INTERNATIONAL
OLIVE
COUNCIL

COI/T.20/Doc. No 28/Rev.2
2017

ENGLISH
Original: ENGLISH

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METHOD OF ANALYSIS

DETERMINATION OF THE CONTENT OF WAXES, FATTY ACID METHYL ESTERS AND FATTY ACID ETHYL ESTERS BY CAPILLARY GAS CHROMATOGRAPHY

1. PURPOSE

This method is for the determination of the content of waxes, and fatty acid methyl and ethyl esters in olive oils. The individual waxes and alkyl esters are separated according to the number of carbon atoms. The method is recommended as a tool for distinguishing between olive oil and olive-pomace oil and as a quality parameter for extra virgin olive oils enabling the detection of fraudulent mixtures of extra virgin olive oils with lower quality oils whether they are virgin, ordinary, lampante or some deodorised oils.

2. PRINCIPLE

Addition of suitable internal standards to the oil and fractionation by chromatography on a hydrated silica gel column. Recovery of the fraction eluted under the test conditions (with a lower polarity than that of the triacylglycerols) and direct analysis by capillary gas chromatography.

3. APPARATUS

3.1. Erlenmeyer flask, 25 ml.

3.2. Glass column for liquid chromatography, internal diameter 15 mm, length 30-40 cm, fitted with a suitable stopcock.

3.3. Gas chromatograph suitable for use with a capillary column, equipped with a system for direct, on-column injection comprising:

3.3.1. Thermostat-controlled oven with temperature programming.

3.3.2. Cold injector for direct on-column injection

3.3.3. Flame ionisation detector and **converter-amplifier.**

3.3.4. Recorder-integrator (*Note 1*) for use with the converter-amplifier (3.3.3), with a response time of not more than 1 s and a variable paper speed.

3.3.5. Capillary column, fused silica (for analysis of the waxes and methyl and ethyl esters), length 8-12 m, internal diameter 0.25-0.32 mm, internally coated with liquid phase (*Note 2*) to a uniform thickness of 0.10-0.30 μm.

3.4. Microsyringe, 10 μl, with hardened needle, for direct on-column injection.

3.5. Electric shaker.

3.6. Rotary evaporator.

3.7. Muffle oven.

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

3.9. Usual laboratory glassware.

4. REAGENTS

4.1. Silica gel, 60-200 μm mesh. Place the silica gel in the muffle oven at 500 °C for at least 4 h. Allow to cool and then add 2% water in relation to the quantity of silica gel used. Shake well to homogenise slurry and keep in the desiccator for at least 12 h prior to use.

4.2. n-hexane, chromatography grade or residue grade (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 in page 11), Solvents with higher boiling point than n-hexane take longer to evaporate. However, they are preferred due to the toxicity of hexane.) (the purity must by checked for example the residue after evaporation of 100ml of solvent may be controlled.).

Note 1: Computerised systems may also be used where the gas chromatography data are entered through a PC.
Note 2: Suitable commercial liquid phases are available for this purpose such as SE52, SE54, etc.

WARNING – Fumes may ignite. Keep away from sources of heat, sparks or naked flames. Make sure the bottles are always properly closed. 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.

Iso-octane is a flammable liquid that presents a fire hazard. Explosion limits in air are 1.1% to 6.0% (volume fraction). It is toxic by ingestion and inhalation. Use a ventilated hood in good operating condition to work with this solvent.

4.3. Ethyl ether, chromatography grade.

WARNING – Highly inflammable and moderately toxic. Irritates the skin. Pernicious if inhaled. May cause damage to eyes. Effects may be delayed. It can form explosive peroxides. Fumes may ignite. Keep away from sources of heat, sparks or naked flames. Make sure the bottles are always properly closed. 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. Do not evaporate to dryness or near dryness. The addition of water or an appropriate reducing agent can reduce peroxide formation. Do not drink. Avoid breathing in the fumes. Avoid prolonged or repeated contact with skin.

4.4. n-heptane, chromatography grade, or **iso-octane.**

WARNING – Inflammable. Pernicious if inhaled. Keep away from sources of heat, sparks or naked flames. Make sure the bottles are always properly closed. Ensure proper ventilation during usage. Avoid breathing in the fumes. Avoid prolonged or repeated contact with skin.

4.5. Standard solution of lauryl arachidate (*Note 3*), at 0.1% (m/V) in heptane (internal standard for waxes).

4.6. Standard solution of methyl heptadecanoate, at 0.02% (m/V) in heptane (internal standard for methyl and ethyl esters).

