 g of product.

III. DETERMINATION OF TOTAL NON-FAT SOLIDS

1. SCOPE AND FIELD OF APPLICATION

This procedure specifies the reference method for the determination of the content of total non-fat solids in heat-treated milk.

2. DEFINITION AND CALCULATION

The total non-fat solids content must be expressed as a percentage by mass.

The content of non-fat solids is:

The content of total solids (see Section I) minus the content of fat (see Section II).

IV. DETERMINATION OF TOTAL NITROGEN CONTENT

1. SCOPE AND FIELD OF APPLICATION

This procedure specifies the reference method for the determination of the total nitrogen content of raw milk and of whole milk, partly skimmed milk and skimmed milk.

2. DEFINITION

The total nitrogen content of milk: the nitrogen content, expressed in per cent by mass, as determined by the specified Kjeldahl method.

3. PRINCIPLE

A weighed quantity of the milk sample is digested with concentrated sulphuric acid and potassium sulphate and copper (II) sulphate as catalyst, in order to convert the nitrogen of the organic compounds into ammonium sulphate. The ammonia is released by the addition of sodium hydroxide solution and then distilled and absorbed in a boric acid solution. This is titrated with an acid solution.

4. REAGENTS

4.1. **Potassium sulphate** (K_2SO_4).

4.2. **Copper sulphate solution.** Dissolve 5,0 g of copper (II) sulphate pentahydrate ($CuSO_4$, $5H_2O$) in water and dilute to 100 ml (at 20 °C) in a volumetric flask.

4.3. **Sulphuric acid,** at least 98,0 % (m/m) H_2SO_4.

4.4. **Sodium hydroxide solution, 47 % (m/m) 704 g NaOH/l (20 °C).**

Note: A less concentrated sodium hydroxide solution may be used for example: 40 % (m/m) 572 g/l, 20 °C; or 30 % (m/m) 399 g/l, 20 °C.

4.5. **Boric acid solution.** Dissolve 40 g of boric acid (H_3BO_3) in one litre of hot water, allow to cool, and store in a borosilicate glass bottle.

4.6. **Indicator solution.** Dissolve 0,01 g methyl red, 0,02 g bromothymol blue and 0,06 g bromocresol green in 100 ml of ethanol. Store the solution in a brown closed bottle, in a cool, dark place.

4.7. **Volumetric solution**

c ($^1/_2$ H_2SO_4) or c (HCl) = 0,1 mol/l standardized to the nearest 0,0001 mol/l.

4.8. **Nitrogen-free sucrose.**

4.9. **Ammonium salt,** pure, such as ammonium oxalate $(NH_4)_2C_2O_4,H_2O$ or ammonium sulfate $(NH_4)_2SO_4$.

4.10. **Tryptophan** ($C_{11}H_{12}N_2O_2$), **phenacetin** ($C_{10}H_7CH_2CONH_2$) or lysine mono- or di-hydrochloride ($C_6H_{14}N_2O_2 \cdot HCl$ or $C_6H_{14}N_2O_2 \cdot 2HCl$).

Note: The purity of reagents in 4.9. and 4.10. should be of higher quality than 'analytical grade'. If available, certified ammonium salt solution (4.9.) should be used.

5. APPARATUS AND GLASSWARE

Usual laboratory equipment and, in particular:

5.1. **Kjeldahl flasks** of capacity 500 ml.

5.2. **Suitable boiling aids,** for example, glass beads of diameter approximately 5 mm, Hengar granules, pumice.

5.3 **Burette or automatic pipette,** to deliver 1,0 ml.

5.4. **Graduated measuring cylinders,** glass, of capacities 50, 100 and 250 ml.

5.5. **Digestion apparatus** in an inclined position (approximately 45°), with electric heaters or gas burners that do not heat the flasks above the level of their contents, with a fume extraction system.

5.6. **Distillation apparatus,** made of borosilicate glass, to which a Kjeldahl flask (5.1.) can be fitted, consisting of an efficient splash-head connected to an efficient condenser with straight inner tube and an outlet tube attached to its lower end; the connecting tubing and stopper (s) shall be close-fitting and preferably of neoprene rubber.

