INTERNATIONAL
OLIVE
COUNCIL

COI/T.20/Doc. No 11/Rev. 4
June 2021

ENGLISH
Original: ENGLISH

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METHOD OF ANALYSIS

DETERMINATION OF STIGMASTADIENES IN VEGETABLE OILS

1. PURPOSE

Determination of stigmastadienes in vegetable oils containing low concentrations of these hydrocarbons, particularly in virgin olive oils and crude olive pomace oil.

2. SCOPE

The standard may be applied to all vegetable oils although determinations are reliable only where the content of these hydrocarbons lies between 0.01 and 4.0 mg/kg. The method is particularly suited to detecting the presence of refined vegetable oils (olive, olive pomace, sunflower, soybean, palm, etc.) in virgin olive oil since refined oils contain stigmastadienes and virgin oils do not.

3. PRINCIPLE

Isolation of unsaponifiable matter. Separation of steroidal hydrocarbon fraction by column chromatography on silica gel and analysis by capillary gas chromatography.

The stigmastadienes can be analyzed using either the reference method of part A or the simplified method of part B. Both methods share the same principle, but the simplified method uses a reduced quantity of sample, reagents and solvents. In case of disputes the results obtained using the reference method should prevail.

PART A: REFERENCE METHOD

A.4. APPARATUS

A.4.1. 250 mL flat-bottom flasks with reflux condenser.

A.4.2. Separating funnels of 500 mL capacity.

A.4.3. 100 mL round-bottom flasks.

A.4.4. Rotary evaporator.

A.4.5. Glass chromatography column (1.5 cm i.d. by 50 cm length) with Teflon cock and a plug of glass wool fiber at the bottom. To prepare silica gel column, pour hexane into the chromatography column to a depth of approximately 5 cm and then fill with a slurry of silica gel in hexane (15 g in 40 mL) with the help of hexane portions. Allow to settle and finish settling by applying slight vibration. Add anhydrous sodium sulphate to a height of 0.5 cm, finally elute the excess hexane.

A.4.6. Gas chromatograph with flame ionization detector, split or on-column injector and oven programmable to within ± 1 ºC.

A.4.7. Fused silica capillary columns for gas chromatography (0.25 or 0.32 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.

A.4.8. Integrator-recorder with possibility of valley-valley integration mode.

A.4.9. 5-10 μL microsyringe for gas chromatography with cemented needle.

A.5. REAGENTS

All reagents should be of analytical grade unless otherwise specified. The water used should be distilled water, or water of at least equivalent purity.

A.5.1. Hexane or mixture of alkanes of b.p. interval 65-70 ºC, distilled with rectifying column. (Hexane may be replaced by iso-octane (2,2,4-trimethyl pentane in chromatography grade), provided that comparable precision values are achieved (see Precision values of the method with the used of isooctane), the residue after evaporation of 100 mL of solvent may be controlled. Solvents with higher boiling point than n-hexane take longer to evaporate. However, they are preferred due to the toxicity of hexane.)

Note 2. The hexane must be distilled to remove impurities.

A.5.2. 96 V/V ethanol.

A.5.3. Anhydrous sodium sulphate.

A.5.4. Alcoholic potassium hydroxide solution at 10% W/V. Add 10 mL of water to 50 g potassium hydroxide, stir, and then dissolve the mixture in ethanol to 500 mL.

Note 3. Alcoholic potash turns brown on standing. It should be prepared freshly each day and kept in well stoppered dark glass bottles.

A.5.5. Silica gel 60 for column chromatography, 70-230 mesh, (Merck ref. 7734 or similar).

Note 4. Usually, silica gel can be used directly from the original container without any treatment. However, some batches of silica may show low activity resulting in bad chromatographic separations. Under these circumstances, the silica gel should be treated in the following way: Deactivate the silica gel by heating for a minimum of 4 h at 550 ºC. After heating, place the silica gel in a desiccator while the gel is cooling and then transfer the silica gel to a stoppered flask. Add 2% of water and shake until no lumps can be seen and the powder flows freely.

If batches of silica gel result in chromatograms with interfering peaks, the silica gel should be treated as above. An alternative could be the use of extra pure silica gel 60 (Merck, ref. 7754).

A.5.6. Stock solution (200 mg/L) of cholesta-3,5-diene (Sigma, 99% purity) in hexane (10 mg in 50 mL).

A.5.7. Standard solution of cholesta-3,5-diene in hexane at concentration of 20 mg/L, obtained by dilution of above solution.

