



METHOD OF ANALYSIS

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

1. SCOPE

The method describes a procedure for determining the individual and total sterols content of fatty substances.

2. PRINCIPLE OF THE METHOD

The fatty substance, with added α -cholestanol as an internal standard, is saponified with potassium hydroxide in ethanolic solution and the unsaponifiables are then extracted with ethyl ether.

The sterol fraction is separated from the unsaponifiable extract by chromatography on a basic silica gel plate. The sterols recovered from the silica gel are transformed into trimethyl-silyl ethers and are analyzed by capillary-column gas chromatography.

3. APPARATUS

- 3.1. 250 ml flask fitted with a reflux condenser having 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 having a wavelength of 366 or 254 nm.
- 3.6. 100 μ l and 500 μ l microsyringes.
- 3.7. A cylindrical filter funnel with a G3 porous septum porosity 15 to 40 μ m) of diameter approximately 2 cm and a depth of some 5 cm, with an attachment suitable for filtration under vacuum and a 12/21 male ground-glass joint.

- 3.8. 50 ml vacuum conical flask with a 12/21 ground-glass female joint which can be fitted to the filter funnel(3.7).
- 3.9. A 10 ml test tube with a tapering bottom and a sealing stopper
- 3.10 Gas chromatograph suitable for use with a capillary column, consisting of:
 - 3.10.1. a thermostatic chamber for columns capable of maintaining the desired temperature with an accuracy of $\pm 1^{\circ}\text{C}$;
 - 3.10.2. a temperature-adjustable injection unit with a persilanized glass vapourizing element and splitting system;
 - 3.10.3. a flame ionization detector and electrometer;
 - 3.10.4. an integrator-recorder suitable for use with the electrometer (3.10.3.) having a response time of not more than one second and a variable paper speed.
- 3.11. A glass or fused-silica capillary column of length 20 to 30 m, internal diameter 0.25 to 0.32 mm, entirely coated with SE-52 or SE-54 stationary phase or equivalent in a uniform thickness between 0.10 and 0.30 μm .
- 3.12. A 10 μl gas chromatography microsyringe with a hardened needle.

4. REAGENTS

- 4.1. Potassium hydroxide, approximately 2 N ethanolic solution. Dissolve 130 g of potassium hydroxide(minimum titre 85%) with cooling in 200 ml of distilled water and then make up to one litre with ethanol. Keep the solution in well-stoppered dark glass bottles (ethanol 95% V/V).
- 4.2. Ethyl ether, analytical purity.
- 4.3. Anhydrous sodium sulphate, analytical purity.
- 4.4. Glass plates coated with silica gel, without fluorescence indicator, thickness 0.25 mm (commercially available ready for use)
- 4.5. Potassium hydroxide, 0.2 N ethanolic solution. Dissolve 13 g of potassium hydroxide in 20 ml of distilled water and make up to one litre with ethanol.
- 4.6. Toluene, for chromatography. (See 5.2.2.)
- 4.7. Acetone, for chromatography. (See 5.2.2.)
- 4.8. Hexane, for chromatography. (See 5.2.2.)
- 4.9. Ethyl ether, for chromatography. (See 5.2.2.)

- 4.10. Chloroform, analytical purity. (See 5.2.2.)
- 4.11. Reference solution for thin-layer chromatography: cholesterol or phytosterols, 5% solution in chloroform.
- 4.12. 2,7-dichlorofluorescein, 0.2% ethanolic solution. Make slightly basic by adding a few drops of 2 N alcoholic potassium hydroxide solution.
- 4.13. Anhydrous pyridine, for chromatography.
- 4.14. Hexamethyl disilazane.
- 4.15. Trimethylchlorosilane.
- 4.16. Sample solutions of sterol trimethylsilyl ethers. To be prepared at the time of use from sterols obtained from oils containing them.
- 4.17. α -cholestanol, 0.2% solution (m/V) in chloroform (internal standard).
- 4.18. Carrier gas: hydrogen or helium, gas-chromatographic purity.
- 4.19. Auxiliary gases:
 - hydrogen or helium, gas-chromatographic purity,
 - air, gas-chromatographic purity.

