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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 USING 3 GRAMS OF SILICA

1. PURPOSE

This method is for the determination of the content of waxes, fatty acid methyl esters and fatty acid 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 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. **Test tube,** 10 ml.

3.2. **Glass column** for liquid chromatography, internal diameter 10 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, length 8-12 m, internal diameter 0.25-0.32 mm, internally coated with liquid phase (*Note 2*) to a uniform thickness of 0.10-0.25 µm.

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

3.5. Microsyringe, 10 µl, with hardened needle, for injection through a silicone septum.

3.6. Electric shaker.

3.7. Rotary evaporator.

3.8. Muffle oven.

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

3.10. Usual laboratory glassware.

3.11. Round-bottomed flasks, 100 ml

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 (each batch must be checked).
Test purity by evaporating 200 ml of n-hexane and subsequently dissolving the residue in 200 µl of n-heptane. Inject 1 µl into the gas chromatograph according to the same conditions as those reported in this method. No peak should appear in the pertinent areas 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. 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.

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.

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 (or arachidyl laurate) (*Note 3*), at 0,002% (m/V) (2mg/100ml) in n-heptane (internal standard for waxes).

4.6. Standard solution of methyl heptadecanoate, at 0.0005% (m/V) (0.5mg/100ml) in n-heptane (internal standard for methyl and ethyl esters).

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

4.9. 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 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.

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

4.10. 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 3 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 (3.6) to make the chromatographic bed more homogeneous. Percolate 10 ml of n-hexane to remove any impurities. Weigh exactly about 100 mg of the sample into the 10-ml flask (3.1), using the analytical balance (3.9), and add a suitable amount of internal standard (4.5) depending on the assumed wax content, e.g. add 0.02 mg (1 ml) of lauryl arachidate in the case of olive oil, 0.05-0.10 mg in the case of olive-pomace oil and 0.005 mg (1 ml) of methyl heptadecanoate for olive oils (4.6).

Transfer the prepared sample to the chromatography column with the aid of two 1-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 10 ml of n-hexane to remove any n-alkanes naturally present, then change the eluent mixture (*Note 4*) (*Note 5*) to a mixture of n-hexane/ethyl ether (99:1) and collect 30-32 ml at a flow of about 15 drops every 10 seconds in a 100 ml round-bottomed flask. (**This fraction contains the methyl and ethyl esters and waxes** - *Note 6*)**.**

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.

Note 6: The L.C. conditions usually produce optimum values; however they are for guidance purposes and should be modified, as required, until performance is satisfactory.

Evaporate the alkyl ester and waxes fraction in a rotary evaporator (3.7) 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 and dilute with 0.5-1 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.

5.2.2. Choice of operating conditions for waxes and methyl and ethyl esters (*Note 7*)

The operating conditions are generally as follows:

- Column temperature for alkyl esters and waxes together:

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

- Column temperature for waxes only:

$$80\ ^\circ C \text{ at first (1')} \xrightarrow{20\ ^\circ C/min} 220\ ^\circ C \xrightarrow{5\ ^\circ C/min} 335\ ^\circ C\ (20)$$

- Detector temperature: 350 °C.
- Amount injected: 1 µl of n-heptane or isooctane solution (0.5-1 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.

Note 7: Due to the high final temperature, positive drift is allowed but may not exceed more than 10% of the full-scale value.

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) and a retention time of 18 ± 3 minutes for the lauryl arachidate internal standard. The most representative peak of the waxes must be over 50% 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.

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, depending on the fraction being analysed.

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).
The Figures provide chromatograms of a refined olive oil spiked with alkyl esters (Fig 1), alkyl esters and waxes in an old virgin olive oil (Fig. 2) and the same oil analysed in wax conditions (Fig.3).

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 C42 to C46 with the aid of the integrator.

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

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

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.

5.6. 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 esters, 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 C42 to C46 (*Note 8*) in milligrams per kilograms of fat (ppm) without decimals.

Report the sum of the contents of the methyl esters and ethyl esters from C16 to C18 and the total of the two in mg/kg as well as the ratio of ethyl esters/methyl esters. All results should be expressed to one decimal place.

