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THE DETERMINATION OF STIGMASTADIENES IN VEGETABLE OILS

(Technical Report)

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The determination of stigmastadienes in vegetable oils (Technical Report)

Abstract: The development by collaborative study of a standardised method for the determination of the stigmastadienes content in vegetable oils is described. The procedure involves the isolation of the unsaponifiable matter in the oil ands its fractioning on a silica gel column. The fraction containing the steroidal hydrocarbons is then analysed by capillary gas chromatography and the quantitative assessment made by the use of an internal standard/ cholestan-3,5-stadiene. The procedure is very sensitive allowing determination at levels of 0.01–4.0 mg/kg.

INTRODUCTION

Significant amounts of hydrocarbons are formed in vegetable oils as a consequence of thermal treatments during the refining processes [1]. Among these hydrocarbons, the stigmasta-3,5-diene, a steroidal compound, is the most abundant in all refined vegetable oils since it derives from the β-sitosterol by dehydration [2]. The 3,5-stigmastadiene is produced together with minor amounts of the 2,4-isomer and both substances originate a single signal and well defined gas chromatographic peak, when the hydrocarbon fraction is analysed on a low polar column [3]. Therefore the sum of both isomers can be easily quantified by gas-chromatographic analysis of the steroidal hydrocarbon fraction [2,4].

For virgin olive oil, the usual production processes (pressure or centrifuging) do not produce measurable amounts of stigmastadienes (less than 0.01 mg/kg). In crude olive residue oil, small concentrations of stigmastadienes are found (ranging between 0.2 and 3 mg/kg) due to the high temperatures applied during the drying of the raw olive residue.

In the refining processes, stigmastadienes are formed in all steps involving high temperatures, such as bleaching and deodorising, but originating more amounts in the former step than in the latter [2]. Depending on the conditions applied during the refining process, commercial refined vegetable oils show stigmastadienes concentrations ranging between 1 and 29 mg/kg, and therefore the assessment of stigmastadienes allows not only the identification of thermally treated oils but also the detection of minor amounts of refined vegetable oils in virgin olive oils.

A method for the determination of stigmastadienes and the results of a collaborative study carried out under the auspices of the International Olive Oil Council were presented to IUPAC Commission on Oils, Fats and Derivatives by the Spanish representative. The Commission considered the question of desirability of introducing the stigmastadiene content as an identity criterion for virgin olive oils and put forward the proposal of study the analytical method which could be adopted as an international standard method.

COLLABORATIVE STUDY

The adopted analytical method [2] consists of three stages: (i) obtention of unsaponifiable matter by hexane, (ii) fractioning of unsaponifiable matter by column chromatography on silica gel, (iii) analysis of the steroidal fraction by capillary gas-chromatography using an internal standard (cholesta-3,5-diene).

Each laboratory was provided with the method and data sheets to report the stigmastadienes concentration, silica gel type and treatment, volume of the first fraction from the silica gel column, gas-chromatographic injection mode, gas-chromatographic column, oven temperature programming and carrier gas conditions.

Samples consisted on duplicates of three oils: (i) virgin olive oil (samples oz and dg), (ii) mixture of virgin olive oil with 0.7% of refined olive oil containing 23 mg/kg of stigmastadienes (samples ab and lp),

and a mixture of virgin olive oil with 1.2 of refined sunflower oil containing 80 mg/kg of stigmastadienes (samples ce and fh).

An additional sample of refined oil was sent in order to obtain suitable reference for the gas-chromatographic peak of stigmastadienes and to practice runs to become familiar with the method. A simpler analytical method to be used with this sample was enclosed.

Samples were sent by November 1993 and results had to reported to the co-ordinator by April 1994.

RESULTS

Materials and operating conditions

Data shown in Tables 1 and 2 were reported by collaborators.