4.7. Sudan 1 (1-phenylazo-2-naphthol)

4.7. Carrier gas: hydrogen or helium, pure, gas chromatography grade.

Note 3: Palmityl palmitate, myristyl stearate or arachidyl laureate may also be used.

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 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 unlabelled 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.

4. 9. 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.

5. PROCEDURE

5.1. Preparation of the chromatography column

Suspend 15 g of silica gel (4.1) in n-hexane (4.2) and introduce into the column (3.2). Allow to settle spontaneously. Complete settling with the aid of an electric shaker (3.5) to make the chromatographic bed more homogeneous. Percolate 70 ml of n-hexane to remove any impurities. Weigh exactly about 500 mg of the sample into the 25-ml flask (3.1), using the analytical balance (3.8), and add a suitable amount of internal standard (4.5)**,** depending on the assumed wax content, e.g. add 0.1 mg of lauryl arachidate in the

case of olive oil, 0.25-0.50 mg in the case of olive-pomace oil and 0.05 mg of methyl heptadecanoate for olive oils (4.6).
Transfer the prepared sample to the chromatography column with the aid of two 2-ml portions of n-hexane (4.2).

Allow the solvent to flow to 1 mm above the upper level of the absorbent. Percolate a further 70 ml of n-hexane to remove any n-alkanes naturally present. Then start chromatographic elution of n-hexane/ethyl ether (99:1) and collect 220 ml at a flow of about 15 drops every 10 seconds. (**This fraction contains the methyl and ethyl esters and waxes).** (*Note 4*) (*Note 5*).

Evaporate the resultant fractions in a rotary evaporator (3.6) until the solvent is almost removed. Remove the last 2 ml under a weak current of nitrogen. Collect the fraction containing the methyl and ethyl esters is diluted with 2-4 ml of n-heptane or iso-octane.

5.2. Gas chromatography analysis

5.2.1. Preliminary procedure

Fit the column to the gas chromatograph (3.3), connecting the inlet port to the on-column system and the outlet port to the detector. Check the gas chromatography apparatus (operation of gas loops, efficiency of detector and recorder system, etc.).

If the column is being used for the first time, it is advisable to condition it. Run a light flow of gas through the column, then switch on the gas chromatography apparatus. Gradually heat until a temperature of 350 °C is reached after approximately 4 h.

Maintain this temperature for at least 2 h, then regulate the apparatus to the operating conditions (regulate gas flow, light flame, connect to electronic recorder (3.3.4), regulate oven temperature for column, regulate detector, etc.). Record the signal at a sensitivity at least twice as high as that required for the analysis. The base line should be linear, with no peaks of any kind, and must not have any drift.

Negative straight-line drift indicates that the column connections are not correct while positive drift indicates that the column has not been properly conditioned.

Note 4: The n-hexane/ethyl ether (99:1) mixture should be freshly prepared every day

Note 5: 100µl of Sudan I dye at 1% in the elution mixture can be added to the sample solution to check visually that the waxes are eluted properly.
The retention time of the dye lies in between that of the waxes and triacylglycerols. Hence, when the dye reaches the bottom of the chromatography column, elution has to be suspended because all the waxes have been eluted.

5.2.2. Choice of operating conditions (*Note 6*)

The operating conditions are generally as follows:

5.2.2.1. For Waxes only:

- Column temperature:

$$80\ ^\circ C \text{ at first } (1') \xrightarrow{20\ ^\circ C/min} 240\ ^\circ C \xrightarrow{5\ ^\circ C/min} 325\ ^\circ C\ (6') \xrightarrow{20\ ^\circ C/min} 340\ ^\circ C\ (10')$$

- Detector temperature: 350 ºC.

- Amount injected: 1 µl of n-heptane solution (2-4 ml).

- Carrier gas: helium or hydrogen at the optimal linear speed for the gas chosen (see Appendix A).

- Instrument sensitivity: suitable for fulfilling the above conditions

5.2.2.2. For waxes and methyl and ethyl esters:

- Column temperature:

$$80\ ^\circ C \text{ at first } (1') \xrightarrow{20\ ^\circ C/min} 140\ ^\circ C \xrightarrow{5\ ^\circ C/min} 335\ ^\circ C\ (20')$$

- Detector temperature: 350 °C.
- Amount injected: 1 µl of n-heptane solution (2-4 ml).
- Carrier gas: helium or hydrogen at the optimal linear speed for the gas chosen (see Appendix A).
- Instrument sensitivity: suitable for fulfilling the above conditions.