Note 8: The components for quantification refer to the peaks with even carbon numbers amongst the C42 - 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 sample where the C46 peak is distinguishable because it is clearly predominant.

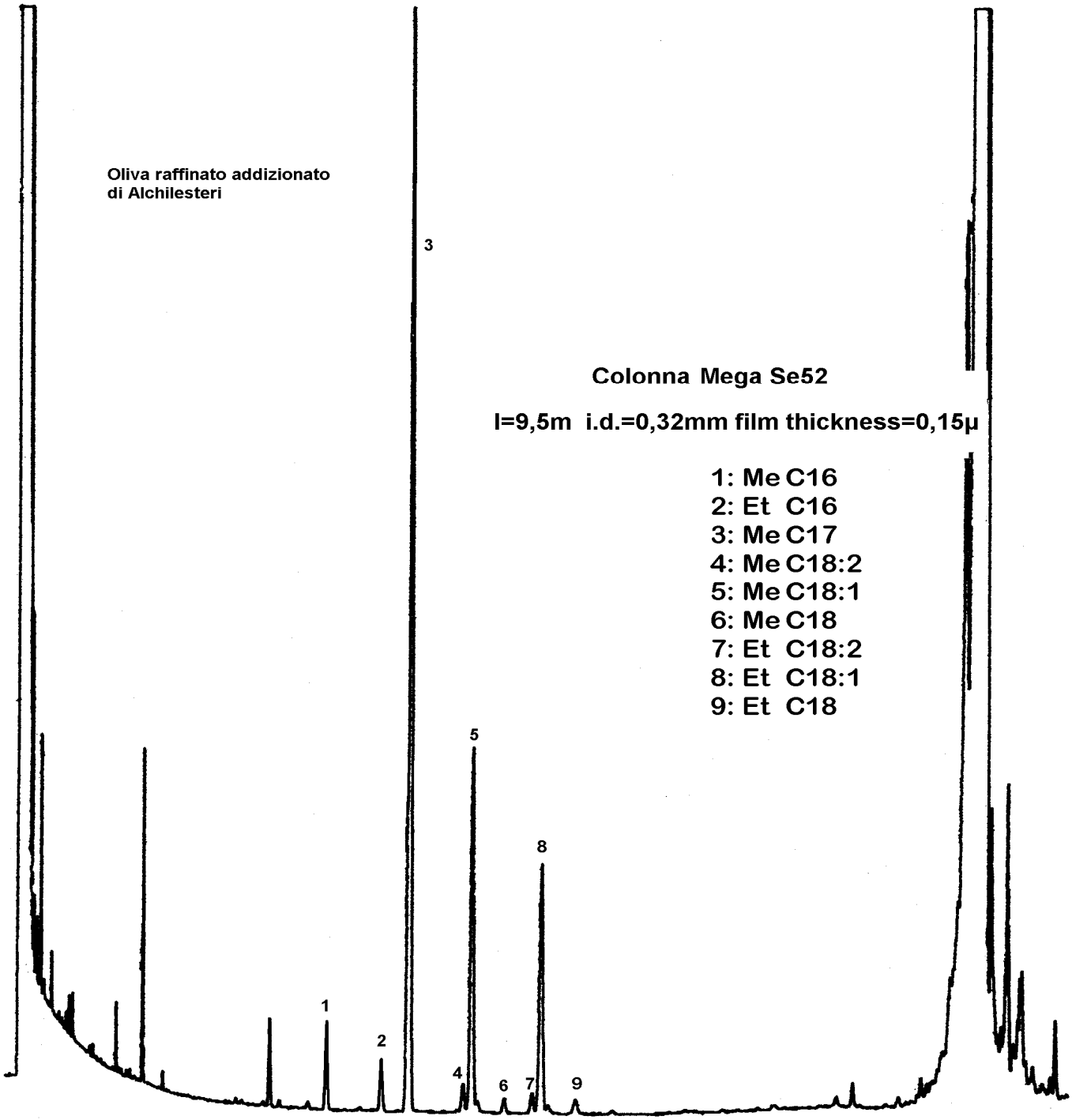

Figure 1 - Chromatogram of a refined olive oil spiked with alkyl esters
The small peak co-eluted just after Me C18:1 (peak 5) should be integrated with Me C18:1. Same remark for the small peak co-eluted just after EtC18:1 (peak 8)