Table 1 Silica gel chromatographic conditions reported by collaborators

Laboratory	Silica gel 60 type	Treatment	Volume of 1st fraction in mL
1	70–230 mesh	None	30
2	Merck no. 7734	None	30
3	Merck no. 7734	None	30
4	Merck no. 7734	2% water after activation	30–35
5	Merck no. 7734	None	40
6	Merck no. 7734	None	35
7	Wakogel C-200	Drying (?) 110 °C, 1 h	30
8	Merck no. 7734	2% water after activation	35
9	Merck no. 7734	2% water after activation	40
10	Merck no. 7734	2% water after activation	

No comments were made by participants. In laboratory 4, the chromatograms of sample 1 showed a large peak of squalene and small peak of cholestadiene. This was probably due to the use of an excessively deactivated silica gel, and therefore, the most of the steroidal hydrocarbons was eluted in the first fraction.

Table 2 Gas-chromatographic conditions reported by collaborators

			Coating		Initial			Final	Carrier gas	
Lab.	Injec. mode	Column ($l \times i.d$) (m × mm)	phase	thickness (μm)	temp. (°C)	time (min)	Ramp (°C/min)	temp. (°C)	gas	Press. (psi*)
1	split	30 × 0.25	SPB-5	0.25	220	6	6	290	H_2	13
2	split	30 × 0.32	SPB-5	0.25	235	6	2	285	He	12
3	split	30 × 0.32	SPB-5	0.25	235	6	12	285	H_2	4
4	split	30 × 0.31	methyl - silicone	0.52	235	6	2	285	H_2	7.3
5	split	30 × 0.32	SE52/54	0.25	90	0	10	300	H_2	14.5
6	split	25 × 0.25	SGL-5	0.25	235	6	2	285	He	17
7	split	30 × 0.32	DB-5	0.25	235	6	2	285	He	13
8	split	25 × 0.25	SGL-5	0.25	235	6	2	285	He	17
9	split	60 × 0.25	RSL 150	0.25	235	5	6	285	H_2	27
10	split	30 × 0.32	SE 54	0.25	235	8	2	285	He	12

* 1 psi = 6.895 kPa

Several gas-chromatographic conditions were applied by collaborators and those of laboratory 9 (splitless injection and 60 m of column length) were far from the recommended ones.

Analytical data

Results reported by collaborators are shown in Table 3. It can be seen that some discordant values correspond to sample 2 from laboratory 4, and samples 2 and 3 from laboratory coinciding with the unusual operating conditions above commented.

Table 3

	Sample 1		Sample 2		Sample 3	
Lab.	oz	dg	ab	lp	ce	fh
1	0.01†	0.04†	0.15	0.16	0.93	0.88
2	0.02	0.02	0.18	0.19	1.02	0.097
3	< 00.01	0.02	0.16	0.16	0.85	1.05
4	ND*	ND	0.31†	0.43 †	0.98	0.98
5	< 0.02	< 0.02	0.19	0.20	1.17	1.08
6	< 0.01	0.01	0.16	0.15	0.93	0.94
7	ND	ND	0.19	0.20	1.26	–‡
8	< 0.02	< 0.02	0.17	0.16	1.01	0.96
9	0.01	0.01	0.22	0.23	1.45	1.25
10	ND	ND	0.17	0.15	0.96	0.89

* Non-detected.
†Outlier identified by Cochran's test.
‡Bottle broken during transportation.

For sample 3, laboratory 7 gave a single result as the duplicate was broken during transportation.

STATISTICAL EVALUATION

Data screening

From 10 laboratories data were received for evaluation. For sample 1, the results 'non-detected' (ND) and < 0.01 were considered as 0.01 and the results < 0.02 as 0.02 since the stigmastadiene content was in the range of the detection limit of the method. Results of sample 3 from laboratory 7 was unsuitable for inclusion in the test as it had no duplicate. The remaining data were subjected to statistical analysis according to the international standard ISO 5725-1986 (E).

Cochran tests

As the six samples analysed were in fact three pairs of corresponding samples, the differences between these blind (hidden) duplicates have been tested for stragglers and outliers according to Cochran's procedure. One laboratory in sample 1 and another in sample 2 were eliminated.