Note 5. if kept at under 4 ºC, solutions 5.6 and 5.7 will not deteriorate over a period of at least 4 months.

A.5.8. Carrier gas for chromatography: N-50 helium or hydrogen.

A.5.9. Auxiliary gases for flame ionization detector: N-50 hydrogen and purified air.

A.5.10 Solution of n-nonacosane in hexane at concentration of approx. 100 mg/L.

A.6. PROCEDURE

A.6.1. Preparation of unsaponifiable matter:

A.6.1.1. Weigh 20 ± 0.1 g of oil into a 250-mL flask, add 1 mL of the standard solution of cholesta-3,5-diene (20 µg) and 75 mL of alcoholic potash at 10%, fit reflux condenser, and heat to slight boiling for 30 min. Remove the flask containing the sample from the heat and allow the solution to cool slightly (do not allow to cool completely as the sample will set). Add 100 mL of water and transfer the solution to a decanting funnel with the aid of 100 mL of hexane. Shake the mixture vigorously for 30 s and leave to stratify.

Note 6. If an emulsion is produced, wait as it disappears rapidly or add small quantities of ethanol.

A.6.1.2. Transfer the aqueous phase beneath to a second separating funnel and extract again with 100 mL of hexane. Once more run off the lower phase and wash the hexane extracts (combined in another separating funnel) three times with 100 mL each time of a mixture of ethanol-water (1:1, V/V) until neutral pH is reached.

A.6.1.3. Pass the hexane solution through anhydrous sodium sulphate (50 g), wash with 20 mL of hexane and evaporate in a rotary evaporator at 30 ºC and low pressure until dryness.

A.6.2. Separation of steroidal hydrocarbon fraction:

A.6.2.1. Take the residue to the fractioning column with the aid of two 1-mL portions of hexane, run the sample onto the column by allowing the solution level to drop to the top of the sodium sulphate and start chromatographic elution with hexane at a flow rate of 1 mL/min. approx. Discard the first 25-30 mL of the elution and then collect the following 40 mL fraction.

Note 7. The first fraction contains saturated hydrocarbons (**Figure 1a**) and the second fraction the steroidal ones. Further elution provides squalene and related compounds. To achieve a good separation between saturated and steroidal hydrocarbons, the optimization of fraction volumes is required. For this, the volume of the first fraction should be adjusted so that when the second fraction is analyzed the peaks representing the saturated hydrocarbons are low (see **Figure 1c**); if they do not appear but the intensity of the standard peak is low, the volume should be reduced. Anyway, a complete separation between the components of the first and second fractions is unnecessary, as there is no overlapping of peaks during GC analysis. The optimization of the volume of the second fraction is generally not needed as a good separation exists with the further components. Nevertheless, the presence of a great peak at approx. 1.5 min lower retention time than the standard is due to squalene, and it is indicative of a bad separation.

A.6.2.2. Evaporate the second fraction in an evaporator at 30 ºC and low pressure until dryness, and immediately dissolve the residue in 0.2 mL of hexane. Keep the solution in the refrigerator until analysis.

Note 8. Residues A.6.1.3. and A.6.2.2 should not be kept dry and at room temperature. As soon as they are obtained, the solvent should be added and the solutions should be kept in the refrigerator.

A.6.3. Gas chromatography

A.6.3.1. Working conditions:

- Injector temperature: 300 ºC.
- Detector temperature: 320 ºC.
- Integrator-recorder: The parameters for integration should be fixed so as to give a correct assessment of the areas.
  Valley-valley integration mode is recommended.
- Sensitivity: About 16 times the minimum attenuation.
- Amount of solution injected: 1 µL.
- Oven programming temperatures: Initial 235 ºC for 6 min and then rising at 2 ºC/min up to 285 ºC.

- Injector with 1:15 flow divider.
- Carrier gas: Helium or hydrogen at about 120 kPa pressure.

These conditions may be adjusted in accordance with the characteristics of the chromatograph and the column to give chromatograms meeting the following requirements: internal standard peak within approx. 5 min. of the time given in A.6.3.2; 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 (A.5.6) and n-nonacosane solution (A.5.10). The cholesta-3,5-diene peak has to appear before the n-nonacosane (**Figure 1c**); if this does not occur, two steps can be taken: to bring down the initial oven temperature or to replace the GC-column by a less polar one.

A.6.3.2. Peak identification

The internal standard peak appears at approx. 19 min. and the stigmasta-3,5-diene at a relative retention time of 1.29 (see Figure 1b).

