INTERNATIONAL
OLIVE
COUNCIL

COI/T.20/Doc. No 28/Rev. 4
November 2024

ENGLISH
Original: ENGLISH

Príncipe de Vergara, 154 – 28002 Madrid – Spain Tel.: +34 915 903 638 Fax: +34 915 631 263 - E-mail: iooc@internationaloliveoil.org – http://www.internationaloliveoil.org/

METHOD OF ANALYSIS

DETERMINATION OF THE CONTENT OF WAXES 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 ethyl esters in olive oils. The individual waxes and alkyl esters are separated according to the number of carbon atoms.

2 - SCOPE

The method is recommended as a tool for distinguishing between olive oil and olive-pomace oil, and as a quality parameter for extra virgin oils, as it facilitates the identification of false blends of extra virgin olive oils and low-quality oils whether they are virgin, lampante or low quality oils that may have underwent a deodorization process. This document presents two methods that can be used both for official control.

3 - PRINCIPLE

Addition of suitable internal standards to the oil and fractionation by chromatography on 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. Waxes and alkyl esters can be analysed using either the method A or the method B. Both methods share the same principle, but the simplified method uses a reduced quantity of sample, reagents, and solvents. Method A is the reference method for counter assessment.

METHOD A – 15 GRAMS OF SILICA

A4 - APPARATUS

A4.1. Test tube, 10 ml.

A4.2 Glass column for liquid chromatography, internal diameter 15 mm, length 40 cm, fitted with a suitable stopcock.

A4.3 Gas chromatograph suitable for use with a capillary column, equipped with a system for direct, on-column injection comprising:

A4.3.1 Thermostat-controlled oven with temperature programming.

A4.3.2 Cold injector for direct on-column injection

A4.3.3 Flame ionisation detector and **converter-amplifier**.

A4.3.4 Computerized systems recorder-integrator (Note[1])

A4.3.5 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 0.10-0.25 µm.

A4.4 Microsyringe, 10 µl, for on-column injection.

A4.5 Electric shaker.

A4.6 Rotary evaporator.

A4.7 Muffle oven.

A4.8 Analytical balance for weighing to an accuracy of ± 0.1 mg.

A4.9 Usual laboratory glassware.

A5 – REAGENTS

A5.1 Silica gel, 60-200 µm mesh. Place the silica gel in the muffle oven at 500 °C for at least 4 hours. 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 hours prior to use. If the silica gel is ultra-pure grade, the muffle oven treatment may not be necessary. In any case each batch of silica must be checked for suitability.

A5.2 n-Hexane, chromatography (or residue) grade – the purity must be checked as follows: 100 ml of n-hexane are evaporated to dryness, residue is re-dissolved in 100 µl n-heptane, and

---

Note[1]: *also to use with the converter-amplifier (A4.3.3) a recorder with a response time of not more than 1 s and a variable paper speed can be used.*

Note[2]: *Suitable commercial liquid phases are available for this purpose such as SE52, SE54 (methyl silicon with 5% phenyl), etc. or other phase with similar or lower polarity.*

analysed as solvent blank applying the same gas chromatographic conditions. There must be no peak in the elution alkyl esters area. (Hexane can be replaced by Isooctane) Hexane – Chromosolv Pestanal is available from Honeywell-Riedel-de Haen (code 34484). This reference is an example of suitable products, which are available commercially. This information is given for the convenience of users of this Standard and does not constitute an endorsement of these products.

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. Harmful 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.

A5.3 **Ethyl ether**, chromatography grade.

WARNING – Highly inflammable and moderately toxic. Irritates the skin. Harmful 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.

A5.4 **n-Heptane**, chromatography grade, or **isooctane**

WARNING – Inflammable. Harmful 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.

A5.5 **Standard solution of lauryl arachidate** (Note[3]) at 0.02% (m/V) in heptane (internal standard for waxes).

A5.6 **Standard solution of methyl heptadecanoate** at 0.005% (m/V) in heptane (internal standard for methyl and ethyl esters).

