
**Animal and vegetable fats and oils —
Determination of individual and total sterols
contents — Gas chromatographic method**

*Corps gras d'origines animale et végétale — Détermination de la teneur en
stérois individuels et totaux — Méthode par chromatographie en phase
gazeuse*



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Foreword

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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 12228 was prepared by ISO/TC 34, *Agricultural food products*, Subcommittee SC 11, *Animal and vegetable fats and oils*.

Annexes A and B of this International Standard are for information only.

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Animal and vegetable fats and oils — Determination of individual and total sterols contents — Gas chromatographic method

1 Scope

This International Standard specifies a method for the gas chromatographic determination of the contents and compositions of sterols in animal and vegetable fats and oils.

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 samples*.

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

3 Definitions

For the purposes of this International Standard, the following definitions apply.

3.1

composition of sterols

composition of individual sterols in the sample, beginning with cholesterol and ending with Δ^7 -avenasterol (see table 1) under the conditions specified in this International Standard

NOTE The composition is expressed as peak area, in percent, and normalized to 100 %.

3.2

total sterol content

mass of the sum of all individual sterols, as determined in accordance with the method specified in this International Standard, beginning with cholesterol and ending with Δ^7 -avenasterol (see table 1), divided by the mass of the test portion

NOTE The content is expressed in milligrams per 100 g.

4 Principle

A test portion is saponified by boiling under reflux with an ethanolic potassium hydroxide solution. The unsaponifiable matter is isolated by solid-phase extraction on an aluminium oxide column. The aluminium oxide column is used to retain the fatty acid anions; sterols pass through the column. The sterol fraction from the unsaponifiable matter is separated by thin-layer chromatography. The qualitative and quantitative compositions of the sterol fraction are determined by gas chromatography using betulin as an internal standard.

5 Reagents

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

5.1 Potassium hydroxide, ethanolic solution, $c(\text{KOH}) \approx 0,5 \text{ mol/l}$.

Dissolve 3 g of potassium hydroxide in 5 ml of water and dilute to 100 ml with ethanol (5.3). The solution should be colourless or straw-coloured.

5.2 Internal standard solution, betulin, 1,0 mg/ml solution in acetone (see note to 5.10).

NOTE In the case of olive pomace oils which may contain betulin, the use of 5α -cholestan- 3β -ol (peak 2 in table 1) as internal standard is recommended.

5.3 Ethanol, of minimum purity 95 % (V/V).

5.4 Aluminium oxide, neutral, 0,063 mm to 0,200 mm, activity step I.

5.5 Diethyl ether, freshly distilled, free from peroxides and residue.

WARNING: Diethyl ether is highly flammable and can form explosive peroxides. Explosive limits in air are 1,7 % (V/V) to 48 % (V/V). Take special precautions when using it.

5.6 Silica gel thin-layer chromatography (TLC) plates, commercially available, dimensions 20 cm x 20 cm, thickness of layer 0,25 mm.

5.7 Developing solvent, hexane/diethyl ether [1/1 (V/V)].

5.8 Standard solution for thin-layer chromatography, 1,0 mg/ml cholesterol and 5,0 mg/ml betulin in acetone.

5.9 Spraying reagent, methanol.

5.10 Silylating reagent, prepared by adding 50 μl of 1-methyl imidazole to 1 ml of *N*-methyl-*N*-(trimethylsilyl)-hepta-fluorobutyramide (MSHFBA).

NOTE Other silylating reagents should normally not be used unless special precautions are taken to ensure that both hydroxyl groups of betulin are silylated. If not, betulin may show two peaks in the GLC.

6 Apparatus

Usual laboratory apparatus and, in particular, the following.

6.1 Round-bottomed flasks, of 25 ml and 50 ml capacity, with ground neck.

6.2 Reflux condenser, with ground joint to fit a flask (6.1).

6.3 Glass column, with PTFE stopper, sintered glass frit and reservoir for 100 ml, length 25 cm, internal diameter 1,5 cm.

