INTERNATIONAL OLIVE COUNCIL

COI/ T.20/ Doc. no. 30
November 2011

ENGLISH
Original: ENGLISH

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METHOD OF ANALYSIS

DETERMINATION OF THE COMPOSITION AND CONTENT OF STEROLS AND TRITERPENE DIALCOHOLS BY CAPILLARY COLUMN GAS CHROMATOGRAPHY

1. SCOPE

The method describes a procedure for determining the individual and total sterols and triterpene dialcohols content of olive oils and olive pomace oils.

2. PRINCIPLE

The oils, with added α-cholestanol as an internal standard, are saponified with potassium hydroxide in ethanolic solution and the unsaponifiable matter are then extracted with ethyl ether.

The sterols and triterpene dialcohols fraction are separated from the unsaponifiable matter by thin-layer chromatography on a basic silica gel plate. The fractions recovered from the silica gel are transformed into trimethylsilyl ethers and then analysed by capillary column gas chromatography.

3. APPARATUS

The usual laboratory equipment and in particular the following:

3.1. 250 ml flask fitted with a reflux condenser with ground-glass joints.

3.2. 500 ml separating funnel.

3.3. 250 ml flasks.

3.4. Complete apparatus for analysis by thin-layer chromatography using 20 x 20 cm glass plates.

3.5. Ultraviolet lamp with a wavelength of 366 or 254 nm.

3.6. 100 µl and 500 µl microsyringes.

3.7. Cylindrical filter funnel with a G3 porous septum (porosity 15-40 µm) of diameter approximately 2 cm and a depth of 5 cm, suitable for filtration under vacuum with male ground-glass joint.

3.8. 50 ml vacuum conical flask with ground-glass female joint, which can be fitted to the filter funnel (3.7).

3.9. 10 ml test tube with a tapering bottom and a sealing glass stopper.

3.10 Gas chromatograph suitable for use with a capillary column with split injection system, consisting of:

3.10.1. A thermostatic chamber for columns capable of maintaining the desired temperature with an accuracy of ± 1ºC;

3.10.2. A temperature-adjustable injection unit with a persilanised glass vaporising element and split system;

3.10.3. A flame ionisation detector (FID);

3.10.4. Data acquisition system suitable for use with the FID detector (3.10.3.), capable of manual integration.

3.11. Fused-silica capillary column of length 20 to 30 m, internal diameter 0.25 to 0.32 mm, coated with 5 % Diphenyl - 95 % Dimethylpolysiloxane (SE-52 or SE-54 stationary phase or equivalent), to a uniform thickness between 0.10 and 0.30 µm.

3.12. Microsyringe, of 10 µl capacity, for gas chromatography, with cemented needle suitable for split injection.

3.13. Calcium dichloride desiccator.

4. REAGENTS

4.1. Potassium hydroxide minimum titre 85 %.

4.2. Potassium hydroxide ethanolic solution, approximately 2 N.

Dissolve 130 g of potassium hydroxide (4.1) with cooling in 200 ml of distilled water and then make up to one litre with ethanol (4.10). Keep the solution in well-stoppered dark glass bottles and stored max. 2 days.

4.3. Ethyl ether, for analysis quality.

4.4. Potassium hydroxide ethanolic solution, approximately 0.2 N.

Dissolve 13 g of potassium hydroxide (4.1) in 20 ml of distilled water and make up to one litre with ethanol (4.10).

4.5. Anhydrous sodium sulphate, for analysis quality.

4.6. Glass plates coated with silica gel, without fluorescence indicator, thickness 0.25 mm (commercially available ready for use).

4.7. Toluene, for chromatography quality.

4.8. Acetone, for chromatography quality.

4.9. n-Hexane, for chromatography quality.

4.10. Ethyl ether, for chromatography quality.

4.11. Ethanol of analytical quality.

4.12. Ethyl acetate of analytical quality.

4.13. Reference solution for thin-layer chromatography: cholesterol or phytosterols, and Erythrodiol 5% solution in Ethyl acetate (4.11).

4.14. 2,7-dichlorofluorescein, 0.2% in ethanolic solution. Make slightly basic by adding a few drops of 2 N alcoholic potassium hydroxide solution (4.2).

4.15. Anhydrous pyridine, for chromatography quality (see Note 5).

4.16. Hexamethyl disilazane of analytical quality.

4.17. Trimethylchlorosilane of analytical quality.

4.18. Sample solutions of sterol trimethylsilyl ethers.
To be prepared at the time of use from sterols and erythrodiol obtained from oils containing them.

4.19. α-cholestanol, purity more than 99% (purity must be checked by GC analysis).

4.20. α-cholestanol internal standard solution, 0.2% solution (m/V) in ethyl acetate (4.11).

