METHOD OF ANALYSIS

DETERMINATION OF STERENES IN REFINED VEGETABLE OILS

1. PURPOSE

Determination of sterenes (campestadienes and stigmastadienes), the hydrocarbons originated from sterols during refining or desterolising treatments applied to vegetable oils.

2. SCOPE

The standard may be used to detect desterolised seed oils in refined olive oil, refined olive-pomace oil and admixtures of these oils with virgin olive oil. The procedure may be also used to quantify stigmastadienes in oils where they occur at a concentration greater than 4.0 mg/kg.

3. PRINCIPLE

Isolation of steroidal hydrocarbon fraction by column chromatography on silica gel impregnated with silver nitrate, and analysis by capillary gas chromatography.

4. APPARATUS

4.1   100 ml round-bottomed flasks.

4.2   500 ml round-bottomed flasks.

4.3   25 ml Erlenmeyer flasks.

4.4   Desiccator containing activated silica gel.

4.5   Rotary evaporator.

4.6   Glass chromatography column (1.5-2.0 cm i.d. by 50 cm length) with Teflon stopcock and a plug of glass wool fibre or sintered glass disc at the bottom. To prepare the silica gel column, pour elution liquid (5.3) into the column to a depth of approximately 5 cm and then fill with a slurry of silver-impregnated silica gel (5.7) in elution liquid (20 g in 40 ml) with the help of portions of elution liquid. Allow to settle and finish settling by applying slight vibration. Add anhydrous sodium sulphate to a height of approximately 0.5 cm, finally elute the excess hexane. Keep the silver-impregnated silica gel away from light by wrapping the column in black paper. Light exposure decreases sterene recovery.

4.7   Gas chromatograph with flame ionisation detector, split injector (or cold on-column) and oven programmable to within ±1 °C.

4.8   Fused silica capillary column for gas chromatography (0.25 mm i.d. by 25 m length) coated with 5%-phenylmethylsilicone phase, 0.25 µm film thickness.

Note 1   Other columns of similar or lower polarity can be used.

4.9   Integrator-recorder with possibility of valley-valley integration mode.

4.10   5-10 µl microsyringe for gas chromatography with hardened needle.

5.   REAGENTS

All the reagents should be of analytical grade unless otherwise specified. The water used should be distilled water, or water of at least equivalent purity. In order to check the purity of the reagents, a blank assay should be done.

5.1   Hexane or mixture of alkanes of b.p. interval 65-70°C, distilled with rectifying column.

5.2   Absolute ethanol.

5.3   Elution liquid for column chromatography. Mixture of hexane or alkanes (5.1) with absolute ethanol at 0.2%.

5.4   Silver nitrate.

5.5   Anhydrous sodium sulphate.

5.6   Silica gel 60 for column chromatography, 70-230 mesh (Merck ref. 7734 or similar), heated in oven at 110°C during two or more hours and allowed to cool in desiccator (4.4).

5.7   Preparation of silver-impregnated silica gel for two columns.

5.8

In a 500-ml round-bottomed flask (4.2) wrapped in black paper, weigh 30 g of activated silica gel (5.6). In an 25-ml Erlenmeyer flask (4.3), dissolve 3.0 g of silver nitrate in 7 ml of distilled water, and with a pipette add the solution drop by drop onto the silica gel, shaking from time to time. Put the stopper on the flask and shake vigorously for 20 s, place the flask in the rotary evaporator and rotate for 30 min under atmospheric pressure and room temperature. The silica gel turns slightly grey and can be kept in stoppered flask in the dark.

5.8   Stock solution (200 ppm) of cholesta-3,5-diene (Sigma) in hexane (10 mg in 50 ml).

5.9   Standard solution of cholesta-3,5-diene in hexane at a concentration of 20 ppm, obtained by dilution of the stock solution (5.8).

Note 2   If kept at under 4°C, solutions 5.8 and 5.9 will not deteriorate over a period of at least 4 months.

5.10   Solution of n-nonacosane in hexane at a concentration of approx. 100 ppm.

5.11   Carrier gas for chromatography: helium or hydrogen of 99.9990% purity.

5.12   Auxiliary gases for flame ionisation detector: hydrogen of 99.9990% purity and purified air.

6.   PROCEDURE

6.1   Separation of steroidal hydrocarbon fraction.

6.1.1   Weigh 1 ± 0.01 g of oil in a 10-ml beaker, add 1 ml of standard solution of cholesta-3,5-diene (20 μg), take the mixture to the fractionating column with the aid of two 1-ml portions of hexane and run the sample onto the column by allowing the solution level to drop to the top of the sodium sulphate.

6.1.2   Start the chromatographic elution with the elution liquid (5.3) at the flow rate of 1 ml/min approx. Discard the first 40 ml of elution and then collect the next 70 ml and, separately, the following 20 ml.

