International Standard 6799

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Animal and vegetable fats and oils — Determination of composition of the sterol fraction — Method by gas-liquid chromatography

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

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Foreword

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It has been approved by the member bodies of the following countries:

Australia	India	Romania
Brazil	Iran	South Africa, Rep. of
Canada	Israel	Spain
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Dominican Republic	Korea, Rep. of	Tanzania
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The member bodies of the following countries expressed disapproval of the document on technical grounds:

Netherlands
USA

This International Standard has also been approved by the International Union of Pure and Applied Chemistry (IUPAC).

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Animal and vegetable fats and oils — Determination of composition of the sterol fraction — Method by gas-liquid chromatography

0 Introduction

Sterols form a part of the characteristic constituents of fats and oils. Animal fats always contain cholesterol, whereas vegetable fats and oils contain a mixture of sterols, for example stigmasterol and β-sitosterol, called phytosterols. However, various vegetable fats and oils, in particular palm oil, contain a small percentage of cholesterol in the total sterols.

1 Scope and field of application

This International Standard specifies a gas-liquid column chromatographic method for the determination of the composition of the sterol fraction of animal and vegetable fats and oils.

The method is used for checking the purity of a fat or oil, or for checking the conformity of a fat or oil to its sales description or advertized composition, and applies to animal and vegetable fats and oils and to mixtures of the two.

If this method is used 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-liquid 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 and oils.

2 References

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

ISO 5509, *Animal and vegetable fats and oils — Preparation of methyl esters of fatty acids.*

3 Principle

Saponification of a test portion, extraction of the unsaponifiable matter, then separation of sterols from the unsaponifiable matter by thin-layer chromatography. Analysis of the separated sterols, or of prepared derivatives, by gas-liquid column chromatography.

4 Reagents

All reagents shall be of recognized analytical quality. The water used shall be distilled water or water of at least equivalent purity.

4.1 Reagents for the preparation of unsaponifiable matter

These will be specified in ISO 3569 which will specify a method for the preparation and determination of unsaponifiable matter.

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 to 60 °C.

4.2.3 Acetone.

4.2.4 Chloroform or, if the use of chloroform is considered to present a safety hazard, **any other suitable solvent or mixture of suitable solvents,** for example a hexane/ethyl acetate [80 + 20 (V/V)] mixture or a toluene/acetone [95 + 5 (V/V)] mixture.

4.2.5 Diethyl ether, free from peroxides and residues.

Alternatively, ethyl acetate or carbon disulphide may be used to dissolve the sterols.

4.2.6 Silica powder with binder, thin-layer chromatographic quality.[1)]

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

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

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

4.2.8 Cholesterol, 100 g/l solution in chloroform.

4.3 Reagents for 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, 0,5 mol/l solution.

4.3.8 Sodium sulphate, anhydrous.

4.4 Reagents for analysis by gas-liquid chromatography

4.4.1 Carrier gas : an inert gas such as nitrogen, helium or argon.

4.4.2 Pure sterols[1], 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 – 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.

5 Apparatus

5.1 Apparatus for the preparation of unsaponifiable matter

This will be specified in ISO 3596 (see 4.1).

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.

5.2.3 Glass plates, of dimensions 200 mm × 200 mm.

5.2.4 Micro-pipettes or **micro-syringes,** capable of delivering droplets of 0,3 to 0,4 µl. An automatic drop applicator can 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 controlled at 103 ± 2 °C.

5.2.8 Boiling water bath.

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

5.2.10 Conical flasks, of capacity 25 ml.

5.2.11 Conical flask, of capacity 250 ml.

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

5.2.13 Desiccator, containing an efficient desiccant.

5.2.14 Ultraviolet lamp.

5.3 Apparatus for preparation of sterol derivatives (if required)

5.3.1 Reaction tube, internally conical, of capacity 5 ml, with a screw top, or a **haemolysis tube,** of capacity 5 ml.

5.3.2 Haemolysis tube, of capacity 10 ml.

5.3.3 Graduated micropipettes.

5.4 Apparatus for analysis of sterols

5.4.1 Gas-liquid chromatograph, with **flame ionization detector** and **recorder,** comprising a **glass column,** of length 180 to 200 cm and 2 to 4 mm in diameter, filled with 2 to 5 % of methylpolysiloxanes[2] or methylphenylpolysiloxanes[3] on diatomaceous earth of particle size 150 to 180 µm or 120 to 150 µm[4], acid-washed and silanized.

NOTES

1 As sterols tend to decompose at high temperatures if they come into contact with metals (except silver), it is recommended that an en-

1) Most pure sterols are not commercially available.

2) SE 30, JXR or OV 1 are suitable.

3) OV 17 is suitable.

4) 80 to 100 mesh or 100 to 120 mesh.

tirely glass system is 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 absorption of sterols present in small quantities.

2 The separation of the sterols depends, to a large extent, on the stationary phase used (see 6.4.3).

6 Procedure

6.1 Test portion and preparation of unsaponifiable matter

Weigh[1], to the nearest 0,01 g, 5 g of the test sample and extract the unsaponifiable matter. Remove the solvent until about 1 ml of solution remains.

NOTES

1 A method for the extraction of unsaponifiable matter will be specified in ISO 3596.

2 For oils having low sterol contents (less than 0,1 %), such as palm oil, it is advisable to increase the size of the test portion in order to obtain at least 5 mg of sterols; consequently, the quantities of reagents need to be modified.