4.21. Phenolphthalein solution, 10 g/L in ethanol (4.10).

4.22. Carrier gas: hydrogen or helium, gas-chromatographic purity.

4.23. Auxiliary gases: hydrogen, helium, nitrogen and air, of gas-chromatographic purity.

4.24. n-Hexane (4.9)/ethyl ether (4.10) mixture 65:35 (V/V).

4.25. Silylation reagent, consisting of a 9:3:1 (V/V/V) mixture of pyridine/hexamethyl disilazane/trimethylchlorosilane.

5. PROCEDURE

5.1. Preparation of the unsaponifiable matter.

5.1.1. Using a 500 µl microsyringe (3.6) introduce into the 250 ml flask (3.1) a volume of the α-cholestanol internal standard solution (4.20) containing an amount of cholestanol corresponding to approximately 10% of the sterol content of the sample. For example, for 5 g of olive oil sample add 500 µl of the α-cholestanol solution (4.20) and 1500 µl for olive-pomace oil. Evaporate until dryness with a gentle current of nitrogen in a warm water bath, after cooling the flask, weigh 5±0.01 g of the dry filtered sample into the same flask.

Note 1. Animal or vegetable oils and fats containing appreciable quantities of cholesterol may show a peak having a retention time identical to cholestanol. If this cases occurs that the sterol fraction will have to be analysed in duplicate with and without internal standard.

5.1.2. Add 50 ml of 2 N ethanolic potassium hydroxide solution (4.2) and some pumice, fit the reflux condenser and heat to gentle boiling until saponification takes place (the solution becomes clear). Continue heating for a further 20 minutes, then add 50 ml of distilled water from the top of the condenser, detach the condenser and cool the flask to approximately 30ºC.

5.1.3. Transfer the contents of the flask quantitatively into a 500 ml separating funnel (3.2) using several portions of distilled water (50 ml). Add approximately 80 ml of ethyl ether (4.10), shake vigorously for approximately 60 seconds, periodically releasing the pressure by inverting the separating funnel and opening the stopcock. Allow standing until there is complete separation of the two phases (Note 2).

Then draw off the soap solution as completely as possible into a second separating funnel. Perform two further extractions on the water-alcohol phase in the same way using 60 to 70 ml of ethyl ether (4.10).

Note 2: Any emulsion can be destroyed by adding small quantities of ethanol (4.11).

5.1.4. Combine the three ether extracts in one separating funnel containing 50 mL of water. Continue to wash with water (50 mL) until the wash water no longer gives a pink colour on the addition of a drop of phenolphthalein solution (4.21).

When the wash water has been removed, filter on anhydrous sodium sulphate (4.5) into a previously weighed 250 ml flask, washing the funnel and filter with small quantities of ethyl ether (4.10).

5.1.5. Evaporate the solvent by distillation on a rotary evaporator at 30 ºC under vacuum. Add 5mL of acetone and remove the volatile solvent completely in a gentle current of air. Dry the residue in the oven at 103±2 ºC for 15 min. Cool in desiccators and weigh to the nearest 0.1 mg.

5.2. Separation of the sterol and triterpene dialcohols fraction (erythrodiol + uvaol)

5.2.1. Preparation of the basic thin layer chromatography plates. Immerse the silica gel plates (4.6) about 4 cm in the 0.2 N ethanolic potassium hydroxide solution (4.5) for 10 seconds, then allow to dry in a fume cupboard for two hours and finally place in an oven at 100º C for one hour.

Remove from the oven and keep in a calcium chloride desiccator (3.13) until required for use (plates treated in this way must be used within 15 days).

Note 3: When basic silica gel plates are used to separate the sterol fraction there is no need to treat the unsaponifiable fraction with alumina. In this way all compounds of an acid nature (fatty acids and others) are retained on the spotting line and the sterols band is clearly separated from the aliphatic and triterpene alcohols band.

5.2.2. Place hexane/ethyl ether mixture (4.24) (Note 4) into the development chamber, to a depth of approximately 1 cm. Close the chamber with the appropriate cover and leave thus for at least half an hour, in a cool place, so that liquid-vapour equilibrium is established strips of filter paper dipping into the eluent may be placed on the internal surfaces of the chamber. This reduces developing time by approximately one-third and brings about more uniform and regular elution of the components.

Note 4: The developing mixture should have replaced for every test, in order to achieve perfectly reproducible elution conditions, alternative solvent 50:50 (V/V) n-hexane/ethyl ether may be used.

5.2.3. Prepare an approximately 5% solution of the unsaponifiable (5.1.5) in ethyl acetate (4.12) and, using the 100 µl microsyringe, depose 0.3 ml of the solution on a narrow and uniform streak on the lower end (2 cm) of the chromatographic plate (5.2.1). In line with the streak, place 2 to 3 µl of the material reference solution (4.13), so that the sterol and triterpene dialchols band can be identified after developing.