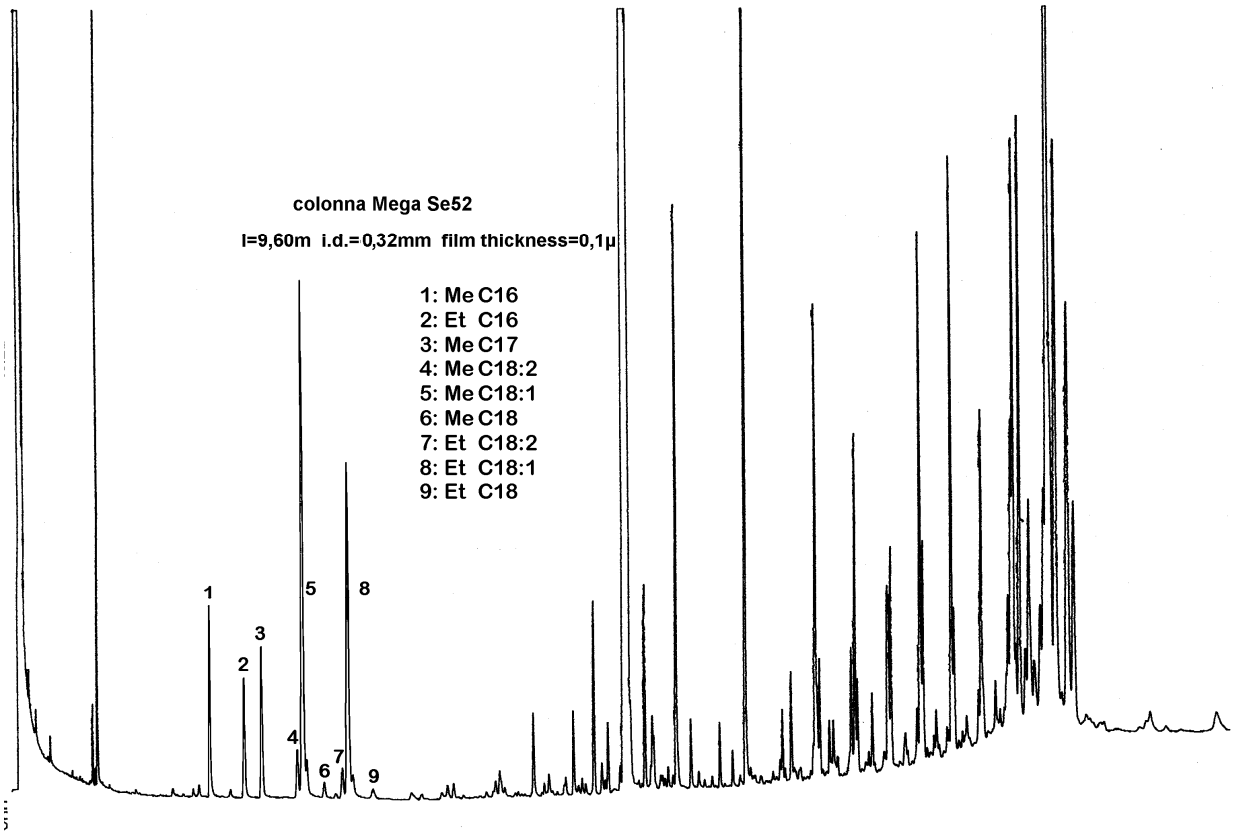

Figure 2 - Chromatogram of virgin olive oil alkyl esters and waxes together

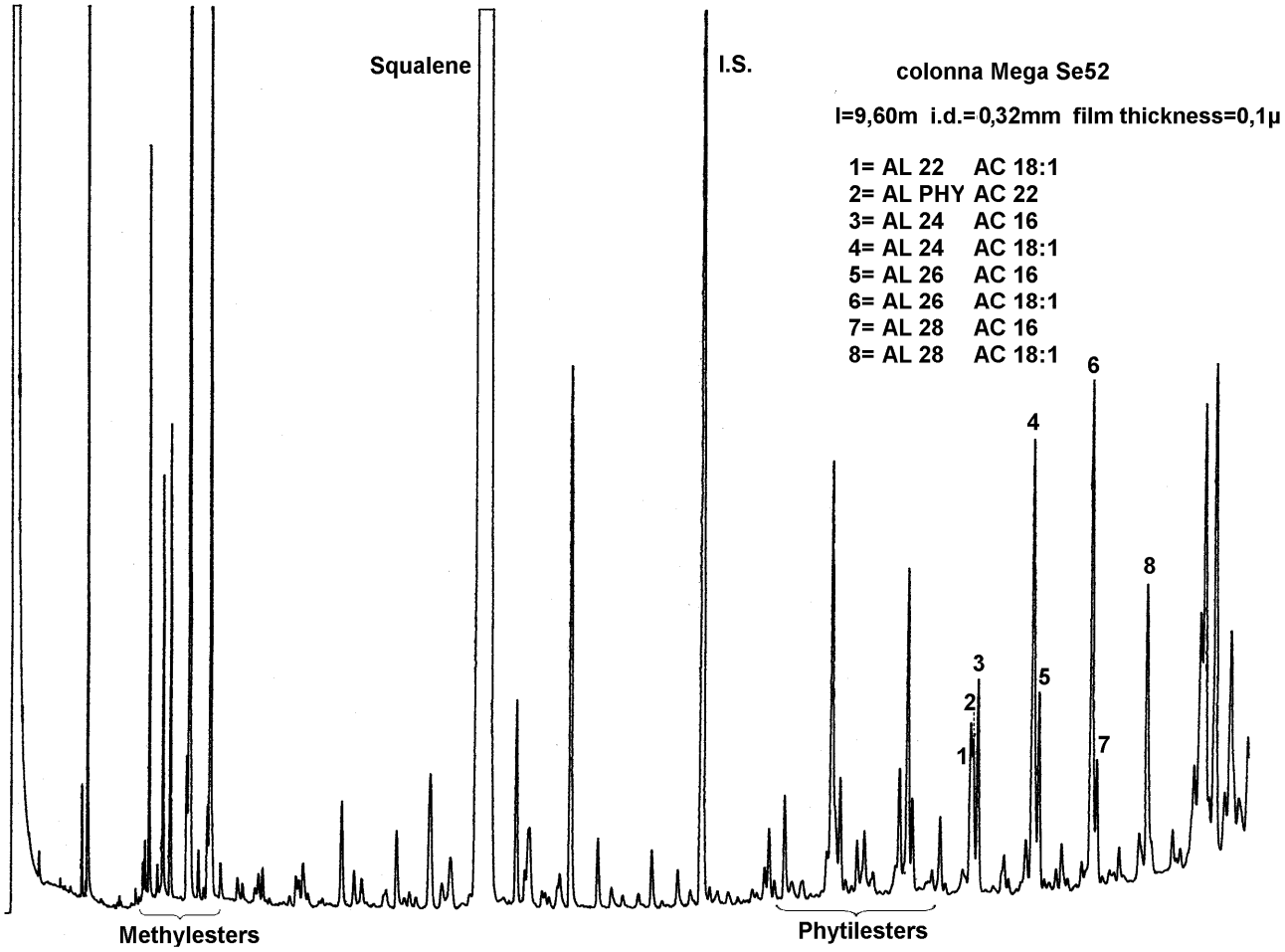

Figure 3 - Methyl esters, ethyl esters and waxes in a virgin olive oil (wax conditions)

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

Analysis of the collaborative test results

Twenty-one laboratories, including some holding IOC recognition at the time, took part in the collaborative test arranged by the International Olive Council in 2012.