5. PROCEDURE

5.1. Preparation of the unsaponifiables

- 5.1.1. Using the 500 μ l microsyringe introduce into the 250 ml flask a volume of 0.2% α -cholestanol solution in chloroform (4.17) containing an amount of cholestanol corresponding to approximately 10% of the sterol content of the sample aliquot taken for the determination. For example, for 5 g of sample add 500 μ l of the 0.2% α -cholestanol solution in the case of an olive oil and 1500 μ l for seed oils or olive-pomace oil. Evaporate to dryness in a current of nitrogen and then weigh accurately 5 g of the dry filtered sample into the same flask. Animal or vegetable oils and fats containing appreciable quantities of cholesterol may show a peak having a retention time identical to cholestanol. If this occurs the sterol fraction will have to be analyzed in duplicate with and without internal standard.
- 5.1.2. Add 50 ml of 2 N ethanolic potassium hydroxide solution, fit the reflux condenser and heat to gentle boiling on a water bath with continuous vigorous stirring 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 30°C.

- 5.1.3. Transfer the contents of the flask quantitatively into a 500 ml separating funnel using several rinses of distilled water, amounting in all to about 50 ml. Add approximately 80 ml of ethyl ether, shake vigorously for approximately 30 seconds and allow to settle (Note 1).

Separate off the lower aqueous phase collecting it in 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 on each occasion.

Note 1: Any emulsion can be destroyed by adding small quantities of ethyl or methyl alcohol by means of a spray.

- 5.1.4. Pool the ether extracts into a single separating funnel and wash with distilled water (50 ml at a time) until the wash water gives a neutral reaction.

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

- 5.1.5. Distil the ether down to a few ml, then bring to dryness under a slight vacuum or in a current of nitrogen, completing drying in a stove at 100°C for approximately a quarter of an hour, and then weigh after cooling in a desiccator.

5.2. Separation of the sterol fraction

- 5.2.1. Preparation of the basic plates. Immerse the silica gel plates (4.4.) completely 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 a stove at 100 ° C for one hour.

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

Note 2: When basic silica gel plates are used to separate the sterol fraction there is no need to treat the unsaponifiables 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 a toluene-acetone mixture in the plate-developing chamber 95:5 (V/V) to a depth of approximately 1 cm. As an alternative a 65:35 (V/V) hexane/ethyl ether mixture may be used. Close the chamber with the appropriate cover and leave thus for at least half an hour 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 3: The developing mixture should be replaced for every test in order to achieve perfectly reproducible elution conditions.

- 5.2.3. Prepare an approximately 5% solution of the unsaponifiables (5.1.5) in chloroform and, using the 100 μ l microsyringe, streak a chromatographic plate (5.2.1) with 0.3 ml approximately 2 cm from one end in a streak which is as thin and as uniform as possible. In line with the streak place 2 to 3 μ l of the sterol reference solution (4.11.) at one end of the plate so that the sterol 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 20° C. Immediately close the chamber with the cover and allow to elute 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.
- 5.2.5. Spray the plate lightly and uniformly with the 2,7-dichlorofluorescein solution. When the plate is observed under ultraviolet light the sterol band can be identified through being aligned with the stain obtained from the reference solution. Mark the limits of the band along the edges of the fluorescence with a black pencil.
- 5.2.6. Using a metal spatula scrape off the silica gel in the marked area. Place the finely comminuted material removed into the filter funnel (3.7). Add 10 ml of hot chloroform, 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 (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, evaporate again to dryness, place in a stove at 105°C for approximately 10 minutes and then allow to cool in a desiccator and weigh.

The residue contained in the test tube consists of the sterol fraction.

5.3. Preparation of the trimethylsilyl ethers

- 5.3.1. Add the silylation reagent, consisting of a 9:3:1 (V/V/V) mixture of pyridine/hexamethyl disilazane/trimethyl chlorosilane (Note 4) in the ratio of 50 μ l for every milligram of sterols to the test tube containing the sterol fraction, avoiding any uptake of moisture (Note 5).

Note 4: Solutions which are ready for use are available commercially. Other silanizing reagents such as, for example, bistrimethylsilyl trifluor acetamide + 1% trimethyl chlorosilane, which has to be diluted with an equal volume of anhydrous pyridine, are also available.

- 5.3.2. Stopper the test tube, shake carefully (without overturning) until the sterols are completely dissolved. 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 5: The slight opalescence which may form is normal and does not cause any anomaly. The formation of a white floc or the appearance of a pink colour are indicative of the presence of moisture or deterioration of the reagent. If these occur the test must be repeated.

