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Anhydrous milk fat — Determination of sterol composition by gas liquid chromatography (Reference method)

Matières grasses laitières anhydres — Détermination de la composition stérolique par chromatographie en phase gazeuse (Méthode de référence)

Reference numbers
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Contents

Page

Foreword

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International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 12078 | IDF 159 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF). It is being published jointly by ISO and IDF.

Foreword

IDF (the International Dairy Federation) is a worldwide federation of the dairy sector with a National Committee in every member country. Every National Committee has the right to be represented on the IDF Standing Committees carrying out the technical work. IDF collaborates with ISO in the development of standard methods of analysis and sampling for milk and milk products.

Draft International Standards adopted by the Action Teams and Standing Committees are circulated to the National Committees for voting. Publication as an International Standard requires approval by at least 50 % of the IDF National Committees casting a vote.

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ISO 12078 | IDF 159 was prepared by the International Dairy Federation (IDF) and Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*. It is being published jointly by IDF and ISO.

All work was carried out by the Joint ISO-IDF Action Team on *Fat*, of the Standing Committee on *Main components in milk*, under the aegis of its project leaders, Mrs M. Juarez (ES) and Mrs G. Contarini (IT).

Anhydrous milk fat — Determination of sterol composition by gas liquid chromatography (Reference method)

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

1 Scope

This International Standard specifies a gas liquid chromatographic reference method for the determination of the sterol composition of anhydrous milk fat extracted from dairy products.

In the case of analysis of milk fat in a mixture of vegetable fats, the specified procedure allows the evaluation of the most important phytosterols. The procedure has been validated on milk fat samples containing approximately 28 % to 32 % of vegetable fat.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 3696, *Water for analytical use — Specification and test methods.*

ISO 6799, *Animal and vegetable fats and oils — Determination of composition of the sterol fraction — Method using gas-liquid chromatography.*

ISO 14156 | IDF 172, *Milk and milk products — Extraction methods for lipids and liposoluble compounds*.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
sterol composition
mass fraction of substances determined by the procedure specified in this International Standard

NOTE The sterol composition can be expressed either as milligrams per 100 g of fat, or as percent of total sterol content.

4 Principle

A suitable standard solution (betulin) is added to the test portion. The fat is saponified with methanolic potassium hydroxide. The unsaponifiable matter is extracted by diethyl ether. The sterols in the unsaponifiable matter are separated by thin-layer chromatography. The sterols are converted into silyl derivatives. Gas-liquid chromatography is carried out using a capillary column. Individual sterols are identified by comparison with the retention time of a reference standard sample. Sterols are quantified by reference to an internal standard method.

By using this International Standard, the adoption of thin-layer chromatography (TLC), as the purification procedure, guarantees the removal of the possible impurities included in the unsaponifiable matter.

5 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified.

5.1 Water, complying with grade 2 in accordance with ISO 3696.

5.2 Methanol (CH_3OH), containing a mass fraction of water of $\leqslant$ 0,5 %.

5.3 Saponification reagent, potassium hydroxide methanolic solution, c(KOH) = 2 mol/l.

Dissolve 11,2 g of KOH in 100 ml of methanol (5.2) and mix well.

5.4 Sodium sulfate (Na_2SO_4), anhydrous.

5.5 *n*-Hexane [$CH_3(CH_2)_4CH_3$].

5.6 Di-isopropyl ether, [$(CH_3)_2CH]_2O$, free from peroxides and residues.

5.7 Betulin, of purity 99 %.

5.8 Betulin standard solution

Accurately weigh about 60 mg of betulin (5.7) into a 100 ml one-mark volumetric flask (6.3). Add about 60 ml of di-isopropyl ether (5.6) and mix carefully until completely dissolved. To help the betulin dissolution, gently warm in a 50 °C water bath. Wait for the temperature of the solution to decrease to room temperature. Dilute to the 100 ml mark with di-isopropyl ether.

The betulin standard solution may be stored in a refrigerator for one month.

5.9 Cholesterol, of purity 99 %.

5.10 Cholesterol standard solution

Accurately weigh about 60 mg of cholesterol (5.9) into a 100 ml one-mark volumetric flask (6.3). Dilute to the 100 ml mark with *n*-hexane (5.5) and mix.

The cholesterol standard solution may be stored in a refrigerator for one month.