6.4 Rotary evaporator, attached to a vacuum pump and water bath maintained at 40 °C.

6.5 Developing tank, made of glass, with a ground glass lid, suitable for use with plates of dimensions 20 cm x 20 cm.

6.6 Microsyringe or micropipette, to deliver 100 μl .

6.7 Oven, maintained at $105 \text{ }^{\circ}\text{C} \pm 3 \text{ }^{\circ}\text{C}$.

6.8 Desiccator, containing an efficient desiccant, for storing the plates.

6.9 Reaction vials, of 0,3 ml capacity, with screw caps and PTFE-lined seals, for preparation of sterol derivatives.

6.10 Gas chromatograph, for capillary columns, with split injector, flame ionization detector and suitable recorder.

6.11 Capillary column, made of fused silica or glass, length 25 m to 60 m, internal diameter 0,2 mm to 0,25 mm, stationary phase SE-54 (or equivalent non-polar phase with a temperature limit of at least 280 °C to 300 °C); film thickness about 0,1 µm.

6.12 Microsyringe for gas chromatography, for injecting volumes of 1 µl.

6.13 Analytical balance, capable of weighing to the nearest 0,001 g and displaying 0,0001 g.

7 Sampling

It is important that the laboratory receive a sample which is truly representative and has not 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 5555 [1].

8 Preparation of test sample

Prepare the test samples in accordance with ISO 661.

9 Procedure

9.1 Test portion

Weigh, to the nearest 1 mg, about 250 mg of the test sample into a 25 ml flask (6.1).

For fats and oils with a sterol content of less than 100 mg per 100 g, proceed using a three-fold amount of the test sample. Adjust reagents and apparatus accordingly.

9.2 Determination

9.2.1 Preparation of the aluminium oxide column

Suspend 10 g of aluminium oxide (5.4) in 20 ml of ethanol (5.3) and pour the slurry into the glass column (6.3). Allow the aluminium oxide to settle and let the solvent run out of the column until the level of the solvent reaches the top level of the aluminium oxide layer.

9.2.2 Extraction of the unsaponifiable matter

Add exactly 1,00 ml of internal standard solution (5.2) to the test portion (9.1).

Add 5 ml of ethanolic potassium hydroxide solution (5.1) and a few anti-bumping granules. Attach the reflux condenser (6.2) to the flask and keep the contents gently boiling for 15 min. Stop heating. **Immediately** dilute the contents of the flask while still hot with 5 ml of ethanol (5.3) and swirl or shake to homogenize.

Pipette 5 ml of this solution onto the prepared aluminium oxide column (9.2.1). Collect the eluate in a 50 ml round-bottom flask (6.1) and allow the column to run off until the solvent level has reached the top level of the aluminium oxide layer. Elute the unsaponifiable matter first with 5 ml of ethanol (5.3) and then with 30 ml of diethyl ether (5.5), with a flowrate of about 2 ml/min. Remove the solvents from the flask by means of the rotary evaporator (6.4).

WARNING: The aluminium oxide column is essential for this procedure. It shall not be replaced by silica or other columns or by solvent extraction.

9.2.3 Thin-layer chromatography

Dissolve the unsaponifiable matter obtained in 9.2.2 in a small amount of diethyl ether (5.5). Apply the solution as a line at a distance of 2 cm from the lower edge onto a TLC plate (5.6) using the microsyringe (6.6). Leave a gap of at least 3 cm from each side edge of the plate. Apply a spot of 5 μ l of the TLC standard solution (5.8) at 1,5 cm from the edge. Fill the developing tank (6.5) with about 100 ml of developing solvent (5.7). Place the plate into the tank and develop it until the solvent reaches the upper edge. Remove the plate from the tank and allow the solvent to evaporate in a fume cupboard.

NOTE Quantitative transfer of the material (9.2.2) to the TLC plate is not necessary in this step. Automatic apparatus for applying streaks may be used. No saturation chamber is used.