Dixon tests

After elimination of significant outliers (i.e. significant at the 1% level) according to Cochran's test the three batch averages were tested for stragglers and outliers according to Dixon's procedure. No laboratory was eliminated.

The impaired data of laboratories 7 for sample 3 was omitted from further evaluation. All other data were maintained in the evaluation after discarding the outliers. Hence, for the final calculation of repeatability and reproducibility, there remained a total of nine laboratories.

Precision

In Table 4, the number of participants, number of accepted result sets, mean values, repeatability and reproducibility at each concentration level have been listed.

When the values for r (repeatability) and R (reproducibility) as given in Table 4 are expressed as functions of their corresponding mean concentration values (m), the following equations are obtained:

$$r = 0.207 \times m - 0.0035, R = 0.408 \times m + 0.0041$$

And for values of m in the range 0.10–1.0, approximately equal to

$$r = 0.20 \times mR = 0.41 \times m, \text{respectively,}$$

Table 4 Statistical analysis of results

	Sample 1	Sample 2	Sample 3
Number of participants	10	10	10
Number of accepted result sets	9	9	9
Mean mass fraction m (mg/kg)	0.014	0.177	1.017
Repeatability			
Standard deviation S_r	0.0032	0.0078	0.0747
Repeatability r	0.0089	0.0218	0.2092
Coefficient of variation CV_r (%)	23	4.4	7.3
Reproducibility			
Standard deviation S_R	0.0055	0.0249	0.150
Reproducibility R	0.0153	0.0697	0.420
Coefficient of variation CV_R (%)	39.6	14.1	14.7

DISCUSSION

From the above equations, describing the precision of the stigmastadienes concentrations it can be concluded that the straight lines corresponding with these equations pass through the origin.

The high coefficient of variation found from results of sample 1 is due to the content of stigmastadienes which was in the range of the detection limit of the method. For samples 2 and 3, the relative repeatability is good. According to Pocklington [5] for an analytical method to become acceptable, the relative reproducibility (CV_R) should be about 23% at the 0.1 mg/kg level and 16% at the 1.0 mg/kg level. For samples 2 and 3 this criterion is fully met (14.1% and 14.7%, respectively).

CONCLUSION

The analytical method is a sensitive procedure which allows reliable determination of minor amounts of stigmastadienes in vegetable oils.

After this collaborative study it was concluded that the method meets the criterion for an analytical method to determine trace amounts of analyte as stated by Pocklington [5].

Based on the repeatability and reproducibility of the results obtained in the collaborative study, the Commission decided to adopt the method. The text of the standardised procedure is given in the following pages.

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The nomenclature of the mentioned organic compounds has been revised according to IUPAC guidelines [6]

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APPENDIX. DETERMINATION OF STIGMASTADIENES IN VEGETABLE OILS

1. Scope

Method for the determination of stigmastadienes in vegetable oils containing low concentrations of these hydrocarbons, particularly in virgin olive oil and crude residue oil.

2. Field of Application

This standard may be applied to all vegetable oils although measurements are reliable only where the content of these hydrocarbons lies between 0.01 and 4.0 mg/kg. The method is suited to detecting the presence of refined vegetable oils (olive oil, olive pomace, sunflower, palm, etc.) in virgin olive oils since refined oils contain stigmastadienes and those obtained by cold pressing 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 flasks suitable for use with a reflux condenser.

4.2 Separating funnels of 500 mL capacity.

4.3 100 mL round-bottomed flasks.

4.4 Rotary evaporator.

4.5 Glass chromatographic column (1.5 cm internal diameter by 50 cm length) with Teflon tap and a plug of glass wool fibre or a sintered glass disc 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 the settling by applying slight vibrations. Add anhydrous sodium sulphate to a height of approximately 0.5 cm, finally elute the excess hexane.

4.6 Gas chromatograph with a flame ionisation 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 internal diameter by 25 m length) coated with 5% phenylmethylsilicone phase, 0.25 μm film thickness (Note 1).

