INTERNATIONAL STANDARD

ISO 6799

Second edition
1991-12-15

Animal and vegetable fats and oils — Determination of composition of the sterol fraction — Method using gas chromatography

Corps gras d'origines animale et végétale — Détermination de la composition de la fraction stérolique — Méthode par chromatographie en phase gazeuse

Reference number
ISO 6799:1991(E)

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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.

International Standard ISO 6799 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Sub-Committee SC 11, *Animal and vegetable fats and oils*.

This second edition cancels and replaces the first edition (ISO 6799:1983), of which it constitutes a technical revision.

Annex A of this International Standard is for information only.

International Organization for Standardization
Case Postale 56 • CH-1211 Genève 20 • Switzerland

Printed in Switzerland

Introduction

Sterols are characteristic constituents of fats whether these are liquid or solid at ambient temperature. Animal fats always contain cholesterol, whereas vegetable fats contain a mixture of sterols, for example stigmasterol and β-sitosterol, called phytosterols. Meanwhile, some vegetable fats, e.g. colza, maize and palm, contain a small percentage of cholesterol in the total sterols.

The results of the method specified in this International Standard can be used for checking the purity of a fat, for checking conformity of a fat with a sales description or advertised composition, or for checking a fat for nutritional purposes.

For detecting the presence of animal fats in vegetable fats, the small quantities of cholesterol, which may be present in vegetable fats, need to be taken into account. In this case, it is necessary to carry out other methods of analysis in addition to the identification of sterols, for example the determination of the composition of fatty acids by gas chromatography (see ISO 5508 and ISO 5509). In practice, branched fatty acids and fatty acids with an odd number of carbon atoms are only present in appreciable quantities in animal fats.

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Animal and vegetable fats and oils — Determination of composition of the sterol fraction — Method using gas chromatography

1 Scope

This International Standard specifies a gas chromatographic method using packed or capillary columns for the determination of the composition of the sterol fraction of animal and vegetable fats and oils.

Des-sterols are determined but substituted sterols (i.e. methyl and dimethyl) may interfere and care is needed in interpreting the results.

2 Normative references

The following standards contain provisions which, through reference in this text, constitute provisions of this International Standard. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 661:1989, *Animal and vegetable fats and oils — Preparation of test sample.*

ISO 3596-1:1988, *Animal and vegetable fats and oils — Determination of unsaponifiable matter — Part 1: Method using diethyl ether extraction (Reference method).*

ISO 3596-2:1988, *Animal and vegetable fats and oils — Determination of unsaponifiable matter — Part 2: Rapid method using hexane extraction.*

ISO 3696:1987, *Water for analytical laboratory use — Specification and test methods.*

ISO 5508:1990, *Animal and vegetable fats and oils — Analysis by gas chromatography of methyl esters of fatty acids.*

3 Principle

Saponification of a test portion, extraction of the unsaponifiable matter and separation of sterols in the unsaponifiable matter by thin-layer chromatography. Analysis of the separated sterols, or of prepared derivatives, by gas chromatography using packed or capillary columns.

4 Reagents

Hazardous compounds are marked with an asterisk.

Use only reagents of recognized analytical grade, unless otherwise stated, and water complying with grade 3 of ISO 3696.

4.1 Reagents for the preparation of unsaponifiable matter

See ISO 3596-1 or ISO 3596-2.

4.2 Reagents for the separation of sterols

4.2.1 Ethanol, 95 % (V/V).

4.2.2 Light petroleum, distilling in the range 30 °C to 60 °C.

4.2.3 Acetone

4.2.4 Developing solvent, such as chloroform* or hexane/ethyl acetate [80 + 20 (V/V)] mixture or toluene*/acetone [95 + 5 (V/V)] mixture.

4.2.5 Ethyl acetate or **diethyl ether**, free from peroxides and residues.

4.2.6 Silica powder with binder, thin-layer chro-

matographic quality[1] (if necessary; see 8.2.1).

4.2.7 Indicator solution, for example rhodamine 6G, 0,5 g/l solution.

Any other colouring product or detection agent, such as a 0,5 g/l solution of 2′,7′-dichlorofluorescein in 95 % (*V*/*V*) ethanol, may be used provided that it does not react with the sterols.

4.2.8 Cholesterol, 100 g/l solution in chloroform.

4.3 Reagents for the preparation of sterol derivatives

4.3.1 Pyridine, anhydrous.*

4.3.2 Hexamethyldisilazane

4.3.3 Trimethylchlorosilane

4.3.4 Acetic anhydride

4.3.5 Hexane

4.3.6 Sodium hydrogen carbonate, 10 g/l solution.

4.3.7 Hydrochloric acid, $c(HCl) = 0,5$ mol/l.

4.3.8 Sodium sulfate, anhydrous.

4.4 Reagents for analysis by gas chromatography

4.4.1 Carrier gas

Inert gas (nitrogen, helium, argon, hydrogen, etc.), thoroughly dried and with an oxygen content of less than 10 mg/kg.