The test was performed on six samples:

- AEC1: virgin olive oil with an alkyl ester content of 75-150 mg (Spain)
- AEC2: extra olive oil with an alkyl ester content of 10-50 mg (Spain)
- AEC3: virgin olive oil with an alkyl ester content of 130 mg (Spain)
- AEC4: olive oil with a wax content of 500 – 600 ppm (Tunisia)
- AEC5: extra virgin olive oil from 2011/12 crop year - 1st prize, Intense green fruity section, IOC Mario Solinas Quality Award 2012 (Spain)
- AEC6: extra virgin olive oil from 2009/10 crop year – 2nd prize, Intense green fruity section, IOC Mario Solinas Quality Award 2009 (Spain).

The results of the collaborative test have been statistically processed according to the rules laid down in the international standards ISO 5725-2. 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

S_r Repeatability standard deviation

RDS_r (%) Relative standard deviation for repeatability (Sr x 100/mean)

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% (Sr multiplied by 2.8)

S_R Reproducibility standard deviation

RDS_R (%) Relative standard deviation for reproducibility (SR 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% (SR multiplied by 2.8)

Methyl esters concentration (mg/kg)						
sample	**AEC 1**	**AEC 2**	**AEC 3**	**AEC 4**	**AEC 5**	**AEC 6**
Mean	51.57	14.04	59.54	63.03	5.31	11.25
n	20	20	20	20	19	19
outliers	0	3	0	3	2	1
Sr	3.49	0.86	1.80	1.53	0.46	1.12
RSD_r(%)	6.77	6.12	3.02	2.43	8.74	9.92
r	9.77	2.41	5.03	4.29	1.30	3.13
S_R	8.89	5.09	16.22	7.38	4.30	9.69
RSD_R(%)	17.25	36.25	27.24	11.71	80.99	86.17
R	24.90	14.26	45.41	20.67	12.05	27.14

Ethyl esters concentration (mg/kg)						
sample	**AEC 1**	**AEC 2**	**AEC 3**	**AEC 4**	**AEC 5**	**AEC 6**
Mean	53.39	21.20	87.02	151.25	2.98	4.52
n	20	20	20	20	19	19
outliers	0	0	0	2	2	2
Sr	1.81	0.91	2.10	4.78	0.64	1.30
RSD_r(%)	3.39	4.31	2.41	3.16	21.59	28.83
r	5.07	2.56	5.87	13.39	1.80	3.65
S_R	9.14	6.03	21.58	16.76	2.38	2.82
RSD_R(%)	17.12	28.44	24.80	11.08	79.88	62.41
R	25.60	16.88	60.42	46.92	6.67	7.90

Alkyl esters concentration (sum of methyl esters and ethyl esters) (mg/kg)						
sample	**AEC 1**	**AEC 2**	**AEC 3**	**AEC 4**	**AEC 5**	**AEC 6**
Mean	104.96	34.52	144.87	218.79	11.60	13.99
n	20	20	20	20	19	19
outliers	0	2	0	2	0	2
Sr	4.41	1.35	3.64	6.04	1.30	2.58
RSD_r(%)	4.20	3.90	2.51	2.76	11.17	18.43
r	12.35	3.77	10.20	16.90	3.63	7.22
S_R	15.28	8.15	33.44	16.27	9.06	7.60
RSD_R(%)	14.55	23.62	23.09	7.44	78.05	54.35
R	42.78	22.83	93.64	45.55	25.36	21.29

Waxes concentration (sum of waxes from C42 to C46) (mg/kg)						
sample	**AEC 1**	**AEC 2**	**AEC 3**	**AEC 4**	**AEC 5**	**AEC 6**
Mean	210.07	36.32	54.02	310.95	34.10	39.08
n	20	20	20	20	19	19
outliers	1	2	2	0	1	1
Sr	3.97	1.64	3.28	7.80	1.83	3.56
RSD_r(%)	1.89	4.53	6.08	2.51	5.36	9.11
r	11.11	4.61	9.19	21.83	5.12	9.97
S_R	32.65	7.19	7.36	84.36	10.34	12.12
RSD_R(%)	15.54	19.79	13.63	27.13	30.33	31.02
R	91.43	20.12	20.62	236.22	28.96	33.95