These conditions may be modified to suit the characteristics of the column and the gas chromatograph in order to separate all the waxes and fatty acid methyl and ethyl esters and to obtain satisfactory peak separation (see Figures 2, 3 and 4) and a retention time of 18 ± 3 minutes for the lauryl arachidate internal standard. The most representative peak of the waxes must be over 60% of the full-scale value while the methyl heptadecanoate internal standard for the methyl and ethyl esters must reach the full-scale value.

The peak integration parameters should be determined in such a way as to obtain a correct evaluation of the peak areas considered.

Note 6: Due to the high final temperature, positive drift is allowed but may not exceed more than 10% of the full-scale value.

5.3. Performance of the 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 waxes are completely eluted,

The base line must always meet the required conditions.

5.4. Peak identification

Identify the peaks from the retention times by comparing them with mixtures of waxes with known retention times, analysed under the same conditions. The alkyl esters are identified from mixtures of methyl and ethyl esters of the chief fatty acids in olive oils (palmitic and oleic).
Figure 1 provides a chromatogram of the waxes in a virgin olive oil. Figures 2 and 3 show the chromatograms of two retail extra virgin olive oils, one with methyl and ethyl esters and the other without them. Figure 4 gives the chromatograms for a top-quality extra virgin olive oil and the same oil spiked with 20% deodorised oil.

5.5. Quantitative analysis of the waxes

Determine the area of the peaks corresponding to the lauryl arachidate internal standard and the aliphatic esters from C40 to C46 with the aid of the integrator.

Determine the individual wax, in mg/kg of fat, as follows:

$$C_x \text{ Wax, mg/kg} = \frac{A_x \cdot m_s \cdot 1000}{A_s \cdot m}$$

where:

A_x = area corresponding to the peak for the individual ester, in computer counts
A_s = area corresponding to the peak for the lauryl arachidate internal standard, in computer counts
m_s = mass of the lauryl arachidate internal standard added, in milligrams;
m = mass of the sample taken for determination, in grams.

5.5.1 Quantitative analysis of the methyl and ethyl esters

With the aid of the integrator, determine the areas of the peaks corresponding to the methyl heptadecanoate internal standard, the methyl esters of the C16 and C18 fatty acids and the ethyl esters of the C16 and C18 fatty acids.

Determine the content of each alkyl ester, in mg/kg of fat, as follows:

$$\text{Ester, mg/kg} = \frac{A_x \cdot m_s \cdot 1000}{A_s \cdot m}$$

where:

A_x = area corresponding to the peak for the individual C16 and C18 ester, in computer counts

A_s = area corresponding to the peak for the methyl heptadecanoate internal standard, in computer counts

m_s = mass of the methyl heptadecanoate internal standard added, in milligrams;

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

6 EXPRESSION OF RESULTS

Report the sum of the contents of the different waxes from C40 to C46 (*Note 7*) in milligrams per kilograms of fat for Ordinary Olive oil (OOO), Lampante olive oil (LOO), Refined olive oil (ROO) , Olive oil (ROO+ VOOs), Crude olive pomace oil (COPO), Refined olive pomace oil and Olive pomace oil (ROPO+ VOOs).

Report the sum of the contents of the different waxes from C42 to C46 (*Note 7*) in milligrams per kilograms of fat for Extra virgin Olive oil (EVOO) and Virgin Olive oil (VOO)

Report the sum of the contents of the methyl esters and ethyl esters from C16 to C18 and the total of the two.

Results should be expressed to the nearest mg/kg.

Report the ratio between ethyl esters and methyl esters

Note 7: The components for quantification refer to the peaks with even carbon numbers amongst the C40 - C46 esters, according to the specimen chromatogram of the waxes in olive oil provided in the attached figure. For identification purposes, if the C46 ester is split, it is recommended to analyse the wax fraction of an olive-pomace oil where the C46 peak is distinguishable because it is clearly predominant.