9.2.4 Isolation of the sterols

Spray the plates with methanol (5.9) until the sterol and betulin zones appear white on a translucent (darker) background. The betulin zone appears slightly below the sterol zone. Mark the zones at the height of the standard spots 2 mm above and 4 mm below the visible zones (see figure 1). Scratch off this part of the layer completely using a spatula and quantitatively collect the silica in a small beaker.

NOTE The wider margin at the lower edge of the visible zones (4 mm vs. 2 mm at the upper edge) is a precaution to avoid partial loss of betulin in this step. Sunflowerseed oil may show three bands (Δ^5 -sterols, Δ^7 -sterols and betulin).

Add 0,5 ml of ethanol to the collected silica gel. Digest the silica gel in the beaker three times with 5 ml of diethyl ether (5.5) and filter into a flask (6.1). Reduce the combined ether extracts to about 1 ml in the rotary evaporator (6.4) and transfer the remaining solution into the reaction vial (6.9). Blow off the solvent in the reaction vial with a stream of nitrogen.

9.2.5 Preparation of sterol trimethylsilyl ethers

Add 100 μ l of the silylation reagent (5.10) to the reaction vial (6.9) containing the isolated sterols. Seal and heat the vial for 15 min in the oven set at 105°C. Allow the reaction vial to cool to room temperature and inject the solution directly into the gas chromatograph (6.10).

9.2.6 Gas chromatography

Optimize the temperature programme and the carrier gas flowrate so that chromatograms similar to figure 2 are obtained. Test the separation with silylated sterol fractions obtained from known oils, as shown in figure 2.

NOTE 1 The following parameters were tested and found useful: GC column: SE-54, 50 m length, 0,25 mm internal diameter, 0,10 μ m film thickness; carrier gas H_2 , carrier gas flowrate 36 cm/s, split 1:20, detector/injector 320° C, temperature programme 240 °C to 255 °C at 4 °C/min, injection volume 1 μ l. Capillary columns of equivalent quality can be used.

NOTE 2 A standard solution containing cholesterol, campesterol, stigmasterol and sitosterol may be used to check the retention times. Use a blank run to test for possible contamination (e.g. cholesterol) from solvents, glass walls, filter, fingerprints, etc.

10 Expression of results

10.1 Identification of sterols

To identify the sterols present in the test sample, determine the relative retention times (RRT) by dividing the retention time (RT) of the sterol in question by the RT of cholesterol and/or betulin. Table 1 shows the RRT of the various sterols corresponding to cholesterol (RRTC) and betulin (RRTB), with SE-54 stationary phase.

NOTE The RRT in table 1 (determined under the conditions of note 1 in 9.2.6) are mentioned as an aid for the identification of the individual sterols only, and to illustrate the elution sequence (cf. also figure. 2). The actual RRT found may deviate slightly from the RRT given in table 1 because the RRT depends on the experimental conditions (type and length of GLC column, temperature programme, and quality of stationary phase).

10.2 Composition of sterols

Calculate peak area, C_i , of sterol i , in percent, according to the following equation:

$$C_i = \frac{A_i}{\sum A} \times 100\%$$

where

A_i is the area of the peak of sterol i ;

$\sum A$ is the sum of the peak areas of all sterols (peaks 1 to 16).

Table 1 — GLC peak identification of individual sterols and betulin by RRT (SE-54 stationary phase)