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

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

4.10 Electrical heating mantle or hot plate.

5. Reagents

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

5.1 Hexane or mixture of alkanes of b.p. interval 65–70 °C, distilled with a rectifying column (Note 2).

5.2 96 v/v Ethanol

5.3 Anhydrous sodium sulphate.

5.4 Alcoholic potassium hydroxide solution at 10%. Add 10 mL of water to 50 g potassium hydroxide pellets, stir, and then dilute the mixture with ethanol to 500 mL (Note 3).

5.5 Silica gel 60 for column chromatography, 70–230 mesh (Merck, 7734or similar) (Note 4)

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

5.7 Standard solution of cholesta-3,5-diene in hexane at a concentration of 20 mg/kg, obtained by dilution of above solution. (note 5).

5.8 Solution of *n*-nonacosane in hexane at concentration of approx. 100 mg/kg.

5.9 Solution of cholesta-3,5-diene (24-ethylcholesta-3,5-diene) from Chiron A.S. Heimdal, Norway) in hexane at concentration of approx. 100 mg/kg.

5.10 Carrier gas for the chromatography: helium or hydrogen of 99.999% purity.

5.11 Auxiliary gases for flame ionisation detector: hydrogen of 99.999% purity and purified air.

6. Procedure

6.1 Preparation of unsaponifiable matter

6.1.1 Weigh 20 ± 0.1 g of oil into a 250-mL flask (4.1), add 1 mL of the standard solution of cholesta-3,5-diene (20 μg)(5.7) and 75 mL alcoholic potassium hydroxide solution at 10% (5.4), 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 separating funnel (4.2) with the aid of 100 mL hexane. Shake the mixture vigorously for 30 s and allow to separate (Note 6).

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 of hexane and evaporate in a rotary evaporator at 30 °C under reduced 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 ml portions of hexane, run the sample onto the column by allowing the solution level to droop to the top of the sodium sulphate and start the chromatographic elution with hexane at a flow rate of 1 mL/min approximately. Discard the first 25–30 mL of the eluate and then collect the following 40 mL fraction. After collection transfer this fraction to a 100-mL round-bottomed flask (4.3) (Note 7).

6.2.2 Evaporate the second fraction in a rotary evaporator at 30 °C under reduced pressure until dryness, and immediately dissolve the residue in 0.2 mL of hexane. Keep the solution in the refrigerator until analysis (Note 8).

6.3 Gas chromatography

6.3.1 Working conditions for split injection

—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 peak areas. Valley-valley integration 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 and 85 kPa of pressure, respectively.

These conditions may be adjusted in accordance with the characteristics of the gas chromatograph apparatus 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 3,5-cholestadiene (5.6) and *n*-nonacosane solution (5.8). The 3,5-cholestadiene should appear before the *n*-nonacosane (Fig. 1c); if that does not occur, two actions can be undertaken: reduce the oven temperature and/or use less polar column.

6.3.2 Peak identification

If helium is used as carrier gas, the internal standard peak appears at approx. 19 min and the stigmastadienes peak at a relative retention time of 1.29 (see Fig. 1b). If hydrogen is used, the internal standard peak appears at approx. 13 min and the stigmastadienes peak at a relative time of 1.35. Reference for the stigmastadienes peak can be obtained analysing a solution of cholesta-3,5-diene (5.9) (Note 9). The stigmastadienes peak is a single chromatographic peak originated by a mixture of cholesta-3,5-diene and minor amounts of the 2,4-isomer. Nevertheless, if the column is too polar or shows a high resolving power, the 2,4-isomer can appear as a small peak before and close to that of the cholesta-3,5-diene (Fig. 2). In order to ensure that both stigmastadienes are eluted as one peak, it is advisable to replace the column by one which is less polar or with a wider internal diameter.