5.7. **Pipette or automatic pipette,** to deliver 0,10 ml.

5.8. **Conical flasks,** of capacity 500 ml, graduated at 200 ml.

5.9. **Burette** of capacity 50 ml, gratuated in 0,1 ml, maximum error ± 0,05 ml.

5.10. **Magnifying lens,** for reading the burette (5.9.).

5.11. **pH meter**

5.12. **Automatic burette.**

6. PROCEDURE

6.1. To the Kjeldahl flask (5.1.) add boiling aid (5.2.) (eg. three glass beads), 15 g of potassium sulphate (4.1.), 1,0 ml of copper sulphate solution (4.2.), approximately 5 g of milk sample (weighed to the nearest 0,001 g) and 25 ml of sulphuric acid (4.3.). Use the acid to wash down any copper sulphate solution, potassium sulphate or milk on the neck of the flask, and gently mix the contents of the flask.

Note: Because organic matter consumes sulphuric acid during boiling, use 30 ml of H_2SO_4 (4.3.), instead of 25 ml for digestion, if the milk contains more than 5,0% (m/m) of fat. This should also be done in the blank test.

6.2. Heat each Kjeldahl flask on the digestion apparatus (5.5.), very gently at first so that all the black froth stays within the bulb. When the initial frothing has ceased and copious white vapour appears, boil vigorously (acid vapour will condense half-way up the neck of the flask) until no black particles remain and until the digest becomes clear pale blue-green in colour. Then boil gently for at least 1,5 hours. Note the following requirements:

(a) The time for the digest to become clear should not be more than one hour, and the total digestion time shall not be less than 2,5 hours. If more than one hour is necessary to achieve clearing, the total digestion time shall be increased accordingly.

(b) The added potassium sulphate promotes the digestion as it raises the boiling temperature of the mixture. If the residual volume of H_2SO_4 is less than approximately 15 ml at the end of the digestion time, nitrogen may have been lost because of excessive heating. If heating by gas, heat the flask on a plate of heat-insulating material, provided with a circular opening of such a diameter that the free flame only touches the part of the flask that is below the surface of the liquid contents (5.5.).

(c) If black particles enter the neck of the flask and are not all washed down into the bulb by the acid refluxing during the inital stages of the vigorous boiling period (this may be facilitated by rotating the flask) allow the flask to cool sufficiently and carefully wash with the minimum of water. Then continue the digestion as described above.

6.3. When the Kjeldahl flasks are cool, add 300 ml of water (see note) to each so as to wash carefully down the neck of the flask, and mix the contents thoroughly ensuring that the crystals which have separated out are dissolved. Add some boiling aid (5.2.) to ensure uniform boiling. Then to each flask, add 70 ml of sodium hydroxide solution (4.4.) (see note) by gently pouring the solution down the inclined neck of the flask to form a bottom layer in the bulb; do not wet the top of the neck with the sodium hydroxide solution.

Note: It is necessary that the combined volume of water and sodium hydroxide solution total 370 ml to enable approximately 150 ml of distillate to be collected just before irregular boiling ('bumping') ensues (6.4.). Thus, if a larger equivalent volume of a sodium hydroxide solution which is less concentrated than 47 % (m/m) is added, the volume of water added shall be reduced accordingly. For example, if 85 ml of 40 % (m/m) or 125 ml of 30 % (m/m) sodium hydroxide solution are to be added, the volume of water added shall be 285 ml or 245 ml respectively.

6.4. Immediately connect each Kjeldahl flask to a distillation apparatus (5.6.). Ensure that the tip of the condenser outlet-tube is immersed in 50 ml of boric acid solution (4.5.) together with 0,20 ml (5 – 6 drops) of indicator solution (4.6.) all contained in a conical flask (5.8.). Swirl the contents of each Kjeldahl flask to mix thoroughly and boil, but gently at first to prevent excessive frothing. When 100 to 125 ml of distillate have been collected, lower each conical flask until the tip of the condenser outlet-tube is approximately 40 mm above the 200 ml mark. Continue each distillation until irregular boiling ('bumping') starts and then immediately stop the heating. Disconnect each Kjeldahl flask and rinse the tip of each condenser outlet-tube with a little water, collecting the rinsings in the conical flask. Note the following requirements:

(a) The distillation rate shall be such that approximately 150 ml of distillate are collected when irregular boiling ('bumping') starts, the volume of the contents of each conical flask will then be approximately 200 ml.