The stigmasta-3,5-diene goes with small quantities of an isomer, and usually both originate a single chromatographic peak. Nevertheless, if the column is too polar or shows a great resolving power, the isomer can appear as a small peak before and close to that of stigmasta-3,5-diene. In this case, the two areas have to be summed (**Figure 2**). It is advisable to eliminate the stigmastadiene split by replacing the column by a less polar or wider int. dia. one.

Note 9. Stigmastadienes for reference can be obtained from the analysis of a refined vegetable oil by applying the method for the determination of steroidal hydrocarbons. Stigmastadienes originate a significant and easily identifiable peak.

Note 10. Care must be taken that there is no overlap between the internal standard and stigmastadienes with any of the peaks which appear in the first fraction eluted from the silica gel column.

A.6.3.3. Quantitative analysis

The stigmastadienes content is determined according to the formula:

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

where:
A_s = area of stigmastadienes peak (if the peak is split up, sum of the areas of the two isomers)

A_c= area of internal standard (cholestadiene)

M_c = mass of standard added, in micrograms

M_o = mass of oil taken, in grams.

The result is expressed to two decimal places.

Detection limit: About 0.01 mg/kg.

Note 11. When stigmastadienes appear in concentrations of more than 4 mg/kg, if quantifying is required, the method for determination of sterenes in refined oil must be applied.