5.2.4. Place the plate in the developing chamber prepared as specified in 5.2.2. The ambient temperature should be maintained between 15 and 20ºC (Note 5). Immediately close the chamber with the cover and allow eluting until the solvent front reaches approximately 1 cm from the upper edge of the plate. Remove the plate from the developing chamber and evaporate the solvent in a flow of hot air or by leaving the plate for a short while, under a hood.

Note 5 . Higher temperature could worsen the separation.

5.2.5. Spray the plate lightly and uniformly with the 2,7-dichlorofluorescein solution (4.14) and then leave to dry. When the plate is observed under ultraviolet light, the sterols and triterpene dialcohols bands can be identified through being aligned with the spots obtained from the reference solution (4.13). Mark the limits of the bands along the edges of the fluorescence with a black pencil (see TLC plate figure 3).

5.2.6. By using a metal spatula, scrape off the silica gel of the marked area. Place the finely comminuted material removed into the filter funnel (3.7). Add 10 ml of hot ethyl acetate (4.12), mix carefully with the metal spatula and filter under vacuum, collecting the filtrate in the conical flask (3.8.) attached to the filter funnel.

Wash the residue in the flask three times with ethyl ether (4.3) (approximately 10 ml each time), collecting the filtrate in the same flask attached to the funnel, evaporate the filtrate to a volume of 4 to 5 ml, transfer the residual solution to the previously weighed 10 ml test tube (3.9), evaporate to dryness by mild heating, in a gentle flow of nitrogen, make up again using a few drops of acetone (4.8), evaporate again to dryness,

The residue contained in the test tube consists of the sterol and triterpene dialchols fractions.

5.3. Preparation of the trimethylsilyl ethers.

5.3.1. Add the silylation reagent (4.25) (Note 6), in the ratio of 50 µl for every milligram of sterols and triterpene dialcohols, in the test tube containing the sterol and triterpene fraction, avoiding any uptake of moisture (Note 7).

Note 6: Ready for use solutions are available commercially. Other silylation reagents, such as, for example, bistrimethylsilyl trifluor acetamide + 1% trimethylchlorosilane, which has to be diluted with an equal volume of anhydrous pyridine, are also available.
Pyridine can be replaced by the same amount of acetonitrile.

5.3.2. Stopper the test tube, shake carefully (without overturning) until the compounds are completely dissolved. Leave to stand for at least 15 minutes at ambient temperature and then centrifuge for a few minutes. The clear solution is ready for gas chromatographic analysis.

Note 7: The slight opalescence, which may form, is normal and does not cause any anomaly. The formation of a white flock or the appearance of a pink colour is indicative of the presence of moisture or deterioration of the reagent. If these occur the test must be repeated (only if hexamethyldisilazane/trimethylchlorosilane is used).

5.4 Gas chromatographic analysis.

5.4.1. Preliminary operations, capillary column conditioning.

5.4.1.1. Fit the column (3.11) in the gas chromatograph, by attaching the inlet end to the split injector and the outlet end to the detector.

Carry out general checks on the gas chromatograph unit (leaks from the gas circuits, detector efficiency, efficiency of the splitting system and recording system, etc.).

5.4.1.2. If the column is being used for the first time, it is recommended that it should be subjected to conditioning: passing a gentle flow of gas through the column itself, then switch on the gas chromatography unit and begin a gradual heating, up to a temperature of at least 20 ºC above the operating temperature (Note 8). Hold this temperature for at least two hours, then place the entire unit in operating mode (adjustment of gas flows and splitting, ignition of the flame, connection with the computing system, adjustment of the column, detector and injector temperature, etc.) and then record the signal with a sensitivity at least two times greater than that one intended for the analysis. The course of the base line must be linear, without peaks of any kind, and must not show drift.

A negative straight-line drift indicates leakage from the column connections; a positive drift indicates inadequate conditioning of the column.

Note 8: The conditioning temperature must always be at least 20ºC less than the maximum temperature specified for the stationary phase used.

5.4.2. Choice of operating conditions.

5.4.2.1 The operating conditions are as follows:

- Column temperature: 260 ± 5 ºC;

- Injector temperature: 280-300 ºC;

- Detector temperature: 280-300 ºC;

- Linear velocity of the carrier gas: helium 20 to 35 cm/s; hydrogen 30 to 50 cm/s;

- Splitting ratio: from 1:50 to 1:100;

- Instrument sensitivity: from 4 to 16 times the minimum attenuation;

- Recording sensitivity: 1 to 2 mV f.s.;

- Amount of substance injected: 0.5 to 1 µl of TMSE solution.