(b) The efficiency of each condenser should be such that the temperature of the contents of each conical flask does not exceed 25 °C during the distillation.

6.5 Titrate each distillate with standard volumetric solution (4.7.) until the pH is 4,6 ± 0,1, using a pH meter and if desired an automatic burette. Addition of an indicator helps to check whether the titration is proceeding correctly. Take each burette reading to the nearest 0.01 ml with the aid of a magnifying lens (5.10.) avoiding errors of parallax.

The titrating may be carried out with the indicator only. Titrate until the colour of the distillate corresponds to that of a solution recently prepared from 150 ml of water to which has been added 50 ml of the boric acid solution and 0,20 ml of the indicator contained in a conical flask (5.8).

6.6. Carry out a blank test according to 6.1. to 6.5. inclusive, taking 5 ml of distilled water together with about 0,1 g of sucrose (4.8.) through the procedure instead of the milk sample.

Note: The titration of the blank distillate will require only a very small volume of the standard volumetric solution (4.7.).

6.7. Regularly check the accuracy of the procedure by using two recovery trials following the procedure according to 6.1. to 6.5. inclusive.

6.7.1. Check that no loss of nitrogen occurs as a result of excessive heat or mechanical leaks during distillation, by using a test portion of 0,15 g of ammonium oxalate or sulphate (4.9.) weighed to the nearest 0,001 g together with 0,1 g of sucrose (4.8.).

The percentage of nitrogen recovered shall be between 99,0 and 100,0 %.

Lower or higher results will indicate failures in the procedure and/or inaccurate concentration of the standard solution (4.7.).

6.7.2. Check that the digestion procedure is sufficient to release all the protein nitrogen by using a test portion of 0,20 g of pure tryptophan, 0,35 g of phenacetin or 0,20 g of lysine hydrochloride (4.10.). All weighings should be to the nearest 0,001 g. At least 98—99 % of the nitrogen should be recovered.

7. SAFETY PRECAUTIONS

When working with concentrated sulphuric acid and sodium hydroxide and when handling Kjeldahl flasks, always wear a laboratory coat, safety goggles and acid resistant gloves.

During distillation, never leave Kjeldahl flasks unattended. Because of potential danger, stop distillation immediately if flask contents 'bump' too vigorously. If the power goes off for more than two to three minutes, lower the collecting flask so that the distillation tip is out of the liquid.

8. EXPRESSION OF RESULTS

8.1. **Calculation and formula:**

Calculate the nitrogen content (W_N), expressed in grams of nitrogen per 100 g of product by:

$$W_N = \frac{1{,}40\,(V - V_O)\,c}{m}$$

where:

W_N = the nitrogen content.
V = the volume in millilitres of the standard volumetric solution of acid used in the determination.
V_O = the volume in millilitres of the standard volumetric solution of acid used in the blank test.
c = the concentration, expressed as moles per litre of the acid standard volumetric solution (4.7.).
m = the mass in grams of the test portion.

Round off the result to the nearest 0,001 g per 100 g.

8.2. **Precision**

8.2.1. *Repeatability (r):* 0,007 g per 100 g.

8.2.2. *Reproducibility (R):* 0,015 g per 100 g.

9. MODIFIED PROCEDURES

9.1. Use a block digestion apparatus fitted with cylindrical flasks, instead of the digestion apparatus and the Kjeldahl flasks described in 5.5. and 5.1. In this case to identify potential trouble, each spot has to be checked individually (6.7.).

9.2. Use of steam distillation instead of direct heating of the flasks (6.4.). When the apparatus does not allow the use of distilled water, care should be taken that the water does not contain acid or alkaline volatiles.