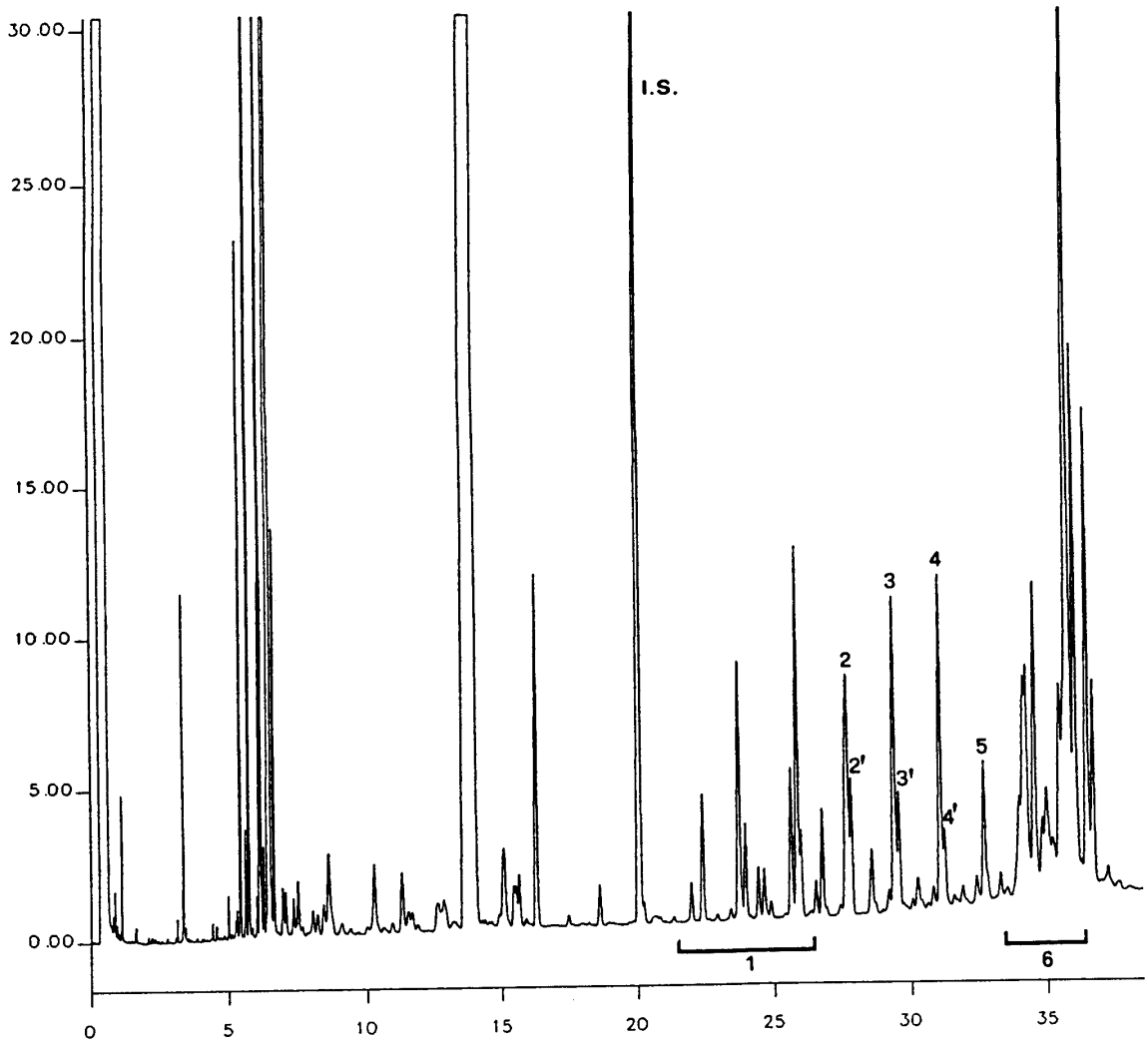

Figure 1 - Example of a gas chromatogram of the wax fraction of an olive oil (*).

Keys:

Peaks with a retention time from 5 to 8 min of the fatty acid methyl and ethyl esters

I.S. Lauryl arachidate

1 = Diterpenic esters
2+2' = C40 esters
3+3' = C42 esters
4+4' = C44 esters
5 = C46 esters
6 = Sterol esters and triterpene alcohols

(*) After elution of the sterol esters, the chromatogram should not show any significant peaks (triacylglycerols).