These conditions may be changed according to the characteristics of the column and gas chromatograph, so as to obtain chromatograms, which meet the following requirements:

- The retention time for the ß-sitosterol peak should be at 20 ± 5 min.;

- The campesterol peak should be: for olive oil (mean content 3 %) 20 ± 5 % of full scale; for soybean oil (average content 20 %) 80 ± 10 % of full scale;

- All the present sterols must be separated. In addition to being separated the peaks, they must also be completely resolved, i.e. the peak trace should return to the base line before leaving for the next peak. Incomplete resolution is, however, tolerated, provided that the peak at RRT 1.02 (Sitostanol) can be quantified using the perpendicular.

5.4.3. Analytical procedure

5.4.3.1 By using the 10 µl microsyringe, take 1 µl of hexane, draw in 0.5 µl of air and then 0.5 to 1 µl of the sample solution. Raise the plunger of the syringe further, so the needle is emptied. Push the needle through the membrane of the injector and after one to two seconds, inject rapidly, and then slowly remove the needle after around five seconds.

An automatic injector can be used as well.

5.4.3.2. Carry out the recording until the TMSE of the present triterpene dialcohols are completely eluted. The base line must continue to meet the requirements (5.4.1.2).

5.4.4. Peak identification

Identify individual peaks on the basis of retention times and by comparison with the mixture of sterol and triterpene dialcohols TMSE, analysed under the same conditions.

The sterols and triterpene dialcohols are eluted in the following order: cholesterol, brassicasterol, ergosterol, 24-methylen-cholesterol, campesterol, campestanol, stigmasterol, Δ7-campesterol, Δ5,23-stigmastadienol, clerosterol, ß-sistosterol, sitostanol, Δ5-avenasterol, Δ5,24-stigmastadienol, Δ7-stigmastenol, Δ7-avenasterol, erythrodiol and uvaol.

The retention times for ß-sitosterol, for SE-52 and SE-54 columns, are shown in Table 1.

Figures 1 and 2 show typical chromatograms for some oils.

5.4.5. Quantitative evaluation.

5.4.5.1 Calculate the areas of the α-cholestanol and the sterol and triterpene dialcohols peaks by using the computing system. Ignore peaks for any compound which are not included (ergosterol must not be calculated) among those listed in Table 1. The response factor for α-cholestanol should be considered equal to 1.

5.4.5.2 Calculate the concentration of each individual sterol, in mg/kg of fatty material, as follows:

$$\text{sterol } x = \frac{A_x \cdot m_s \cdot 1000}{A_s \cdot m}$$

where:

A_x = peak area for sterol x, in computing system counts.
A_s = area of the α-cholestanol peak, in computing system counts.
m_s = mass of added α-cholestanol, in milligrams;
m = mass of the sample used for determination, in grams.

6. EXPRESSION OF THE RESULTS

6.1. Report individual sterol concentrations as mg/kg of fatty material and their sum as "total sterols".

The composition of each of the individual sterols and of the erythrodiol and uvaol should be expressed to one decimal point.

Total sterol composition should be expressed without any decimal point.

6.2. Calculate the percentage of each individual sterol from the ratio of the relevant peak area to the total peak area for sterols:

$$\text{sterol x} = \frac{A_x}{\Sigma A} \cdot 100$$

where:

A_x = peak area for x;
ΣA = total peak area for sterols

6.3. Apparent b-sitosterol: Δ5-23-stigmastadienol + clerosterol + β-sitosterol + sitostanol + Δ5-avenasterol + Δ5-24-stigmastadienol.

6.4. Calculate the percentage of erythrodiol and uvaol:

$$\text{Erytrodiol +Uvaol} = \frac{\text{Er.} + \text{Uv.}}{\text{Er.} + \text{Uv.} + \Sigma A} \times 100$$

Where

ΣA = sum area for sterol in computing system counts.
Er = area of Erythrodiol in computing system counts.
Uv = area of Uvaol in computing system counts.

APPENDIX

Determination of the linear speed of the gas

With the gas chromatograph set to normal operating conditions, inject 1 to 3 µl of methane (or propane) and measure the time taken by the gas to pass through the column, from the time of injection to the time at which the peak appears (tM).

The linear speed in cm/s is given by L/tM, where L is the length of the column in centimetres and tM is the measured time, in seconds.