9.3. A test portion of 1 g of milk (semi-macro Kjeldahl) can be used instead of 5 g (6.1.) provided:

— the amounts of the reagents used for mineralization (6.1.): H_2SO_4, $CuSO_4 \cdot 5\,H_2O$, K_2SO_4, are reduced to the same ratio (1/5).

— the total digestion time (6.2.) is reduced to 75 minutes.

— the amount of sodium hydroxide solution (6.3.) is reduced to the same ratio (1/5).

— an acid standard solution (4.7.) of lower concentration (0,02 to 0,03 mol/l) has to be used.

Note: Using one or more of these options is acceptable only if the repeatability value (8.2.1.) and the two accuracy tests results (6.7.) are in accordance with the requirements given in this method.

V. DETERMINATION OF PROTEIN CONTENT

1. SCOPE AND FIELD OF APPLICATION

This procedure specifies the reference method for the determination of the content of protein in heat treated milk (Article 3 A3 in Directive 85/397/EEC).

2. DEFINITION

Protein content: The value obtained by multiplying the total nitrogen content, expressed as a percentage by mass, determined in accordance with the method described in Section IV(3) by an appropriate factor (3.).

3. CALCULATION

Protein content of milk as percentage by mass = 6,38 x total N content of milk %.

VI. DETERMINATION OF SPECIFIC MASS

1. SCOPE AND FIELD OF APPLICATION

This procedure specifies the reference method for the determination of the specific mass at 20 °C of raw milk and of whole milk, partly skimmed milk and skimmed milk.

2. DEFINITION

The specific mass of the milk is the ratio of the mass of a certain volume of milk at 20 °C to that of the mass of the same volume of water at 20 °C.

3. PRINCIPLE

The specific mass at 20 °C is determined by a hydrometer.

4. APPARATUS AND GLASSWARE

Usual laboratory equipment and, in particular:

4.1. **Hydrometer**

The specific gravity hydrometer is an instrument consisting of a glass float, which, at its lower end, is wide and heavy. There is a cylindrically shaped glass rod attached to the upper and of the float and coaxially orientated to it; the upper end of the rod is closed.

The glass float contains the load (lead, mercury, etc.) intended to adjust the hydrometer mass. The rod includes a graduated scale from 1,025 to 1,035 g/ml.

The hydrometer should he checked by the pycnometric method, using a pycnometer of a capacity of approximately 100 ml equipped with a precision thermometer.

4.2. **Cylinders** (glass or stainless steel).

Minimum dimensions should be:

— internal diameter approximately 35 mm

— internal height approximately 225 mm.

4.3. **Water bath** regulated at 20 ± 0,1 °C.

4.4. **Water bath** regulated at 40 ± 2 °C.

4.5. **Thermometer,** graduated to 0,5 °C.

5. PROCEDURE

5.1. Mix the sample by inversion to disperse the fat and place in the water bath (4.4.). Allow the sample to reach a temperature of 40 °C and maintain at that temperature for five minutes. Mix thoroughly by careful inversion to ensure that the fat is homogeneously distributed. Cool to 20 °C in the second water bath (4.3).

5.2. Mix the sample thoroughly by careful inversion to avoid inclusion of air. Pour the milk into the cylinder (4.2.), held inclined so as to avoid the formation of foam or of bubbles. Use sufficient milk sample to ensure that some will overflow from the cylinder when the hydrometer (4.1.) is placed in it. Carefully lower the hydrometer into the milk and allow to float freely when it reaches its balance position. The cylinder is to be placed vertical. The hydrometer must be positioned in the middle of the liquid column and should not touch the sides of the cylinder.

5.3. When the hydrometer reaches equilibrium read the graduation at the top of the meniscus.

5.4. Immediately after taking the hydrometer reading introduce the thermometer (4.5.) into the sample and read the temperature with an accuracy of 0,5 °C. The temperature must not differ more than ± 2 °C from ± 20 °C.

6. TEMPERATURE CORRECTION

6.1. If the temperature of the milk sample is not exactly 20 °C when the measurement of its specific mass is made, then the result obtained must be corrected adding to the determined specific mass 0,0002 for each degree Celcius above 20 °C, or subtracting 0,0002 for every degree Celsius below 20 °C. This correction is only valid if the temperature of the milk sample differs by not more than 5 °C from 20 °C.

