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METHOD FOR THE 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.

4. APPARATUS

4.1. 250 ml flat-bottom flasks with reflux condenser.

4.2. Separating funnels of 500 ml capacity.

4.3. 100 ml round-bottom flasks.

4.4. Rotary evaporator.

4.5. Glass chromatography column (1.5 cm i.d. by 50 cm length) with Teflon cock and a plug of glass wool fibre 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.

4.6. Gas chromatograph with flame ionization detector, split or on-column injector and oven

programmable to within ± 1°C.

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.

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

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

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.

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

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

5.2. 96 v/v ethanol.

5.3. Anhydrous sodium sulphate.

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.

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 four hours at 550°C. 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).

5.6. Stock solution (200 ppm) of cholesta-3,5-diene (Sigma, 99% purity) in hexane (10 mg in 50 ml).

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

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

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

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

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

6. PROCEDURE

6.1. Preparation of unsaponifiable matter:

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 minutes. 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 seconds and leave to stratify.

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

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) until neutral pH is reached.

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

6.2. Separation of steroidal hydrocarbon fraction:

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 1 a) 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.

6.2.2. Evaporate the second fraction in an evaporator at 30°C 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 6.1.3. and 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.

6.3. Gas chromatography

6.3.1. Working conditions:

- Injector temperature: 300°C.
- Detector temperature: 320°C.
- 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°C for 6 min. and then rising at 2°C/min. up to 285°C.
- Injector with 1:15 flow divider.
- Carrier: 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 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 (5.6) and n-nonacosane solution (5.10). The cholesta-3,5-diene peak has to appear before the n-nonacosane (Fig. 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.

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.

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.

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 steroidal hydrocarbons must be applied.

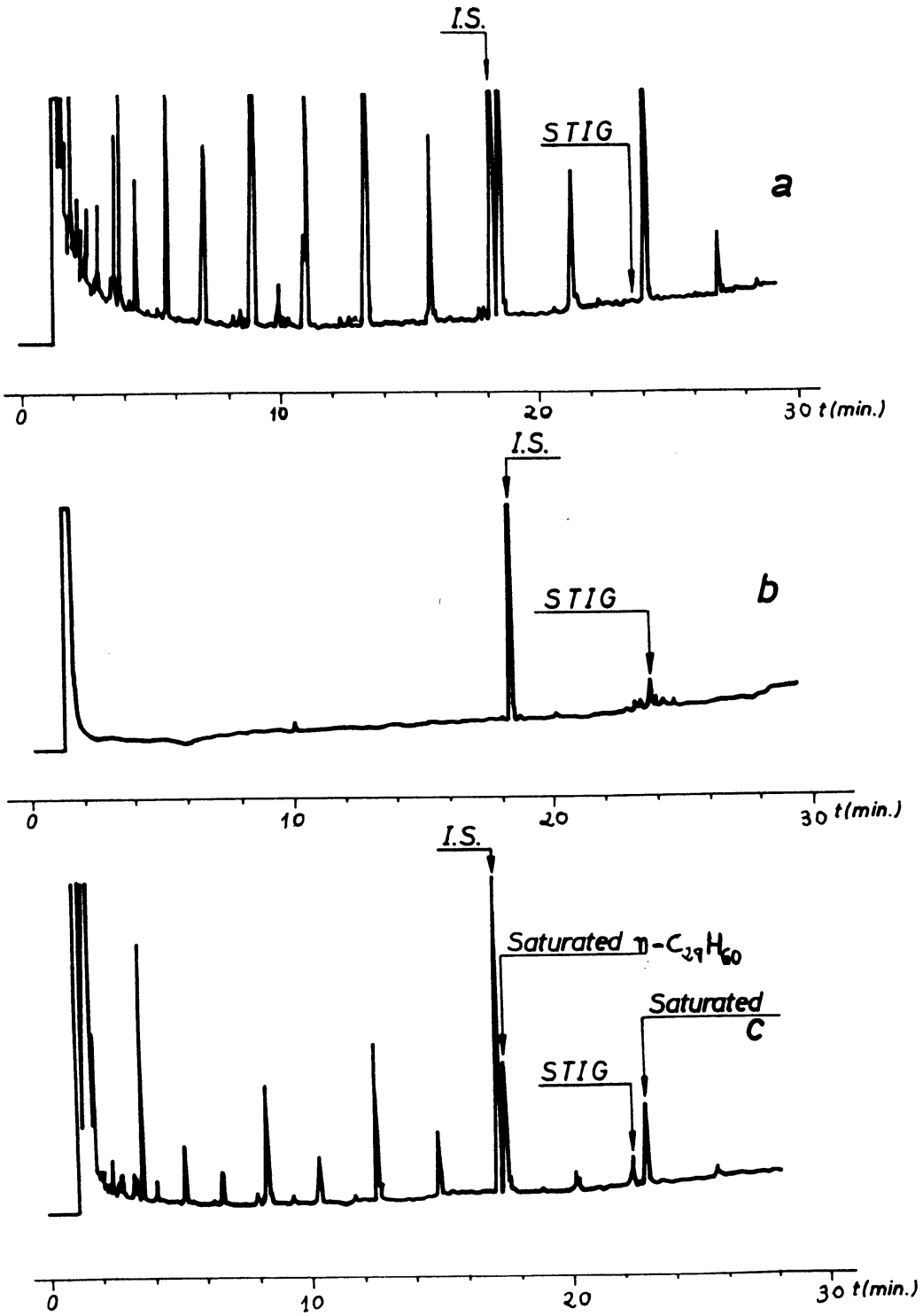
I.S.
STIG
a
0
10
20
30 t(min.)
I.S.
STIG
b
0
10
20
30 t(min.)
I.S.
Saturated n-C29H60
Saturated
C
STIG
0
10
20
30 t(min.)

