

Commission Regulation (EU) No 61/2011 of 24 January 2011
amending Regulation (EEC) No 2568/91 on the characteristics of
olive oil and olive-residue oil and on the relevant methods of analysis

Changes to legislation: There are currently no known outstanding effects for the Commission Regulation (EU) No 61/2011, ANNEX II. (See end of Document for details)

ANNEX II

ANNEX XX

Method for the 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, 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, 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 (point 3.3.3), with a response time of not more than 1 s and a variable paper speed.

Note 1: Computerised systems may also be used where the gas chromatography data are entered through a PC.

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 .

Note 2: Suitable commercial liquid phases are available for this purpose such as SE52, SE54, etc.

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 $\pm 0,1$ 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 (the purity must be checked).

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.

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 0,05 % (m/V) in heptane (internal standard for waxes).

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

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).**

4.8. **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

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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 (point 4.1) in n-hexane (point 4.2) and introduce into the column (point 3.2). Allow to settle spontaneously. Complete settling with the aid of an electric shaker to make the chromatographic bed more homogeneous. Percolate 30 ml of n-hexane to remove any impurities. Weigh exactly about 500 mg of the sample into the 25-ml flask (point 3.1), using the analytical balance (point 3.8), and add a suitable amount of internal standard (point 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 (point 4.6).

Transfer the prepared sample to the chromatography column with the aid of two 2-ml portions of n-hexane (point 4.2).

Allow the solvent to flow to 1 mm above the upper level of the absorbent. Percolate a further 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).

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

Note 5: 100 µl 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.

Evaporate the resultant fractions in a rotary evaporator 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 (point 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 (point 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 6).

The operating conditions are generally as follows:

- Column : 20 °C/min 5 °C/min
temperature
- 80 °C at first (1') ————— 140 °C ————— 335 °C (20)
- Detector : 350 °C.
temperature
- Amount : 1 µl of n-heptane solution (2-4 ml).
injected
- Carrier gas : helium or hydrogen at the optimal linear speed for the gas chosen (see Appendix A).
- Instrument : suitable for fulfilling the above conditions.
sensitivity

Note 6: 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 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.

5.3. Performance of the analysis

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Take up 10 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 or stigmastadienes 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).

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 total waxes content by adding each individual wax, in mg/kg of fat, as follows:

$$\text{Waxes, mg/kg} = \frac{(\sum A_x) \cdot m_s \cdot 1\,000}{A_s \cdot m}$$

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:

$$\text{Ester, mg/kg} = \frac{A_x \cdot m_s \cdot 1\,000}{A_s \cdot m}$$

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.

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.

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.