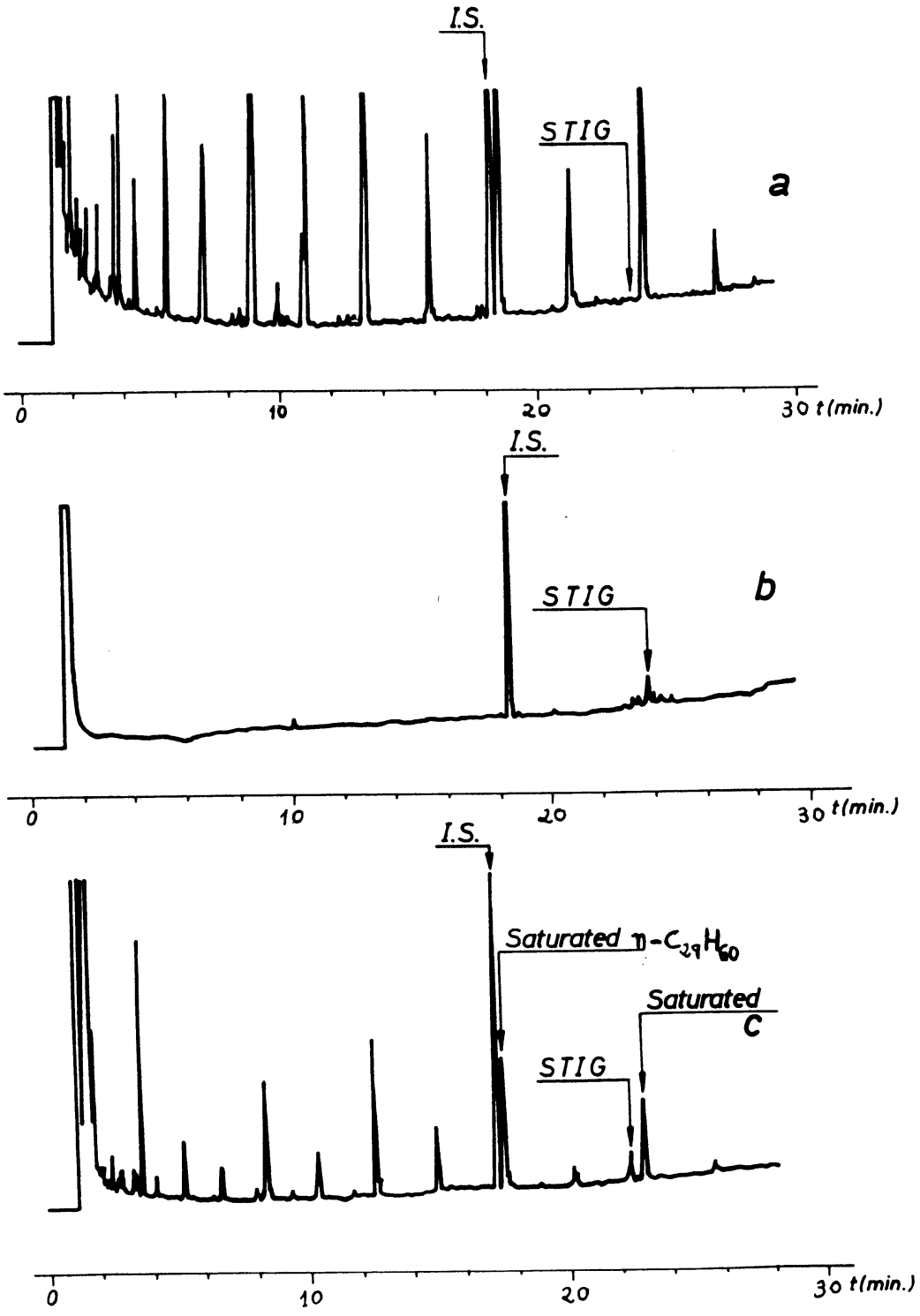

Figure 1. Gas chromatograms obtained from olive oil samples analyzed on a fused silica capillary column (0.25 mm i.d. by 25 m) coated with 5%-phenylmethylsilicone, 0.25 μm film thickness.

(a) First fraction (30 mL) from a virgin oil, spiked with standard.

(b) Second fraction (40 mL) from an olive oil containing 0.10 mg/kg of stigmastadienes.

(c) Second fraction (40 mL) containing a small proportion of the first fraction.

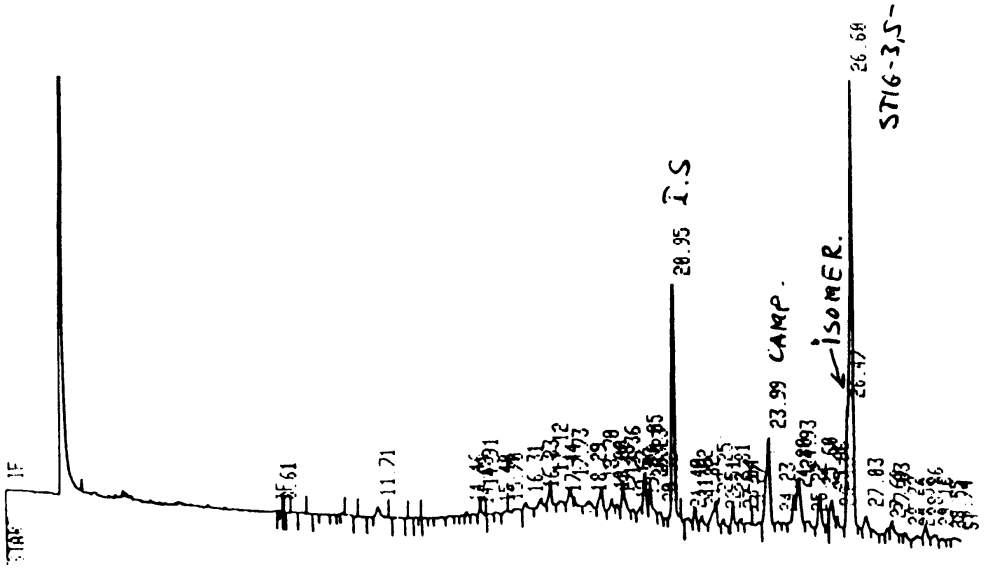

Figure 2. Gas chromatogram obtained from refined olive oil sample analyzed on DB-5 column showing the isomer of stigmasta-3,5-diene

PART B: ALTERNATIVE METHOD

B.4 APPARATUS

B.4.1. 50 mL flasks suitable for use with a reflux condenser.

B.4.2. Glass column for liquid chromatography, internal diameter 10 mm, length 20-40 cm, fitted with a suitable stopcock.

B.4.3. Gas chromatograph suitable for use with a capillary column having the following parts:

B.4.3.1. Thermostat-controlled oven with temperature programming.

B.4.3.2. Injector one of the following two injectors:

B.4.3.2.1. Cold injector for direct on-column injection

B.4.3.2.1. S/SL, vaporizing injector operating in split-less mode with a closing time of 1 min at a temperature of 300 °C

B.4.3.3. Flame ionization detector and converter-amplifier.

B.4.3.4. Computerized acquisition system or a recorder-integrator for use with the converter amplifier (B.3.3.3), with a response time of not more than 1 s and a variable paper speed.

B.4.3.5. Capillary column

Fused silica capillary columns for gas chromatography (0.25 or 0.32 mm i.d. by 25-30 m length) coated with 5% phenylmethylsilossane phase, 0.10-0,25 µm film thickness[1].

B.4.4. Microsyringe, 10 µL, for direct on-column injection.