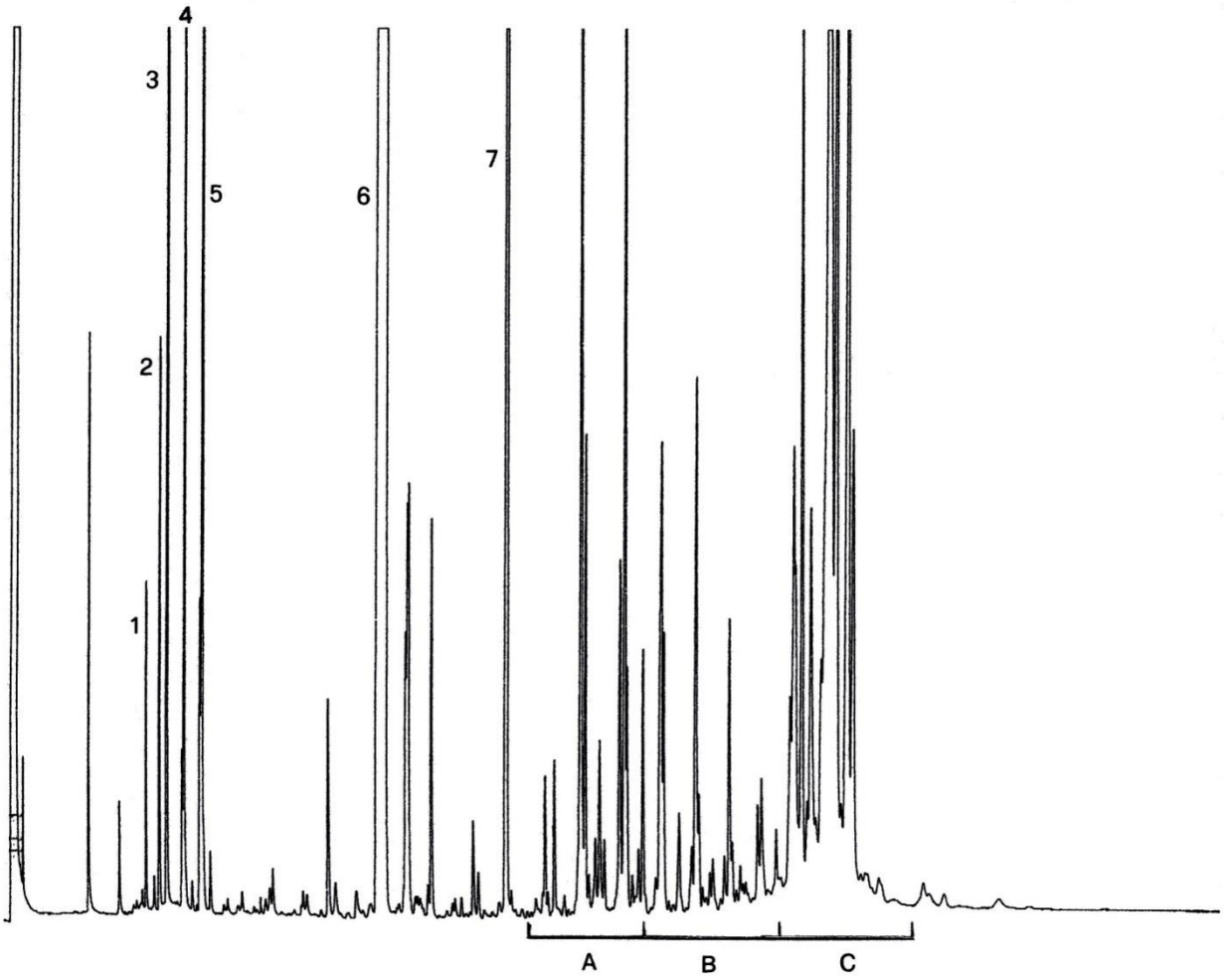

Figure 2 – Methyl esters, ethyl esters and waxes in a **virgin olive oil.**

Keys:

1 – Methyl C16
2 – Ethyl C16
3 – Methyl heptadecanoate I.S.
4 – Methyl C18
5 – Ethyl C18
6 – Squalene
7 – Lauryl arachidate I.S.
A – Diterpenic esters
B – Waxes
C – Sterol esters and triterpenic esters

