INTERNATIONAL STANDARD ISO 3595

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Milk fat — Detection of vegetable fat by the phytosteryl acetate test

Matières grasses du lait — Détection des matières grasses végétales au moyen de l'essai à l'acétate de phytostérol

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FOREWORD

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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.

International Standard ISO 3595 was drawn up by Technical Committee ISO/TC 34, *Agricultural food products*, and was circulated to the Member Bodies in August 1974.

It has been approved by the Member Bodies of the following countries :

Australia	Hungary	South Africa, Rep. of
Austria	India	Spain
Belgium	Ireland	Sweden
Bulgaria	Israel	Turkey
Chile	Mexico	United Kingdom
Czechoslovakia	Netherlands	U.S.S.R.
France	New Zealand	
Germany	Poland	

No Member Body expressed disapproval of the document.

NOTE – This International Standard has been developed jointly with the IDF (International Dairy Federation) and the AOAC (Association of Official Analytical Chemists, U.S.A.) on the basis of an IDF Standard for the purpose of being included in the FAO/WHO Code of Principles concerning Milk and Milk Products and Associated Standards.

The text as approved by the above organizations has also been published by the IDF (IDF Standard No. 32A) and by the AOAC (Official Methods of Analysis).

Printed in Switzerland

Milk fat — Detection of vegetable fat by the phytosteryl acetate test

1 SCOPE AND FIELD OF APPLICATION

This International Standard specifies a method for the detection in milk fat of the presence of the more common vegetable fats, using the phytosteryl acetate test.

2 REFERENCES

ISO/R 707, *Milk and milk products — Sampling.*

ISO 3594, *Milk fat — Detection of vegetable fat by gas-liquid chromatography of sterols (Reference method).*

3 DEFINITION

sterols content of fat: The content of compounds precipitable as digitonides, expressed as a percentage by mass, as determined by the procedure described (see note under 8.2.9).

4 PRINCIPLE

4.1 Saponification of the fat and precipitation of the sterols as sterol digitonides by addition of an ethanolic digitonin solution.

4.2 Determination of the melting point of the steryl acetate after acetylation of the steryl digitonides with acetic anhydride.

4.3 Microscopical examination of the crystal form of the sterols after conversion of the steryl acetates into the sterols by saponification with a potassium hydroxide solution.

5 REAGENTS

All reagents shall be of analytical quality. Water shall be distilled water or water of at least equivalent purity.

5.1 Potassium hydroxide solution.

Dissolve 400 g of potassium hydroxide in 600 ml of water.

5.2 Digitonin, 10 g/l ethanolic solution.

Dissolve 10 g of digitonin in 1 l of ethanol (5.3).

5.3 Ethanol, 95 to 96 % (*V*/*V*).

5.4 Ethanol, 80 % (*V*/*V*).

5.5 Diethyl ether.

5.6 Acetic anhydride.

5.7 Pentane or **light petroleum,** boiling range 40 to 60 °C.

5.8 Copper(II) sulphate, 70 g/l solution.

Dissolve 70 g of copper(II) sulphate pentahydrate ($CuSO_4.5H_2O$) in 1 l of water.

5.9 Sodium sulphate, anhydrous.

6 APPARATUS

Usual laboratory equipment and

6.1 Analytical balance.

6.2 Conical flasks, capacity 500 ml, with ground joints and fitted with air condensers.

6.3 Glass micro-filtering device, as shown in figure 1. (See also P.C. den Herder, *Neth. Milk and Dairy J.*, **9** (1955), p. 261.)

Dimensions in millimetres

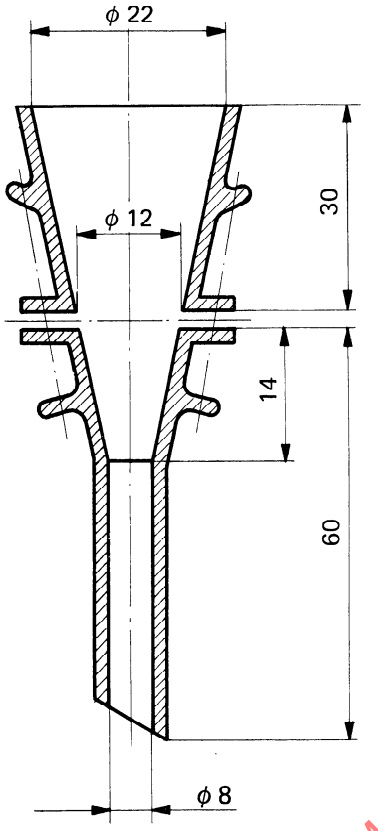

FIGURE 1 — **Micro-filtering device**

6.4 Melting point apparatus.

6.5 Melting point tubes, internal diameter 0,8 to 1,0 mm, length 50 mm.

6.6 Test tubes, of heat-resistant glass, diameter 12 mm, length 35 mm.

6.7 Microscope slides and **cover slips.**

6.8 Ordinary or polarizing microscope, linear magnification 200 X.

6.9 Drying oven, capable of being controlled at 102 ± 2 °C.

6.10 Glycerol bath, capable of being controlled at 130 to 145 °C.

7 SAMPLING

See ISO/R 707.