B.4.5. Microsyringe, 10 µL, for injection through a silicone septum.

B.4.6. Electrical heating mantle or hot plate.

B.4.7. Rotary evaporator.

B.4.8. Muffle oven.

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1 Suitable commercial liquid phases are available for this purpose such as SE52, SE54, DB-5 etc.

B.4.9. Analytical balance for weighing to an accuracy of ± 0.1 mg.

B.4.10. Microsyringe, 100 µL, for internal standard addition.

B.4.11. Separating funnel fitted with a suitable stopcock.

B.4.12. Usual laboratory glassware.

B.5. REAGENTS

All reagents should be of analytical grade unless otherwise specified. The water used should be distilled water, or water of at least equivalent purity.

B.5.1. Silica gel, 60-200 mesh. Place the silica gel in the muffle oven at 550 °C for at least 4 h. Allow to cool, transfer in an Erlenmeyer flask with a ground glass stopper and then add 2% water in relation to the quantity of silica gel used. Shake well, preferably with a rotary agitator to homogenize slurry and wait at least 12 h prior to use.

B.5.2. Extraction and elution solvent. One of the following three alternative solvents can be used.

B.5.2.1. n-hexane, chromatography grade (or residue grade) the purity must be checked. Evaporate 50 mL of n-hexane, dissolve the residue in 100 µL and analyze the chromatographic conditions of the method. No peaks shall be present in the area of interest of the chromatogram.

WARNING –Fumes may ignite. Keep away from sources of heat, sparks or naked flames. Make sure the bottles are always properly closed. Vapors may cause drowsiness or dizziness. Ensure proper ventilation during usage. Avoid build-up of fumes and remove any possible fire risk, such as heaters or electric apparatus not manufactured from non-inflammable material. Pernicious if inhaled, because it may cause nerve cell damage. Avoid breathing in the fumes. Use a suitable respiratory apparatus if necessary. Avoid contact with eyes and skin.

B.5.2.2. n-heptane, chromatography grade, the purity must be checked: evaporate 50 mL of solvent and dissolve the residue in 100 µL and analyze the chromatographic conditions of the method. No peaks shall be present in the area of interest of the chromatogram.

WARNING – Fumes may ignite. Keep away from sources of heat, sparks or naked flames. Make sure the bottles are always properly closed. Vapours may cause drowsiness or dizziness. Ensure proper ventilation during usage. Avoid build-up of fumes and remove any possible fire risk, such as heaters or electric apparatus not manufactured from non-inflammable material.

B.5.2.3. iso-octane (2,2,4-trumethylpentane), chromatography grade, the purity must be checked: evaporate 50 mL of solvent and dissolve the residue in 100 µL and analyze the chromatographic conditions of the method. No peaks shall be present

in the area of interest of the chromatogram.

WARNING –Fumes may ignite. Keep away from sources of heat, sparks or naked flames. Make sure the bottles are always properly closed. Vapours may cause drowsiness or dizziness. Ensure proper ventilation during usage. Avoid build-up of fumes and remove any possible fire risk, such as heaters or electric apparatus not manufactured from non-inflammable material. Avoid contact with skin and eyes. Avoid inhalation of vapour or mist.

B.5.3. Potassium Hydroxide pellets 85% min.

WARNING-Caution: Very corrosive. After inhalation move the patient to ventilated fresh air place and call a physician. After contact with the skin wash thoroughly with water and remove contaminated clothing. In case of contact with eyes, rinse immediately with plenty of water and consult an eye specialist. Avoid contact with eyes and skin.

B.5.4. 96% V/V ethanol

WARNING - Flammable. Keep away from oxidizers, heat and flames. Keep away from heat, sparks and open flame. Avoid spilling, skin and eye contact. Ventilate well avoid breathing vapors.

B.5.5. Sodium Sulphate Anhydrous

B.5.7. Cholesta-3,5-diene purity > 93% (es. Sigma cod. C6012)

B.5.7.1. Stock solution (200 ppm) of cholesta-3,5-diene (Sigma > 93%) in elution solvent (B.5.2) (10 mg in 50 mL).

B.5.7.2. Working standard solution (20 ppm) of cholesta-3,5-diene in elution solvent (B.5.2), obtained by dilution of the above indicated solution[2].

B.5.8. Solution of n-nonacosane in extraction solvent at concentration of approximately 100 ppm.