7. EXPRESSION OF RESULTS

The method of calculation and formula specific mass of the sample is expressed in g/ml of skimmed milk at 20 °C according to the following formula:

$$\frac{1\,000 \cdot mv - MG \cdot mv}{1\,000 - \frac{MG \cdot mv}{0{,}92}} = \frac{0{,}92\ mv\ (1\,000 - MG)}{920 - MG \cdot mv}$$

where

mv = the specific mass of the sample read on the hydrometer (5.4) in g/l
MG = the fat content of the sample in g/l
0,92 = the density of fat.

8. PRECISION

8.1. **Repeatability** (r): 0,0003 g/ml.

8.2. **Reproducibility** (R): 0,0015 g/ml.

Appendix

(to Annex II)

ALTERNATIVE PROCEDURE USING FAT-EXTRACTION TUBES WITH SIPHON OR WASH-BOTTLE FITTINGS

A.1. PROCEDURE

A.1.1. **Preparation of the test sample**

See 6.1.

A.1.2. **Test portion**

Proceed as specified in 6.2. but using the fat-extraction tubes (see 5.6.).

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

A.1.3. **Blank test**

See 6.3.

A.1.4. **Preparation of fat-collecting vessel**

See 6.4.

A.1.5. **Determination**

A.1.5.1. Add 2 ml of the ammonia solution (4.1.), or an equivalent volume of a more concentrated ammonia solution, and mix thoroughly with the pretreated test portion at the bottom of the tube. After the addition of the ammonia, carry out the determination without delay.

A.1.5.2. Add 10 ml of the ethanol (4.2.) and mix gently but thoroughly at the bottom of the tube. *If desired,* add two drops of the Congo red or Cresol red solution (4.3.).

A.1.5.3. Add 25 ml of diethyl ether (4.4.), close the tube with a cork saturated with water or with a stopper wetted with water (5.6.), and shake the tube vigorously, but not excessively (in order to avoid the formation of persistent emulsions), with repeated inversions for one minute. 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 (4.6.) using the wash bottle (5.8.) so that the rinsings run into the tube.

A.1.5.4. Add 25 ml of light petroleum (4.5.), close the tube with the rewetted cork or stopper (rewet by dipping in water), and shake the tube gently for 30 seconds as described in A.1.5.3.

A.1.5.5. Centrifuge the closed tube for one to five minutes at a rotational frequency of 500 to 600 rev min^{-1} (5.2.). If the centrifuge is not available (see note to 5.2.), allow the closed tube to stand in the rack (5.7.) for at least 30 minutes until the supernatant layer is clear and distincly separated from the aqueous layer. If necessary, cool the tube in running water.

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

A.1.5.7. Insert a siphon fitting or a wash-bottle fitting into the tube and push down the long inner limb of the fitting until the inlet is approximately 3 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 (6.4.) containing a few boiling aids (5.10.) in the case of flasks (optional with metal dishes), avoiding the transfer of any of the aqueous layer. Rinse the outlet of the fitting with a little of the mixed solvent, collecting the rinsings in the fat-collecting vessel.

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

Rinse the outlet of the fitting with a little of the mixed solvent, collecting the rinsings in the vessel. If desired, the solvent or part of the solvent may be removed from the vessel by distillation or evaporation as described in 6.5.12.

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

A.1.5.10. Carry out the *second extraction* by repeating the operations described in A.1.5.3. to A.1.5.8., but using only 15 ml of diethyl ether (4.4.) and 15 ml of light petroleum (4.5.). Use the ether to rinse the long inner limb of the fitting during the removal of the fitting from the tube after the previous extraction.

A.1.5.11. Carry out a *third extraction* by again repeating the operations described in A.1.5.3. to A.1.5.8. using 15 ml of the diethyl ether and 15 ml of the light petroleum and rinsing the long inner limb of the fitting as described in A.15.10.

The third extraction can be omitted for skimmed milk.

A.1.5.12. Proceed as described in 6.5.12. to 6.5.16.