5.4 Gas chromatographic analysis.

5.4.1. Preliminary operations, column conditioning

5.4.1.1. Fit the column in the gas chromatograph, attaching the inlet end to the 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. Pass a gentle flow of gas through the column and then switch on the gas chromatography unit and begin gradual heating up to a temperature of at least 20 °C above the operating temperature (Note 6). 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 electronic recorder, adjustment of the column, detector and injector temperature, etc.) and then record the signal with a sensitivity at least two times greater than that intended for the analysis. The course of the base line must be linear, without peaks of any kind, and must not drift.

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

Note 6: The conditioning temperature must always be at least 20° C less than the maximum temperature specified for the stationary phase used.

5.4.2. Choice of operating conditions

5.4.2.1 The guideline operating conditions are as follows:

- column temperature: $260 \pm 5^{\circ}$ C,
- injector temperature: 280° C,
- detector temperature: 290° C,
- 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.,
- paper speed: 30 to 60 cm/hour,
- amount of substance injected: 0.5 to 1 μ l of TMSE solution .

These conditions may be varied in the light of column and gas-chromatograph characteristics so as to obtain chromatograms which meet the following requirements:

- the retention time for β -sitosterol should be 20 ± 5 min.,
- the campesterol peak should be: for olive oil (mean content 3%) $15 \pm 5\%$ of full scale; for soya oil (mean content 20%) $80 \pm 10\%$ of full scale,
- all the sterols present must be separated. In addition to being separated the peaks 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 TRR 1.02 can be quantified using the perpendicular.

5.4.3. Analytical procedure

5.4.3.1 Using the 10 μ l microsyringe take 1 μ l hexane, draw in 0.5 μ l of air and then 0.5 to 1 μ l 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, then slowly remove the needle after some five seconds.

5.4.3.2. Continue recording until the TMSE of the sterols present 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 mixture of sterol TMSE analyzed under the same conditions.

The sterols are eluted in the following order: cholesterol, brassicasterol, 24-methylene cholesterol, campesterol, campestanol, stigmasterol, Δ^7 - campesterol, $\Delta^5,23$ -stigmastadienol, clerosterol, β -sitosterol, sitostanol, Δ^5 -avenasterol, $\Delta^5, 24$ -stigmastadienol, Δ^7 -stigmastenol, Δ^7 -avenasterol.

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

Figures 1 and 2 illustrate typical chromatograms for some oils.

5.4.5. Quantitative evaluation

5.4.5.1 Calculate the areas of the α -cholestanol and the sterol peaks using the integrator. Ignore peaks for any compound which are not included among those listed in Table 1. The response coefficient for α -cholestanol is to be 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 square millimetres;

A_s = area of the α -cholestanol peak, in square millimetres;

m_s = mass of α -cholestanol added, in milligrams;

m = mass of the sample used for determination, in grams.

6. EXPRESSION OF THE RESULTS

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

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

$$\% \text{ of sterol x} = \frac{A_x}{\sum A} \cdot 1000$$

where:

A_x = peak area for x;

$\sum A$ = total peak area for sterols

APPENDIX

Determination of the linear velocity 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 (t_M).

The linear velocity in cm/s is given by L/t_M , where L is the length of the column in centimetres and t_M 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- 3β -ol	0,67	0,63
2	cholestanol	5α -cholestan- 3β -ol	0,68	0,64
3	brassicasterol	(24S)-24-methyl- $\Delta^5,22$ -cholestadien- 3β -ol	0,73	0,71
4	24-methylene-cholesterol	24-methylene- $\Delta^5,24$ -cholestadien- 3β -ol	0,82	0,80
5	campesterol	(24R)-24-methyl- Δ^5 -cholesten- 3β -ol	0,83	0,81
6	campestanol	(24R)-24-methyl-cholestan- 3β -ol	0,85	0,82
7	stigmasterol	(24S)-24-ethyl- $\Delta^5,22$ -cholestadien- 3β -ol	0,88	0,87
8	Δ^7 -campesterol	(24R)-24-methyl- Δ^7 -cholesten- 3β -ol	0,93	0,92
9	$\Delta^5,23$ -stigmastadienol	(24R,S)-24-ethyl- $\Delta^5,23$ -cholestadien- 3β -ol	0,95	0,95
10	clerosterol	(24S)-24-ethyl- $\Delta^5,25$ -cholestadien- 3β -ol	0,96	0,96
11	β -sitosterol	(24R)-24-ethyl- Δ^5 -cholesten- 3β -ol	1,00	1,00
12	sitostanol	24-ethyl-cholestan- 3β -ol	1,02	1,02
13	Δ^5 -avenasterol	(24Z)-24-ethylidene- Δ^5 -cholesten- 3β -ol	1,03	1,03
14	$\Delta^5,24$ -stigmastadienol	(24R,S)-24-ethyl- $\Delta^5,24$ -cholestadien- 3β -ol	1,08	1,08
15	Δ^7 -stigmastenol	(24R,S)-24-ethyl- Δ^7 -cholesten- 3β -ol	1,12	1,12
16	Δ^7 -avenasterol	(24Z)-24-ethylidene- Δ^7 -cholesten- 3β -ol	1,16	1,16