5.11 Campesterol, of purity 65 %.

5.12 Campesterol standard solution

Accurately weigh about 10 mg of campesterol (5.11) into a 100 ml one-mark volumetric flask (6.3). Dilute to the 100 ml mark with *n*-hexane (5.5) and mix.

The campesterol standard solution may be stored in a refrigerator for one month.

5.13 Stigmasterol, of purity 95 %.

5.14 Stigmasterol standard solution

Accurately weigh about 10 mg of stigmasterol (5.13) into a 100 ml one-mark volumetric flask (6.3). Dilute to the 100 ml mark with *n*-hexane (5.5) and mix.

The stigmasterol standard solution may be stored in a refrigerator for one month.

5.15 β-Sitosterol, of purity 95 %.

5.16 β- Sitosterol standard solution

Accurately weigh about 10 mg of β-sitosterol (5.15) into a 100 ml one-mark volumetric flask (6.3). Dilute to the 100 ml mark with *n*-hexane (5.5) and mix.

The β-sitosterol standard solution may be stored in a refrigerator for one month.

Since the phytosterol standard solutions (5.12, 5.14 and 5.16) are used only for a qualitative evaluation, they may be replaced by sterols prepared from soya oil having campesterol, stigmasterol and β-sitosterol as the major components.

5.17 Diethyl ether ($C_2H_5OC_2H_5$), free from peroxides and residues.

5.18 Chloroform ($CHCl_3$).

5.19 Ethanol (C_2H_5OH), absolute.

5.20 Ammonia solution (NH_3), containing a mass fraction of NH_3 of approximately 25 % [($\rho_{20}(NH_3)$ = 910 g/l)].

5.21 Pyridine ($C_5H_5N{\cdot}3H_2O$), dry.

5.22 Silylating agents, such as trimethychlorosilane, hexamethyldisilazane or equivalent.

5.23 2′,7′-Dichlorofluorescein solution, with a concentration of 5 g/l absolute ethanol or, alternatively, **rodamine G solution**, with a concentration of 1,5 g/l absolute ethanol.

6 Apparatus

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

Usual laboratory apparatus and, in particular, the following.

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

6.2 Round-bottomed flasks, with ground neck, of capacity 200 ml to 250 ml.

6.3 One-mark volumetric flasks, of capacity 100 ml.

6.4 Reflux condenser, to fit the round-bottomed flasks (6.2).

6.5 Water bath, capable of being maintainied at 50 °C ± 2 °C and of boiling.

6.6 One-mark pipettes, of capacity 10 ml.

6.7 One-mark pipettes, of capacity 1 ml.

6.8 Separating funnels, of capacity 500 ml.

6.9 Glass funnels, of diameter 100 mm.

6.10 Dry filter paper, folded, fast grade, of diameter 250 mm.

6.11 Rotary vacuum evaporator, with water bath capable of maintaining all temperatures up to boiling.

6.12 Graduated cylinders, of capacities 100 ml and 250 ml.

6.13 Nitrogen supply, with gas purity of at least 99 %.

6.14 Micropipette or **microsyringe**, capable of delivering drops of about 0,3 µl to 0,4 µl.

Alternatively, an automatic drop applicator may be used.

6.15 Drying oven, capable of maintaining a temperature of 103 °C ± 2 °C.

6.16 Precoated silica plates[1)], for thin-layer chromatography, of size 200 mm × 200 mm.

6.17 Glass plate-developing tank, with a ground-glass lid, suitably sized for the precoated silica plates (6.16).

6.18 Spraying device

6.19 Ultraviolet (UV) lamp, capable of working at wavelength 254 nm.

6.20 Micro-spatula

6.21 Round-bottomed flasks, with ground neck, of capacity 25 ml.

6.22 Water vacuum pump

6.23 Cylindrical filter funnels, with porous glass filter of diameter 40 µm, to fit the 25 ml round-bottomed flask (6.21), with vacuum adapter.

6.24 Desiccator

6.25 Graduated cylinder, with ground neck, of capacity 100 ml.

6.26 Graduated pipette, of capacity 1 ml.

6.27 Vials, cone-shaped inside, of capacities 5 ml and 10 ml.

6.28 Gas-liquid chromatograph, equipped with flame ionization detector and capillary injection system (splitter, or on column).