3 If it is desired to perform a quantitative determination, the unsaponifiable matter should be dried at not more than 50 °C under reduced pressure, in order to avoid any undesirable oxidative deterioration, weighed and dissolved in 0,5 to 1 ml of the solvent (4.2.4).

6.2 Separation of sterols by thin-layer chromatography

6.2.1 Preparation of plates[2]

Carefully clean the glass plates (5.2.3) by means of the ethanol (4.2.1), the light petroleum (4.2.2) and the acetone (4.2.3), until fatty matter is totally eliminated.

Put 30 g of the silica (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), controlled at 103 ± 2 °C, for 1 h. Allow the plates to cool to ambient temperature in the desiccator (5.2.13) before use.

6.2.2 Preparation of the tank

Introduce a sufficient quantity of the solvent (4.2.4) into the developing tank (5.2.1) and put on the lid. Leave for a few hours in order to obtain liquid-vapour equilibrium; this state may be reached more quickly by placing a sheet of filter paper, covering three internal walls, and dipping into the developing solvent, in the tank.

6.2.3 Separation of sterols

By means of the micro-pipette or micro-syringe (5.2.4), transfer 50 to 60 µl of the solution obtained in 6.1 onto a prepared plate (see 6.2.1), 20 mm from one of the edges, in a continuous line of droplets which are as fine as possible, leaving a width of 25 mm on the right and left sides of the plate unused.

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

Immediately introduce the plate into the prepared developing tank (6.2.2), 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.

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

NOTE — The sterol fraction spot should be completely separate from spots of other components.

Collect the silica containing the sterol spots 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 chloroform (4.2.4) or the diethyl ether (4.2.5) (see the note below), fit the reflux condenser (5.2.12) to the flask and boil gently in the water bath (5.2.8) for 15 min.

Allow to cool and filter through the fluted filter paper (5.2.9) into a 25 ml conical flask (5.2.10). Remove the silica deposited on the filter and again treat it with 5 ml of boiling chloroform. Repeat this procedure three times.

NOTE — In place of the chloroform, the diethyl ether may be used with certain advantages, for example rhodamine 6 G is soluble in chloroform and insoluble in diethyl ether.

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 the diethyl ether (4.2.5) or the chloroform (4.2.4).

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

a) directly with the free sterols in the solution;

b) with derivatives of the sterols [silylethers or acetates (see 6.3)]. With certain stationary phases, these derivatives give less tailing than the free sterols.

1) Very precise weighing is not necessary if a qualitative determination only is envisaged.

2) Prepared plates are commercially available.

6.3 Preparation of sterol derivatives (if required)

Prepare the silylethers (6.3.1) or, failing this, the acetates (6.3.2). Once they have been prepared, carry out chromatography immediately.

6.3.1 Formation of silylethers

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 the pyridine (4.3.1), 0,1 ml of the hexamethyldisilazane (4.3.2) and 0,04 ml of the trimethylchlorosilane (4.3.3). Allow to stand for 5 min. Use between 0,5 and 1,0 µl of the upper layer for injection.

6.3.2 Microacetylation of sterols

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

6.4 Analysis by gas-liquid chromatography of isolated sterols or their derivatives

6.4.1 Setting up the apparatus

6.4.1.1 Injector

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

6.4.1.2 Oven and column

— Temperature : 230 to 240 °C.

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

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

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.

6.4.2 Test

Inject into the column an appropriate volume of each of the standard solutions (4.4.2) in order to determine the retention distance (or time) for each sterol.

Then inject the appropriate quantity of the mixture of isolated sterols (6.2.3) or their derivatives (6.3.1 or 6.3.2) into the apparatus.

6.4.3 *Examination of chromatograms*

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

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.

For information only, under the operating conditions described, and when using methylpolysiloxanes (SE 30) or methylphenyl polysiloxanes (OV 17) as the stationary phase, the retention times for the free sterols (the origin being the peak for air) relative to that for cholesterol, are as given in the following table.

Sterol	Stationary phase	
	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

7 Expression of results

7.1 The method makes it possible to determine the composition of the sterols for a given stationary phase, with certain restrictions as follows :

a) the response of each sterol is a function of the type of detector used;

b) the precise quantitative interpretation of the chromatograms is only possible if the individual sterols used for the calibration are pure or, failing this, if the exact composition of the solution of sterols indicated in the note to 4.4.2 has been previously definitively determined by this method by calibration with pure sterols. If not, the results shall be expressed as a percentage of the peak areas.

NOTE — In certain cases, it may be desirable to group the sterols determined individually to facilitate interpretation.

7.2 Use the method of internal normalization, i.e. assume that all the sterols present in the sample are represented on the chromatogram, and that the total area of the peaks represents 100 % of the constituents (total elution).

If pure sterols can be tested, determine the correction factor K_i in relation to cholesterol under the same operating conditions as those used for the test.

The content of sterol i, expressed as a percentage by mass of the total sterols, is given by the formula :

$$\frac{A_i \times K_i}{\Sigma (A_i \times K_i)} \times 100$$

where

A_i is the area of the peak corresponding to sterol i;

K_i is the correction factor, in relation to cholesterol, corresponding to sterol i.

In the absence of pure sterols or of a solution of sterols as indicated in the note to 4.4.2, of exactly known composition, it is reasonable to assume that the correction factors K_i are all equal to 1; the results shall then be expressed as a percentage of the peak areas.

Express the results to the nearest unit.

8 Test report

The test report shall show the method and the stationary phase used and the results obtained. It shall also mention any operating conditions not specified in this International Standard or regarded as optional, as well as any circumstances that may have influenced the results.

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