TABLE I

RELATIVE RETENTION TIMES FOR STEROLS

Peak	Identification		Relative retention Time	
			SE 54 column	SE 52 Column
1	Cholesterol	Δ-5-cholesten-3*β*-ol	0.67	0.63
2	Cholestanol	5*α*-cholestan-3*β*-ol	0.68	0.64
3	Brassicasterol	[24S]-24-methyl-Δ-5,22-cholestadien-3*β*-ol	0.73	0.71
*	Ergosterol	[24S] 24 methy Δ5-7-22 cholestatrien 3βol	0,78	0,76
4	24-methylene-cholesterol	24-methylene-Δ-5,24-cholestadien-3*β*-o1	0.82	0.80
5	Campesterol	(24R)-24-methyl-Δ-5-cholesten-3*β*-ol	0.83	0.81
6	Campestanol	(24R)-24-methyl-cholestan-3*β*-ol	0.85	0.82
7	Stigmasterol	(24S)-24-ethyl-Δ-5,22-cholestadien-3*β*-ol	0.88	0.87
8	Δ-7-campesterol	(24R)-24-methyl-Δ-7-cholesten-3*β*-ol	0.93	0.92
9	Δ-5,23-stigmastadienol	(24R,S)-24-ethyl-Δ-5,23-cholestadien-3*β*-ol	0.95	0.95
10	Clerosterol	(24S)-24-ethyl-Δ-5,25-cholestadien-3*β*-ol	0.96	0.96
11	*β*-sitosterol	(24R)-24-ethyl-Δ-5-cholesten-3*β*-ol	1.00	1.00
12	Sitostanol	24-ethyl-cholestan-3*β*-ol	1.02	1.02
13	Δ-5-avenasterol	(24Z)-24-ethylidene-Δ-cholesten-3*β*-ol	1.03	1.03
14	Δ-5-24-stigmastadienol	(24R,S)-24-ethyl-Δ-5,24-cholestadien-3*β*-ol	1.08	1.08
15	Δ-7-stigmastenol	(24R,S)-24-ethyl-Δ-7-cholesten-3*β*-ol	1.12	1.12
16	Δ-7-avenasterol	(24Z)-24-ethylidene-Δ-7-cholesten-3*β*-ol	1.16	1.16
17	Erythrodiol	5α olean-12en-3β28 diol	1,41	1,41
18	Uvaol	Δ12-ursen-3β28 diol	1,52	1,52

FIGURE 1

GAS CHROMATOGRAM OF THE STEROL AND TRITERPENE DIALCHOLS FRACTION OF A LAMPANTE OLIVE OIL
(spiked with internal standard)

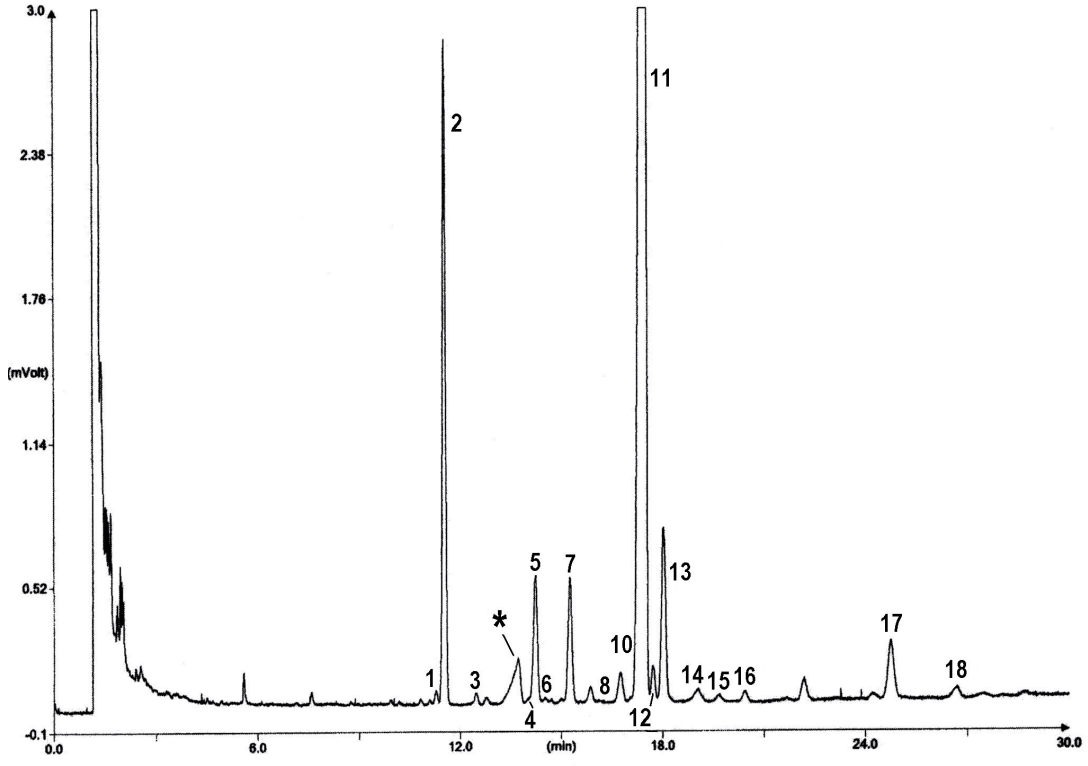


FIGURE 2

GAS CHROMATOGRAM OF THE STEROL AND TRITERPENE DIALCHOLS FRACTION OF A REFINED OLIVE OIL
(spiked with internal standard)