6.28.1 Carrier gas, hydrogen or helium, of purity at least 99,999 %.

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1) Machery Nagel Sirt 25 or Merck 5715 silica 60 are examples of suitable products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO or IDF of these products.

6.28.2 Other gases, free from organic impurities (C_nH_m of below 1 part per million), nitrogen and hydrogen, of purity at least 99,995 %, and synthetic air.

6.28.3 Capillary column, with a stationary phase which has been successfully employed to perform separation of sterols that can have different polarities depending on their chemical composition (methylpolysiloxane, methylphenylpoly-siloxane, polyglycol). Select the stationary phase, the column length (between 10 m and 30 m), the internal diameter (between 0,22 mm and 0,32 mm) and the film thickness (0,12 µm or more) taking into account the laboratory experience and the injection system applied. In any case, the selected column shall produce both a complete separation between the solvent peak and cholesterol, and a baseline resolution between the cholesterol, campesterol, stigmasterol and β-sitosterol peaks. Moreover, no baseline bleeding shall appear during the whole gas chromatographic (GC) run. Examples of a correct GC profile obtained by using the two different operative conditions are shown in Figures A.1 and A.2.

6.28.4 Flame ionization detector, capable of being heated to a temperature of 30 °C above the final temperature of the column oven.

6.28.5 Injector, in the case of using a splitter, capable of being heated to a temperature of 30 °C above the final temperature of the column oven. If using a cold on-column injector, it shall be capable of being maintained at a temperature several degrees below the boiling point of the solvent.

NOTE The oven temperature (isothermal or programmed) and the carrier gas flow depend on the column selected and on the injection system adopted.

6.28.6 Injection syringe, of capacity 1 µl to 10 µl.

6.28.7 Integration system, preferably computerized.

6.29 Gas chromatographic conditions

Follow the manufacturer's instructions for the instrument set-up. The oven temperature and the carrier gas flow depend on the column selected and on the injection system adopted. The examples listed below report applicable conditions for split and on-column injection systems.

6.29.1 Split injector

An example of applicable conditions using a split injector is:

a) Capillary column: fused silica with 5 % phenyl and 95 % methylpolysiloxane, of length 15 m, of internal diameter 0,22 mm, of film thickness 0,24 µm;

b) Injection system: splitter with ratio 1:30, set at 300 °C;

c) Carrier gas: helium, flow set at 0,6 ml/min;

d) Detector: flame ionization detector (FID) set at 300 °C;

e) Oven temperature: isothermal, set at 260 °C;

f) Sample: the sterols extracted by the described method were diluted in 1 ml of *n*-hexane;

g) Retention time: in minutes.

6.29.2 On-column injector

An example of applicable conditions using an on-column injector is:

a) Capillary column: fused silica with 5 % phenyl and 95 % methypolysiloxane, of length 25 m, of internal diameter 0,32 mm, of film thickness 0,12 µm;

b) Injection system: on column;

c) Carrier gas: hydrogen, flow set at 2,4 ml/min;

d) Detector: FID set at 330 °C;

e) Oven temperature: start initial temperature at 60 °C for 1 min; set first gradient at 40 °C per min; stop temperature increase at 250 °C for 1 min; set second gradient at 6 °C per min; stop temperature increase at 320 °C for 10 min;

f) Sample: the sterols extracted by the described method were diluted in 5 ml of *n*-hexane;

g) Retention time: in minutes.

7 Sampling

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 707 | IDF 50.

8 Preparation of test sample

Prepare the test sample in accordance with ISO 14156 | IDF 172. Melt the sample in the drying oven (6.15) set at 50 °C.

9 Procedure

9.1 Sterol standard solutions

9.1.1 Calibrating solution for the determination of cholesterol response factor

Transfer, with a one-mark pipette (6.7), 1 ml of cholesterol standard solution (5.10) to a 5 ml vial (6.27). Add, with another one-mark pipette (6.7), 1 ml of betulin (internal) standard solution (5.8) and mix. Remove the solvents under a gentle stream of nitrogen while warming on the water bath (6.5) set at 50 °C.

9.1.2 Qualitative solution for the determination of sterol retention time

Transfer, with separate one-mark pipettes (6.7), 1 ml of each of the sterol standard solutions prepared in 5.10, 5.12, 5.14 and 5.16, respectively, into a 10 ml vial (6.27). Add, with another one-mark pipette (6.7), 1 ml of betulin (internal) standard solution (5.8) and mix. Remove the solvents under a gentle stream of nitrogen while warming on the water bath (6.5) set at 50 °C.