A5.7 **Sudan 1** (1-phenylazo-2-naphthol) optional (attention: azo-compounds have mutagenic and carcinogenic properties)

A5.8 **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.

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

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.

WARNING - 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.

A5.9 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.

A6 - PROCEDURE

A6.1 Preparation of the chromatographic column

Suspend 15 g of silica gel (A5.1) in n-hexane (A5.2) and introduce into the column (A4.2). Allow to settle spontaneously. Complete settling with the aid of an electric shaker (A4.5) to make the chromatographic bed more homogeneous. Percolate 20 ml of n-hexane to remove any impurities. Weigh exactly, about 500 mg of the sample into the 10-ml test tube (A4.1), using the analytical balance (A4.8), and add a suitable amount of internal standard (A5.5) depending on the assumed wax content, e.g. add 0.10 mg of lauryl arachidate in the case of extra virgin olive oil, virgin olive oil, refined olive oil and olive oil, 0.25-0.50 mg in the case of olive-pomace oil and 0.05 mg of methyl heptadecanoate for extra virgin olive oils (A5.6). Transfer the prepared sample to the chromatography column with the aid of two 2-ml portions of n-hexane (A5.2). Allow the solvent to flow to 1 mm above the upper level of the absorbent. Percolate 50 ml n-hexane/ethyl ether (99:1) to further remove hydrocarbons (alkanes and

sterenes). Then continue the chromatographic elution of n-hexane/ethyl ether (99:1) (Note[4]) and collect 150 ml at a flow of about 15 drops every 10 seconds. (**This fraction contains ethyl esters and waxes**) (Note[5]).

Evaporate the resultant fractions in a rotary evaporator (A4.6) until the solvent is almost removed. Remove the last 2 ml under a weak current of nitrogen. Collect the fraction containing the ethyl esters and waxes diluted with 1-2 ml of n-heptane or isooctane.

A6.2 Gas chromatography analysis

A6.2.1 Preliminary procedure

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 to 350 °C (approximately 4 hours).
Maintain this temperature for at least 2 hours, then regulate the apparatus to the operating conditions (regulate gas flow, light flame, connect to electronic recorder (A4.3.4), regulate oven temperature for column, regulate detector, etc.). Record the signal at a sensitivity at least twice as high as 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.

A6.2.2 Choice of operating conditions for waxes and ethyl esters (Note[6])

The operating conditions are generally as follows:

- Column temperature:

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

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

Note[4]: *The n-hexane/ethyl ether (99:1) mixture should be freshly prepared every day, n-hexane can be replaced with the same amount of isooctane.*

Note[5]: *The elution volumes indicated in the text are those which usually are employed. However, if needed, they can be modified to achieve a satisfactory separation of the fractions. For this purpose, 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 volume of the dye lies in between that of the waxes and triacylglycerols. Hence, when the dye reaches the bottom of the chromatography column, elution must be suspended because all the waxes have been eluted. Verify the correct elution by checking the presence on the chromatogram at the same time of squalene and epoxy squalene.*

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

- Detector temperature: 350 °C.
- Amount injected: 1 µl of n-heptane solution (1-2ml).
- Carrier gas: helium or hydrogen at the optimal linear speed for the gas chosen (see Annex B).
- Instrument sensitivity suitable for the quantification of the analytes

These conditions may be modified to suit the characteristics of the column and the gas chromatograph to separate all the waxes and fatty acid ethyl esters and to obtain satisfactory peak separation (see Figures 1 and 2)

A6.3 Performance of the analysis

Take up 1-2 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 (C40-C46) are completely eluted.
The base line must always meet the required conditions.

A6.4 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 main fatty acids in olive oils (palmitic and oleic).
Figure 1 (Annex A) shows a chromatogram of the FAEE and waxes in an extra virgin olive oil using the method A (15 g).
Figure 2 (Annex A) shows a chromatogram of the FAEE and waxes in a lampante olive oil using the method A (15 g).

A6.5 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 in the case of extra virgin olive oil and virgin olive oil and form C40 to C46 in the case of other oils, with the aid of the integrator.

