Commission Regulation (EEC) No 2568/91 of 11 July 1991 on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis

[F1ANNEX IV

DETERMINATION OF WAX CONTENT BY CAPILLARY COLUMN GAS CHROMATOGRAPHY

Textual Amendments

F1 Substituted by Commission Regulation (EC) No 702/2007 of 21 June 2007 amending Commission Regulation (EEC) No 2568/91 on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis.

1. SUBJECT

This method describes a process for determining the wax content of olive oils. Waxes are separated according to the number of their carbon atoms. The method may be used in particular to distinguish between olive oil obtained by pressing and that obtained by extraction (olive-residue oil).

2. PRINCIPLE

Addition of a suitable internal standard to the fat or oil, then fractionation by chromatography on a hydrated silica gel column. Recovery under the test conditions of the fraction eluted first (the polarity of which is less than that of the triglycerides), then direct analysis by capillary column gas chromatography.

3. EQUIPMENT

3.1. 25 ml Erlenmeyer flask.

3.2. Glass column for gas chromatography, internal diameter 15,0 mm, length 30 to 40 cm, fitted with a stopcock.

3.3. Suitable gas chromatograph with a capillary column, equipped with a system for direct introduction into the column comprising the following:

3.3.1. Thermostatic chamber for the columns, equipped with a temperature programmer.

3.3.2. Cold injector for direct introduction into the column.

3.3.3. Flame ionisation detector and converter-amplifier.

3.3.4. Recorder-integrator capable of working with the converter-amplifier (3.3.3), rate of response no slower than 1 second, with variable paper speed. (It is also possible to use computerised systems that allow the acquisition of gas chromatography data via a PC.)

3.3.5. Glass or fused silica capillary column 8 to 12 m long and with an internal diameter of 0,25 to 0,32 mm, with liquid phase, with a uniform film thickness between 0,10 and 0,30 µm. (There are liquid phases suitable for the purpose of type SE-52 or SE-54 available on the market.)

3.4. 10 µl microsyringe for on-column injection, equipped with a hardened needle.

3.5. Electrovibrator.

3.6. Rotary evaporator.

3.7. Muffle furnace.

3.8. Analytical balance with guaranteed precision of ± 0,1 mg.

3.9. Normal laboratory glassware.

4. REAGENTS

4.1. Silica gel with a granule size of between 60 and 200 µm.

Place the gel in the furnace at 500 °C for at least four hours. After cooling, add 2 % water in relation to the quantity of sampled silica gel. Shake well to homogenise the slurry. Keep in darkness for at least 12 hours prior to use.

4.2. n-hexane, for chromatography.

4.3. Ethyl ether, for chromatography.

4.4. n-heptane, for chromatography.

4.5. Standard solution of lauryl arachidate, at 0,1 % (m/v) in hexane (internal standard). (It is also possible to use palmityl palmitate or myristyl stearate.)

4.5.1. *Sudan 1 (1-phenyl-azo-2-naphthol).*

4.6. Carrier gas: hydrogen or helium, gas-chromatographic purity.

4.7. Auxiliary gases:

— pure hydrogen for gas chromatography,

— pure air for gas chromatography.

5. PROCEDURE

5.1. Preparation of the chromatographic column.

Suspend 15 g of silica gel (4.1) in the n-hexane (4.2) and introduce it into the column (3.2). Allow to settle spontaneously. Complete settling with the aid of an electrovibrator (3.5) to make the chromatographic layer more homogeneous. Percolate 30 ml of n-hexane to remove any impurities. Using the balance (3.8) weigh exactly 500 mg of the sample into the 25 ml Erlenmeyer flask (3.1), add the appropriate quantity of the internal standard (4.5) according to the presumed wax content. For example, add 0,1 mg of lauryl arachidate for olive oil, and 0,25 to 0,5 mg for olive-residue oil. Transfer the prepared sample to the chromotography column using two 2 ml portions of n-hexane (4.2).

Allow the solvent to flow away until it reaches 1 mm above the upper level of the absorbant then percolate a further 70 ml of n-hexane in order to eliminate the n-alkanes naturally present. Then start the chromatographic elution by collecting 180 ml of the mixture of n-hexane/ethyl ether (ratio 99:1), keeping a rate of flow of approximately 15 drops every 10 seconds. Elution of the sample must be carried out at a room temperature of 22 ± 4 °C.
NB:

— The n-hexane/ethyl ether mixture (99:1) must be prepared every day.