Peak	Common names	Sterols	RRTC	RRTB
1	Cholesterol	Cholest-5-en-3 β -ol	1,00	0,44
2	Cholestanol	5 α -Cholestan-3 β -ol	1,02	0,45
3	Brassicasterol	[24S]-24-Methyl cholesta-5,22-dien-3 β -ol	1,09	0,48
4	24-Methylene cholesterol	24-Methylene cholesta-5,24-dien-3 β -ol	1,21	0,53
5	Campesterol	[24R]-24-Methyl cholest-5-en-3 β -ol	1,23	0,54
6	Campestanol	[24R]-24-Methyl cholestan-3 β -ol	1,25	0,55
7	Stigmasterol	[24S]-24-Ethyl cholesta-5,22-dien-3 β -ol	1,31	0,57
8	Δ 7-Campesterol	[24R]-24-Methyl cholest-7-en-3 β -ol	1,38	0,59
9	Δ 5,23-Stigmastadienol	[24R,S]-24-Ethyl cholesta-5,23-dien-3 β -ol	1,40	0,60
10	Clerosterol	[24S]-24-Ethyl cholesta-5,25-dien-3 β -ol	1,42	0,62
11	Sitosterol	[24R]-24-Ethyl cholest-5-en-3 β -ol	1,47	0,64
12	Sitostanol	[24R]-24-Ethyl cholestan-3 β -ol	1,50	0,65
13	Δ 5-Avenasterol	[24Z]-24(28)-Ethylidene cholest-5-en-3 β -ol	1,52	0,66
14	Δ 5,24-Stigmastadienol	[24R,S]-24-Ethyl cholesta-5,24-dien-3 β -ol	1,59	0,69
15	Δ 7-Stigmasterol	[24R,S]-24-Ethyl cholest-7-en-3 β -ol	1,65	0,72
16	Δ 7-Avenasterol	[24Z]-24(28)-Ethylidene cholest-7-en-3 β -ol	1,70	0,74
X	Erythrodiol		2,03	0,88
Y	Uvaol		2,17	0,95
17	Betulin	Lup-20[29]-ene-3 β ,28-diol	2,30	1,00
RRTC: relative retention time based on cholesterol = 1,00				
RRTB: relative retention time based on betulin = 1,00				
NOTE Sitosterol may coelute together with α -spinasterol and Δ 7,22,25-stigmastatrienol. [24R]-24-Ethyl cholesta-7,25(27)-dien-3 β -ol is present in sterols of sunflower and pumpkin seed oil and may coelute with peak 14 (Δ 5,24-stigmastadienol).				

10.3 Determination of the total sterol content

For the purposes of this method it is assumed that the response factors of all sterols and of betulin are equal.

NOTE In several tests silylated sterols and silylated betulin in equal amounts gave the same detector response using an FID detector under these conditions.

Calculate the total sterol content S , in milligrams per 100 g of fat, according to the following equation:

$$S = \frac{\Sigma(A) \times m_B}{A_B \times m_T} \times 100$$

where

- m_B is the mass of betulin, in milligrams;
- $\Sigma(A)$ is the sum of the peak areas of the individual sterols present;
- A_B is the peak area of the betulin internal standard;
- m_T is the mass of the test sample, in grams.

For calculation of the total sterol content, consider all peaks of sterols beginning with cholesterol and ending with Δ^7 -avenasterol (peak 16), but without erythrodiol and uvaol (peaks X and Y).

11 Precision

11.1 Interlaboratory test

Details of an interlaboratory test on the precision of the method are summarized in annex A. The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given.

11.2 Repeatability limit, r

The repeatability limit (r) is the value less than or equal to which the absolute difference between two test results obtained under repeatability conditions may be expected to be with a probability of 95 %.

Repeatability conditions are conditions where independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time.