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

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

where:

Ax = area corresponding to the peak for the individual ester, in computer counts (sum of peaks: C42, C44, C46 for EVOO and VOO; sum of peaks: C40, C42, C44 and C46 for the other oils)

As = area corresponding to the peak for the lauryl arachidate internal standard, in computer counts
ms = mass of the lauryl arachidate internal standard added, in milligrams
m = mass of the sample taken for determination, in grams

A6.6 Quantitative analysis of the ethyl esters

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

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

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

where:

Ax = area corresponding to all the peak for the individual C16 and C18 ethyl ester, in computer counts.
As = area corresponding to the peak for the methyl heptadecanoate internal standard, in computer counts.
ms = mass of the methyl heptadecanoate internal standard added, in milligrams.
m = mass of the sample taken for determination, in grams.

A7 - EXPRESSION OF RESULTS

Report the sum of the contents of the different waxes from C42 to C46 [7] in milligrams per kilograms of fat for Extra virgin Olive oil (EVOO) and Virgin Olive oil (VOO). Report the sum of the content of C40 to C46 in the case of the other categories of olive or olive pomace oils.
Report the sum of the contents of the 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. Peaks identification of the individual waxes, especially if C46 peak is splitted, can be made by comparing the retention time of the wax fraction of an olive-pomace oil.*

METHOD B - 3 GRAMS OF SILICA

B4 - APPARATUS

B4.1. Test tube, 10 ml.

B 4.2 Glass column for liquid chromatography, internal diameter 10 mm, length 40 cm, fitted with a suitable stopcock.

B4.3 Gas chromatograph suitable for use with a capillary column, equipped with a system for direct, on-column injection comprising:

B4.3.1 Thermostat-controlled oven with temperature programming.

B4.3.2 Cold injector for direct on-column injection

B4.3.3 Flame ionisation detector and **converter-amplifier**.

B4.3.4 Computerized systems recorder-integrator (Note[8])

B4.3.5 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[9]) to a uniform thickness of 0.10-0.25 µm.

B4.4 Microsyringe, 10 µl, for on-column injection.

B4.5 Electric shaker.

B4.6 Rotary evaporator.

B4.7 Muffle oven.

B4.8 Analytical balance for weighing to an accuracy of ± 0.1 mg.

B4.9 Usual laboratory glassware.

B5 – REAGENTS

B5.1 Silica gel, 60-200 µm mesh. Place the silica gel in the muffle oven at 500 °C for at least 4 hours. 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 hours prior to use. If the silica gel is ultra-pure grade, the muffle oven treatment may not be necessary. In any case each batch of silica must be checked for suitability.

Note[8]: *also to use with the converter-amplifier (B4.3.3). A recorder with a response time of not more than 1 s and a variable paper speed can be used.*

Note[9]: *Suitable commercial liquid phases are available for this purpose such as SE52, SE54 (methyl silicon with 5% phenyl), etc. or other phase with similar or lower polarity.*

B5.2 **n-Hexane**, chromatography (or residue) grade – the purity must be checked as follows: 100 ml of n-hexane are evaporated to dryness, residue is re-dissolved in 100 µl n-heptane, and analysed as solvent blank applying the same gas chromatographic conditions. There must be no peak in the elution alkyl esters area. (Hexane can be replaced by Isooctane) Hexane – Chromosolv Pestanal is available from Honeywell-Riedel-de Haen (code 34484). This reference is an example of suitable products, which are available commercially. This information is given for the convenience of users of this method and does not constitute an endorsement of these products.

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. Harmful 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.

B5.3 **Ethyl ether**, chromatography grade.

WARNING – Highly inflammable and moderately toxic. Irritates the skin. Harmful 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.

B5.4 **n-Heptane**, chromatography grade, or **isooctane**

WARNING – Inflammable. Harmful 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.

B5.5 **Standard solution of lauryl arachidate** (Note[10]) at 0.002% (m/V) in heptane (internal standard for waxes).

B5.6 **Standard solution of methyl heptadecanoate** at 0.0005% (m/V) in heptane (internal standard for methyl and ethyl esters).