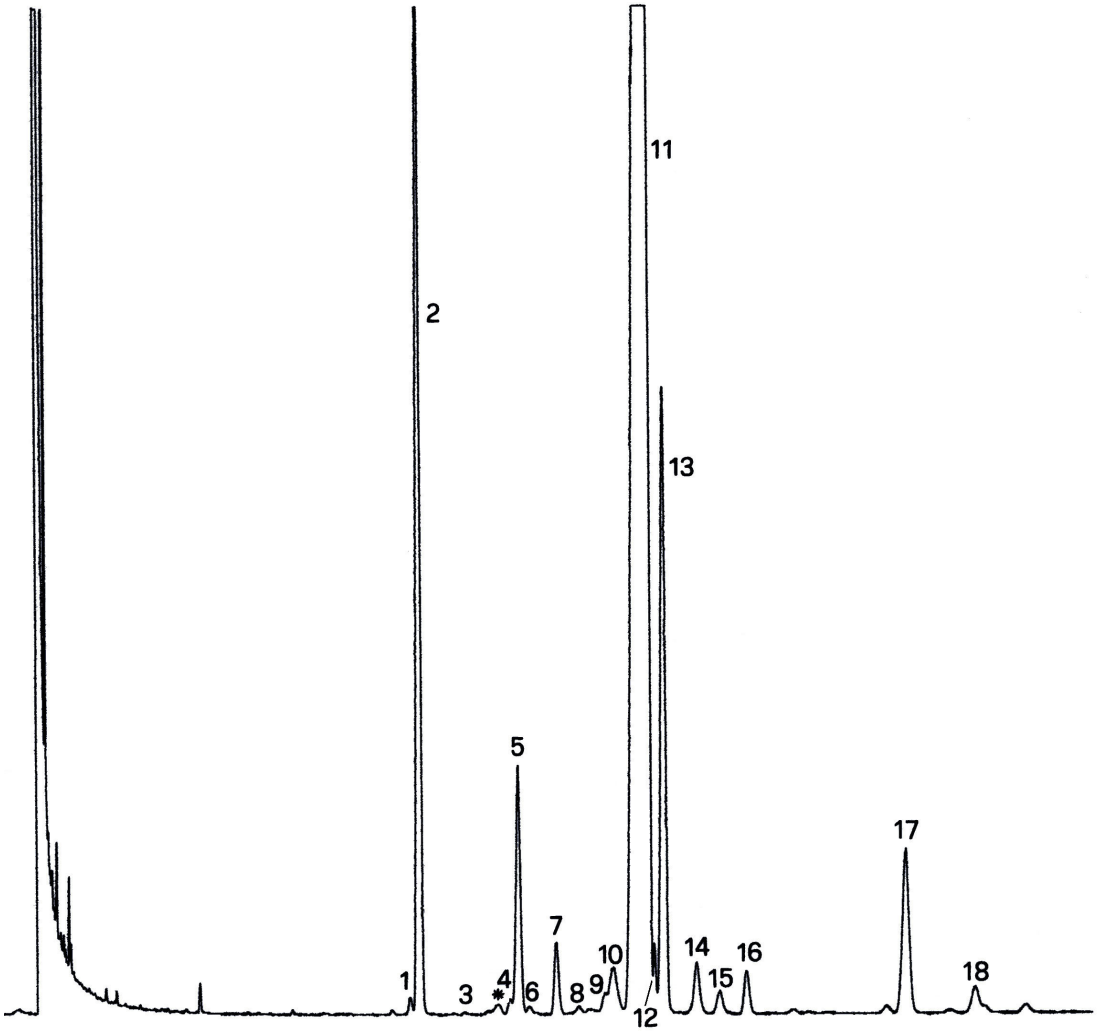
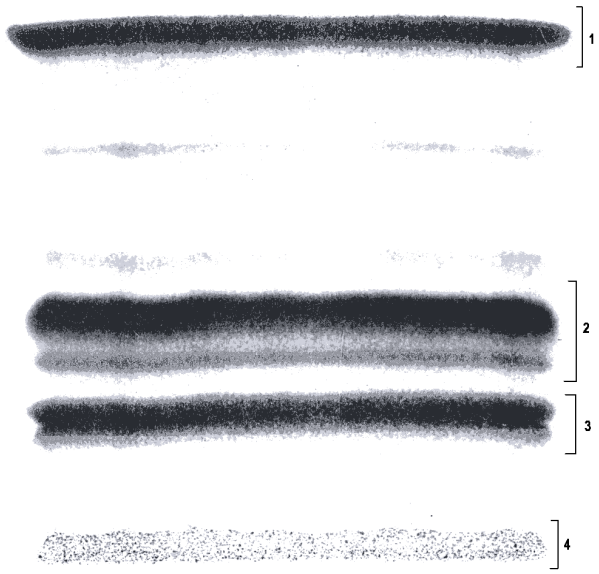


Figure 3

TLC plate olive pomace oil with the zone that should be scraped for sterols and triterpenic dialcohols determination.