Note 3 Optimisation of the volumes of the first and second fractions can be done by examination of the gas chromatograms obtained from each fraction. The first fraction must contain saturated hydrocarbons, and does not contain cholestadiene; the second fraction must show steroidal hydrocarbons but not saturated ones and the third fraction should not contain sterenes (Figure 1).

Note 4. At the end of the chromatographic process, the column should be drained and the dry silver-impregnated silica gel poured into a container and stored for silver recycling.

6.1.3   Transfer the second fraction to a 100-ml round-bottomed flask (4.1) and evaporate in a rotary evaporator at 30°C under reduced pressure until dryness and immediately dissolve the residue in 0.2 ml of elution liquid. Keep the solution in the refrigerator until analysis.

Note 5. The residue (6.1.3) should not be kept dry at room temperature. As soon as it is obtained, the solvent should be added and the solutions should be kept in the refrigerator.

6.2   Gas chromatography.

6.2.1   Working conditions for split injector.
   -   Injector temperature: 300°C.
   -   Detector temperature: 320°C.
   -   Oven programming temperatures: initial 235°C for 6 min and then rising at 2°C/min up to 285°C.
   -   Split injector with 1:15 flow divider.
   -   Amount of solution injected 2 μl.
   -   Sensitivity: about 16 times the minimum attenuation.
   -   Gas carrier: helium or hydrogen at about 120 and 80 kPa of pressure, respectively.
   -   Integrator-recorder. The parameters for integration should be fixed so as to give a correct assessment of the areas.

These conditions should be adjusted in accordance with the characteristics of the chromatograph and the column to give chromatograms meeting the following requirements: internal standard peak should appear within approx. ± 3 min of the time given in 6.2.2; the internal standard peak should be at least 80% of the full scale.

The gas chromatographic system must be checked by injecting a mixture of the stock solution of cholestadiene (5.8) and n-nonacosane solution (5.10). The cholesta-3,5-diene peak must appear as a resolved peak just before the n-nonacosane (**Figure 2**); if it does not, two steps can be taken: change the oven temperature and/or use a less polar column. Under these conditions, the peaks of saturated hydrocarbons do not interfere with the sterene peaks (**Figure 2**).

6.2.2   Peak identification.

Using helium as carrier gas, the internal standard peak appears at approx. 19 min and that of the stigmasta-3,5-diene at a relative retention time of approx. 1.28 (**Figure 1b**). The campesta-3,5-diene appears at a retention time of approx. 1.15 relative to the standard and approx. 0.90 relative to stigmasta-3,5-diene.

Using hydrogen as carrier gas, the internal standard peak appears at approx. 15 min and that of the stigmasta-3,5-diene at a relative retention time of approx. 1.33. The campesta-3,5-diene appears at a retention time of approx. 1.18 relative to the standard and approx. 0.88 relative to stigmasta-3,5-diene.

Campesta-3,5-diene and stigmasta-3,5-diene could occur with small quantities of the respective 2,4-isomers. Usually, the two groups of compounds elute together as two

chromatographic peaks. Nevertheless, if the column is too polar or shows a high resolving power, the 2,4-isomer can appear as small peaks before and close to those of the respective 3,5-isomers (**Figure 3**). In order to ensure that the stigmastadienes are eluted as a single peak, it is advisable to replace the column by one which is either less polar or has a wider internal diameter.

Note 6. **Gas chromatographic references of these steroidal hydrocarbons can be obtained by analysing samples of refined palm or sunflower oils or of sunflower oil heated with 10% v/w of sulphuric acid:water (1:1) at 150°C for 5 min.**

6.2.3 Quantitative analysis.

The content in stigmastadienes is determined according to the formula:

$$\text{mg/kg of stigmastadienes} = \frac{A_s * M_c}{A_c * M_o}$$

where: A_s = area of peak corresponding to stigmastadienes.

A_c = area of internal standard

M_c = mass of standard added, in micrograms.

M_o = mass of oil taken, in grams.

The ratios between the hydrocarbons are calculated as follows:

$$R1 = \frac{\text{area of stigmastadienes peak}}{\text{area of campestadienes peak}}$$

Usually each pair of isomers originates a single chromatographic peak, but if the two isomers yield separated peaks, both areas have to be summed.

The ratios are valid only if the stigmastadiene peak reaches at least 50% of the full scale.