When using soya oil instead of pure standards, use the same procedure as described for the test portion in 9.2.

9.2 Test portion

Shake the melted test sample (Clause 8) for 1 min to obtain a homogeneous test sample. Weigh, to the nearest 1 mg, about 2 g of the sample into a round-bottomed flask (6.2).

9.3 Saponification

Add, with a one-mark pipette (6.6), 10 ml of betulin (internal) standard solution (5.8) and 100 ml of saponification reagent (5.3) to the test portion (9.2). Fit the round-bottomed flask to the reflux condenser (6.4). Boil gently on the water bath (6.5) set at 80 ºC for approximately 1 h.

NOTE When bumping is expected, anti-bumping granules can be added.

9.4 Extraction of the unsaponifiable matter

Cool the round-bottomed flask to 35 °C. Transfer the test solution quantitatively to the separating funnel (6.8). Add 100 ml of diethyl ether (5.17) and 200 ml of distilled water. Use a portion of each of the formerly mentioned solvents to rinse the flask in order to avoid sample losses. Shake vigorously while degassing frequently. Allow layers to separate and to clarify completely.

Drain the lower aqueous layer into a second separating funnel (6.8). Extract the obtained soap solution with 100 ml of diethyl ether (5.17) in the same manner as described above. Carry out a third extraction with 50 ml of diethyl ether.

Combine the ether extracts in another separating funnel (6.8). Add 100 ml of water and shake gently (violent shaking at this stage may cause formation of an emulsion). After the separation of layers, drain the water layer. Wash the ether solution twice with 50 ml of water each time. If an emulsion forms during washing, add several drops of ethanol (5.19).

Filter the ether solution through the fluted dry filter paper (6.10), filled with at least 30 g of sodium sulfate (5.4), into a round-bottomed flask (6.2). Rinse the separating funnel with a small volume of diethyl ether (5.17). Evaporate the solvent by using the rotary vacuum evaporator (6.11) while gently warming the flask by setting the water bath at 50 °C.

9.5 Thin-layer chromatography (TLC)

9.5.1 Preparation of TLC plates

Activate precoated silica plates (6.16) before use in the drying oven (6.15) set at 103 °C for 1 h. Store the plates in the desiccator (6.24) before use.

9.5.2 Preparation of glass plate-developing tank

Prepare, in a 100 ml graduated cylinder (6.25), the developing mixture of diethyl ether/chloroform with ratio 10:90 (volume fraction). Add 0,5 ml of ammonia solution (5.20) with a graduated pipette (6.26). Stopper and shake gently.

Pour the mixture into the glass plate-developing tank (6.17). Fit the lid and allow liquid/vapour equilibrium to be attained.

9.5.3 TLC of unsaponifiable matter

Dissolve the unsaponifiable matter in 1 ml of chloroform (5.18). Use a micropipette or microsyringe (6.14) to load 400 µl onto the silica plate in a continuous line. Leave unused a width of 20 mm on the left-hand side of the plate, and a width of 40 mm on the right-hand side of the plate. Add 40 µl of the betulin standard solution (5.8) and 40 µl of the cholesterol standard solution (5.10) at the same level as the sample loading line to the middle of the right-hand 40 mm band of the plate.

Transfer the plate to the glass plate-developing tank (6.17). Fit the lid and wait until the solvent is 10 mm below the top edge. Remove the plate from the tank and allow the solvent to evaporate at room temperature.

Spray the plate with 2',7'-dichlorofluorescein solution (5.23). Allow the solvent to evaporate and examine the plate under the ultraviolet (UV) lamp (6.19).

Identify each sterol band by comparison with the reference spot of the cholesterol on the right-hand side of the plate. Remove the sterol bands using a micro-spatula (6.20) or another suitable tool. Transfer the powder to the cylindrical filter funnel (6.23).

Fit the funnel to a round-bottomed flask (6.21) and add 5 ml of chloroform (5.18). Connect the water vacuum pump (6.22) and filter the solvent entirely through the sterol-containing material into the flask.