B.5.9. Potassium hydroxide, approximately 2 mol/L ethanolic solution. Dissolve 130 g of potassium hydroxide with cooling in 200 mL of distilled water and then make up to one liter with ethanol. Keep the solution in well-stoppered dark glass bottles. [3].

B.5.10. Carrier gas: hydrogen or helium, pure, gas chromatography grade.

WARNING - Hydrogen – Highly inflammable, under pressure. Keep away from sources of heat, sparks, naked flames or electric apparatus not manufactured from non-inflammable material. Make sure the bottle valve is shut when not in use. Always use with a pressure reducer. Release the tension of the reducer spring before opening the bottle valve. Do not stand in front of the bottle outlet when opening the valve. Ensure proper ventilation during

2 The solutions B.5.7.1 and B.5.7.2 are stable for a period of at least four months if kept at less than 4 °C.

3 Alcoholic potash turns brown on standing. It should be prepared freshly each day and kept in well stoppered dark glass bottles

usage. Do not transfer hydrogen from one bottle to another. Do not mix gas in the bottle. Make sure the bottles cannot be knocked over. Keep them away from sunlight and sources of heat. Store in a corrosive-free environment. Do not use damaged or unlabeled bottles.

Helium. - Compressed gas at high pressure. It reduces the amount of oxygen available for breathing. Keep the bottle shut. Ensure proper ventilation during usage. Do not enter storage areas unless they are properly ventilated. Always use with a pressure reducer. Release the tension of the reducer spring before opening the bottle valve. Do not transfer gas from one bottle to another. Make sure the bottles cannot be knocked over. Do not stand in front of the bottle outlet when opening the valve. Keep them away from sunlight and sources of heat. Store in a corrosive-free environment. Do not use damaged or unlabelled bottles. Do not inhale. Use solely for technical purposes.

B.5.11. Auxiliary gases:

- Hydrogen, pure, gas chromatography grade.
- Air, pure, gas chromatography grade.

WARNING

Air. - Compressed gas at high pressure. Use with caution in the presence of combustible substances as the self-ignition temperature of most of the organic compounds in the air is considerably lower under high pressure. Make sure the bottle valve is shut when not in use. Always use a pressure reducer. Release the tension of the reducer spring before opening the bottle valve. Do not stand in front of the bottle outlet when opening the valve. Do not transfer gas from one bottle to another. Do not mix gas in the bottle. Make sure the bottles cannot be knocked over. Keep them away from sunlight and sources of heat. Store in a corrosive-free environment. Do not use damaged or unlabelled bottles. Air intended for technical purposes must not be used for inhaling or respiratory apparatus.

B.6. PROCEDURE

B.6.1. Preparation of the chromatography column

B.6.1.1. Suspend 5 g of silica gel (B.5.1) in the elution solvent (B.5.2) and introduce into the column (B.4.2). Allow to settle spontaneously. Complete settling with the aid of an electric shaker to make the chromatographic bed more homogeneous. Percolate 10 mL of the elution solvent (B.5.2) to remove any impurities.

B.6.1.2. Preparation of unsaponifiable matter:

B.6.1.3. Weigh 2,0 ± 0.1 g of oil into a 50-mL round bottomed flask and add using a glass syringe, 100 µL of the working standard solution of cholesta-3,5-diene (B.5.7.2) and 10 mL of 2 mol/L alcoholic potash. Fit reflux condenser, and heat to slight boiling for 30 min. Remove the flask containing the sample from the heat and allow the solution to cool slightly. Add 20 mL of water and transfer the solution to a separating funnel with the aid of 20 mL of extraction solvent . Shake the mixture

vigorously for 30 s and leave to stratify.

Discard the aqueous layer. Wash twice the extraction solvent with 50 mL of a mixture of ethanol- water (1:1) until neutral pH is reached (discard the washing phase) . If an emulsion is produced, wait as it disappears rapidly or add small quantities of ethanol.

B.6.1.4. Pass the extraction solvent solution through anhydrous sodium sulphate (20 g), wash with 20 mL extraction solvent and evaporate in a rotary evaporator at 30 ºC and low pressure until dryness.

B.6.2. Separation of steroidal hydrocarbon fraction:

B.6.2.1. Take the residue to the fractionating column with the aid of two 1-mL portions of extraction solvent, run the sample onto the column.

Allow the solvent to flow to 1 mm above the upper level of the absorbent, and then percolate 10 ± 5 mL of extraction solvent in order to elute n-alkanes naturally occurring [4].

Then percolate further 10 ± 5 mL of hexane and collect it in a test tube (this fraction contains the sterenes)[5].