FIGURE 1
GAS CHROMATOGRAM OF THE STEROL FRACTION OF A CRUDE OLIVE OIL
(spiked with internal standard)

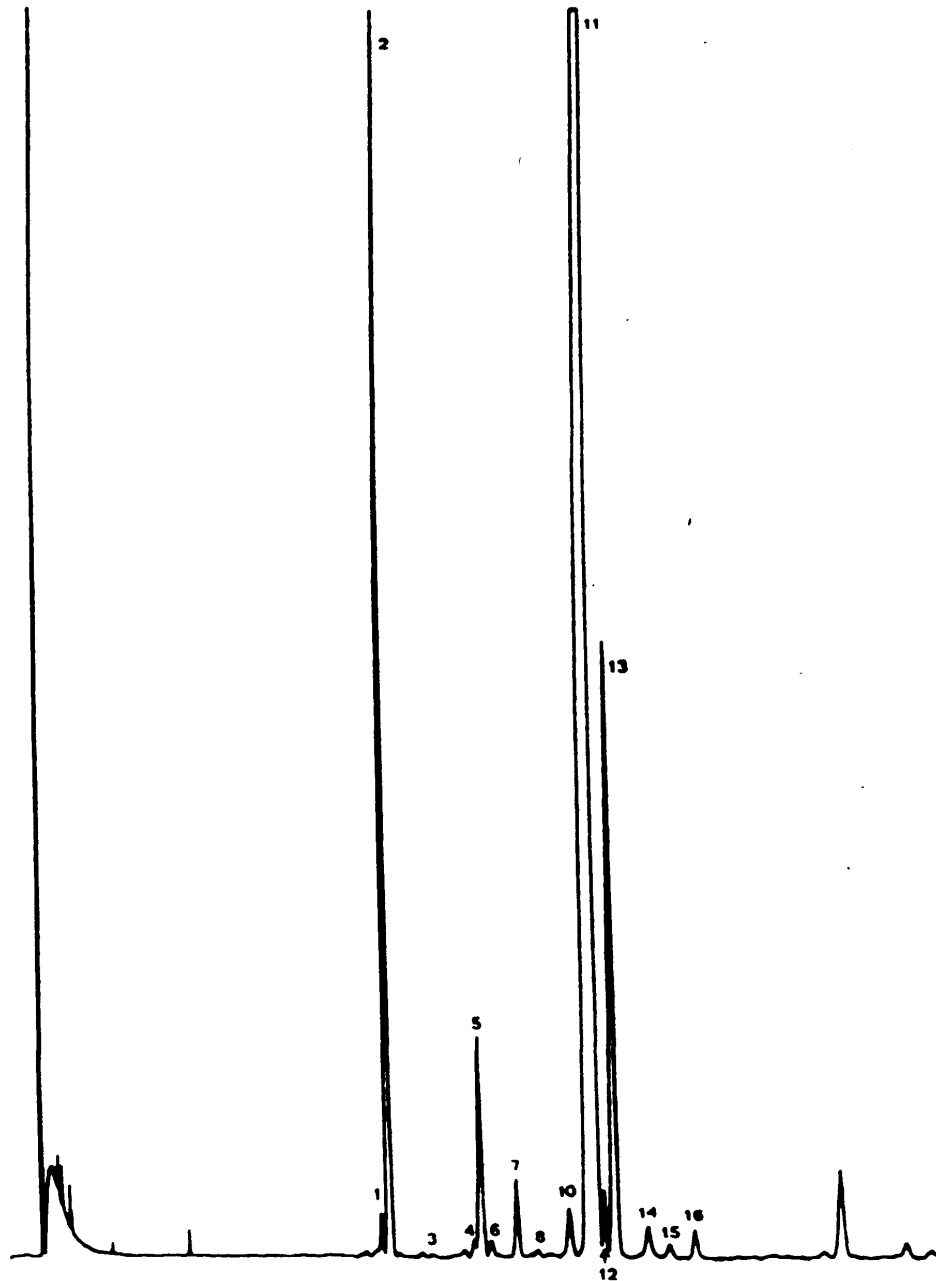
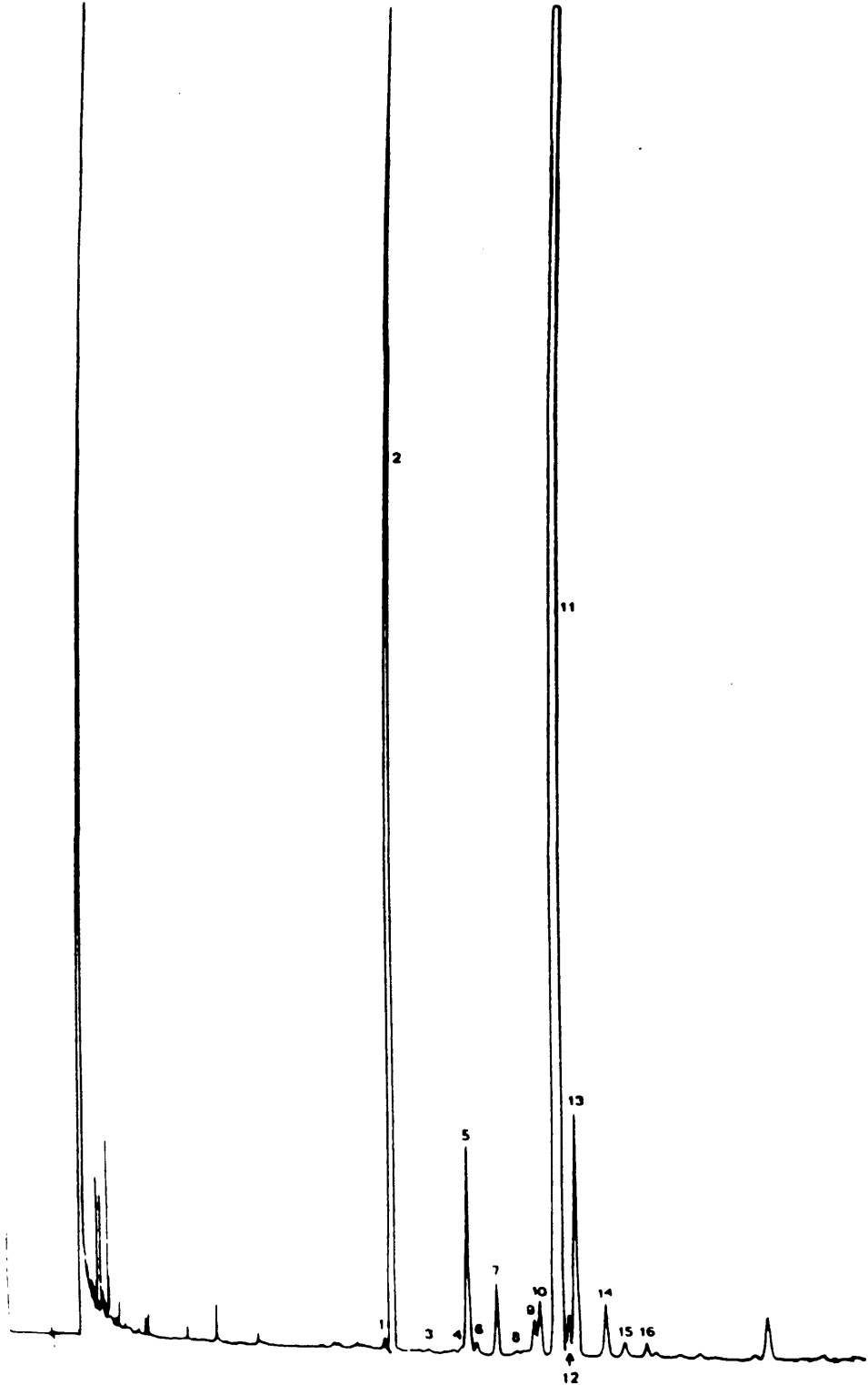


FIGURE 2
GAS CHROMATOGRAM OF THE STEROL FRACTION OF A REFINED OLIVE OIL
(spiked with internal standard)



PRECISION VALUES OF THE METHOD

1. Analysis of the collaborative test results

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

Nineteen laboratories holding IOOC recognition at the time took part in the collaborative test arranged by the Executive Secretariat in 1999. The laboratories were from eight countries.