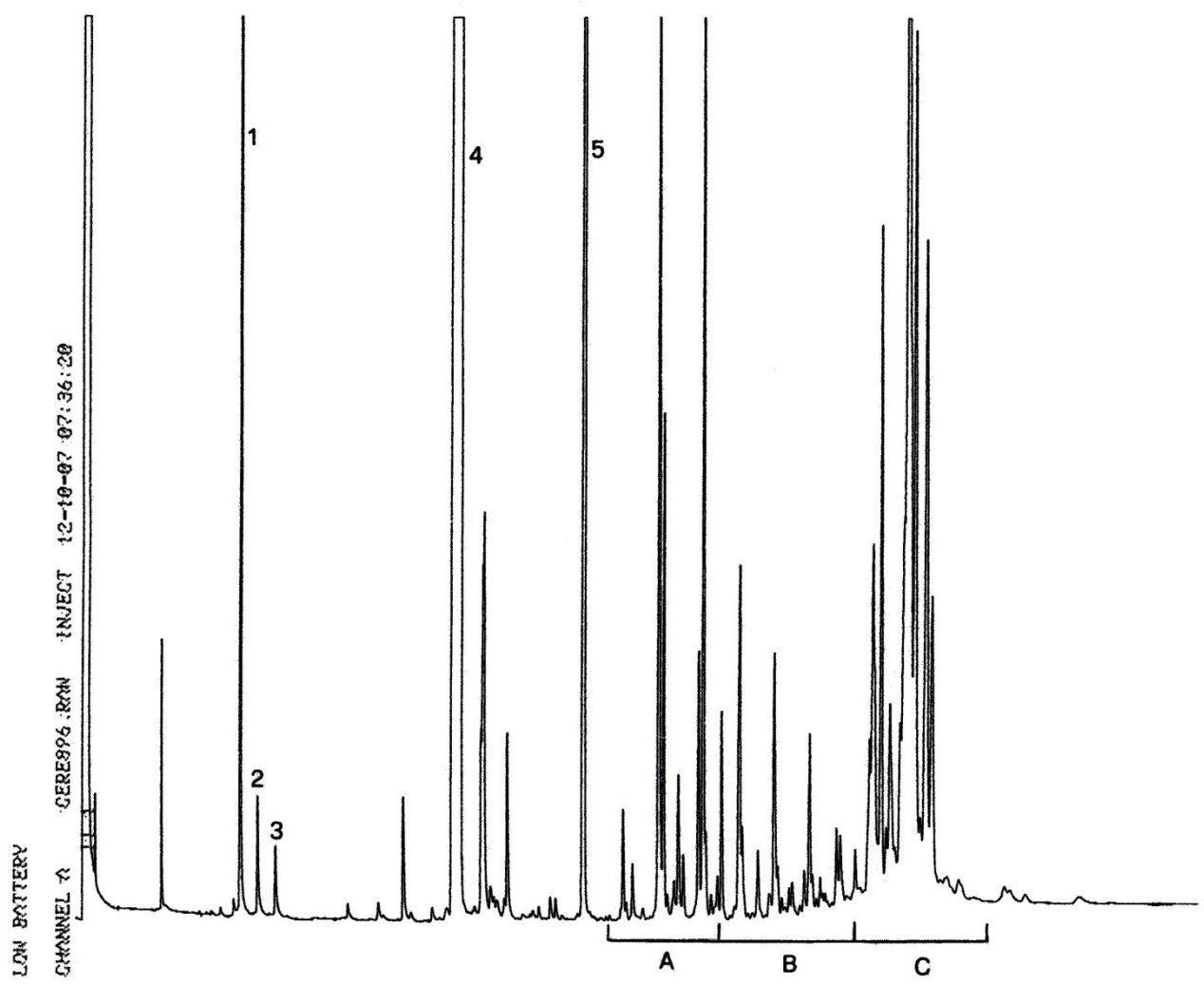

Figure 3 – Methyl esters, ethyl esters and waxes in an **extra virgin olive oil.**

Keys:

1 – Methyl heptadecanoate I.S.
2 – Methyl C18
3 – Ethyl C18
4 – Squalene
5 – Lauryl arachidate I.S.