Regulate the flow in order to have about 15 drops every 10 s. All operation must be done at room temperature (below 28 °C).

Evaporate the stigmastadiene fraction with the rotary evaporator or in a heated dry block with the aid of a gentle flush of nitrogen and dissolve in 50-100 µL of elution solvent (B.5.2).

B.7. GAS CHROMATOGRAPHY

B.7.1. Operating conditions

The operating conditions are generally as follows:

- Column temperature:

	20 °C/min		5°C/min	
80 °C at first (1')	⟶	200 °C	⟶	260 °C (20)

4 Usually elution with 10 mL produces an efficient separation, however to ensure a good separation of the saturated and steroid hydrocarbons the volume of each fraction has to be carefully optimized because if saturated hydrocarbons coelute with stigmastadienes false results may be achieved. It is recommended to calibrate the elution volume by using a nonacosane - cholesta-3,5-diene solution and measuring the elution volumes that gives the best separation of the two components.

5 If a large peak at approximately 1.5 min lower retention time than the standard appears is due to squalene and it is indicative of a bad separation.

- Detector temperature: 300 °C.
- Amount injected: 1–2 µL of B.5.2 solution (50-100 µL)
- Carrier gas: helium or hydrogen at the optimal linear speed for the gas chosen.
- Instrument sensitivity: suitable for achieving a detection limit of about 0,01 mg/kg for stigmastadiene.

These conditions may be modified according to the characteristics of the column and the gas chromatograph in order to achieve an elution without stigmastadiene overlapping

B.7.2. Chromatographic analysis

Take up 1 µL of the solution with the aid of the 10 µL micro-syringe, drawing back the plunger until the needle is empty. Introduce the needle into the injection system and inject quickly after 1– 2 s. After about 5 s, gently extract the needle.

Perform the recording until the stigmastadienes are completely eluted.

The gas chromatographic system must be checked injecting a mixture of the stock solution of cholestadiene and n-nonacosane solution. The two peaks must be fully resolved. If not, change the temperature program or use a column with a different polarity.

B.7.3. Peak identification

Identify peaks from the retention times by comparing the obtained chromatogram with a reference chromatogram obtained from the analysis of a refined vegetable oil[5].

B.7.4. Quantitative analysis.

Determine the areas of the peaks corresponding to the cholesta-3,5-diene internal standard and of the stigmastadienes:

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

where:

A_s = area corresponding to the peak of the stigmastadienes, in computer counts
A_c= area corresponding to the internal standard (cholesta,3-5,diene) in computer counts
M_c = mass of the standard in µg
M_o = mass of the sample, in grams.

5 Chromatograms obtained from the analysis of a refined olive oil or from an olive pomace oil usually originate a predominate and easily identifiable peak stigmastadiene peak.

B.8. EXPRESSION OF RESULTS

Report the stigmastadienes content in mg/kg.

Results should be expressed to two decimal place up to 0.99 mg/kg; concentration higher than 1.0 mg/kg should be expressed with one decimal place.

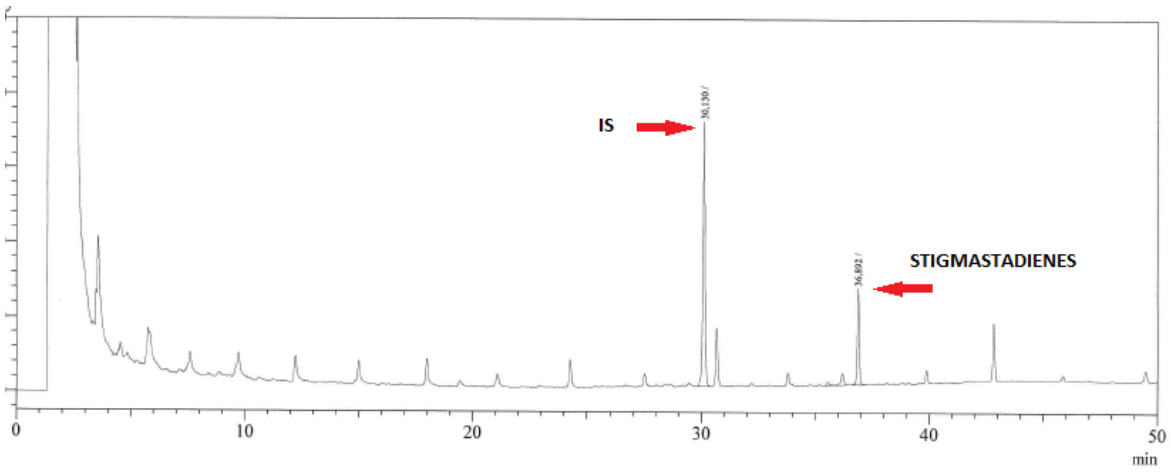

Figure 3. Gas chromatogram obtained from a virgin olive oil containing 5% refined olive oil analyzed with the method of part B.