Disconnect the vacuum pump and repeat the filtration with 5 ml of diethyl ether (5.17). Carry out this filtration preparation once more, with 5 ml of chloroform (5.18) and 5 ml of diethyl ether, respectively.

Remove the solvent by using the rotary vacuum evaporator (6.11), while gently warming the flask by setting the water bath at 50 °C.

9.6 Preparation of silyl derivatives

Convert the sterols obtained in 9.5.3 into trimethyl-silyl derivatives.

NOTE Many reagents, such as those specified in 5.22, are available to convert the sterols into trimethyl-silyl derivatives (TMS). The volume and proportions of these reagents to be used depend on their degree of reaction, and consequently it is impossible to lay down any standard conditions. In any case, the following procedure can represent an example: add to the sterols obtained in 9.5.3, 200 µl of a solution of bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane (99:1) and 200 µl of pyridine (5.21) and mix. Warm the obtained solution in a water bath set at 70 °C for 30 min.

Remove solvents under a gentle stream of nitrogen while warming on the water bath (6.5) set at 50 °C. Dilute with *n*-hexane (5.5) to a concentration suitable for the adopted gas-liquid chromatographic injection system (e.g. 0,5 ml and 5 ml for split and on-column injector, respectively).

The solution obtained in this way is ready for injection into the gas chromatograph.

NOTE An incomplete silylation is indicated by the presence of a second peak next to betulin.

9.7 Qualitative analysis

Prepare the trimethyl-silyl derivatives of the sterol solution (9.1.2) as described in 9.6. Inject the solution of derivatives into the gas chromatograph. Record the retention times of the reference sterols. Analyse the test portion (9.6) under the same conditions as used in the sterol solution.

NOTE 1 The order of elution of the major sterols is the following: cholesterol, campesterol, stigmasterol and β-sitosterol. The internal standard betulin elutes after the β-sitosterol. The chromatogram given in Figure A.1 is an aid for the identification of these sterols.

NOTE 2 If soya oil is used to identify the phytosterols, analysing the solution in 9.1.1 can permit the determination of the retention time of cholesterol.

Identify the peaks of the test sample by comparison of the retention data obtained with the sterol standard solution.

9.8 Quantitative analysis

9.8.1 Calculation of response factor

Prepare the trimethyl-silyl derivatives of the calibrating solution (9.1.1) as described in 9.6. Inject the solution of derivates into the gas chromatograph. Determine the area of peaks attributable to betulin and cholesterol.

Calculate the response factor, F_r, expressed to two decimal places, by using the following equation:

$$F_r = \frac{(w_c \times P_c) \times A_b}{(w_b \times P_b) \times A_c}$$

where

w_c is the mass fraction of cholesterol in the calibrating solution (9.1.1);

w_b is the mass fraction of betulin in the calibrating solution (9.1.1);

A_b is the numerical value of the peak area of betulin;

A_c is the numerical value of the peak area of cholesterol;

P_c is the numerical value of the purity of the cholesterol (5.9), (e.g. P_c = 0,99);

P_b is the numerical value of the purity of the betulin (5.7), (e.g. P_b = 0,99).

NOTE The response factor calculated for cholesterol will also be applied for the other sterols (campesterol, stigmasterol and β-sitosterol).

9.8.2 Determination of the test portion

Analyse the test portion under the same conditions as used in the calibrating solution. Determine the area of peaks attributable to betulin, cholesterol and other sterols, if detectable.

Repeat the injection of the calibrating solution and the calculation of F_r as described in 9.8.1.

9.8.3 Calculation and expression of results

9.8.3.1 Calculation of sterols as mass fraction

Calculate the average response factor for cholesterol, $F_{r,a}$, the standard deviation and the coefficient of variation between the values. A successful determination gives a response factor close to 1 and a coefficient of variation less than 2.

Calculate the mass fraction of each sterol, w_i, by using the following equation:

$$w_i = \frac{(w_b \times P_b) \times A_i \times F_{r,a}}{A_b \times m_s} \times 100$$

where

w_i is the mass fraction of each sterol (cholesterol, campesterol, stigmasterol, β-sitosterol) in the sample, in milligrams per 100 g of fat;

w_b is the mass fraction of betulin added to the test portion, in milligrams (9.3);

P_b is the numerical value of the purity of the betulin (5.7), (e.g. P_b = 0,99);

A_i is the numerical value of the peak area of each sterol in the test portion (9.8.2);

$F_{r,a}$ is the average value of the cholesterol response factor (9.8.3.1);

A_b is the numerical value of the peak area of betulin (9.8.2);

m_s is the mass of the test portion, in grams (9.2).