Legenda:
1 – Squalene
2 – Triterpene and Aliphatic alcohols
3 – Sterols and Triterpenic dialcohols
4 – Start and free fatty acids

PRECISION VALUES OF THE METHOD FOR STEROLS

1. Analysis of the collaborative test results

The precision values of the method are given in the table overleaf.

Nineteen Laboratories took part in the collaborative test arranged by the Executive Secretariat in 2009.

The test was performed on five samples:

ST-1 Crude olive pomace oil.
ST-2 Refined olive pomace oil.
ST-3 Extra virgin olive oil.
ST-4 Blend of 20% high oleic sunfloweroil, 70% extra virgin olive oil and 10% rapeseed oil.
ST-5 Blend of 15% soybean oil and 85% olive oil.

The results of the collaborative test organised by the IOOC Executive Secretariat have been statistically processed according to the rules laid down in the international standards ISO 5725 **Accuracy (trueness and precision) of measurement methods and results.** Outliers were examined by applying Cochran's and Grubbs' test to the laboratory results for each determination (replicates a and b) and each sample.

The table lists:

n_p number of participating Laboratories

outliers number of laboratories with outlying values

mean mean of the accepted results

r value below which the absolute difference between two single independent test results obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time may be expected to lie with a probability of 95%

S_r Repeatability standard deviation

RDS_r (%) Repeatability coefficient of variation (S_r x 100/average)

R value below which the absolute difference between two single test results obtained with the same method on identical test material in different Laboratories with different operators using different equipment may be expected to lie with a probability of 95%

S_R Reproducibility standard deviation

RDS_R (%) Reproducibility coefficient of variation (S_R x 100/mean)

Sterol composition

Cholesterol

Sample	ST-1	ST-2	ST-3	ST-4	ST-5
No. participating laboratories, n_P	19	19	19	19	19
No. laboratories retained after eliminating outliers, n_p	19	14	16	18	18
No. test results in all labs, n_t	38	28	32	36	36
Mean (%)	0,13	0,13	0,13	0,16	0,21
Repeatability standard deviation, s_r	0,024	0,018	0,016	0,025	0,015
Coefficient of variation of repeatability, CV(r), %	18,8	14,0	12,3	15,1	7,2
Repeatability limit, r	0,07	0,05	0,04	0,07	0,04
Reproducibility standard deviation, s_R	0,041	0,023	0,038	0,047	0,058
Coefficient of variation of reproducibility, CV(R), %	31,9	17,8	29,5	29,1	27,7
Reproducibility limit, R	0,12	0,07	0,11	0,13	0,16

Brassicasterol

Sample	ST-1	ST-2	ST-3	ST-4	ST-5
No. participating laboratories, n_P	19	19	19	19	19
No. laboratories retained after eliminating outliers, n_p	19	15	17	19	18
No. test results in all labs, n_t	38	30	34	38	36
Mean (%)	0,05	0,02	0,000	1,46	0,02
Repeatability standard deviation, s_r	0,013	0,004	----	0,039	0,007
Coefficient of variation of repeatability, CV(r), %	25,9	21,1	----	2,7	32,7
Repeatability limit, r	0,04	0,01	----	0,11	0,02
Reproducibility standard deviation, s_R	0,039	0,020	----	0,052	0,024
Coefficient of variation of reproducibility, CV(R), %	75,1	115,2	----	3,6	107,2
Reproducibility limit, R	0,11	0,06	----	0,15	0,07

Campesterol

Sample	ST-1	ST-2	ST-3	ST-4	ST-5
No. participating laboratories, n_P	19	19	19	19	19
No. laboratories retained after eliminating outliers, n_p	18	18	17	18	18
No. test results in all labs, n_t	36	36	34	36	36
Mean (%)	3,22	3,13	2,98	10,74	6,99
Repeatability standard deviation, s_r	0,044	0,045	0,029	0,100	0,061
Coefficient of variation of repeatability, CV(r), %	1,3	1,4	1,0	1,0	0,9
Repeatability limit, r	0,12	0,13	0,08	0,28	0,17
Reproducibility standard deviation, s_R	0,085	0,087	0,086	0,259	0,167
Coefficient of variation of reproducibility, CV(R), %	2,6	2,8	2,9	2,4	2,4
Reproducibility limit, R	0,24	0,24	0,24	0,73	0,47