11.3 Reproducibility limit, R

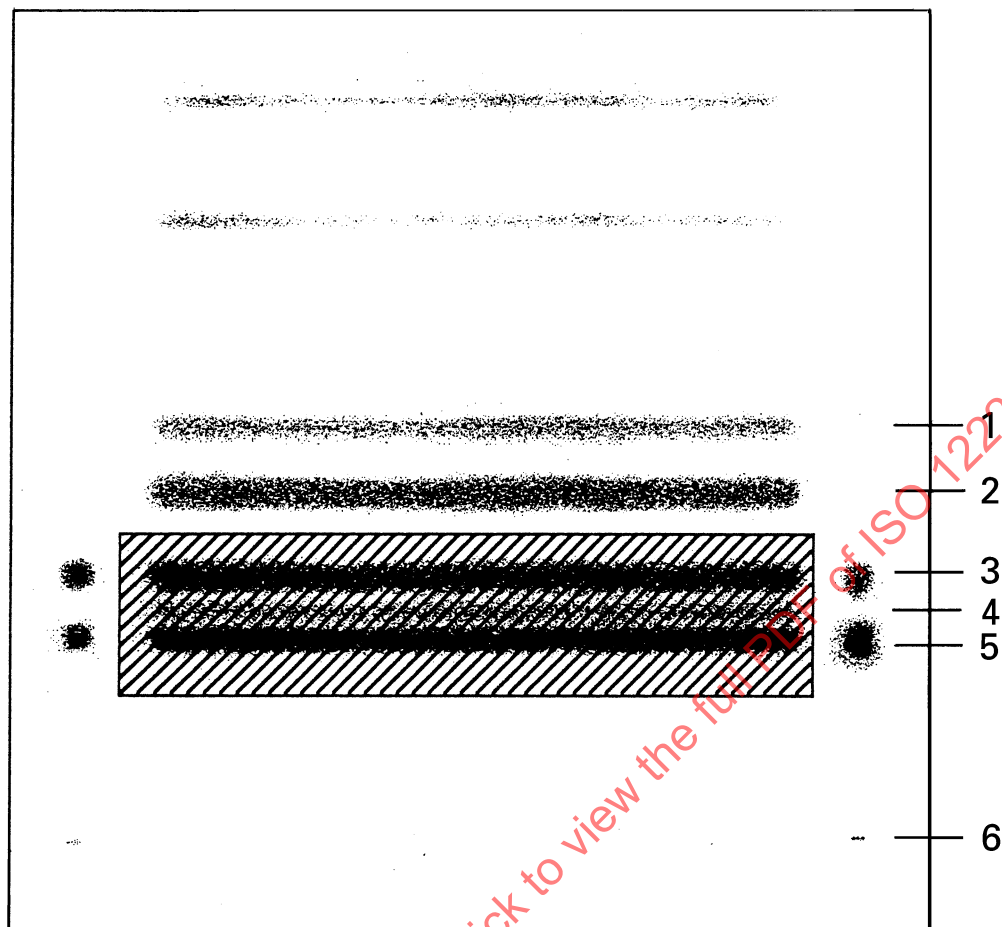
The reproducibility limit (R) is the value less than or equal to which the absolute difference between two test results obtained under reproducibility conditions may be expected to be with a probability of 95 %.

Reproducibility conditions are conditions where independent test results are obtained with the same method on identical test items in different laboratories with different operators using different equipment within short intervals of time.

12 Test report

The test report shall specify:

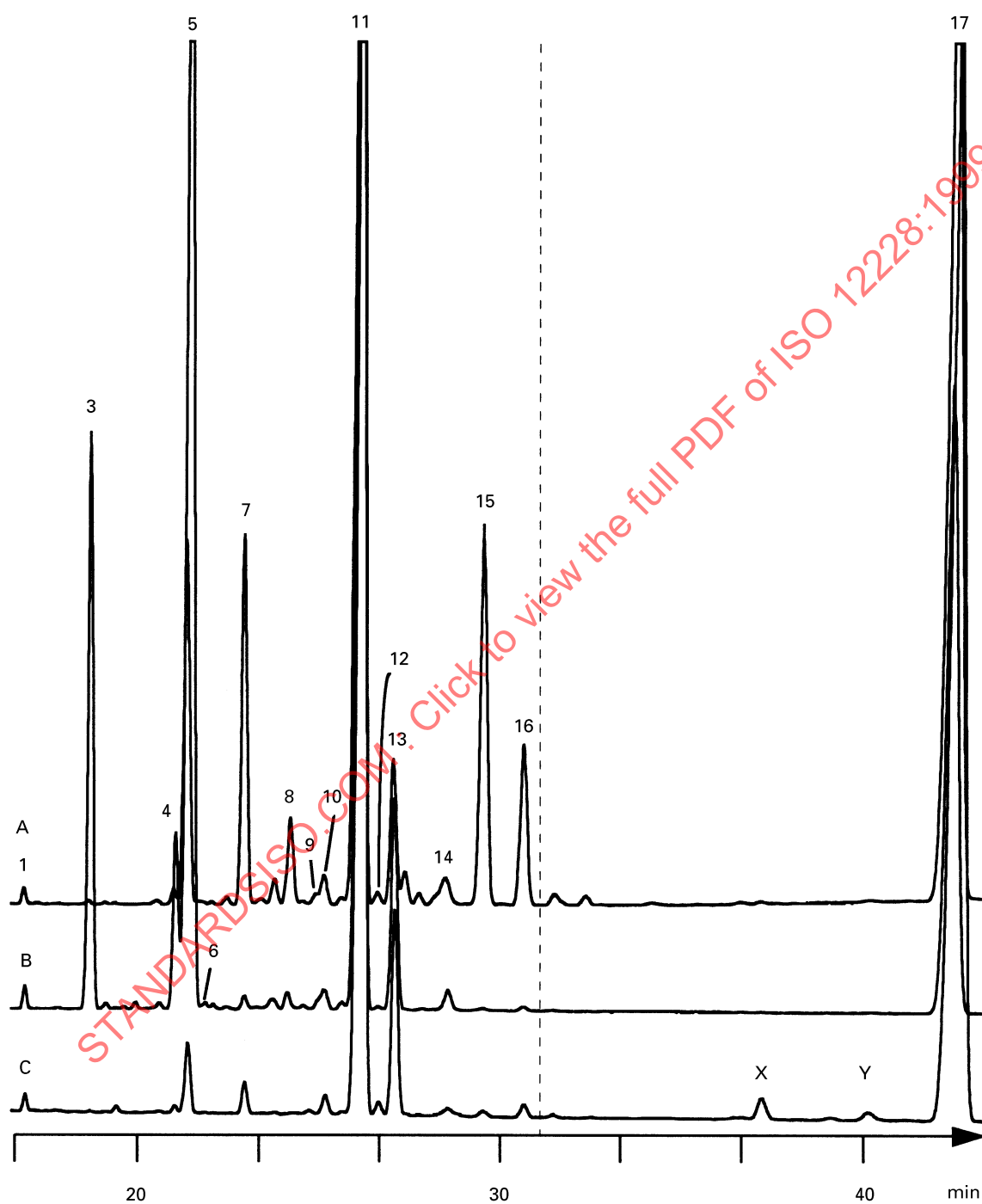
- all information necessary for the complete identification of the sample;
- the sampling method used, if known;
- the test method used, together with reference to this International Standard;
- all operating details not specified in this International Standard, or regarded as optional, together with details any incidents which may have influenced the test result(s);
- the test result(s) obtained; or
- if the repeatability has been checked, the final quoted result obtained.

**Key**

- 1 Triterpenes
- 2 Methyl sterols
- 3 Δ^5 -sterols
- 4 Δ^7 -sterols
- 5 Betulin
- 6 Start

NOTE Zones appear white on a transparent background. Hatched area is scratched off; note wider margin at the bottom (4 mm vs. 2 mm at the top). Rf values of the bands: betulin 0,30; Δ^7 -sterols 0,33; Δ^5 -sterols 0,35; methyl sterols 0,45; triterpenes 0,53.

Figure 1 — TLC isolation of sterols from unsaponifiable matter (steps 9.2.3 and 9.2.4)



NOTE Identification of the peak numbers is according to table 1. Conditions are as in note 1 of 9.2.6.

Figure 2 — GLC of sterols from sunflower (A), rapeseed (B) and olive oil (C)

Annex A (informative)

Interlaboratory test

The precision of the method has been established by an international interlaboratory test organized by DIN in 1996/97, on samples of olive oil, sunflowerseed oil and rapeseed oil, and carried out in accordance with ISO 5725-1 and ISO 5725-2.