B5.7 **Sudan 1** (1-phenylazo-2-naphthol) optional (attention: azo-compounds have mutagenic and carcinogenic properties)

B5.8 **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

Note[10]: *Palmityl palmitate, myristyl stearate or arachidyl laureate may also be used.*

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.

WARNING - 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.

B5.9 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.

B6 - PROCEDURE

B6.1 Preparation of the chromatographic column

Suspend 3 g of silica gel (B5.1) in n-hexane (B5.2) and introduce into the column (B4.2). Allow to settle spontaneously. Complete settling with the aid of an electric shaker (B4.5) 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 test tube (B4.1), using the analytical balance (B4.8), and add a suitable amount of internal standard (B5.5) 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). Transfer the prepared sample to the chromatography column with the aid of two 1-ml portions of n-hexane (B5.4).
Allow the solvent to flow to 1 mm above the upper level of the absorbent. Percolate a 12-15 ml n-hexane (*) to further remove hydrocarbons (alkanes and sterenes). Then start the

chromatographic elution of n-hexane/ethyl ether (99:1) and collect 40-45 ml at a flow of about 15 drops every 10 seconds.

This fraction contains the ethyl esters and waxes (Note[11] and Note 10[12]).

(*) Not necessary for the determination of waxes only.

Evaporate the resultant fractions in a rotary evaporator (B4.6) 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 diluted with 0.5-1 ml of n-heptane or isooctane.

B6.2 Gas chromatography analysis

B6.2.1 Preliminary procedure

Fit the column to the gas chromatograph (B4.3), 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 to 350 °C (approximately 4 hours).

Maintain this temperature for at least 2 hours, then regulate the apparatus to the operating conditions (regulate gas flow, light flame, connect to electronic recorder (B4.3.4), regulate oven temperature for column, regulate detector, etc.). Record the signal at a sensitivity at least twice as high as 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.

B6.2.2 Choice of operating conditions for waxes and ethyl esters (Note[13])

The operating conditions are generally as follows:

- Column temperature:

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

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

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

Note[11]:The n-hexane/ethyl ether (99:1) mixture should be freshly prepared every day, n-hexane can be replaced with the same amount of isooctane.

Note[12]:*The elution volumes indicated in the text are those which usually are employed. However, if needed, they can be modified to achieve a satisfactory separation of the fractions. For this purpose, 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 volume of the dye lies in between that of the waxes and triacylglycerols. Hence, when the dye reaches the bottom of the chromatography column, elution must be suspended because all the waxes have been eluted.*

Note[13]: *Due to the high final temperature, positive drift is allowed but may not exceed more than 10% of the full-scale value.*

- Carrier gas: helium or hydrogen at the optimal linear speed for the gas chosen (see Annex B).
- Instrument sensitivity: suitable for fulfilling the above suitable for the quantification of the analytes

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 ethyl esters and to obtain satisfactory peak separation (see Figures1-2).

B6.3 Performance of the analysis

Take up 1-2 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 (C40-C46) are completely eluted.
The base line must always meet the required conditions.

B6.4 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 main fatty acids in olive oils (palmitic and oleic).
Annex A reports some examples of chromatograms of ethyl esters and waxes suitable to identify related peaks.
Figure 3. Shows a chromatogram of the FAEE and waxes in an extra virgin olive oil using the method B (3 g).
Figure 4. Shows the chromatograms of FAEE and waxes in a virgin olive oil using the method B (3 g).