Stigmasterol

Sample	ST-1	ST-2	ST-3	ST-4	ST-5
No. participating laboratories, n_P	19	19	19	19	19
No. laboratories retained after eliminating outliers, n_p	17	16	18	18	19
No. test results in all labs, n_t	34	32	36	36	38
Mean (%)	1,23	1,05	0,41	2,83	5,40
Repeatability standard deviation, s_r	0,027	0,040	0,046	0,043	0,087
Coefficient of variation of repeatability, CV(r), %	2,2	3,8	11,1	1,5	1,6
Repeatability limit, r	0,08	0,11	0,13	0,12	0,24
Reproducibility standard deviation, s_R	0,039	0,059	0,064	0,111	0,158
Coefficient of variation of reproducibility, CV(R), %	3,2	5,7	15,6	3,9	2,9
Reproducibility limit, R	0,11	0,17	0,18	0,31	0,44

Apparent β-Sitosterol

Sample	ST-1	ST-2	ST-3	ST-4	ST-5
No. participating laboratories, n_P	19	19	19	19	19
No. laboratories retained after eliminating outliers, n_p	16	17	17	18	17
No. test results in all labs, n_t	32	34	34	36	34
Mean (%)	93,9	93,8	95,2	78,6	84,8
Repeatability standard deviation, s_r	0,141	0,223	0,091	0,195	0,220
Coefficient of variation of repeatability, CV(r), %	0,15	0,24	0,10	0,25	0,26
Repeatability limit, r	0,4	0,6	0,3	0,6	0,6
Reproducibility standard deviation, s_R	0,355	0,471	0,340	1,372	0,819
Coefficient of variation of reproducibility, CV(R), %	0,38	0,50	0,36	1,75	1,00
Reproducibility limit, R	1,0	1,3	1,0	4,0	2,2

Δ7-Stigmastenol

Sample	ST-1	ST-2	ST-3	ST-4	ST-5
No. participating laboratories, n_P	19	19	19	19	19
No. laboratories retained after eliminating outliers, n_p	18	19	19	17	18
No. test results in all labs, n_t	36	38	38	34	36
Mean (%)	0,70	0,94	0,27	3,52	1,10
Repeatability standard deviation, s_r	0,028	0,056	0,025	0,090	0,040
Coefficient of variation of repeatability, CV(r), %	4,1	6,0	9,5	2,6	3,6
Repeatability limit, r	0,08	0,16	0,07	0,25	0,11
Reproducibility standard deviation, s_R	0,086	0,084	0,067	0,171	0,087
Coefficient of variation of reproducibility, CV(R), %	12,3	8,9	25,3	4,9	7,9
Reproducibility limit, R	0,24	0,24	0,19	0,48	0,24

Triterpenic dialcohols (Erythrodiol + Uvaol)

Sample	ST-1	ST-2	ST-3	ST-4	ST-5
No. participating laboratories, n_P	19	19	19	19	19
No. laboratories retained after eliminating outliers, n_p	17	15	19	18	15
No. test results in all labs, n_t	34	30	38	36	30
Mean (mg/kg)	22,38	27,17	1,80	1,06	2,9
Repeatability standard deviation, s_r	0,214	0,333	0,215	0,162	0,108
Coefficient of variation of repeatability, CV(r), %	1,0	1,2	11,9	15,3	3,8
Repeatability limit, r	0,60	0,93	0,60	0,46	0,30
Reproducibility standard deviation, s_R	1,028	1,257	0,450	0,342	0,210
Coefficient of variation of reproducibility, CV(R), %	4,6	4,6	25,0	32,2	7,3
Reproducibility limit, R	2,88	3,52	1,26	0,96	0,59

Total sterol content

Sample	ST-1	ST-2	ST-3	ST-4	ST-5
No. participating laboratories, n_P	18	18	18	18	18
No. laboratories retained after eliminating outliers, n_p	17	18	18	17	17
No. test results in all labs, n_t	34	36	36	34	34
Mean (mg/kg)	4487,0	3169,8	1359,8	2066,5	1552,1
Repeatability standard deviation, s_r	71,76	49,15	45,19	30,14	31,75
Coefficient of variation of repeatability, CV(r), %	1,6	1,6	3,3	1,5	2,1
Repeatability limit, r	200,9	137,6	126,5	84,4	88,9
Reproducibility standard deviation, s_R	378,89	234,65	82,93	131,47	89,54
Coefficient of variation of reproducibility, CV(R), %	8,4	7,4	6,1	6,4	5,8
Reproducibility limit, R	1060,9	657,0	232,2	368,1	250,7

2. References

ISO 5725-1: 1994 Accuracy (trueness and precision) of measurement methods and results – Part 1: General principles and definitions

ISO 5725-2: 1994 Accuracy (trueness and precision) of measurement methods and results – Part 2: Basic method for the determination of the repeatability and reproducibility of a standard measurement method

ISO 5725-5: 1994 Accuracy (trueness and precision) of measurement methods and results – Part 5: Alternative methods for the determination of the precision of a standard measurement method

ISO 5725-6: 1994 Accuracy (trueness and precision) of measurement methods and results – Part 6: Use in practice of accuracy values