9.8.3.2 Calculation of sterols as a percentage of total

Calculate the mass fraction of each sterol, w_i, as a percentage of the total mass fraction of sterols by using the following equation

$$w_i = \frac{A_i \times F_{r,a}}{\sum(A_i \times F_{r,a})} \times 100\ \%$$

9.8.3.3 Expression of results

Express the results to one decimal place.

10 Precision

10.1 Interlaboratory test

Details of an interlaboratory test, in accordance with ISO 5725-1 and ISO 5725-2, on the precision of the method are summarized in Annex B.

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

10.2 Repeatability

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5 % of cases be greater than:

— for cholesterol: r = 21,2 mg/100 g of fat; s_r = 7,57 mg/100 g of fat;

— for campesterol: r = 3,4 mg/100 g of fat; s_r = 1,21 mg/100 g of fat;

— for stigmasterol: r = 2,8 mg/100 g of fat; s_r = 1,01 mg/100 g of fat;

— for β-sitosterol: r = 7,5 mg/100 g of fat; s_r = 2,70 mg/100 g of fat.

10.3 Reproducibility

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

— for cholesterol: R = 40,4 mg/100 g of fat; s_R = 14,44 mg/100 g of fat;

— for campesterol: R = 5,6 mg/100 g of fat; s_R = 2,00 mg/100 g of fat;

— for stigmasterol: R = 4,3 mg/100 g of fat; s_R = 1,52 mg/100 g of fat;

— for β-sitosterol: R = 11,4 mg/100 g of fat; s_R = 4,60 mg/100 g of fat.

11 Test report

The test report shall specify:

a) all information necessary for the complete identification of the sample;

b) the sampling method used, if known;

c) the method used, with reference to this International Standard;

d) all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents that may have influenced the test result(s);

e) the test result(s) obtained or, if the repeatability has been checked, the final quoted results obtained.

Annex A
(informative)