A – Diterpenic esters
B – Waxes
C – Sterol esters and triterpenic esters

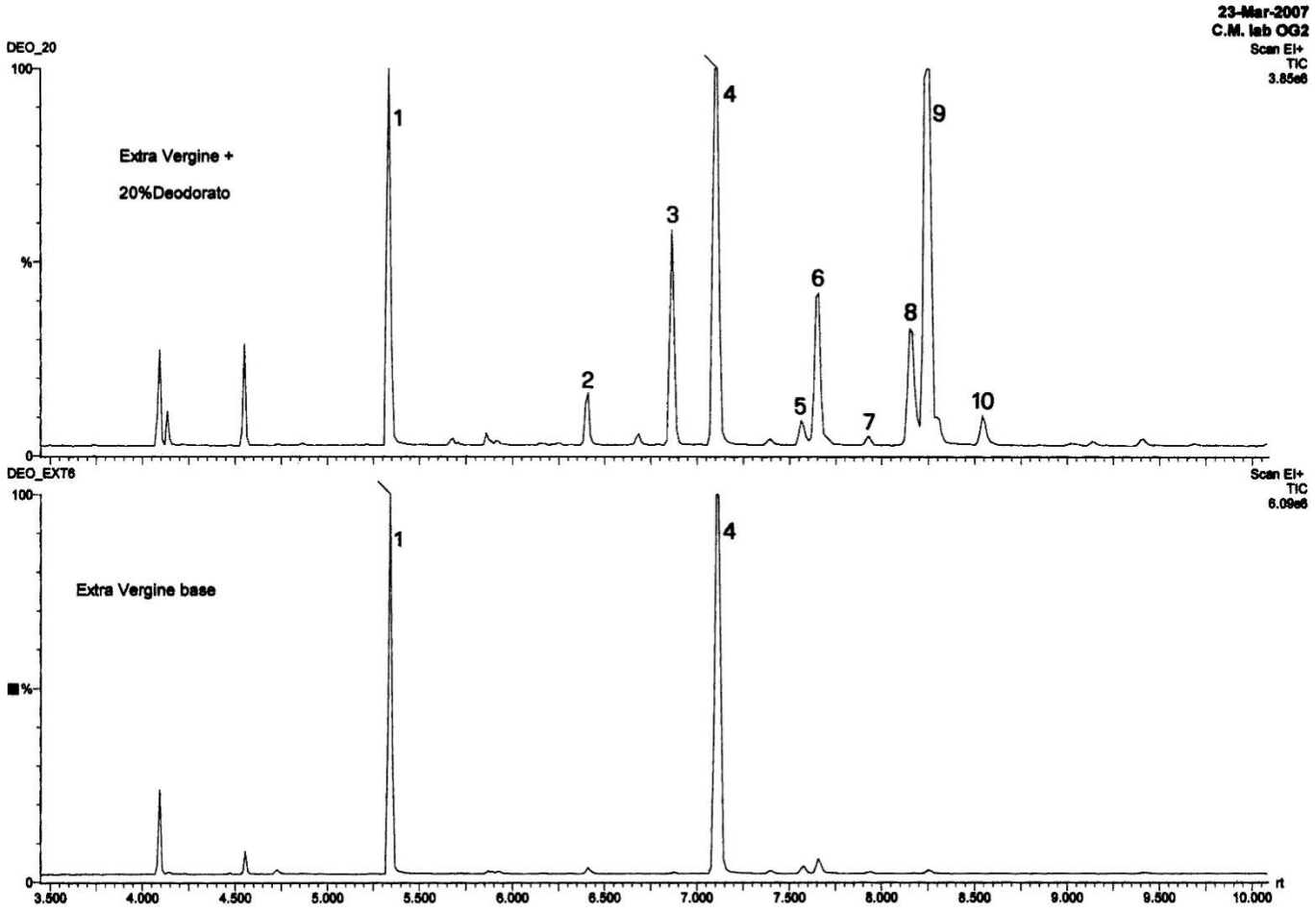

Figure 4 – Part of a chromatogram of an **extra virgin olive oil** and **the same oil spiked with deodorised oil.**

Keys:

1 – Methyl myristate I.S.
2 – Methyl palmitate
3 – Ethyl palmitate
4 – Methyl heptadecanoate I.S.
5 – Methyl linoleate
6 – Methyl oleate
7 – Methyl stearate
8 – Ethyl linoleate
9 – Ethyl oleate
10 – Ethyl stearate

APPENDIX A

Determination of linear gas speed

Inject 1:3 µl of methane (or propane) into the gas chromatograph after adjusting it to the normal operating conditions. Measure the time the gas takes to run through the column from the moment it is injected until the peak emerges (tM).

The linear speed in cm/s is given by L/tM where L is the length of the column, in cm, and tM is the time measured in s.

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PRECISION VALUES OF THE METHOD

FOR WAXES AND ALKYL ESTERS

1. Analysis of the collaborative test results

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

Twenty laboratories took part in the collaborative test arranged by the Executive Secretariat in 2008. The laboratories were from seven countries.

The test was performed on five samples: Olive Oil and Extra Virgin Olive Oil

A: extra virgin olive retail Italy
B: extra virgin olive retail Italy
: C extra virgin olive retail + refined
D: extra virgin olive oil + lampante
E: extra virgin olive oil + retail Germany

The results of the collaborative test organised by the IOC Executive Secretariat were 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's test to the laboratory results for each determination (replicates a and b).

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

RSD_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

RSD_r (%) reproducibility coefficient of variation (S_r x 100 / mean)

Wax content (mg/kg)

Ring Test COI 2008

	A	B	C	D	E
n	20	18	19	18	18
outliers	2	1	0	0	0
mean	125	181	199	142	174
r	9,8	13,0	20,1	17,6	12,2
S_r	3,3	4,4	6,8	5,9	4,1
RSD_r(%)	2,7	2,4	3,4	4,2	2,4
R	87,3	75,4	67,9	82,7	44,0
S_R	29,5	25,6	23,0	27,8	14,8
RSD_R(%)	23,7	11,8	11,6	19,6	8,5

ETHYL & METHYL ESTERS content (mg/kg)

Ethyl C16+C18 - Ring Test COI 2010

A: high quality extra virgin year 2001
B: high quality extra virgin year 1991 *C: extra virgin supermarket year 2010*
D: extra virgin supermarket year 2010 *E: extra virgin supermarket year 2010*