Figure 1

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.

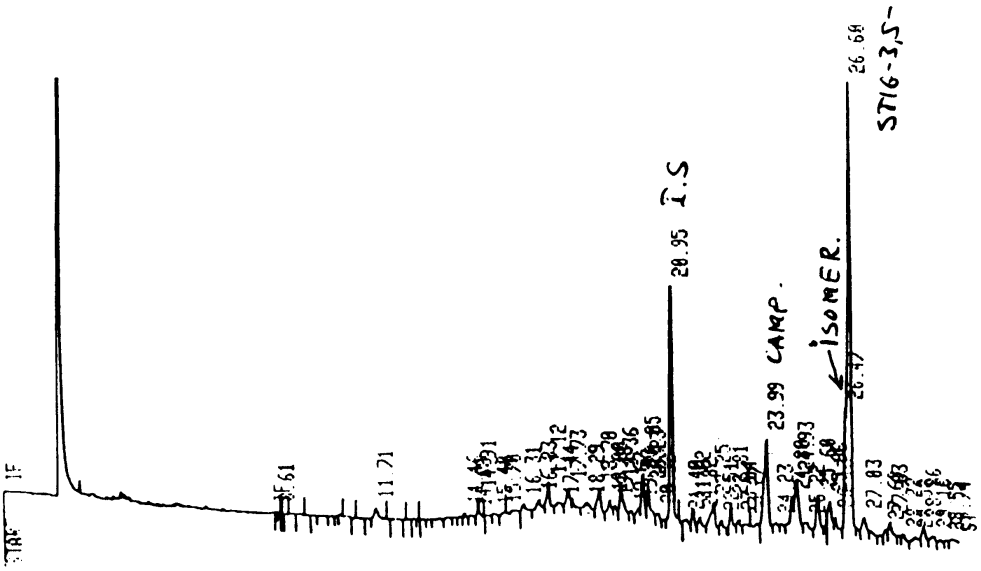

Figure 2

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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)

Stigmastadienne content (ppm)

	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
Sr	0.004	0.03	0.14	0.01	0.17
RSDr (%)	$32.43_{(not\ sig.)}$	3.70	1.5	8.41	2.29
R	0.033	0.15	1.66	0.06	1.59
S_R	0.012	0.05	0.59	0.025	0.57
RSD_R(%)	$98.59_{(not\ sig.)}$	6.73	6.26	11.45	7.55

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 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