Examples of the gas-liquid chromatographic analysis

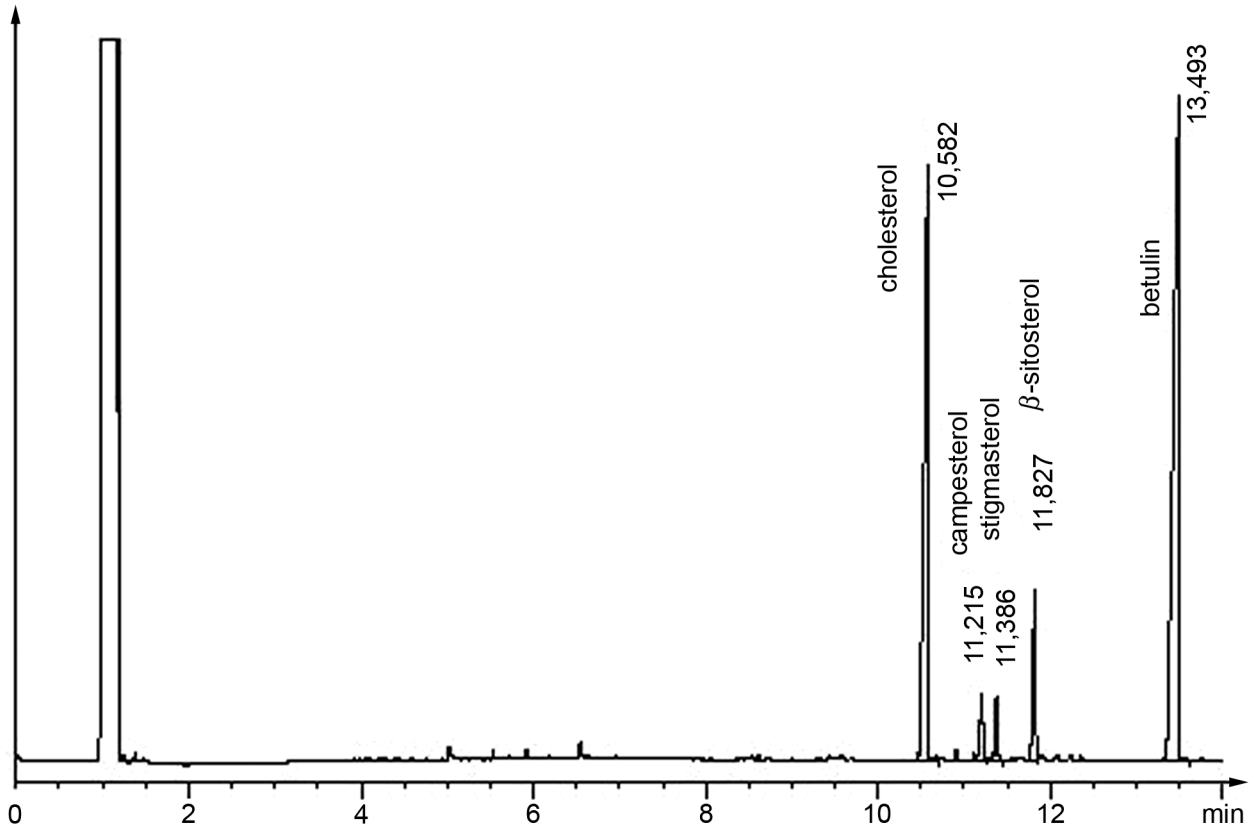

Figure A.1 — Example of a GC profile of sterols obtained by the GC conditions reported in 6.29.1

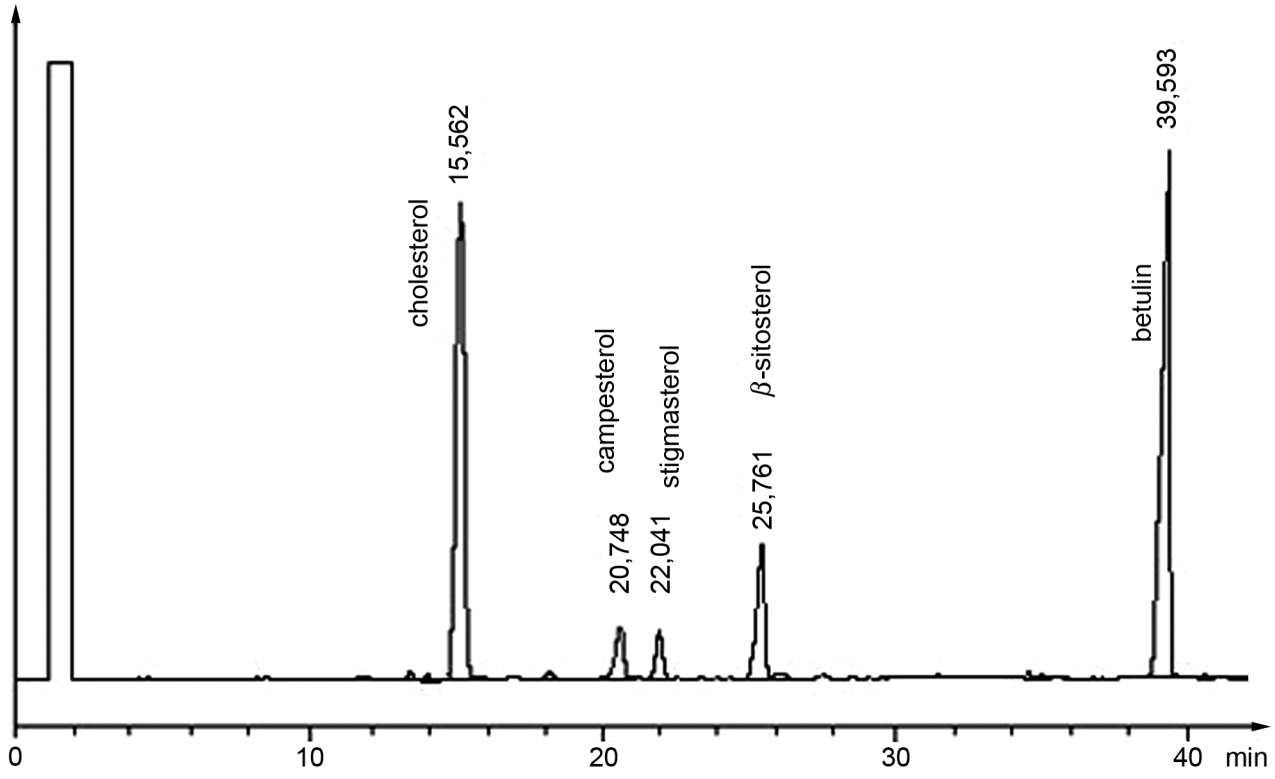

Figure A.2 — Example of a GC profile of sterols obtained by the GC conditions reported in 6.29.2