	A	B	C	D	E
n	15	17	17	17	17
outliers	1	2	1	2	2
mean	5	137	276	96	28
r	2,14	5,36	7,6	6,66	2,66
S_r	0,76	1,91	2,71	2,38	0,95
RSD_r(%)	14,8	1,4	1,0	2,5	3,4
R	6,71	38,82	95,91	29,23	15,50
S_R	2,40	13,86	34,25	10,44	5,54
RSD_R(%)	46,5	10,1	12,4	10,9	19,7

Methyl C16+C18 - Ring Test COI 2010

	A	B	C	D	E
n	15	17	17	17	17
outliers	2	2	1	1	3
mean	33	69	74	44	16
r	5,67	10,1	5,09	7,69	2,71
S_r	2,02	3,61	1,82	2,75	0,97
RSD_r(%)	6,1	5,2	2,5	6,2	6,1
R	13,38	26,85	29,48	18,44	10,52
S_R	4,78	9,59	10,53	6,58	3,76
RSD_R(%)	14,3	13,8	14,2	14,9	23,6

SUM Methyl + Ethyl C16+C18 - Ring Test COI 2010

	A	B	C	D	E
n	15	17	17	17	17
outliers	2	1	2	2	2
mean	38	212	350	139	43
r	6,80	16,83	6,29	7,21	4,09
S_r	2,43	6,01	2,25	2,58	1,46
RSD_r(%)	6,3	2,8	0,6	1,9	3,4
R	17,91	77,26	112,95	38,47	14,12
S_R	6,39	27,59	40,34	13,74	5,04
RSD_R(%)	16,6	13,0	11,5	9,9	11,7

RATIO on Methyl and Ethyl C16+C18 - Ring Test COI 2010

	A	B	C	D	E
n	15	17	17	17	17
outliers	0	1	1	1	1
mean	0,2	2,0	3,8	2,2	1,8
r	0,08	0,21	0,30	0,35	0,42
S_r	0,03	0,08	0,11	0,13	0,15
RSD_r(%)	18,2	3,8	2,8	5,7	8,5
R	0,23	0,57	1,56	0,68	1,38
S_R	0,08	0,20	0,56	0,24	0,49
RSD_R(%)	51,5	10,1	14,7	11,0	28,2

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

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

Ethyl esters (mg/kg)	**n**	**Consensus mean**	**S_r**	**S_R**
Regulatory method	22	26	1,39	2,29
Alternative solvent method	24	27	0,98	2,97
Evaluation	**Calculated**	**Limit**	**Conclusion/Comments**	
Difference (Regulatory method- Alternative solvent method)	1			
Test F repeatability	2,03	2,07	F cal < F limit	
Test F reproducibility	1,68	2,10	F cal < F limit	
Current reproducibility Regulatory	2,97	3,94	S_R obtained is smaller than current S_R	
T Student	0,74	2	t cal < t limit	

Where Regulatory Method is with the use of hexane as solvent;
Where Alternative solvent method is 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.

PRECISION VALUES FOR WAXES ONLY

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)

Wax content (mg/kg)

	A	B	C	D	E
n	19	19	19	19	19
outliers	5	5	4	3	5
mean	120	123	222	174	346
r	9,5	12,6	10,5	12,2	14,9
S_r	3,4	4,5	3,8	4,7	5,3
RSD_r(%)	2,8	3,6	1,7	2,7	1,5
R	38,8	48,9	58,9	25,7	44,4
S_R	13,9	17,5	21,0	9,2	15,9
RSD_R(%)	11,5	14,2	9,5	5,3	4,6

2. **Analysis of the collaborative IOOC test results in 2017 for the aptitude test** Only a sample of Virgin olive oil with a quantifiable content of alkyl ester, adulterated with 10% refined olive oil and 2% animal fat has been tested.

Waxes (mg/kg)	n	Consensus mean	S_r	S_R
Regulatory method	25	479	6,9	28,5
Alternative solvent method	23	485	7,0	25,6

Evaluation	Calculated	Limit	Conclusion/Comments
Difference (Regulatory method- Alternative solvent method)	-6		
Test F repeatability	1,03	2,04	F cal < F limit
Test F reproducibility	1,24	2,06	F cal < F limit
Current reproducibility Regulatory	25,6	43,1	S_R obtained is smaller than current S_R

T Student	0,70	2	t cal < t limit

Where Regulatory Method is with the use of hexane as solvent;
Where Alternative solvent method is 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. 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 5725-5:1998 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

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