NOTE 1 Hydrogen, which is used only with capillary columns, can double the speed of analysis but is hazardous. Safety devices are available.

4.4.2 Pure sterols, such as cholesterol, stigmasterol, brassicasterol, β-sitosterol and campesterol, standard solutions in diethyl ether or any other suitable solvent, corresponding to 20 g of sterol per litre.

NOTE 2 Most pure sterols are not commercially available. The European Community Bureau of Reference (BCR) is preparing sterol reference materials, e.g. RM162 (maize and soya in equal masses).

If it is impossible to obtain the various pure sterols, and particularly if only the qualitative analysis is carried out, the solutions of pure sterols may be replaced by a 25 g/l solution of sterols of sunflower oil, a 25 g/l solution of sterols of rapeseed oil and a 20 g/l solution of cholesterol. The sterols of these solutions shall be prepared as described in 8.1 and 8.2.

5 Apparatus

Usual laboratory apparatus and, in particular, the following.

5.1 Apparatus for the preparation of unsaponifiable matter

See ISO 3596-1 or ISO 3596-2.

5.2 Apparatus for the separation of sterols

5.2.1 Developing tank, made of glass, with a ground glass lid, suitable for use with plates of dimensions 200 mm × 200 mm.

5.2.2 Spreader and platform, for preparation of the plates (if necessary; see 8.2.1).

5.2.3 Prepared plates, of dimensions 200 mm × 200 mm, or **glass plates**, of the same dimensions.

5.2.4 Micropipettes or **microsyringes**, capable of delivering droplets of 0,3 µl to 0,4 µl. An automatic drop applicator may be used.

5.2.5 Apparatus for spraying the indicator solution onto the plates

5.2.6 Microspatula

5.2.7 Oven, capable of being operated at 103 °C ± 2 °C.

5.2.8 Boiling water-bath

5.2.9 Filter paper, of 55 mm diameter, fluted, for retaining fine particles.

5.2.10 Conical flasks, of 25 ml capacity.

5.2.11 Conical flask, of 250 ml capacity.

5.2.12 Reflux condenser, to fit the 25 ml conical flasks (5.2.10).

5.2.13 Desiccator, containing an efficient desiccant.

1) Silica gels containing the indicator solution (4.2.7) are commercially available.

5.2.14 Ultraviolet lamp

5.3 Apparatus for the preparation of sterol derivatives (if required)

5.3.1 Reaction tube, internally conical, of 5 ml capacity, with a screw cap, preferably lined with polytetrafluoroethylene (PTFE) or **haemolysis tube**, of 5 ml capacity.

5.3.2 Haemolysis tube, of 10 ml capacity.

5.3.3 Graduated micropipettes

5.4 Apparatus for analysis of sterols

5.4.1 Gas chromatograph, with **flame ionization detector** and **recorder**, with a packed column (5.4.2) or capillary (5.4.3) column and a suitable injector, such as a split type.

5.4.2 Packed column, made of glass, 180 cm to 200 cm long and 2 mm to 4 mm in diameter, filled with 2 % (*m*/*m*) to 5 % (*m*/*m*) of methylpolysiloxanes[2] or diatomaceous earth of particle size 150 µm to 180 µm or 120 µm to 150 µm[3], coated with methylphenylpolysiloxanes[4], acid-washed and silanized.

NOTES

3 As sterols tend to decompose at high temperatures if they come into contact with metals (except silver), it is recommended that an entirely glass system be used. However, a stainless steel column may be used provided that its efficiency and resolution are tested with a known mixture of sterols and it is confirmed that there is no decomposition or retention of sterols present in small quantities.

4 The separation of the sterols depends, to a large extent, on the stationary phase used (see 8.4.1.3).

5.4.3 Capillary column, 10 m to 25 m long, of internal diameter 0,2 mm to 0,5 mm, deactivated by silanization, then coated with methylpolysiloxanes[2] or methylphenylpolysiloxanes[4] or other suitable phases.

NOTES

5 See table 2 for relative retention times compared with cholesterol.

6 See note 4 to 5.4.2.

6 Sampling

Sampling should have been carried out in accordance with ISO 5555.

7 Preparation of the test sample

Prepare the test sample in accordance with ISO 661.

8 Procedure

WARNING — Avoid prolonged exposure to pyridine or chloroform by, for example, carrying out all operations, so far as possible, in a fume cupboard.

8.1 Preparation of unsaponifiable matter

Using 5 g ± 0,2 g of the test sample (clause 7), prepare the unsaponifiable matter in accordance with ISO 3596-1 or ISO 3596-2. Remove the solvent until about 1 ml of solution remains.