B6.5 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 in the case of extra virgin olive oil and virgin olive oil and form C40 to C46 in the case of other oils, with the aid of the integrator.
Determine the content of each individual wax, in mg/kg of fat, as follows:

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

where:

Ax = area corresponding to the peak for the individual ester, in computer counts (sum of peaks: C42, C44, C46 for EVOO and VOO; sum of peaks: C40, C42, C44 and C46 for the other oils)

As = area corresponding to the peak for the lauryl arachidate internal standard, in computer counts
ms = mass of the lauryl arachidate internal standard added, in milligrams
m = mass of the sample taken for determination, in grams

B6.6 Quantitative analysis of the ethyl esters

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

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

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

where:

Ax = area corresponding to the peak for all the individual C16 and C18 ethyl ester, in computer counts ().
As = area corresponding to the peak for the methyl heptadecanoate internal standard, in computer counts.
ms = mass of the methyl heptadecanoate internal standard added, in milligrams.
m = mass of the sample taken for determination, in grams

B7 - EXPRESSION OF RESULTS

Report the sum of the contents of the different waxes from C42 to C46 in the case of extra virgin olive oil and virgin olive oil and from C40 to C46 in the case of other categories of olive or olive pomace oils in milligrams per kilograms of fat (Note[14]).
Report the sum of the contents of the ethyl esters from C16 to C18 and the total of the two.
Results should be expressed to the nearest mg/kg.

Note[14]: *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. Peaks identification of the individual waxes, can be made by comparing the retention time of the wax fraction of an olive-pomace oil.*

ANNEX A

Examples of chromatograms:

The following chromatograms are reported as an aid to identify peaks as well as to give information about the separation to be obtained.

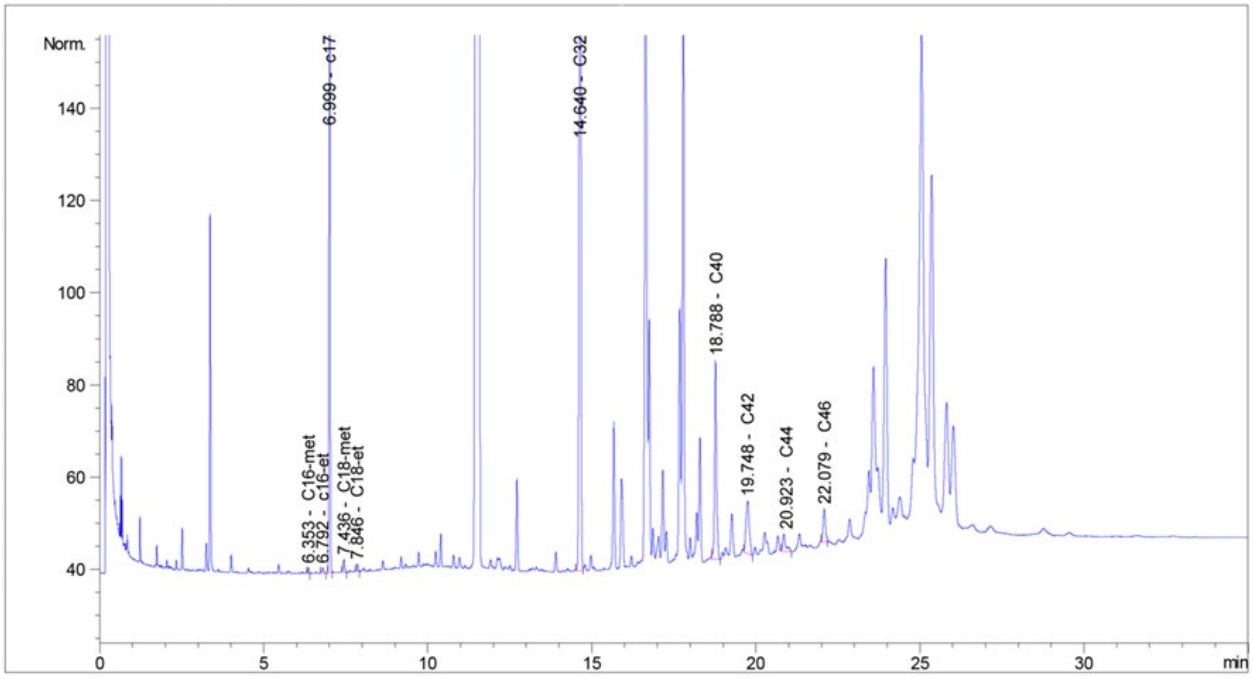

Figure 1: Chromatogram of FAEE and waxes of EVOO using method A (15 g).