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 IOC 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 organized by the IOC 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)

Stigmastadienes content (mg/kg)

	A	B	C	D	E
n	19	19	19	19	19
outliers	3	5	7	2	5
mean	0.01	0.80	9.49	0.22	7.55
r	0.01	0.08	0.39	0.05	0.48
S_r	0.00	0.03	0.14	0.01	0.17
RSD_r(%)	$32.4_{(not\ sig.)}$	3.7	1.5	8.4	2.3
R	0.03	0.15	1.66	0.06	1.59
S_R	0.01	0.05	0.59	0.03	0.57
RSD_R(%)	$98.6_{(not\ sig.)}$	6.7	6.3	11.5	7.6

2. Analysis of the collaborative IOC test results in 2017 for the aptitude test

Only a sample of Virgin olive oil with a quantifiable content of stigmastadienes, adulterated with 10% refined olive oil and 2% animal fat has been tested.

Stigmastadienes (mg/kg)	n	Consensus mean	S_r	S_R
Regulatory method	25	15.70	0.38	1.08
Alternative solvent method	24	15.45	0.26	1.06

Evaluation	Calculated	Limit	Conclusion/Comments
Difference (Regulatory method-Alternative solvent method)	0.25		
Test F repeatability	2.16	2.04	Altought the calculated F is greater than limiting F, the S_r obtained with the alternative solvent is smaller and improves the results
Test F reproducibility	1.04	2.04	F cal < F limit
Current reproducibility Regulatory	1.1	1.3	S_R obtained is smaller than current S_R
T Student	0.81	2	t cal < t limit

Where Regulatory Method is method of part A with the use of hexane as solvent;

Where Alternative Solvent Method is of part B with the use of isooctane as solvent.

The comparison of the results has focused on the comparative evaluation of the variances, both under conditions of reproducibility, as well as the existence of a significant bias or not among the assigned values after applying the regulated method and the obtained after using the alternative solvent.

For this, the F Fisher of two variances obtained, in both conditions, as well as the Student t statistic of the two populations studied, which compares the two means obtained and their respective variances, under conditions of reproducibility, was calculated.

The currently published precision value for the studied level has also been compared, and that obtained with the use of alternative solvents.

3. Analysis of the collaborative IOC test results in 2020 for the comparison of method of part A and B.

Eighteen laboratories took part in the collaborative test arranged by the Executive Secretariat in 2020. The laboratories were from nine countries.

The test was performed using the reference method of part A and the simplified alternative method of part B on a set of five samples:

A: unmodified virgin olive oil
B: mixture of virgin olive oil + refined olive oil (approx. 1%)
C: mixture of virgin olive oil + refined olive oil (approx. 5%)
D: virgin olive oil + refined pomace oil
E: mixture of virgin olive oil + refined sunflower oil (approx. 10%)

PART A : REFERENCE METHOD

SAMPLE	A	B	C	D	E
N	18	18	18	18	17
ouliers	3	7	5	5	4
Mean	0.016	0.075	0.29	1.062	3.156
Median	0.018	0.077	0.290	1.056	3.151
Sr	0.003	0.004	0.010	0.024	0.066
SR	0.008	0.008	0.019	0.058	0.147
Cv r %=	20.0%	5.7%	3.5%	2.2%	2.1%
Cv R %=	46.9%	10.8%	6.6%	5.5%	4.6%

PART B : ALTERNATIVE METHOD

SAMPLE	A	B	C	D	E
N	18	18	18	18	17
ouliers	4	5	5	5	5
Mean	0.018	0.072	0.278	0.944	3.212
Median	0.020	0.075	0.289	1.047	3.168
Sr	0.0027	0.007	0.014	0.032	0.095
SR	0.0075	0.021	0.044	0.156	0.200
Cv r %=	15.0%	10.1%	4.9%	3.4%	3.0%
Cv R %=	41.7%	28.8%	16.0%	16.5%	6.2%

Normative references

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

ISO 5725-2: 2019 Accuracy (trueness and precision) of measurement methods and results – Part 2: Basic method for the determination of 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