— For a visual check on the correct elution of the waxes 100 µl of 1 % Sudan in the elution mixture can be added to the sample in solution. Since the colourant has an intermediate retention, between waxes and triglycerides, when the coloration has reached the bottom of the column the elution should be suspended because all the waxes will have been eluted.

Dry the fraction thus obtained in a rotary evaporator (3.6.) until virtually all the solvent has been eliminated. Eliminate the final 2 ml of solvent with the aid of a weak current of nitrogen; then add 2-4 ml n-heptane.

5.2. Analysis by gas chromatography

5.2.1. Preparatory work

Fit the column to the gas chromatograph (3.3) by connecting the inlet port to the on-column system and the outlet port to the detector. Perform a general check on the GC apparatus (operation of gas circuits, detector and recorder efficiency, etc.).

If the column is being used for the first time it should be conditioned first. Pass a little gas through the column, then turn on the GC apparatus. Heat gradually until 350 °C is reached after about four hours. Maintain that temperature for at least two hours then regulate the apparatus to operating conditions (set gas flow, light flame, connect to the electronic recorder (3.3.4), set temperature of column chamber, detector, etc.) and record the signal at a sensitivity at least twice as high as that required for the analysis. The baseline must be linear, with no peaks of any kind, and must not show any deviation.

A negative straight-line drift indicates that the column connections are not tight; a positive drift that the column has not been sufficiently conditioned.

5.2.2. Choice of operating conditions

The operating conditions are generally as follows:

— column temperature:

	20 °C/ minute		5 °C/ minute		20 °C/ minute	
Initially 80 °C (1′)	→	240 °C	→	325 °C (6′)	→	340 °C (10′)

— detector temperature: 350 °C;

— quantity of substance injected: 1 µl of the n-heptane solution (2-4 ml);

— carrier gas: helium or hydrogen at the correct linear velocity for the gas selected (see Appendix);

— instrument sensitivity: suitable for the following conditions:

The conditions may be modified according to the characteristics of the column and the GC apparatus to obtain separation of all the waxes and a satisfactory peak resolution (see figure); the internal standard C_{32} retention time must be 18 ± 3 minutes. The most representative wax peak must be at least 60 % of the full scale.

The peak integration parameters must be established so as to obtain a correct evaluation of the areas of the peaks in question.

NB: Given the high final temperature, a positive drift of no more than 10 % of the full scale is permitted.

5.3. Performance of the analysis

Sample 1 µl of the solution using the 10 µl microsyringe; withdraw the syringe plunger so that the needle is empty. Place the needle in the injector and after 1-2 seconds inject quickly; remove the needle slowly after about five seconds.

Record until the waxes are completely eluted.

The base line must always satisfy the required conditions.

5.4. Identification of peaks

Identification of the different peaks should be based on retention time by comparison with wax mixtures of known retention times analysed under the same conditions.

The figure is a chromatogram of the waxes of a virgin olive oil.

5.5. Evaluation of quantity

Calculate the areas of the peaks of the internal standard and the aliphatic esters of C_{40} to C_{46} using the integrator.

Calculate the wax content of each of the esters in mg/kg fat using the formula:

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

where:

A_x = area of each ester's peak, in square millimetres;
A_s = area of the internal standard's peak, in square millimetres;
m_s = mass of added internal standard, in milligrams;
m = mass of sample for analysis, in grams.