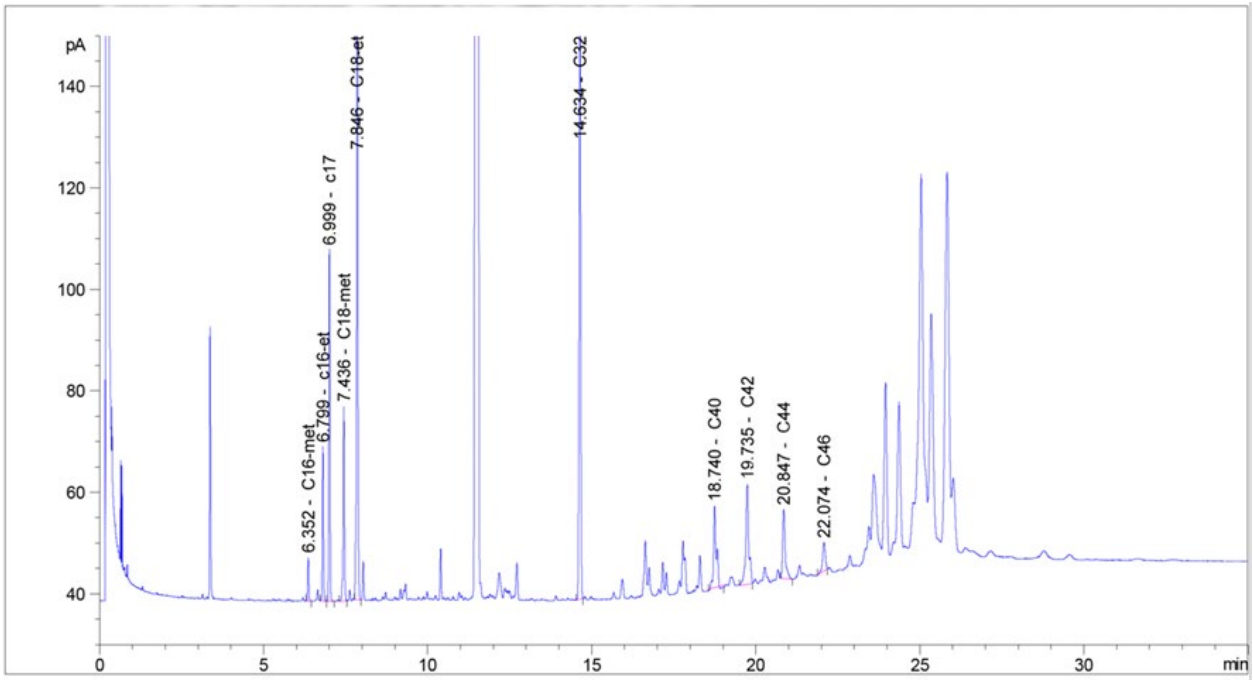

Figure 2: Chromatogram of FAEE and waxes of Lampante olive oil using method A (15 g).