NOTES

7 For fats having low sterol contents [approximately less than 0,1 % (*m*/*m*)], it is advisable to increase the size of the test portion in order to obtain at least 5 mg of sterols; the quantities of reagents would need to be modified as a consequence.

8 If the solvent is completely evaporated and the residue dissolved in hexane, a better separation may be obtained in 8.2.

8.2 Separation of sterols by thin-layer chromatography

8.2.1 Preparation of plates

If prepared plates are not available, prepare them as follows. Carefully clean the glass plates (5.2.3) using the ethanol (4.2.1), the light petroleum (4.2.2) and the acetone (4.2.3), until fatty matter is totally eliminated.

For the preparation of five plates, put 30 g of the silica powder (4.2.6) into the 250 ml conical flask (5.2.11) and add 60 ml of water. Stopper and shake vigorously for 1 min. Immediately introduce the paste into the spreader (5.2.2). Spread a layer of thickness 0,25 mm onto the clean plates.

Allow the plates to dry for 15 min in air, and then dry in the oven (5.2.7) at 103 °C for 1 h. Allow the plates to cool to ambient temperature in the desiccator (5.2.13) before use.

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2) SE 30, JXR or OV 1 are 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 of these products.

3) 80 mesh to 100 mesh or 100 mesh to 120 mesh.

4) OV 17 is a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

8.2.2 Separation of sterols

By means of a micropipette or microsyringe (5.2.4), transfer 50 µl to 60 µl of the solution obtained in 8.1 onto a prepared plate (see 8.2.1), 20 mm from the lower edge, in a continuous line of droplets which are as fine as possible, leaving unused a width of 25 mm on the right- and left-hand sides of the plate.

Place 0,3 µl to 0,4 µl of the cholesterol solution (4.2.8) 10 mm from the left-hand edge and 10 mm from the right-hand edge of the plate.

Place sufficient developing solvent (4.2.4) in the tank (5.2.1) and immediately stand the plate in it. Put on the lid and develop the plate until the solvent front reaches a point about 10 mm from the upper edge. Remove the plate from the tank and allow the solvent to evaporate at laboratory temperature, preferably under a fume hood.

Using the apparatus (5.2.5), spray with the appropriate indicator solution, for example the rhodamine 6G solution (4.2.7), and examine the obtained chromatogram under ultraviolet light. Identify the position of the sterol fraction by means of the two cholesterol reference spots and mark the position with a needle.

NOTE 9 The sterol fraction spot should be completely separate from spots of other components, especially those of methyl- and dimethylsterols.

8.2.3 Recovery of the sterols

Collect the silica containing the sterol fraction by means of the microspatula (5.2.6) or any other suitable means. Transfer the silica into a 25 ml conical flask (5.2.10), add 5 ml of the developing solvent (4.2.4) or ethyl acetate or diethyl ether (4.2.5) (see note 9), fit the reflux condenser (5.2.12) to the flask and boil the mixture gently on the water-bath (5.2.8) for 15 min.

Allow the mixture to cool and filter through the fluted filter paper (5.2.9) into a 25 ml conical flask (5.2.10). Returning any silica on the filter paper to the flask used for extraction, repeat the extraction and filtration. Carry out the extraction and filtration twice more.

NOTE 10 Diethyl ether may be used with advantage over chloroform. One such advantage is that rhodamine 6G is insoluble in diethyl ether and soluble in chloroform.

Combine the filtrates and remove the solvent under a gentle stream of nitrogen. Dissolve the residue in the minimum quantity (normally less than 1 ml) of ethyl acetate or diethyl ether (4.2.5) or the developing solvent (4.2.4).

Analysis of the sterols by gas chromatography may then be carried out

a) directly with the free sterols in the solution, except when polar capillary columns are used;

b) with derivatives of the sterols [silyl ethers or acetates (see 8.3)]. With certain stationary phases, these derivatives give less tailing than the free sterols.

8.3 Preparation of sterol derivatives (if required)

Prepare the silyl ethers in accordance with 8.3.1 or, failing this, the acetates in accordance with 8.3.2.

Once they have been prepared, carry out chromatography immediately.

Alternatively, sterol derivatives may be prepared using *N*-methyl-*N*-trimethylsilylheptafluorobutyramide (MSHFBA), *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) or *N*-trimethylsilylimidazole (MSI) [available as TriSil®"Z"[5]].

8.3.1 Formation of silyl ethers

Place a few milligrams of the sterols in the reaction tube or 5 ml haemolysis tube (5.3.1), and add, successively, 0,5 ml of pyridine (4.3.1), 0,1 ml of hexamethyldisilazane (4.3.2) and 0,04 ml of trimethylchlorosilane (4.3.3). Allow to stand for 5 min. Use a portion of the upper layer for injection.