Report the ratio between ethyl esters and methyl esters

Figure 1

Example of a gas chromatogram of the wax fraction of an olive oil⁽¹⁾

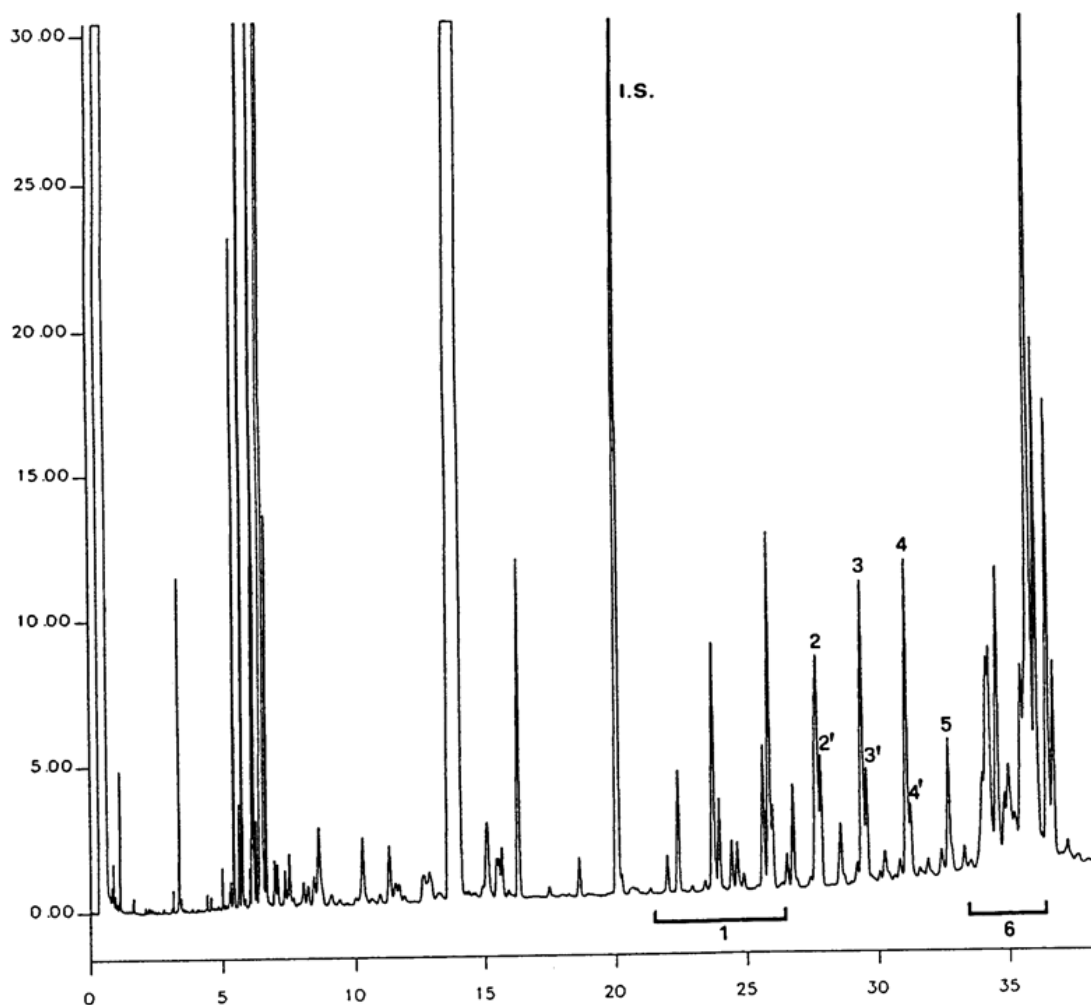


Figure 2

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Methyl esters, ethyl esters and waxes in a virgin olive oil