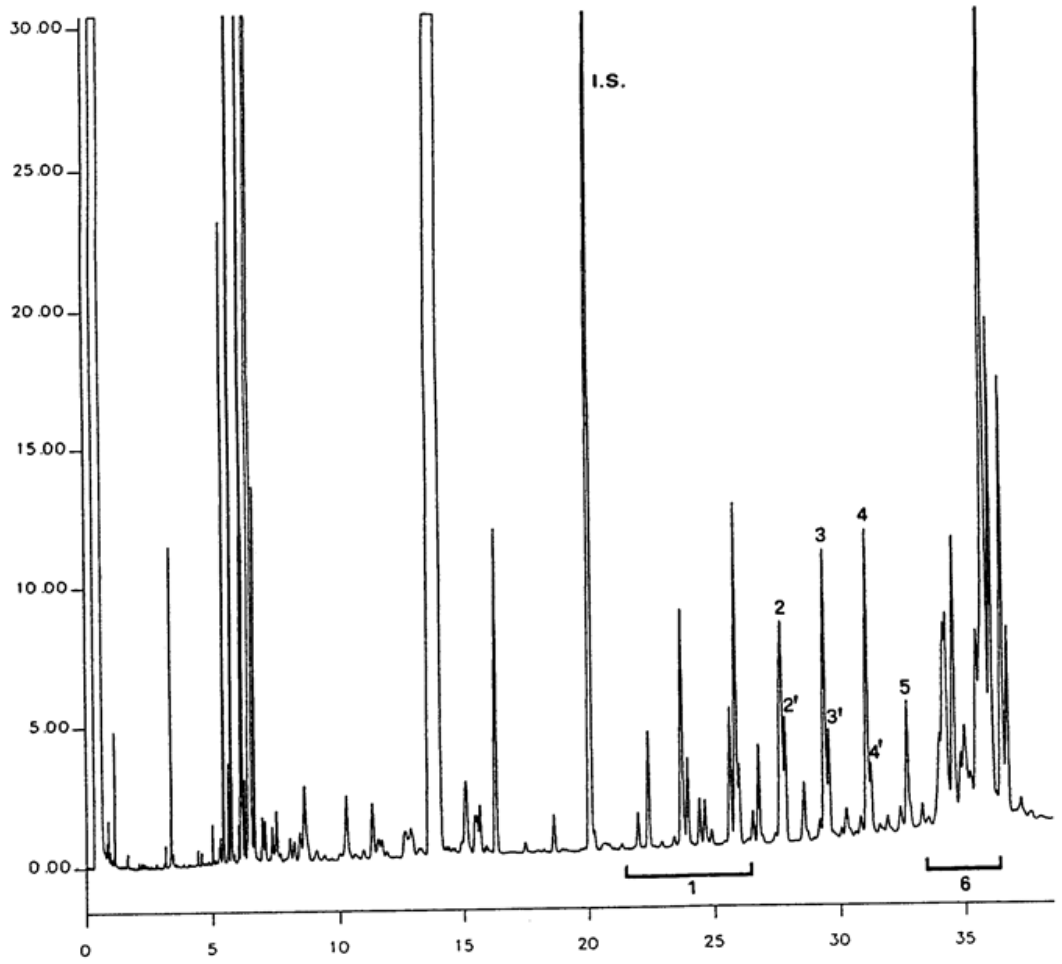
6. EXPRESSION OF RESULTS

Indicate the total of the contents of the various C_{40} to C_{46} waxes in mg/kg fat (ppm).

NB: The components to be quantified refer to the peaks with carbon pair numbers between esters C_{40} and C_{46}, using the example of the olive oil wax chromatogram shown in the figure below. If ester C_{46} appears twice, it is recommended that to identify it the fraction of the waxes of an olive-residue oil should be analysed where the C_{46} peak is easy to identify because it is in the clear majority.

The results should be expressed to one decimal place.

Figure Chromatogram of the waxes of an olive oil[(1)]



Appendix

Determination of the linear velocity of the gas

Inject 1-3 μl methane (or propane) into the GC apparatus after it has been regulated to normal operating conditions. Measure the time it takes for the gas to flow through the column from the time it is injected to the time the peak appears (t_M).

The linear velocity in cm/s is given by the formula L/t_M, where L is the length of the column in cm and t_M the time measured in seconds.**]**

(1) **[**[F1]After elution of the sterol esters the chromatogram trace must not show any significant peaks (triglycerides).**]**

Textual Amendments

F1 Substituted by Commission Regulation (EC) No 702/2007 of 21 June 2007 amending Commission Regulation (EEC) No 2568/91 on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis.

Status:
Point in time view as at 01/10/2008.

Changes to legislation:
There are outstanding changes not yet made to Commission Regulation (EEC) No 2568/91. Any changes that have already been made to the legislation appear in the content and are referenced with annotations.