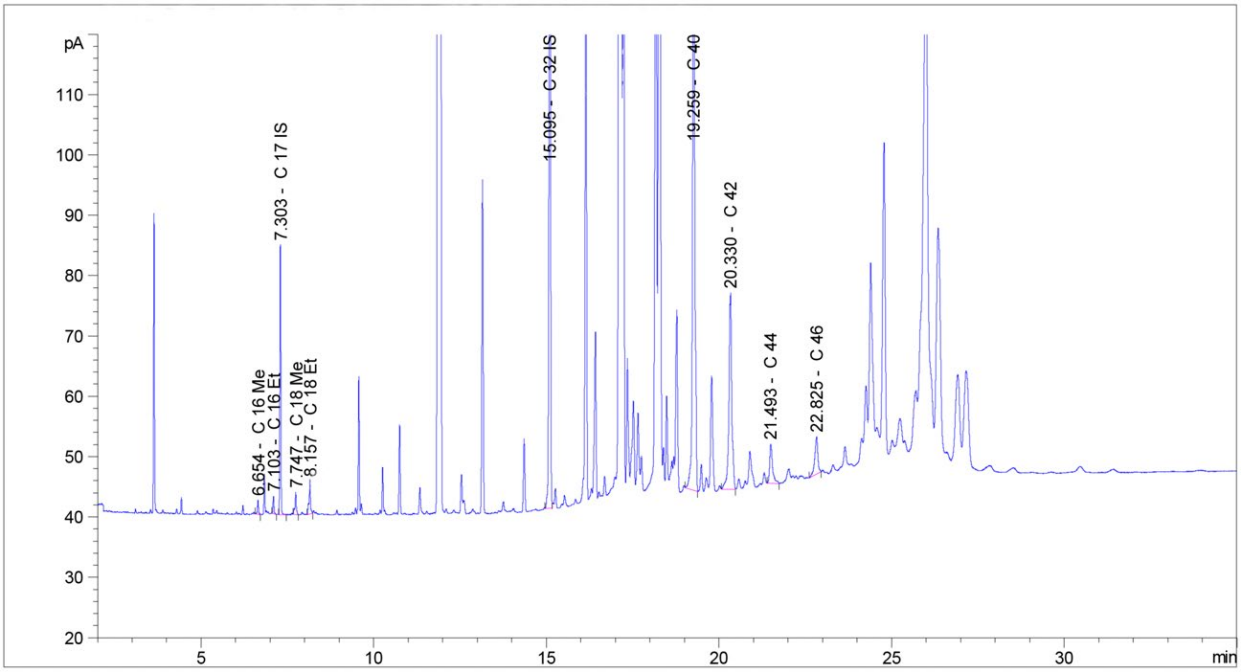

Figure 3: Chromatogram of FAEE and waxes of EVOO using method B (3 g).