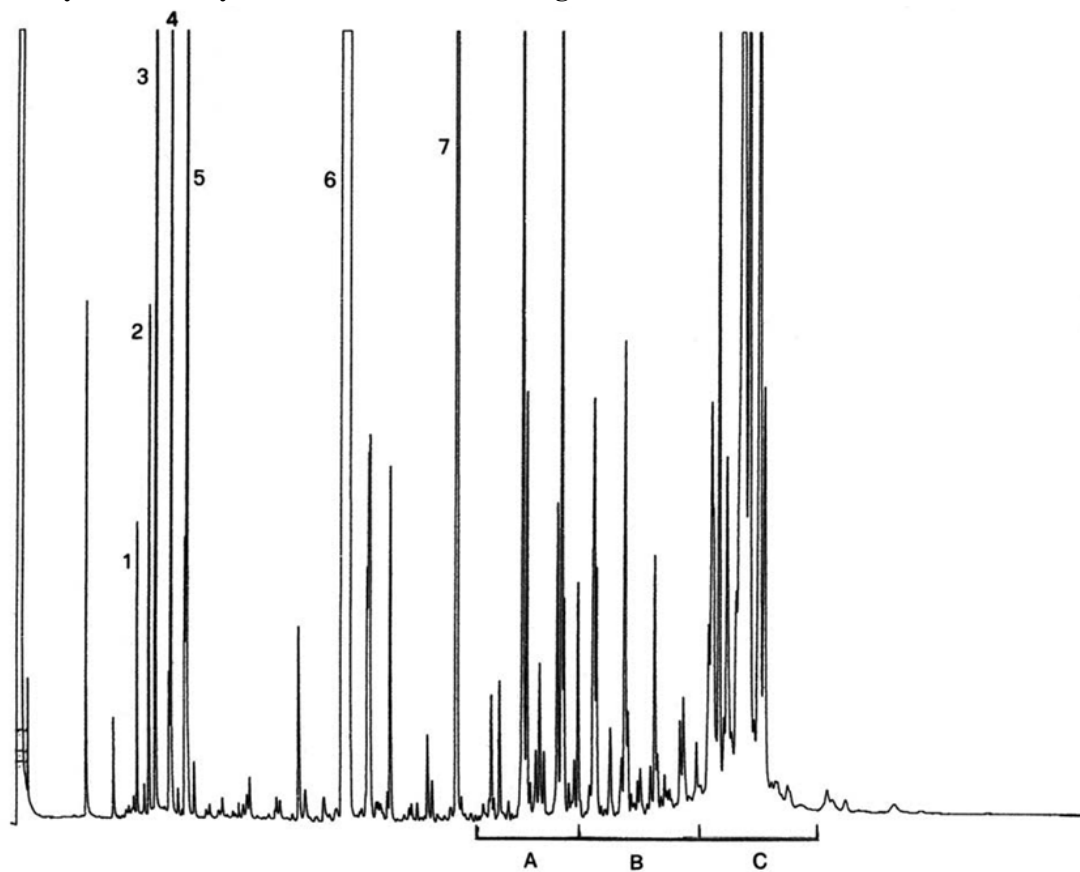


Figure 3

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Methyl esters, ethyl esters and waxes in an extra virgin olive oil