8 PROCEDURE

8.1 Preparation of test sample

8.1.1 *Butter*

Melt about 50 g of the laboratory sample at a temperature below 50 °C until the fat and water layers separate. Remove the fat layer by decantation and clarify the fat at a temperature of about 40 °C by filtering it through a dry filter paper, taking care that no water passes on to the filter.

8.1.2 *Milk and cream*

Centrifuge the laboratory sample to obtain a cream having a fat content of about 40 %. Churn the cream in a laboratory churn. Collect the butter lumps and proceed as described in 8.1.1.

8.1.3 *Cheese*

Grind the laboratory sample in a mortar with anhydrous sodium sulphate (5.9) until a granular mass is produced.

Extract the mass with pentane or light petroleum (5.7) (a continuous extraction apparatus may be used) and evaporate the solvent in a boiling water bath.

8.1.4 *Condensed milk, evaporated milk and ice-cream*

Add to the laboratory sample twice its volume of boiling water and heat the mixture on a boiling water bath to a temperature of 75 °C. Add an amount of copper(II) sulphate solution (5.8) equal to one-tenth of the volume of the mixture and continue heating until the precipitate coagulates.

Filter the precipitate through a filter paper and wash it with warm water until the filtrate is colourless. Carefully drain the precipitate, grind it in a mortar with anhydrous sodium sulphate (5.9) and proceed as described in 8.1.3.

8.1.5 *Dried milk*

Grind the laboratory sample in a mortar with some water so as to obtain a clotted mass. Allow it to stand for about 15 min. Then add anhydrous sodium sulphate (5.9), grind again until a granular mass is produced and proceed as described in 8.1.3.

8.2 Preparation of the sterol digitonides

8.2.1 Weigh, to the nearest 0,1 g, about 15 g of the test sample (8.1) and transfer this test portion into a conical flask (6.2).

8.2.2 Add to the test portion 10 ml of potassium hydroxide solution (5.1) and 20 ml of ethanol (5.3).

8.2.3 Place the air condenser on the flask, heat the flask on a boiling water bath, swirling until the solution has become clear, and continue boiling for 30 min.

8.2.4 Add 60 ml of water and then 180 ml of ethanol (5.3) and raise the temperature to about 40 °C.

8.2.5 Add 30 ml of the ethanolic digitonin solution (5.2), swirl and allow to cool. Place the flask in a refrigerator at about 5 °C for about 12 h (conveniently overnight).

8.2.6 Collect the precipitate of sterol digitonide by filtration through a medium speed filter paper in a Büchner funnel (diameter 80 mm).

8.2.7 Wash the precipitate with water at about 5 °C until the filtrate stops foaming, then once with 25 to 50 ml of ethanol (5.3) and once with 25 to 50 ml of diethyl ether (5.5).

8.2.8 Dry the filter paper and precipitate on a watch-glass in the drying oven (6.9), controlled at 102 ± 2 °C, for 10 to 15 min.

8.2.9 Fold the filter paper in two so that the precipitate comes off as a film and transfer the precipitate into a weighing bottle.

NOTE – If it is desired to know the content of sterols, weigh the precipitate in the weighing bottle to the nearest 0,001 g and calculate this content, as a percentage by mass, using the formula :

$$\frac{0{,}25\,m_1}{m_0} \times 100 = \frac{25\,m_1}{m_0}$$

where

m_0 is the mass, in grams, of the test portion (8.2.1);

m_1 is the mass, in grams, of sterol digitonide precipitate.

Express the result rounded off to the second decimal place.

8.3 Preparation of the steryl acetates and determination of the melting point

8.3.1 Transfer 0,1 ± 0,005 g of the sterol digitonide (8.2.9) to a test tube (6.6), add 1 ml of acetic anhydride (5.6) and heat the tube in the glycerol bath (6.10) between 130 and 145 °C until the precipitate has dissolved. Do not use direct heat, since spattering may occur. Continue heating for 2 min and allow to cool to about 80 °C.

8.3.2 Add 4 ml of ethanol (5.3), mix and heat slightly to dissolve any steryl acetate which may tend to crystallize out.

8.3.3 Filter the solution while still warm through a small medium speed filter paper impregnated with ethanol, and collect the filtrate in another test tube.

8.3.4 Heat the filtrate in the test tube carefully until it boils gently.

8.3.5 Keep the solution boiling and, while shaking the test tube vigorously, add carefully 1 to 1,5 ml of water drop by drop from a pipette until the steryl acetate is just about to precipitate yet remains in solution. Avoid superheating.