Table A.1 — Statistical results for the total sterol content

Sample	Olive oil	Sunflower-seed oil	Rapeseed oil
Level, mg/kg	2 000	4 000	8 000
Number of participating laboratories	14	14	14
Number of acceptable results	13	14	13
Number of individual test results of all laboratories on each sample	2	2	2
Mean value, mg/kg	1 887	3 785	7 585
Standard deviation of repeatability (s_r)	66,75	165,62	174,64
Repeatability limit (r)	186,89	463,73	489,00
Repeatability coefficient of variation, %	3,54	4,37	2,30
Standard deviation of reproducibility (s_R)	212,20	542,38	762,67
Reproducibility limit (R)	594,17	1518,66	2135,48
Reproducibility coefficient of variation, %	11,25	14,33	10,05

Table A.2 — Statistical results for the individual sterol content: Cholesterol

Sample	Olive oil	Sunflower-seed oil	Rapeseed oil
Level, %	1,0	0,5	0,5
Number of participating laboratories	14	14	14
Number of acceptable results	13	12	14
Number of individual test results of all laboratories on each sample	2	2	2
Mean value, %	0,8	0,4	0,4
Standard deviation of repeatability (s_r)	0,15	0,05	0,08
Repeatability limit (r)	0,41	0,13	0,22
Repeatability coefficient of variation, %	19,54	11,53	18,33
Standard deviation of reproducibility (s_R)	0,35	0,21	0,15
Reproducibility limit (R)	0,97	0,58	0,41
Reproducibility coefficient of variation, %	45,89	52,43	34,11

Table A.3 — Statistical results for the individual sterol content: Brassicasterol

Sample	Olive oil	Sunflower-seed oil	Rapeseed oil
Level, %	0,1	0,1	10
Number of participating laboratories	14	14	14
Number of acceptable results	13	13	13
Number of individual test results of all laboratories on each sample	2	2	2
Mean value, %	0,1	0,09	10,8
Standard deviation of repeatability (s_r)	0,17	0,068	0,14
Repeatability limit (r)	0,48	0,190	0,38
Repeatability coefficient of variation, %	135,59	80,289	1,26
Standard deviation of reproducibility (s_R)	0,19	0,089	0,26
Reproducibility limit (R)	0,52	0,249	0,72
Reproducibility coefficient of variation, %	147,53	104,940	2,39

Table A.4 — Statistical results for the individual sterol content: 24-Methylene cholesterol and campesterol

Sample	Olive oil	Sunflower-seed oil	Rapeseed oil
Level, %	5	10	40
Number of participating laboratories	14	14	14
Number of acceptable results	12	11	13
Number of individual test results of all laboratories on each sample	2	2	2
Mean value, %	3,5	9,0	37,5
Standard deviation of repeatability (s_r)	0,50	0,21	0,19
Repeatability limit (r)	1,40	0,58	0,54
Repeatability coefficient of variation, %	14,15	2,31	0,51
Standard deviation of reproducibility (s_R)	0,52	0,26	0,35
Reproducibility limit (R)	1,45	0,72	0,97
Reproducibility coefficient of variation, %	14,67	2,88	0,93

Table A.5 — Statistical results for the individual sterol content: Campestanol

Sample	Olive oil	Sunflower-seed oil	Rapeseed oil
Level, %	< 0,1	0,1	0,1 to 0,5
Number of participating laboratories	14	14	14
Number of acceptable results	9	10	12
Number of individual test results of all laboratories on each sample	2	2	2
Mean value, %	0,03	0,1	0,2
Standard deviation of repeatability (s_r)	0,024	0,03	0,06
Repeatability limit (r)	0,066	0,09	0,16
Repeatability coefficient of variation, %	84,853	35,13	32,78
Standard deviation of reproducibility (s_R)	0,059	0,13	0,18
Reproducibility limit (R)	0,165	0,37	0,50
Reproducibility coefficient of variation, %	212,132	147,45	106,00