NOTE 11 For a series of analyses, it is recommended that the reagents are mixed in advance and that the reacted mixture is evaporated to dryness then dissolved in diethyl ether or hexane.

8.3.2 Microacetylation of sterols

Place a few milligrams of the sterols in the 10 ml haemolysis tube (5.3.2). Add 0,1 ml of acetic anhydride (4.3.4) and 0,1 ml of pyridine (4.3.1). Maintain at 70 °C for 1 h. Add 2 ml of ice-cold water and 5 ml of hexane (4.3.5). Shake vigorously and then remove the water (using a small separating funnel or pipette). Wash successively with 5 ml of sodium hydrogen carbonate solution (4.3.6), 5 ml of hydrochloric acid solution (4.3.7), and then with 5 ml of water. Dry the extract over sodium sulfate (4.3.8) and filter.

5) TriSil®"Z" is an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

8.4 Analysis by gas chromatography of free sterols or their derivatives

NOTE 12 The use of non-polar capillary columns is recommended for the analysis of free sterols.

8.4.1 Using a packed column

8.4.1.1 Setting up the apparatus

8.4.1.1.1 Injector and detector

— Temperature: 20 °C sto 30 °C above that of the column.

8.4.1.1.2 Oven and column

— Temperature: 230 °C to 240 °C.

— Rate of flow of carrier gas (4.4.1): 30 ml/min to 50 ml/min.

Before first use, condition the filled columns for 48 h at 250 °C with the carrier gas flowing.

Calculate the resolution of the column in accordance with ISO 5508. The resolution of the peaks of campesterol and stigmasterol shall be greater than 1,0. If not, the column shall not be used for the determination of sterols.

8.4.1.2 Test

Inject into the column an appropriate volume, e.g. 0,5 µl to 1,0 µl, of each of the standard solutions (4.4.2) in order to determine the retention time (or distance) for each sterol.

Then inject the appropriate quantity, e.g. 0,5 µl to 1,0 µl, of the mixture of isolated sterols (8.2.3) or their derivatives (8.3.1 or 8.3.2) into the apparatus.

8.4.1.3 Examination of chromatograms

Identify the sterols present by determining the relative retention times (or distances).

The stationary phase used will determine which sterols can be separated by the column. Thus, if methylphenylpolysiloxanes are used as the stationary phase, β-sitosterol can be separated from Δ-5-avenasterol and Δ-7-stigmasterol can be separated from Δ-7-avenasterol.

NOTE 13 Under the operating conditions described, and when using methylpolysiloxanes (SE 30) or methylphenylpolysiloxanes (OV 17) as the stationary phase, the relative retention times for the free sterols (the origin being the solvent peak) relative to that for cholesterol are as given in table 1.

Table 1 — Relative retention times for packed columns

Sterol	SE 30	OV 17
Cholesterol	1,00	1,00
Brassicasterol	1,11	1,13
Campesterol	1,26	1,33
Stigmasterol	1,36	1,45
β-Sitosterol	1,56	1,66
Δ-5-Avenasterol	1,56	1,86
Δ-7-Stigmasterol	1,76	1,96
Δ-7-Avenasterol	1,76	2,18

8.4.2 Using a capillary column

8.4.2.1 Setting up the apparatus

— Temperature of the column: 250 °C to 260 °C.

— Temperature of the injector and the detector: 30 °C above that of the column.

8.4.2.2 Test

Inject into the column an appropriate volume, e.g. 0,1 µl to 0,2 µl, of each of the standard solutions (4.4.2) in order to determine the retention time (or distance) for each sterol.

Then inject the appropriate quantity, e.g. 0,1 µl to 0,2 µl, of the mixture of isolated sterols (8.2.3) or their derivatives (8.3.1 or 8.3.2) into the apparatus.

8.4.2.3 Examination of chromatograms

Identify the sterols present by determining the relative retention times (or distances).

NOTE 14 Under the operating conditions described, and when using methylphenylpolysiloxanes (OV 17) as the stationary phase, the relative retention times (the origin being the solvent peak) of the free sterols, silyl ethers and acetates, relative to that for cholesterol, are as given in table 2.

Table 2 — Relative retention times for capillary columns

Sterol	OV 17 free sterols	OV 17 silylethers	OV 17 acetates
Cholesterol	1,00	1,00	1,00
Brassicasterol	1,13	1,10	1,13
Campesterol	1,30	1,23	1,30
Stigmasterol	1,42	1,32	1,42
β-Sitosterol	1,61	1,47	1,61
Δ-5-Avenasterol	1,80	1,60	1,79
Δ-7-Stigmasterol	1,90	1,69	1,90
Δ-7-Avenasterol	2,13	1,85	2,09