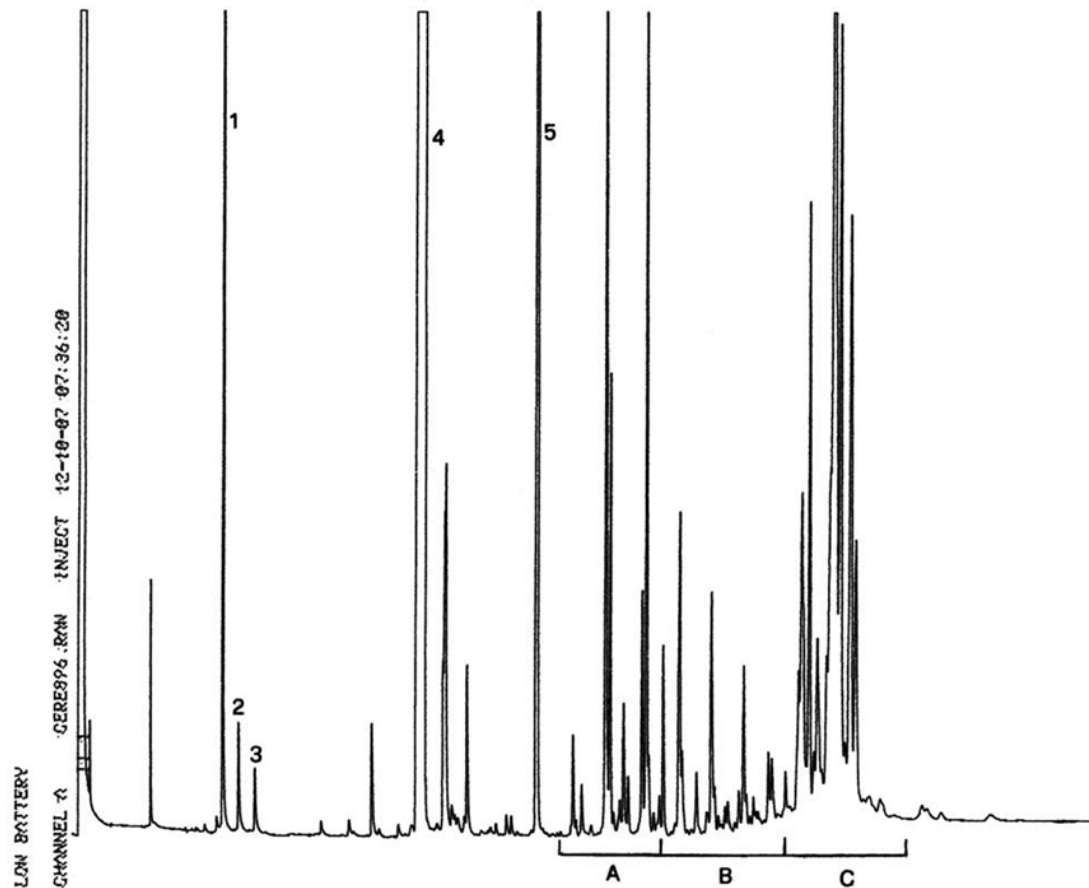
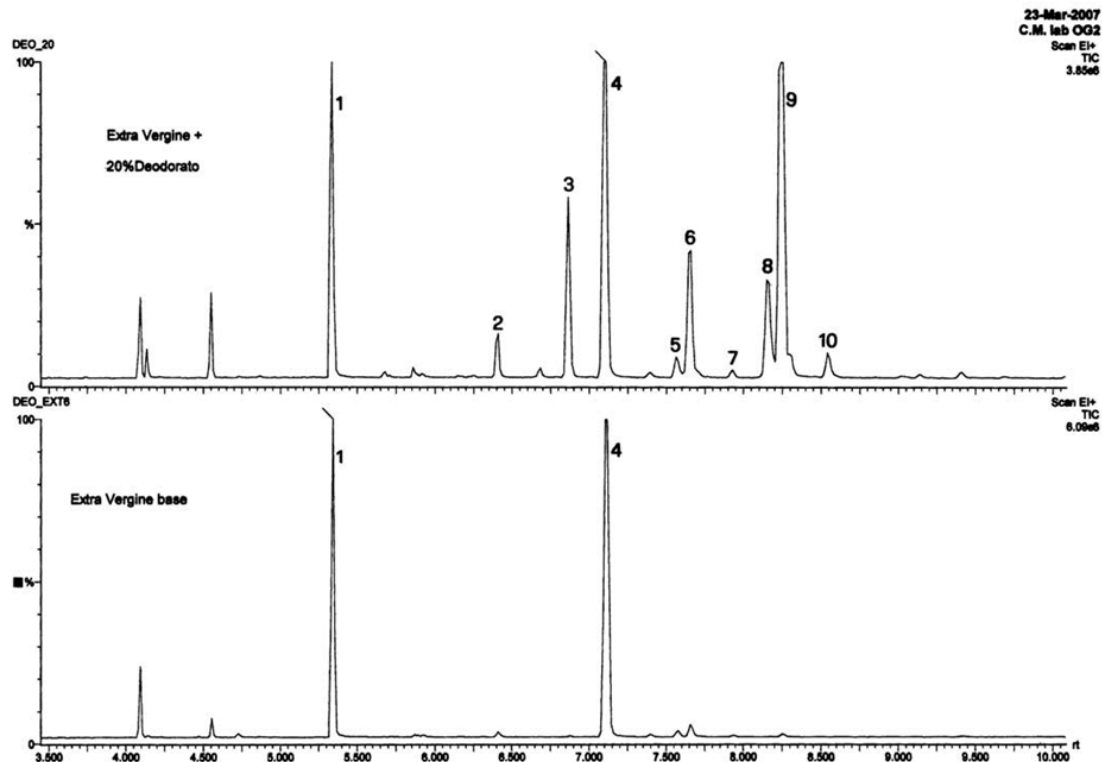


Figure 4

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Part of a chromatogram of an extra virgin olive oil and the same oil spiked with deodorised oil



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

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