Note 7. If the quantity of stigmastadienes is greater than 50 ppm, the analysis should be repeated using a smaller oil sample in order to obtain a more exact quantification.
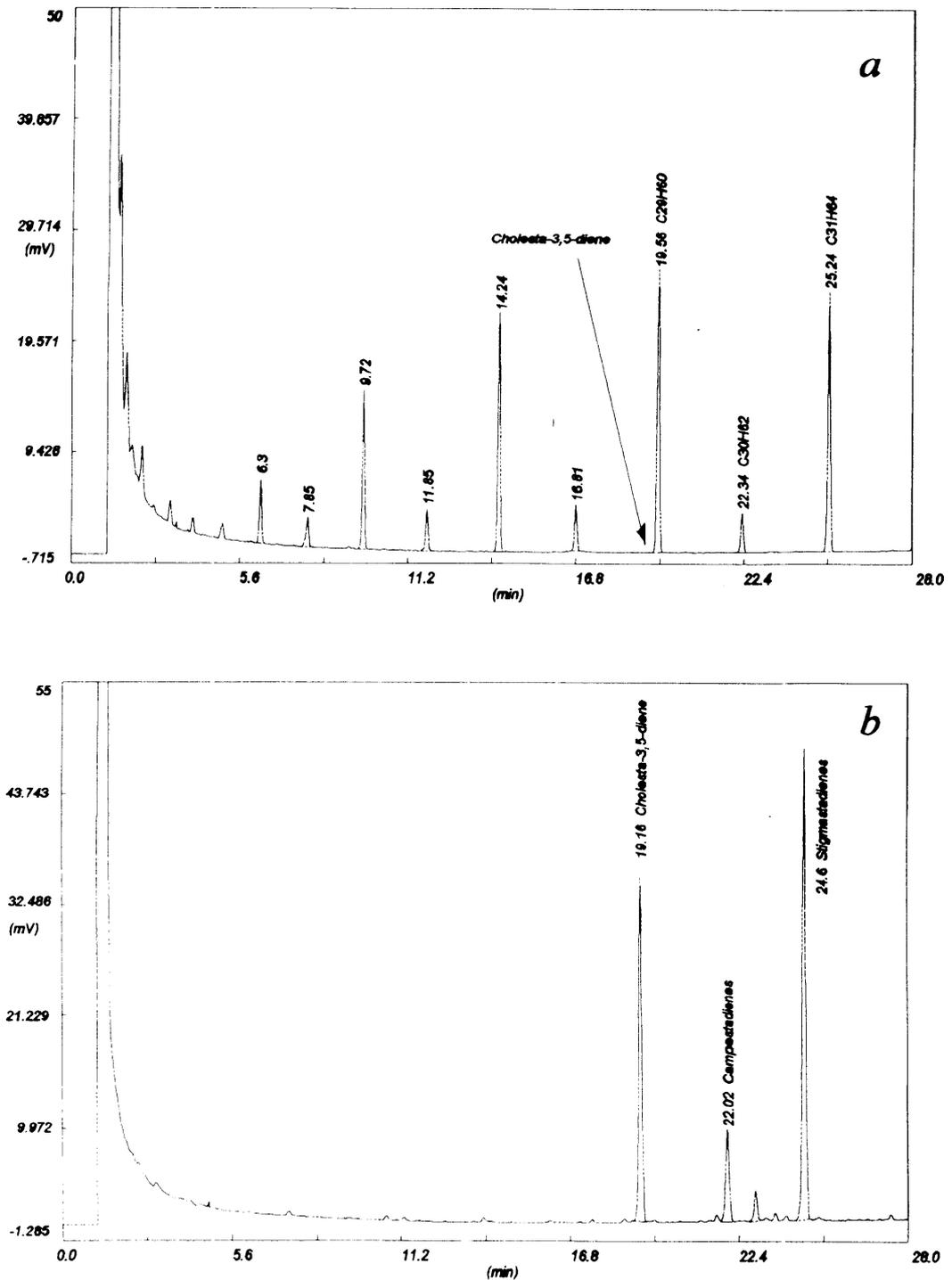
Figure 1. Gas chromatographic profiles of 1st and 2nd fractions obtained on a capillary column (0.25 mm i.d. by 25 m length) coated with 5%-phenylmethylsilicone (0.25 μm film thickness) using helium as carrier gas.
a). 1st fraction from an olive oil sample
b). 2nd fraction from a groundnut oil sample
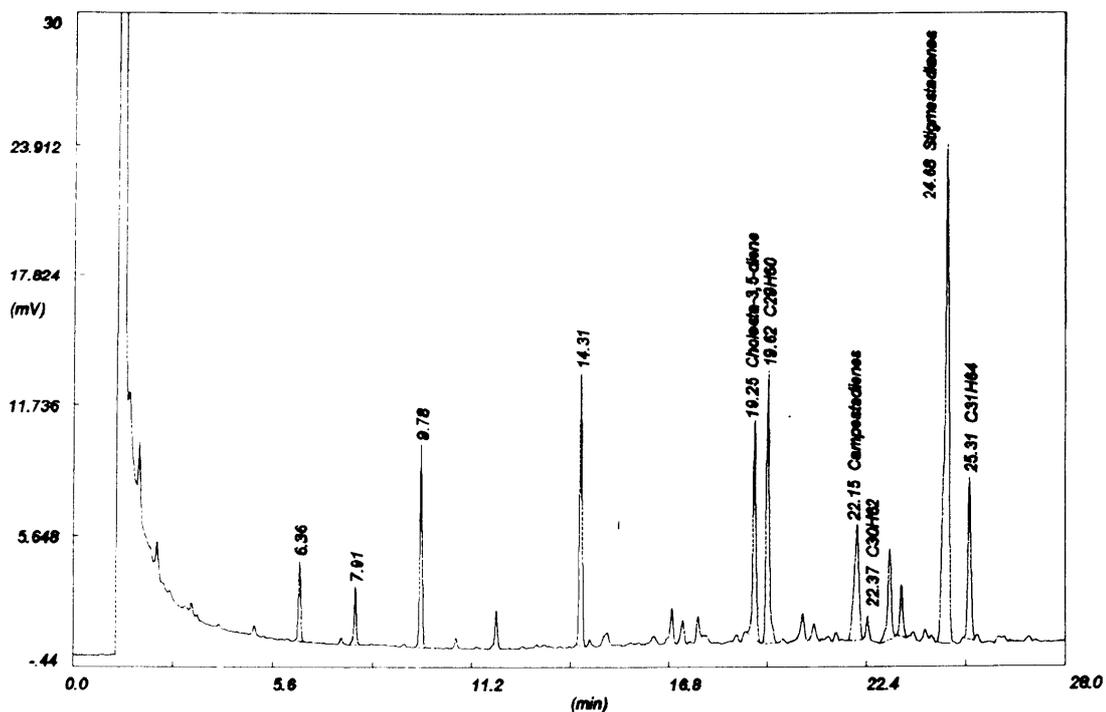
Figure 2. Gas chromatographic profile of 1st + 2nd fraction obtained from a mixture of refined olive oil and desterolised sunflower oil on a