Table A.6 — Statistical results for the individual sterol content: Stigmasterol

Sample	Olive oil	Sunflower-seed oil	Rapeseed oil
Level, %	1	10	0,5
Number of participating laboratories	14	14	14
Number of acceptable results	13	13	13
Number of individual test results of all laboratories on each sample	2	2	2
Mean value, %	1,4	8,1	0,3
Standard deviation of repeatability (s_r)	0,20	0,11	0,05
Repeatability limit (r)	0,55	0,31	0,15
Repeatability coefficient of variation, %	13,81	1,37	15,51
Standard deviation of reproducibility (s_R)	0,22	0,25	0,10
Reproducibility limit (R)	0,61	0,69	0,29
Reproducibility coefficient of variation, %	15,22	3,03	30,87

Table A.7 — Statistical results for the individual sterol content: $\Delta 7$ -Campesterol

Sample	Olive oil	Sunflower-seed oil	Rapeseed oil
Level, %	0,1	1	0,5
Number of participating laboratories	14	14	14
Number of acceptable results	13	14	14
Number of individual test results of all laboratories on each sample	2	2	2
Mean value, %	0,1	1,6	0,3
Standard deviation of repeatability (s_r)	0,11	0,15	0,05
Repeatability limit (r)	0,31	0,43	0,15
Repeatability coefficient of variation, %	81,11	9,38	17,40
Standard deviation of reproducibility (s_R)	0,21	0,91	0,15
Reproducibility limit (R)	0,59	2,56	0,43
Reproducibility coefficient of variation, %	156,63	56,21	50,10

Table A.8 — Statistical results for the individual sterol content: $\Delta 5,23$ -Stigmastadienol

Sample	Olive oil	Sunflower-seed oil	Rapeseed oil
Level, %	0,5	1	0,5
Number of participating laboratories	14	14	14
Number of acceptable results	14	14	14
Number of individual test results of all laboratories on each sample	2	2	2
Mean value, %	0,7	1,2	0,5
Standard deviation of repeatability (s_r)	0,07	0,14	0,06
Repeatability limit (r)	0,20	0,39	0,16
Repeatability coefficient of variation, %	9,63	11,64	11,94
Standard deviation of reproducibility (s_R)	0,57	0,94	0,15
Reproducibility limit (R)	1,59	2,62	0,42
Reproducibility coefficient of variation, %	77,37	78,57	31,69

Table A.9 — Statistical results for the individual sterol content: Clerosterol

Sample	Olive oil	Sunflower-seed oil	Rapeseed oil
Level, %	0,2	< 0,1	0,1 to 0,5
Number of participating laboratories	14	14	14
Number of acceptable results	13	10	12
Number of individual test results of all laboratories on each sample	2	2	2
Mean value, %	0,3	0,07	0,2
Standard deviation of repeatability (s_r)	0,04	0,022	0,03
Repeatability limit (r)	0,12	0,063	0,08
Repeatability coefficient of variation, %	17,02	34,400	16,50
Standard deviation of reproducibility (s_R)	0,37	0,101	0,20
Reproducibility limit (R)	1,03	0,284	0,56
Reproducibility coefficient of variation, %	142,20	155,968	114,56

Table A.10 — Statistical results for the individual sterol content: Sitosterol

Sample	Olive oil	Sunflower-seed oil	Rapeseed oil
Level, %	80	60	45
Number of participating laboratories	14	14	14
Number of acceptable results	12	13	14
Number of individual test results of all laboratories on each sample	2	2	2
Mean value, %	81,4	57,0	44,3
Standard deviation of repeatability (s_r)	0,88	0,41	0,22
Repeatability limit (r)	2,45	1,15	0,63
Repeatability coefficient of variation, %	1,08	0,72	0,51
Standard deviation of reproducibility (s_R)	1,81	1,42	0,73
Reproducibility limit (R)	5,07	3,99	2,05
Reproducibility coefficient of variation, %	2,23	2,50	1,65