8.3.6 Add a few drops of ethanol (5.3) to redissolve any precipitated steryl acetates.

8.3.7 Allow to cool in air for 2 h and finally in ice water for 30 min.

8.3.8 Filter the crystallized steryl acetates on a small disk of hardened fast filter paper by using suction and the micro-filtering device (6.3), and rinse the crystals with 1 ml of ethanol (5.4).

8.3.9 Redissolve the crystal cake by heating it over a micro-burner in a test tube (6.6) with 1 ml of ethanol (5.3).

8.3.10 Allow to cool first in air for 15 min and then in ice water for 5 min. Filter the crystallized steryl acetates as described in 8.3.8.

8.3.11 Repeat the operations described in 8.3.9 and 8.3.10. If necessary (see 9.3), repeat these operations twice more.

8.3.12 Dry the crystal cake on the paper first at about 30 °C and then in the drying oven (6.9), controlled at 102 ± 2 °C, for 10 to 15 min.

8.3.13 Disintegrate the crystal cake, mix the crystals on a watch-glass and fill a melting point tube (6.5) to a height of about 3 mm. Determine the melting point in the melting point apparatus (6.4), raising the temperature in the last phase of the melting process at a rate of 0,5 °C/min. Record as the melting point the reading on the thermometer, to 0,1 °C, at the moment when the last crystal has just disappeared.

8.4 Microscopical examination of the sterols

NOTE – This examination will only be necessary when the melting point of the steryl acetate is found to be 115,5 °C or higher, but lower than 117,0 °C (see 9.3.2).

8.4.1 Dissolve about 0,01 g of the steryl acetates (8.3.13) in 1 ml of ethanol (5.3) in a small test tube and add 1 or 2 drops of potassium hydroxide solution (5.1).

8.4.2 Heat on a boiling water bath until boiling begins and the steryl acetates dissolve.

8.4.3 Add 10 ml of water, transfer the solution to a 125 ml separating funnel and shake with 25 ml of diethyl ether (5.5).

8.4.4 After separation, drain and discard the aqueous layer.

8.4.5 Wash the ether layer with three 5 ml portions of water.

8.4.6 Transfer the ether layer to a 50 ml beaker and evaporate to dryness.

8.4.7 Dissolve the residue in 10 ml of ethanol (5.4). Place a drop of the clear solution on a microscope cover slip and let it spread over the slip. Wait until crystallization starts at the edges of the cover slip, then invert the slip and lay it on a microscope slide.

8.4.8 During further crystallization, examine the crystals under the microscope (6.8) at about 200 × linear magnification.

9 INTERPRETATION OF RESULTS

9.1 If the melting point of the steryl acetate is found to be between 114,0 and 115,5 °C, the laboratory sample shall not be considered to contain vegetable fat.

9.2 If the melting point of the steryl acetate is found to be 117,0 °C or higher, the laboratory sample shall be considered to contain vegetable fat.

9.3 If, however, the melting point of the steryl acetate is found to be 115,5 °C or higher, but lower than 117,0 °C, repeat the redissolving, recrystallization and filtration twice more (see 8.3.11), dry the crystal cake and determine the melting point as described in 8.3.12 and 8.3.13.

9.3.1 If the melting point of the steryl acetate is then found to be 117,0 °C or higher, the laboratory sample shall be considered to contain vegetable fat.

9.3.2 If, however, the melting point of the steryl acetate is found to have remained 115,5 °C or higher, but lower than 117,0 °C, then subject the sterol crystals to microscopical examination as described in 8.4.

9.3.2.1 If, on microscopical examination, the sterol crystals are found to have only the form of a parallelogram with an obtuse angle of 100°, which is characteristic of cholesterol (see figure 2), the laboratory sample shall not be considered to contain vegetable fat.

9.3.2.2 If, on microscopical examination, some of the sterol crystals are found to have an elongated hexagonal form with an apical angle of 108°, which is characteristic of phytosterols, or if some of the crystals have a re-entrant angle (swallow-tail), which is characteristic of mixtures of cholesterol and phytosterols (see figure 2), the laboratory sample shall be considered to contain vegetable fat.

10 SENSITIVITY OF THE TEST

The sensitivity depends upon the nature of the vegetable fat which may have been added, i.e. upon the content and composition of the phytosterol mixture present in the vegetable fat.

11 TEST REPORT

The test report shall give the melting point of the steryl acetates and the number of recrystallizations, a description of the microscopical appearance of the sterol crystals, if relevant, and the method used. It shall also mention any operating conditions not specified in this International Standard, or regarded as optional (see note to 8.2.9), as well as any circumstances that may have influenced the result.

The report shall include all details required for the complete identification of the sample.