7. Calculation and expression of results

7.1 Calculation

The stigmastadienes content will be determined according to the formula:

mg/kg of stigmastadienes $= A_s \times M_c/(A_c \times M_o)$

where:

A_s: area of stigmastadienes peak (if the peak is splited up, sum of areas of the 3,5-and 2,4-isomers); A_c: area of internal standard (cholesta-3,5-diene); G_{MC}: mass of internal standard (cholesta-3,5-diene) added in μg; M_o: mass of oil taken.

7.2 Expression of results

Results to be given to two places of decimals.

8. Quality assurance

8.1 For general principles analytical quality control see the section on Quality Assurance in the introductory part of the Compendium of the Standard Methods (1st supplement to the 7th edn).

8.2 For specific application of analytical quality control see the Annexe to this standard method.

9. Notes

1 Other columns of similar lower polarity can be used.

2 The hexane must be distilled to remove impurities.

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

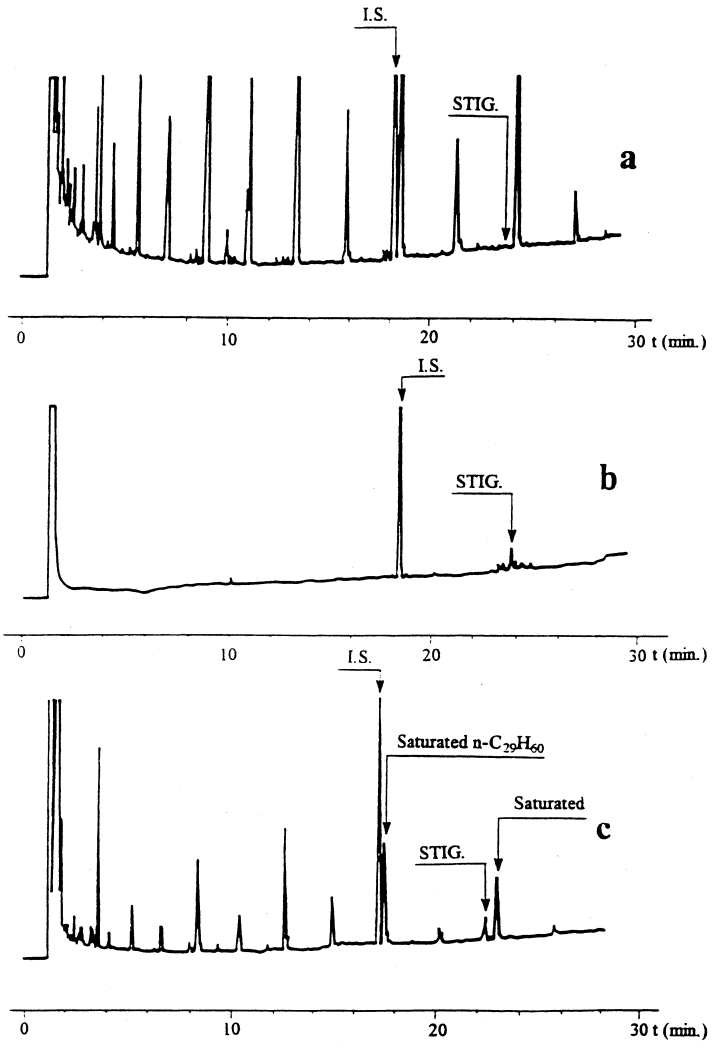

Fig. 1 Gas chromatograms obtained from olive oil samples analysed on a fused silica capillary column (0.25 mm i.d. × 25 m) coated with 5%-phenylmethylsilicone, 0.25 μm film thickness. (a) First fraction (30 mL) from a virgin oil, spiked with standard. (b) Second fraction (40 mL) from an olive oil containing 0.10 mg/kg of stigmastadienes. (c) Second fraction (40 mL) containing a small proportion of the first fraction.