capillary column (0.25 mm i.d. by 25 m length) coated with 5%-phenylmethylsilicone (0.25 µm film thickness) using helium as carrier gas.
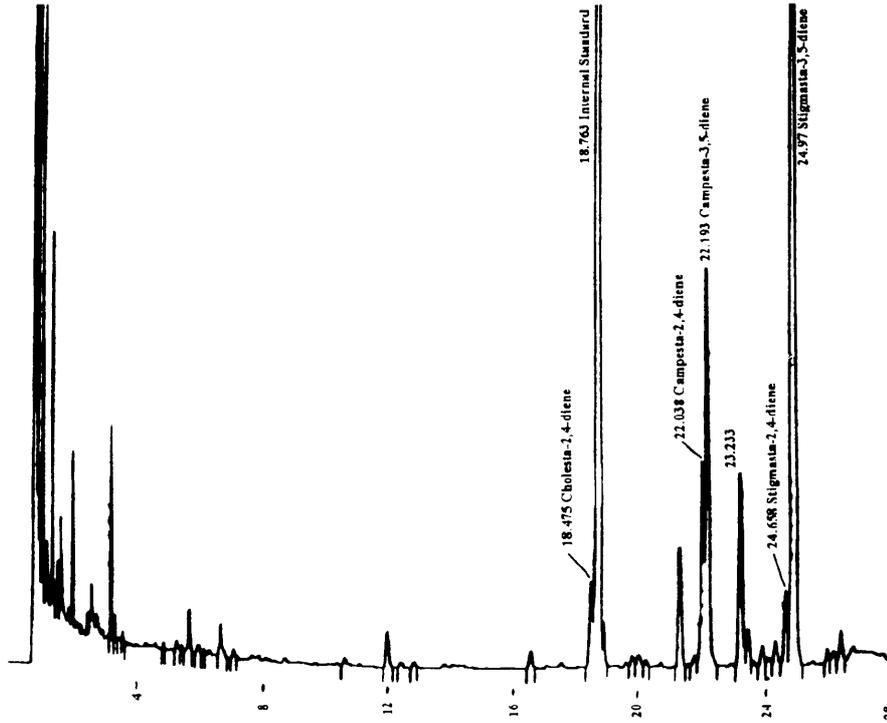
Figure 3. Gas chromatogram of the second fraction of the palm oil sample, obtained on a polar column (65%-phenylmethylsilicone) showing the sterene isomers.

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 x 100/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 x 100/mean)

R1 sterene ratio

	A	B	C	D	E
n			18		19
outliers			5		7
mean			19.96		15.86
r			1.67		1.25
S_r			0.59		0.45
RSD_r (%)			3.00		2.84
R			4.38		3.04
S_R			1.56		1.08
RSD_R(%)			7.85		6.86

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