Annex B
(informative)

Results of an interlaboratory test

An international collaborative test involving 11 laboratories was carried out in accordance with ISO 5725-1 and ISO 5725-2 on three different samples of anhydrous milk fat containing 28 % to 32 % of vegetable fat. The three fat mixtures were divided into six blind duplicated samples.

The test was organized and evaluated by Instituto del Frio (CSIC) (ES) and Instituto Sperimentale Lattiero Caseario (IT). The results for the mass fractions of cholesterol, campesterol, stigmasterol and β-sitosterol have been expressed as milligrams per 100 g of fat.

The obtained results were subjected to statistical analysis in accordance with ISO 5725-2 to give the precision data shown in Table B.1.

Table B.1 — Results of interlaboratory test

Cholesterol			
	Sample 1	**Sample 2**	**Sample 3**
Number of participating laboratories after eliminating outliers	8	7	9
Mean value (mg/100 g fat)	191,1	190,5	181,9
Repeatability standard deviation, s_r (mg/100 g fat)	6,75	4,02	11,96
Coefficient of variation of repeatability (%)	3,5	2,1	6,6
Repeatability limit, r (= 2,8 s_r), (mg/100 g fat)	18,9	11,3	33,5
Reproducibility standard deviation, s_R (mg/100 g fat)	14,74	14,79	13,79
Coefficient of variation of reproducibility (%)	7,7	7,8	7,6
Reproducibility limit, R (= 2,8 s_R) (mg/100 g fat)	41,3	41,4	38,6

Campesterol			
	Sample 1	**Sample 2**	**Sample 3**
Number of participating laboratories after eliminating outliers	8	10	9
Mean value (mg/100 g fat)	15,6	17,6	18,0
Repeatability standard deviation, s_r (mg/100 g fat)	1,10	0,88	1,66
Coefficient of variation of repeatability (%)	7,0	5,0	9,2
Repeatability limit, r (= 2,8 s_r), (mg/100 g fat)	3,1	2,5	4,6
Reproducibility standard deviation, s_R (mg/100 g fat)	1,31	2,57	2,11
Coefficient of variation of reproducibility (%)	8,4	14,6	11,8
Reproducibility limit, R (= 2,8 s_R) (mg/100 g fat)	3,7	7,2	5,9

Table B.1 (*continued*)

Stigmasterol			
	Sample 1	**Sample 2**	**Sample 3**
Number of participating laboratories after eliminating outliers	10	10	9
Mean value (mg/100 g fat)	12,3	13,2	13,9
Repeatability standard deviation, s_r (mg/100 g fat)	1,04	0,75	1,22
Coefficient of variation of repeatability (%)	8,4	5,7	8,8
Repeatability limit, r (= 2,8 s_r), (mg/100 g fat)	2,9	2,1	3,4
Reproducibility standard deviation, s_R (mg/100 g fat)	1,57	1,53	1,46
Coefficient of variation of reproducibility (%)	12,7	11,6	10,5
Reproducibility limit, R (= 2,8 s_R) (mg/100 g fat)	4,4	4,3	4,1

β-sitosterol			
	Sample 1	**Sample 2**	**Sample 3**
Number of participating laboratories after eliminating outliers	10	9	9
Mean value (mg/100 g fat)	37,7	39,7	41,7
Repeatability standard deviation, s_r (mg/100 g fat)	2,26	1,60	4,23
Coefficient of variation of repeatability (%)	6,0	4,0	10,1
Repeatability limit, r (= 2,8 s_r), (mg/100 g fat)	6,3	4,5	11,9
Reproducibility standard deviation, s_R (mg/100 g fat)	3,77	3,97	4,46
Coefficient of variation of reproducibility (%)	10,0	10,0	10,7
Reproducibility limit, R (= 2,8 s_R) (mg/100 g fat)	10,6	11,1	12,5

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