The test was performed on five samples:

- A: extra virgin olive oil
- B: virgin olive oil + refined sunflower oil
- C: virgin olive oil + refined olive-pomace oil
- D: virgin olive oil + refined soybean oil + refined sunflower oil
- E: refined olive oil + refined olive-pomace oil + refined soybean oil + lampante virgin 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** 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 ($S_r \times 100/\text{mean}$)
- 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 ($S_R \times 100/\text{mean}$)

Total sterol content (mg/kg)

	A	B	C	D	E
n	19	19	19	19	19
outliers	5	3	4	5	3
mean	1547	1720	1618	1498	1578
r	85.59	75.06	57.00	52.32	60.83
S_r	30.57	26.81	20.35	18.68	21.72
RSD_r (%)	1.98	1.56	1.26	1.25	1.38
R	95.45	181.85	156.85	164.25	155.08
S_R	34.09	64.94	56.02	58.66	55.38
RSD_R(%)	2.20	3.78	3.46	3.92	3.51

Sterol composition (%)

- Cholesterol

	A	B	C	D	E
n	17	17	17	17	17
outliers	2	2	4	5	3
mean	0.18	0.19	0.16	0.14	0.16
r	0.09	0.09	0.04	0.06	0.04
S_r	0.03	0.03	0.01	0.02	0.01
RSD_r (%)	19.41	17.43	11.11	17.55	10.53
R	0.21	0.23	0.13	0.13	0.15
S_R	0.07	0.08	0.05	0.04	0.05
RSD_R(%)	43.43	42.94	31.82	35.28	34.15

Sterol composition

- Brassicasterol

	A	B	C	D	E
n	17	17	17	17	17
outliers	4	1	0	1	2
mean	0	0.01	0.01	0.02	0.02
r	-	0.01	0.02	0.03	0.01
S_r	-	0.00	0.01	0.01	0
RSD_r (%)	-	75.59 _(not sig.)	83.24 _(not sig.)	73.17 _(not sig.)	30.79 _(not sig.)
R	-	0.05	0.05	0.05	0.06
S_R	-	0.01	0.01	0.01	0.02
RSD_R(%)	-	209.76 _(not sig.)	166.95 _(not sig.)	115.48 _(not sig.)	116.35 _(not sig.)

- Campesterol

	A	B	C	D	E
n	15	15	15	15	15
outliers	0	0	0	0	0
mean	4.05	4.95	3.99	3.95	4.29
r	0.14	0.20	0.20	0.15	0.12
S_r	0.05	0.07	0.07	0.05	0.04
RSD_r (%)	1.29	1.45	1.82	1.43	1.03
R	0.26	0.34	0.27	0.19	0.29
S_R	0.09	0.12	0.09	0.07	0.10
RSD_R(%)	2.30	2.51	2.49	1.79	2.42

- Stigmasterol

	A	B	C	D	E
n	15	15	15	15	15
outliers	1	0	0	1	0
mean	1.03	2.27	1.06	1.39	2.35
r	0.05	0.12	0.09	0.08	0.15
S_r	0.02	0.04	0.03	0.03	0.05
RSD_r (%)	1.90	1.94	30.6	2.16	2.32
R	0.11	0.19	0.13	0.11	0.20
S_R	0.04	0.07	0.04	0.04	0.07
RSD_R(%)	3.87	3.13	4.64	2.93	3.12

- Delta- 7- stigmastenol

	A	B	C	D	E
n	15	15	15	15	15
outliers	0	0	0	1	0
mean	0.14	2.25	0.19	0.51	0.56
r	0.09	0.16	0.08	0.09	0.08
S_r	0.03	0.06	0.02	0.03	0.03
RSD_r (%)	24.67	2.69	15.30	6.37	5.32
R	0.12	0.26	0.11	0.11	0.13
S_R	0.04	0.09	0.04	0.04	0.04
RSD_R(%)	30.66	4.25	22.57	7.89	8.48

- Apparent beta-sitosterol

	A	B	C	D	E
n	15	15	15	15	15
outliers	0	1	0	0	0
mean	93.88	88.7	93.93	93.23	91.63
r	0.27	0.38	0.36	0.29	0.33
S_r	0.10	0.13	0.13	0.10	0.12
RSD_r(%)	0.11	0.15	0.14	0.11	0.13
R	0.87	0.91	0.70	0.90	0.89
S_R	0.31	0.32	0.25	0.32	0.32
RSD_R(%)	0.33	0.37	0.27	0.35	0.35

2. Normative 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