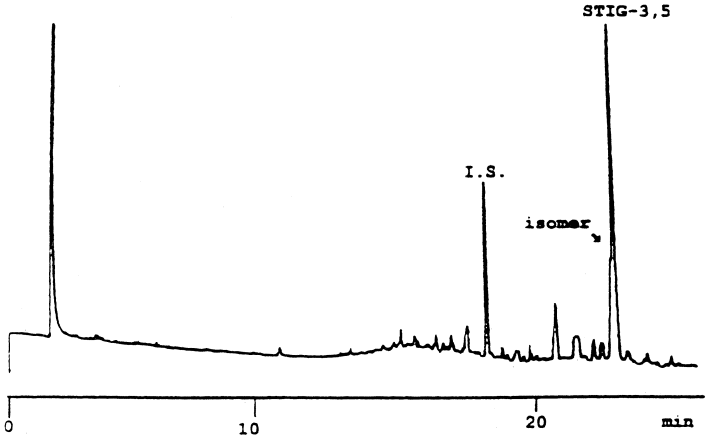

Fig. 2 Gas chromatogram obtained from a refined olive oil sample analysed on DB-5 column showing the isomer of 3,5 sigmastadiene.

4 Usually, silica gel can be used directly from the container without any treatment. However, some batches of silica may show low activity resulting in bad chromatographic separations. Under this circumstance, the silica gel should be treated in the following way: Activate the silica gel by heating for at least four hours at 550 °C. After the 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 (Merck no. 7754).

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

6 If an emulsion is produced which does not rapidly disappear, add small quantities of ethanol.

7 The first fraction contains saturated hydrocarbons (Fig. 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 optimisation of fraction volumes is required. For this, the volume of the first fraction should be adjusted so that when the second fraction is analysed the peaks representing the saturated hydrocarbons are low/see Fig. 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 the second fractions is unnecessary, as there is no overlapping of peaks during gas chromatographic analysis if the gas chromatographic conditions are adjusted as indicated in 6.3.1. The optimisation 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 large peak at approx. 1.5 min lower retention time than the standard is due to the squalene, and it is indicative of a bad separation.

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.

9 If cholesta-3,5-diene (24-ethyl-cholesta-3,5-diene) is not available, a chromatographic reference for stigmastadienes can be obtained analysing 1–2 g of a refined vegetable oil. Stigmastadienes originate a significant and easily identifiable peak.

Appendix

1. Repeatability limit

The absolute difference between two independent single 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 should not be greater than the repeatability limit (r) as calculated by the formula in Table 5.

Table 5 Repeatability (r) and reproducibility (R) limits of the determination of stigmastadienes in virgin olive oils, according to mean mass fraction value (m) in the range 0.10–1.0 mg/kg: $r = 0.20 \times m$; $R = 0.41 \times m$

Sample (virgin olive oil)	1 (low)	2 (medium)	3 (high)
Number of participating laboratories	10	10	10
Number of laboratories retained after elimination of outliers	9	9	9
Number of outliers (laboratories)	1	1	1
Number of accepted results	18	18	18
Mean mass fraction (m) (mg/kg sample)	0.014	0.177	1.017
Repeatability standard deviation (S_r) (mg/kg sample)	0.003	0.008	0.075
Repeatability relative standard deviation (CV_r in %)	23.0	4.4	7.3
Repeatability limit (r)[$2.83 \times S_r$]	0.009	0.022	0.209
Reproducibility standard deviation (S_R) (mg/kg sample)	0.006	0.025	0.150
Reproducibility relative standard deviation (CV_R in %)	39.6	14.1	14.7

2. Reproducibility limit

The difference between two single test results, obtained with the same method on identical test material in different laboratories with different operators using different equipment, should not be greater than the reproducibility limit (*R*) as calculated from the formula in Table 5.

3. Sensitivity

The sensitivity of the method is demonstrated by the low values for r and R at the Low concentrations studied (see table of statistical data below). The limit of detection is 0.01 mg/kg. Statistical and other data derived from the result of the interlaboratory test

4. The interlaboratory test

The interlaboratory test carried out at the international level in 1993–94 by the IUPAC Commission on Oils, Fats and Derivatives, in which 10 laboratories participated, each two results for each sample, gave the statistical results (evaluated in accordance with ISO 5725-1986 E summarised in Table 5.