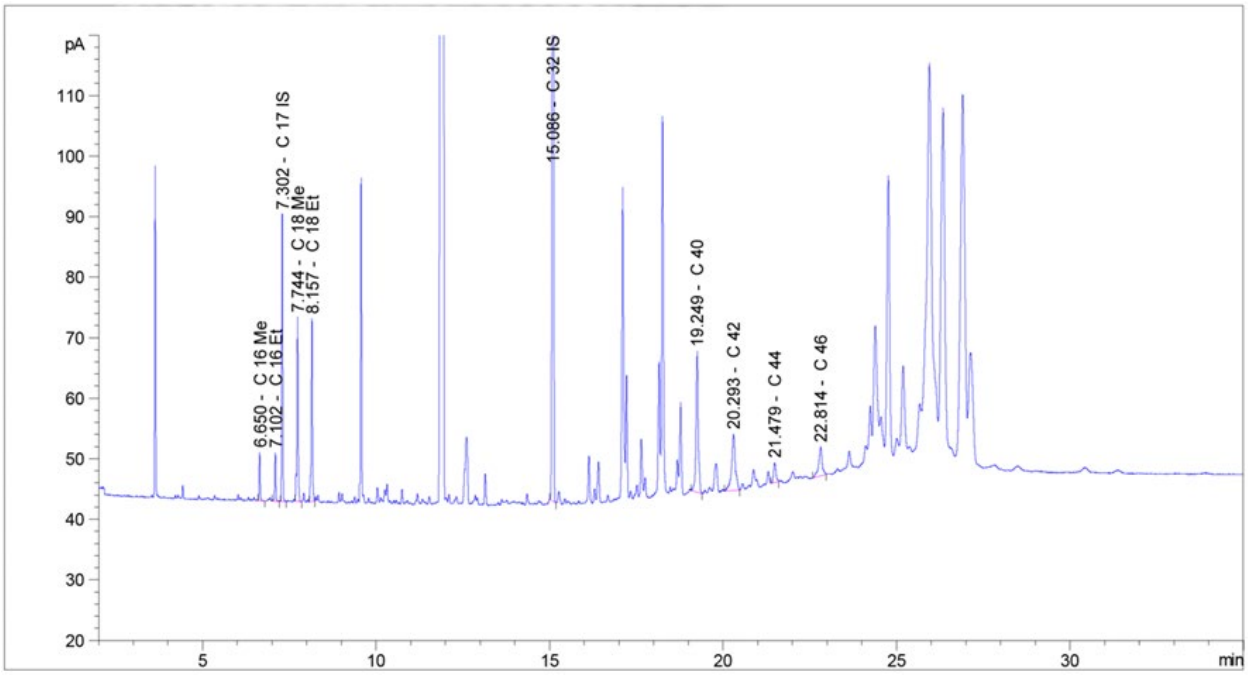

Figure 4: Chromatogram of FAEE and waxes of VOO using method B (3 g).

ANNEX B

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.*

ANNEX C

Precision values of the ethyl esters and wax method - Analysis of the collaborative test results

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.

The test was performed on five samples.

M1: EVOO to determine FAEE and waxes.

M2: EVOO to determine FAEE and waxes.

M3: VOO to determine FAEE and waxes.

M4: LOO to determine FAEE and waxes.

M5: OPO to determine waxes.

Outliers were examined by applying Cochran's and Grubbs's test to the laboratory results for each determination (replicates a and b).

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

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)

Ethyl esters (mg/kg) – Method A 15 g of silica				
Sample	**M1**	**M2**	**M3**	**M4**
Mean	10.00	8.21	36.97	50.83
n	16	16	16	16
outliers	3	2	0	0
Sr	0.574	0.330	1.316	1.934
RSDr (%)	5.74	4.02	3.56	3.80
r	1.61	0.92	3.68	5.41
S_R	0.759	0.915	3.720	6.736
RSD_R (%)	7.59	11.15	10.06	13.25
R	2.12	2.56	10.42	18.86

Ethyl esters (mg/kg) -Method B (3 g of silica)				
Sample	**M1**	**M2**	**M3**	**M4**
Mean	10.00	8.15	39.22	52.46
n	15	15	15	15
Outliers	4	4	0	0
S_r	0.570	0.225	1.234	1.903
RSDr (%)	5.90	2.76	3.15	3.63
r	1.65	0.63	3.46	5.33
S_R	1.681	0.991	4.450	4.691
RSD_R (%)	14.00	12.16	11.34	8.94
R	3.92	2.77	12.46	13.13

For waxes determination, for both methods A and B:
M1, M2 and M3 (sum of waxes from C42 to C46); M4 and M5 (sum of waxes from C40 to C46)

Waxes (mg/kg) Method A 15 g of silica					
Sample	**M1***	**M2***	**M3***	**M4****	**M5****
Mean	93.00	45.17	38.54	323.17	2350.72
n	16	16	16	16	16
outliers	4	1	2	1	0
Sr	0.610	1.406	1.315	4.346	35.728
RSDr (%)	0.66	3.11	3.41	1.34	1.52
r	1.71	3.94	3.68	12.17	100.04
S_R	8.053	4.879	5.590	23.649	247.180
RSD_R (%)	8.66	10.80	14.51	7.32	10.52
R	22.55	13.66	15.65	66.22	692.10

* Sum C42-C46 ; ** Sum C40-C46

Waxes (mg/kg) Method B (3 g of silica)					
Sample	**M1***	**M2***	**M3***	**M4****	**M5****
Mean	90.45	45.53	38.81	325.47	2354.21
n	15	15	15	15	15
Outliers	2	1	2	0	0
S_r	1.770	2.306	1.745	7.758	50.548
RSDr (%)	1.96	5.06	4.50	2.38	2.15
r	4.96	6.46	4.89	21.72	141.54
S_R	15.287	7.066	8.236	23.614	213.990
RSD_R (%)	16.90	15.52	21.22	7.26	9.09
R	42.80	19.79	23.06	66.12	599.17

* Sum C42-C46 ; ** Sum C40-C46

ANNEX D

References

ISO 5725-1:1994 Accuracy (trueness and precision) of measurement methods and results – Part 1: General principles and definitions

ISO 5725-2:2019 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